BEHAVIORAL CHARACTERIZATION AND NEUROBIOLOGICAL CORRELATES OF THE DEVELOPMENT OF STEREOTYPY IN DEER MICE: A MOUSE MODEL OF RESTRICTED REPETITIVE BEHAVIORS IN NEURODEVELOPMENTAL DISORDERS

By

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To my sister and her yet unborn child

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LIST OF ABBREVIATIONS

CNS	central nervous system
CO	cytochrome oxidase
EE	enriched (or complex) environment
DBS	deep brain stimulation
GPe	globus pallidus external segment
GPi	globus pallidus internal segment
ISMP	index of striosome and matrix predominance
MOR1	mu-opioid receptor 1 protein
MSN	medium spiny neurons
OCD	obsessive-compulsive disorder
PBS	phosphate buffered saline
RRB	restricted, repetitive behaviors
SC	standard laboratory cages
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
STN	subthalamic nucleus
STR	striatum

Abstract of Dissertation Presented to the Graduate School
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Restricted, repetitive behaviors are diagnostic for autism and common features of neurodevelopmental disorders. Despite the clinical significance, the etiology, development, and pathophysiology linked to these behaviors are largely unknown. Using *Peromyscus maniculatus* (deer mouse) as an animal model of repetitive behaviors associated with environmental restriction, the current studies characterized the development of stereotyped behaviors associated with restricted laboratory environment and investigated the neurobiological perturbations associated with such behaviors.

The first study evaluated the long-term changes in the indirect basal ganglia pathway associated with stereotypy in adult mice, behaviors of which have been well characterized, using cytochrome oxidase (CO) histochemistry. The activity in the subthalamic nucleus (STN), a key node of the indirect pathway, was found to be negatively correlated with stereotypy. In addition, pharmacological manipulations to increase indirect pathway activity by co-administration of adenosine A_{2A} and A_1 receptor agonists selectively reduced stereotypy dose-dependently.

The second study identified three distinct developmental trajectories of stereotypy. This study used a novel method to evaluate temporal organization of repetitive behaviors and, importantly, the interplay between temporal dynamics and developmental trajectory.

The third study evaluated the alteration of the indirect pathway during the development of stereotypy. Developmental trajectory was significantly associated with STN activity. Conversely, assessment of striatal compartments (striosomal versus matrix), imbalance of which has been suggested to be important in repetitive behaviors, showed no association with the development of stereotypy. Volume and cell counts of striosomes were also not linked to stereotypy.

The fourth study examined the effect of chronic intermittent administration of amphetamine on the development of stereotypy in deer mice. Although deer mice were behaviorally sensitized, chronic administration of amphetamine had no significant effect on the development and expression of spontaneous stereotypy.

Lastly, the fifth study examined effects of chronic administration of adenosine A_{2A} and A_1 receptor agonists on the development of stereotypy. Exposure to this drug combination for seven days early in the post-weaning period resulted in a long-term alteration of the developmental trajectory indexed by a persistent reduction in frequency and less rigid behavioral organization.

The results of these studies collectively support that the development of stereotypy is linked to the reduced activity of indirect basal ganglia pathway, and pharmacological manipulations targeting this pathway attenuated the development and expression of these behaviors. These findings further suggest drug development efforts targeting this

pathway that may be potentially useful in treating repetitive behaviors in neurodevelopmental disorders.

CHAPTER 1
GENERAL INTRODUCTION

Restricted Repetitive Behaviors in Neurodevelopmental Disorders

Restricted repetitive behaviors encompass a wide range of abnormal behaviors, which are characterized by repetition, topographic invariance, and lack of obvious function. These behaviors are common phenotypes of several neurodevelopmental disorders including autism for which they constitute one of three diagnostic domains (American Psychiatric Association, 2000). Repetitive behaviors in autism include motor stereotypies, repetitive manipulation of objects and verbalizations as well as more complex behaviors such as compulsions, rituals, insistence on sameness, and narrow and circumscribed interests (Lewis and Bodfish, 1998; Turner, 1999; Bodfish et al., 2000). Complex repetitive behaviors seem to involve cognitive components, which are often accompanied by some set of rules or a 'just right' criterion for completion. This categorization (repetitive sensorimotor behavior and resistance to change) has been recently validated by factor analyses of the relevant items from the Autism Diagnostic Interview-Revised (ADI-R) (Cuccaro et al., 2003; Szatmari et al., 2006). This wide range of repetitive behaviors is also typical of individuals with severe forms of intellectual and developmental disability (Bodfish et al., 2000).

More specific forms of repetitive behaviors are part of the phenotype of certain genetic disorders. These include repetitive self-biting of lips or digits in Lesch-Nyhan syndrome (Cauwels and Martens, 2005), skin picking in Prader-Willi syndrome (Symons et al., 1999), and symmetric midline hand stereotypies in Rett's syndrome (Carter et al., 2009). Other psychiatric and neurological disorders may also be associated with repetitive behaviors, some of which include childhood onset obsessive-compulsive

disorder (OCD) (repetitive checking or washing), Tourette syndrome (vocal tics), and frontotemporal and Alzheimer's dementias (body rocking, tapping, chewing) (Muller et al., 1997; Cath et al., 2001; Mateen and Josephs., 2009). In general, restricted, repetitive behaviors are considered abnormal and clinically significant because they are stigmatizing, preclude or disrupt goal-directed actions, limit interaction with the environment, and on occasion are self-injurious. The repetitive behaviors have been also reported in individuals without specific neurological and psychiatric disorders (Catellanos et al., 1996; Rafaeli-Mor et al., 1999; Singer, 2009). Importantly a number of stereotyped motor acts (e.g., rocking) and compulsive behaviors (e.g., bedtime rituals) are characteristics of normative development (Thelen, 1980; Evans et al., 1997).

Despite their prevalence in clinical as well as non-clinical populations, little is known about the development of these repetitive behaviors. One exception is the recently reported longitudinal study by Richler et al. (2010) assessing children with autism and with non-spectrum developmental delay at four different developmental time-points in childhood. Children with autism showed persistently high repetitive sensorimotor behaviors, whereas children with developmental delay showed decline of this category of behaviors over time. Conversely, higher-order insistence on sameness was initially low in all subjects, which gradually increased only in children with autism. This group also showed that there were developmental sub-trajectories, which have long been speculated due to the variability of the expression level of these behaviors among the population.

Despite this first systematic longitudinal work, the lack of knowledge on the development of repetitive behavior is obvious. In addition, the pathophysiology of these

behaviors has received limited clinical study, although several neuroimaging studies have pointed the structural and functional changes associated with repetitive behaviors (O'Sullivan et al., 1997; Peterson et al., 1998; Sears et al., 1999; Hollander et al., 2005). For example, increased right caudate volume was noted in individuals with autism spectrum disorders, which was positively correlated with rates of repetitive behaviors (Sears et al., 1999; Hollander et al., 2005). Patients with trichotillomania displaying repetitive hair-pulling had significantly reduced volumes of the left putamen (O'Sullivan et al., 1997). Moreover, tics in Tourette syndrome were inversely correlated with changes of blood flow and oxygen concentration in the basal ganglia and thalamus (Peterson et al., 1998). Although these studies point to the possible alterations in cortico-basal ganglia circuitry in repetitive behavior disorders, the neurobiological perturbations responsible for these behavior disorders are still largely unknown. A more complete understanding of the neurobiological mechanisms associated with such behaviors is required to facilitate the development of efficacious early intervention and prevention strategies, and such an understanding requires the use of appropriate animal models.

Animal Models of Restricted, Repetitive Behaviors

To study the underlying neurobiological basis of repetitive behaviors, several categories of relevant animal models are available, including stereotypy associated with central nervous system (CNS) insults, pharmacologically-induced stereotypy, and stereotypy associated with restricted environments (Lewis et al., 2007 for review).

Stereotypy Associated with Central Nervous System Insults

Recent advances in genomics have provided a wealth of information about the etiology of neuropsychiatric disorders. Some of these new genomic tools include

inducible transgenic mice, where expression of target genes is spatially and temporary controlled by the regulation of tetracycline response elements by tetracyclin-controlled transcactivator, and lentiviral and adeno-viral vectors, which utilize the virus' function to infect other cells by transporting their genome to express genes of interest in a spatially and temporary controlled manner. Of particular interest is that some of these gene-manipulated animals to model neuropsychiatric disorders demonstrate repetitive behaviors, which in some cases resemble the repetitive behavior symptoms in clinical populations.

For example, mice expressing truncated methyl-CpG binding protein 2 (MeCP2), mutation of which is known as a major cause of Rett syndrome (Meloni et al., 2000; Carney et al., 2003), display repetitive forepaw movements. These movements resemble the midline hand stereotypies (hand wringing and waving) commonly seen in patients with Rett syndrome (Shahbazian et al., 2002; Moretti et al., 2005). Excessive grooming resulting in hair removal and tissue damage has been reported in Hoxb8 homozygous knockout mice (Greer and Capecchi, 2002). Expression of the Hoxb8 gene is found in the orbitofrontal and anterior cingulate cortices, and caudate nucleus, which comprise so-called 'OCD circuitry' (Graybiel and Rauch, 2000). Excessive grooming in Hoxb8 knockout mice has particular resemblance to trichotillomania, an OC spectrum disorder. Deletion of the postsynaptic scaffolding protein SAPAP3, which is highly expressed in the striatum and important in regulating glutamatergic cortico-striatal synapses, induced excessive grooming to the point of inducing lesions to the head, neck, and snout in mice (Welch et al., 2007).

In addition to CNS insults via gene manipulations, prenatal exposure to environmental risk factors has been shown to induce similar behavioral phenotypes to those seen in patients with neurodevelopmental disorders. For example, exposure of valproic acid, which has been considered as a potential etiological factor for autistic symptoms (Folstein and Rosen-Sheidley, 2001; Keller and Persico, 2003), to rats on day 12.5 of gestation produced neurological abnormalities similar to those reported in autistic individuals (e.g., altered sensitivities to tactile stimuli, diminished prepulse inhibition, decreased social behaviors, and hyperactivity including stereotypy-like behaviors) (Schneider and Przewlocki, 2005).

More recently, Martin et al. (2008) showed that rhesus monkeys, prenatally exposed to human IgG collected from mothers with multiple children with autism, displayed whole body stereotypies and locomotion. Conversely exposure to human IgG collected from mothers with typically developing children didn't induce repetitive behavior in offspring. This study suggested that prenatal exposure to autoantibodies may be attributed as one of the neurodevelopmental risk factors.

Pharmacologically Induced Stereotypy

Much of what we know about the neurobiological basis of motor stereotypy comes from investigations using pharmacological models. Systematic investigation of anatomical and neurochemical mechanisms underlying drug-induced repetitive behavior implicated cortico-basal ganglia circuitry in the execution of these movements. Intrastriatal and systemic administration of direct and indirect dopamine agonists (e.g., amphetamine, apomorphine, and cocaine) as well as opiate agonists and NMDA receptor antagonists (e.g., MK-801 and PCP) to alter neurochemical transmission in the striatum have consistently induced motor stereotypy (Ernst and Smelik, 1966; Iwamoto

and Way, 1977; Lewis et al., 1990; Segal et al., 1995; Vandebroek and Odberg, 1997; Vandebroek et al., 1998).

Stereotypy Associated with Environmental Restriction

Repetitive behaviors are frequently reported in animals housed in a confined environment, such as zoo, farm, and laboratory environments (Mason, 1991). Such behaviors are typically considered to be an indicator of poor animal welfare for those in captivity. Examples of these species-specific stereotyped behaviors include pacing, route-tracing, and feather-picking in parrots and tits (Jenkins, 2001; Garner et al., 2003a; Garner et al., 2003b; Meehan et al., 2004), bar-mouthing, vertical jumping, and backward somersaulting in rodents (Vandebroek and Odberg, 1997; Powell et al., 1999; Garner and Mason, 2002), pacing, body-rocking, tail-biting, and self-injurious behavior in rhesus monkeys (Lutz et al., 2003; Taylor et al., 2005), pacing, somersaulting, and over-grooming in prosimians (Tarou et al., 2005), crib-biting, boxwalking, and head-shaking in horses (McGreevy et al., 1995; Bachmann et al., 2003), regurgitation and tongue flicking in pandas (Swaisgood et al., 2005), and head-twirling in minks (Mason, 1993). These stereotyped behaviors seem to develop as a consequence of animals' responses to restricted environments, which limit expression of species-typical naturalistic behaviors, because alleviation of environmental deprivation has been consistently reported to reduce rates of these behaviors (Powell et al., 2000; Turner et al., 2002; Garner et al., 2003b; Meehan et al., 2004; Swaisgood et al., 2005; Hadley et al., 2006).

Cortico-Basal Ganglia Circuitry and Repetitive Behaviors

Clinical and animal studies have implicated alterations of cortico-basal ganglia circuitry associated with restricted repetitive behaviors. The basal ganglia are a group of

subcortical nuclei that regulate execution of motor and cognitive programs (Figure 1-1). Among five circuits connecting the cortex and the basal ganglia, the motor circuit that is hypothesized to be responsible for the expression of stereotypy originates from the primary motor cortex and premotor area. Glutamatergic axons from these cortical regions enter the input nucleus of the basal ganglia, especially the dorsolateral striatum, and the output neurons from the basal ganglia regulate activation of thalamocortical neurons. These neurons terminate at the somatosensory cortex, primary motor cortex, and supplementary motor area, providing positive feedback to ongoing motor programs in the primary motor cortex (Parent and Hazrati, 1995; Herrero et al., 2002).

There are two major pathways that travel through the basal ganglia system: the direct pathway and indirect pathway. The direct pathway consists of GABAergic medium spiny neurons directly projecting to the output nucleus of the basal ganglia, the globus pallidus internal (GPi) and substantia nigra pars reticulata (SNpr). These striatonigral neurons express dopamine D_1 receptors and adenosine A_1 receptors that are co-localized with glutamate receptors. They contain the neuropeptides dynorphin and substance P (Steiner and Gerfen, 1998). The indirect pathway consists of GABAergic striatal medium spiny neurons projecting to the globus pallidus external (GPe), GABAergic neurons in GPe projecting to the subthalamic nucleus (STN), and finally glutamatergic neurons sending excitatory projection to GPi and SNpr. The striatopallidal medium spiny neurons express dopamine D_2 receptors and adenosine A_{2A} receptors, and contain the neuropeptide enkephalin (Steiner and Gerfen, 1998).

Normal dopaminergic innervation of the striatum plays a crucial role in execution of movements. Dopamine D_1 receptors in the striatonigral neurons are positively coupled

to adenylyl cyclase (Missale et al., 1998). Therefore, dopamine acts to amplify the glutamatergic corticostriatal inputs, resulting in increased GABAergic inhibition of GPi and SNpr. In contrast, dopamine D_2 receptors in the striatopallidal neurons are negatively coupled to adenylyl cyclase (Missale et al., 1998). Via D_2 receptors, dopamine acts to diminish the corticostriatal inputs, resulting in decreased inhibition of GPe, hence causing this nucleus to exert even more inhibitory control over STN. This increased inhibition of STN removes its excitatory influence on GPi and SNpr. Thus activation of both D_1 and D_2 receptors removes inhibitory tone of the basal ganglia output neurons, ultimately resulting in disinhibition of the thalamocortical neurons to provide positive feedback to motor programs.

Abnormal execution of movements is evident when the balance between the direct and indirect pathway is disrupted. It is postulated that stereotypic behavior is expressed as a consequence of a relative increase in striatonigral tone (Graybiel et al., 2000). This hypothesis is supported by the finding that transgenic mice inducibly overexpressing the transcription factor ΔFosB selectively in dynorphin-containing striatonigral neurons exhibit increased daily wheel running, whereas such behavior was significantly reduced in mice overexpressing ΔFosB in enkephalin-containing striatopallidal neurons (Werme et al., 2002).

Specific manipulations to create this imbalanced activity induce repetitive behaviors in animals. For example, Grabli et al. (2004) have reported that stereotyped behavior (e.g., licking and biting of fingers) was induced in monkeys when the GABA antagonist bicuculline was microinjected into the limbic aspect of GPe, which was reduced by deep brain stimulation (DBS) applied to STN without affecting a control

motor task (Baup et al., 2008). Winter et al. (2008c) have shown that rats that sustained ibotenic acid lesions to STN exhibited an increase in compulsive lever pressing in the signal attenuation model of OCD. This same research group has also shown that bilateral high frequency stimulation of STN as well as pharmacological inactivation of STN reduced quinpirole-induced compulsive checking in rats (Winter et al., 2008a; Winter et al., 2008b; Klavir et al., 2009). These results point to a potentially important role for the indirect pathway function in the expression of repetitive behavior of unknown etiology.

Importantly, alterations to the indirect pathway are linked to repetitive behaviors in a few neurological disorders. The uncontrolled motor movements characteristic of Huntington's disease are attributed to the differential degeneration of striatopallidal neurons (Deng et al., 2004; Starr et al., 2008). DBS applied to STN reduced the severity of symptoms in previously treatment refractory OCD patients (Burdick et al., 2009). Similarly, DBS of STN and GPe has been found to be effective in ameliorating the L-DOPA induced tardive dyskinesia observed in Parkinson's disease (e.g., Kleiner-Fisman et al., 2006).

Deer Mouse Model of Repetitive Behaviors

We have adopted a deer mouse model of stereotypy induced by environmental restriction. These animals exhibit high rates of repetitive vertical jumping or backward somersaulting when housed under standard laboratory conditions (Powell et al., 1999). These behaviors emerge as early as weaning and persist across the life-time. Exposure to a larger, more complex environment following weaning significantly attenuates the development of these behaviors (Powell et al., 2000; Hadley et al., 2006).

Exposure to an enriched environment, especially shortly after weaning, attenuated the expression of stereotypy. Enrichment later in life also attenuated stereotypy, although the degree of attenuation was not as great as that seen in younger animals (Hadley et al., 2006). These studies collectively suggest that repetitive jumping and backward somersaulting seen in deer mice are displayed as a consequence of environmental restriction, especially during the young age. Moreover we have also shown that highly stereotypic deer mice have deficits in a reversal learning task, mirroring inflexibility and resistance to change (Tanimura et al., 2008).

The neurobiological changes associated with environmental restriction, which is also linked to the expression of stereotypy, were found in brain regions comprising cortico-basal ganglia circuitry (e.g., Turner et al. 2003). More specifically, significantly higher dendritic arborization and spine density was seen in the motor cortex and the dorsolateral striatum in low-stereotypy mice reared in an enriched environment as compared to high-stereotypy mice in an enriched environment and mice in a standard-cage condition (Turner et al., 2003). The similar interaction between environmental enrichment and stereotypy was found in metabolic neuronal activity in the motor cortex, striatum, and nucleus accumbens (Turner et al., 2002) as well as BDNF levels in the striatum (Turner and Lewis, 2003).

More direct manipulations to alter the activity of cortico-basal ganglia circuitry induced or attenuated the expression of repetitive behaviors. Intrastriatal administration of the selective D_1 receptor antagonist SCH23390 and the NMDA receptor antagonist MK-801 to increase relative tone of the indirect basal ganglia pathway selectively attenuated stereotypy (Presti et al., 2003). Conversely, the intrastriatal administration of

the direct dopamine receptor agonist apomorphine increased rearing and locomotion, although it didn't exacerbate repetitive jumping and backward somersaulting (Presti et al., 2002). These results collectively implicate that the relatively increased tone of the direct pathway is linked to the expression of stereotypy.

Furthermore, we assessed neuronal activities of the direct and indirect pathways by measuring neuropeptides dynorphin and enkephalin respectively. We found significantly decreased enkephalin content in high-stereotypy mice compared to low-stereotypy mice. Moreover, we saw a significant negative correlation between striatal enkephalin content and frequency of stereotypy but no relationship was found between dynorphin and stereotypy (Presti and Lewis, 2005), suggesting that the imbalance between the basal ganglia pathways linked to stereotypy may be largely due to the decreased indirect pathway tone.

Based on these previous findings in adult deer mice, we continued investigating the role of the indirect pathway activity in the expression as well as development of repetitive behaviors. Our current focus is especially on the development of these behaviors and associated neurobiological changes. Repetitive behaviors in deer mice develop spontaneously shortly after weaning, which provide us advantage to study slowly occurring neuroadaptive changes at different developmental stages of such behaviors. Thus the purpose of this dissertation was to define the development of repetitive behaviors and associated neurobiological correlates in deer mice. This collection of works presented in the following chapters was the first attempt to investigate systematically behaviors and neuroanatomical and neurochemical changes associated with the development of stereotypy in the early post-weaning period.

Aims

The first aim was to determine differential activation of the indirect basal ganglia pathway in adult deer mice exhibiting various rates of stereotypy. We have particularly focused on the subthalamic nucleus (STN) which is a key node of the indirect pathway and has been considered the potential therapeutic target for deer brain stimulation (DBS) for several neuropsychiatric disorders such as obsessive compulsive disorder (OCD), depression, and Parkinson's disease. Previous work from our lab suggests that indirect pathway activation, but not direct pathway activation, may be decreased in high-stereotypy deer mice. Thus using cytochrome oxidase (CO) histochemistry, a marker of neuronal metabolic capacity, as another way to test our hypothesis we assessed the activity of STN as well as other brain regions mediating the expression of stereotypy in adult mice reared under either standard laboratory cages or enriched environment. CO histochemistry was selected as a marker to detect chronic long-term neuronal changes responsible for the development of repetitive abnormal behaviors. Other markers of neuronal activity, such as c-Fos and 2-deoxyglucose, were not appropriate for this model, because they detect rather acute or short-term neuronal excitability.

CO is a respiratory enzyme found in the inner wall of mitochondrial membrane and the final protein complex in the electron transport chain of oxidative phosphorylation. It catalyzes the terminal transfer of electrons from four cytochrome c molecules to oxygen to form two molecules of H_2O while pumping protons out across the membrane. The proton gradient created by CO results in electrostatic energy toward the mitochondrial matrix. A transmembrane enzyme adenosine triphosphate (ATP) synthase allows protons to flow back across the membrane and use this conserving energy to generate ATP from adenosine diphosphate (ADP). Because neurons are heavily dependent on

oxygen metabolism of glucose for ATP synthesis, measurement of CO in brain tissue is a reliable marker of cells' capacity for energy consumption. CO activity can be measured as the accumulation of visible reaction product in a histochemical reaction, which is produced when cytochrome c oxidizes the chromogen (DAB). Because CO limits the rate of reoxidization by cytochrome c, the optical density values indicate the metabolic capacity of the tissue (Gonzalez-Lima and Cada, 1998).

In addition, we sought to determine the effect of pharmacological manipulations on stereotypy, targeting adenosine receptors, which alter striatopallidal neuronal activation. This hypothesis was based on our previous findings (Presti and Lewis, 2003) where the expression of stereotypy was associated with decreased neuropeptide enkephalin, which is selectively expressed in striatopallidal neurons. Adenosine A_{2A} receptors are almost exclusively expressed in the striatum. More specifically A_{2A} receptors are found on presynaptic glutamatergic cortico-striatal neurons and striatopallidal neurons, which comprise the indirect pathway. Stimulation of these receptors activates adenynyl cyclase and ascending transduction cascade. Thus we evaluated the effect of adenosine pharmacology on the expression of stereotypy in deer mice.

The second aim of this dissertation was to characterize the development and temporal organization of repetitive behaviors. Our lab has traditionally assumed that stereotyped behaviors in deer mice mature approximately 60 days post-weaning and used it as the standard protocol. No systematic study, however, has been conducted to characterize the developmental trajectories or to identify those animals, which never develop an appreciable level of stereotypy despite experience in restricted environments. In addition, we typically express rates of stereotypy as the average

frequency of repetitive jumping or backward somersaulting exhibited per hour during the testing period. The frequency of behavior does not provide information about the repetitive nature of behaviors within or between bouts. These analyses became particularly informative and novel because we were able to generate distinct developmental trajectories within a population of animals with longitudinal data sets, and used all the data values collected from each animal for the duration of the entire dark cycle to visually and mathematically describe the bout organization of behaviors.

For the third aim based on the findings Aim#1, we assessed CO changes in the indirect pathway at three developmental time-points based on the findings in Aim#2. This study was particularly important for understanding the neurobiological correlates of the interaction between developmental periods and rates of stereotypy. In addition, we investigated the role of the 'third' basal ganglia pathway, which has been implicated in repetitive behaviors (Canales and Graybiel, 2000b), on the development of stereotypy in deer mice. The striatum is neurochemically distinguished into two compartments: striosome and matrix. Literature suggests that these two compartments are segregated neuroanatomically, mediate different functionalities, and are associated with specific alterations associated with movement disorders (White and Hiroi, 1998; Brown et al., 2002, Lawhorn et al., 2008). In addition to the function of indirect basal ganglia pathway, we assessed the compartmental activation using the CO-stained brain tissue superimposed with striosome-labeled adjacent brain sections.

The fourth aim was to assess whether chronic pharmacological manipulations known to induce behavioral sensitization would alter the development and expression of stereotypy. Intermittent administration of psychostimulants (e.g., amphetamine, cocaine,

apomorphine) induces potentiated behavioral response to acute administration of the same drug (sensitization) or different agents such as stressors (cross-sensitization) (Robinson and Berridge, 2003). Sensitization has been associated with long-lasting functional changes in cortico-basal ganglia circuitry and can thus be viewed as a model of pathological neuroadaptation. For example, preferential activation of striosomes has been reported in sensitized animals at much lower doses of amphetamine than those required to induce this effect in non-sensitized animals (Canales and Graybiel, 2000b). There has been a hypothesis that stereotypy associated with environmental restriction is an animal's response to stressful experience and subsequent alteration in dopamine transmission (Cabib, 2006), although little evidence has been available to evaluate such an assertion. If stereotypy associated with environmental restriction is due to stress-induced sensitization, any stimulus or experience capable of activating the dopamine system should also be able to promote stereotypy by chronic stressful experience, Thus we assessed the effects of chronic amphetamine administration to induce behavioral sensitization on the expression of stereotypy in adult deer mice and the developmental trajectory in younger deer mice.

Lastly, the fifth aim was to examine the effect of chronic administration of adenosine A_{2A} and A_1 receptor agonists during the early post-weaning period on the development of stereotypy. The finding from Aim#1 led us to hypothesize that the pharmacological manipulation during the period when animals are most sensitive to environmental stimulation may prevent the development of high rates of stereotypy and have long-term effects after cessation of drug administration. When administered with L-DOPA, chronic adenosine A_{2A} receptor agonist administration has been shown to

reduce the development of L-DOPA-induced dyskinesia in a animal model of Parkinson's disease (Agnati et al., 2004). Although the underlying neurobiological process resulting in the development of spontaneous stereotypy in deer mice is likely different from that in L-DOPA-induced dyskinesia, the same class of drug to target striatopallidal neurons may exert beneficial effects in both cases. The findings from this aim are particularly informative and significantly expand our future research projects to seek potential early pharmacotheraphy for the development of restricted, repetitive behaviors in neurodevelopmental disorders.

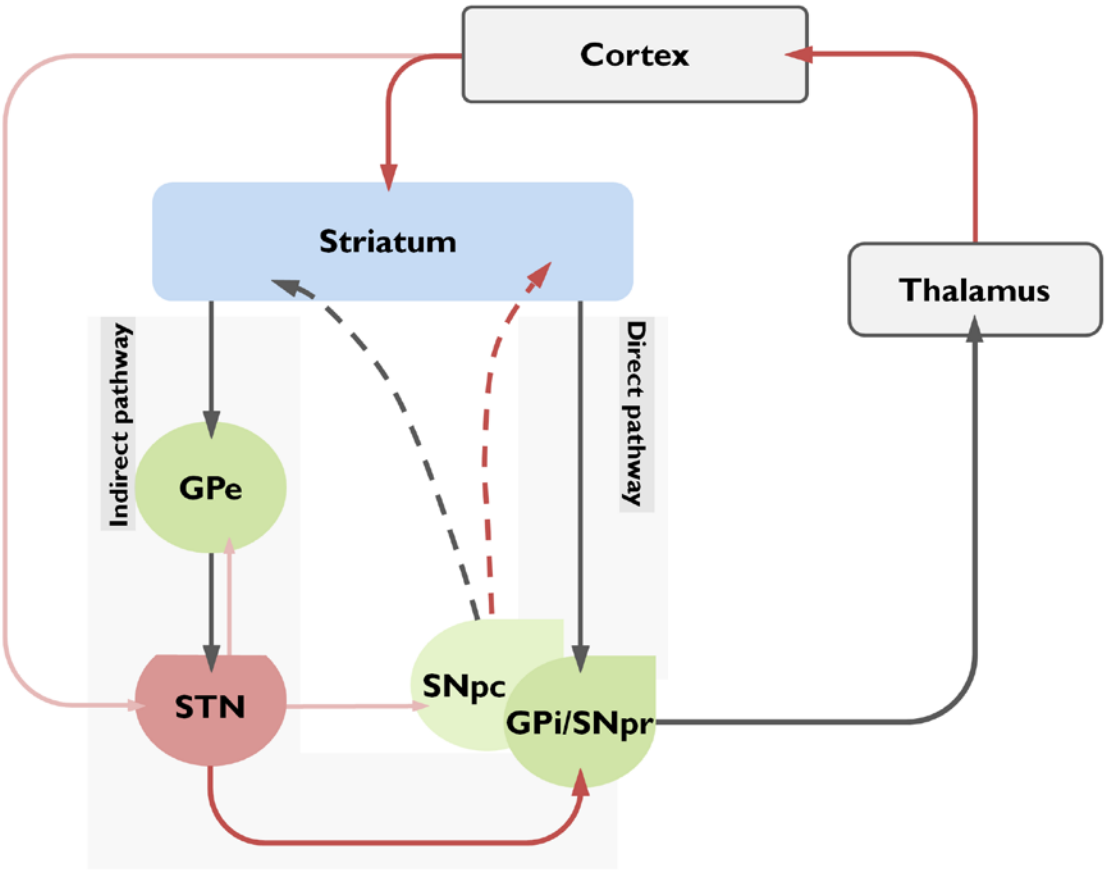


Figure 1-1. Schematic of cortico-basal ganglia circuitry. The black solid lines represent inhibitory GABAergic inputs; the red solid lines represent excitatory glutamatergic inputs; dotted lines represent dopaminergic inputs. Dopamine binds to D1 and D2 receptors which activate (dotted red) and inhibit (dotted black) medium spiny neurons respectively.

CHAPTER 2
INDIRECT BASAL GANGLIA PATHWAY MEDIATION OF REPETITIVE BEHAVIOR: ATTENUATION BY ADENOSINE RECEPTOR AGONISTS

Introduction

Restricted, repetitive behaviors are typically characterized as inflexible, persistent, and apparently functionless. This categorization captures a wide range of behaviors from sensorimotor (e.g., motor stereotypy, repetitive manipulation of objects, compulsions) to more cognitively driven behaviors (e.g., insistence on sameness, restricted interests) (Cuccaro et al., 2003; Szatmari et al., 2006). Restricted, repetitive behaviors constitute one of three diagnostic domains of autism spectrum disorder (Lewis and Bodfish, 1998 for review) and are common features of related neurodevelopmental disorders (e.g., Rett's syndrome, intellectual and developmental disability) as well as several psychiatric disorders (e.g., obsessive-compulsive disorder (OCD), trichotillomania) and neurological diseases (e.g., Tourette syndrome, frontotemporal dementia). In addition, motor stereotypies have been described in adults and children without diagnosed neurodevelopmental, psychiatric, or neurological disorders (Berkson et al., 1999; Berkson et al., 2001; Mahone et al., 2004; Singer, 2009) and are ubiquitous in normative development (Thelen, 1979; Evans et al., 1997).

Despite their clinical importance, the specific neurobiological mechanisms associated with the abnormal expression of these behaviors are largely unidentified. Several neuroimaging studies have identified structural and functional basal ganglia differences linked to the expression of repetitive behaviors (O'Sullivan et al., 1997; Peterson et al., 1998; Sears et al., 1999; Hollander et al., 2005). Experimental manipulations known to induce stereotypies in animals have provided more direct

evidence for the importance of cortico-basal ganglia circuitry in mediating these behaviors (e.g., Welch et al., 2007).

Dysregulation of cortico-striato-thalamo-cortical circuitry associated with motor disorders is thought to be due to an imbalance between the direct and indirect pathways comprising this circuitry (Graybiel, 2000). For example, injection of a GABA antagonist to the external aspect of the globus pallidus (GPe) induced stereotypy in non-human primates, which was attenuated by deep brain stimulation (DBS) of the subthalamic nucleus (STN) (Baup et al., 2008). Similarly inactivation of STN exacerbated the compulsive lever-pressing observed in rats in the signal attenuation model of OCD (Winter et al., 2008c). These results point to a potentially important role for the indirect pathway function in the expression of repetitive behavior of unknown etiology.

Our laboratory has employed deer mice (*Peromyscus maniculatus*) which develop high levels of motor stereotypies (repetitive jumping and/or backward somersaulting) as a consequence of being reared in a standard laboratory environment. These behaviors, which do not require social isolation, specific cues or contexts, pharmacological agents, or specific CNS insult for induction, occur at a high rate, persist across much of the life of the animal and appear relatively early in development. Housing these mice in more complex environments especially early in development attenuates the development and expression of these behaviors (Powell et al., 1999; Hadley et al., 2006). The repetitive hindlimb jumping and backward somersaulting exhibited by deer mice correspond to the repetitive sensorimotor behaviors frequently observed in neurodevelopmental disorders. We have also shown that highly stereotypic deer mice have deficits in a reversal learning task, mirroring inflexibility and resistance to change (Tanimura et al., 2008).

These features plus considerable heterogeneity in individual levels of expression, modulation by early experience, and mediation by cortical-basal ganglia circuitry make deer mice a useful model of restricted, repetitive behavior in neurodevelopmental disorders.

We have previously shown that alterations of cortico-basal ganglia circuitry are linked to the expression of spontaneous repetitive behaviors in deer mice (Turner et al., 2002; Presti et al., 2004). In particular, we found a significant inverse correlation between striatal enkephalin content and stereotypy. Conversely, there was no association between dynorphin and repetitive behavior (Presti and Lewis, 2005). This finding lead to the hypothesis that the expression of spontaneous stereotypy is a result of imbalanced activity of striatal pathways driven by reduced activity of the indirect pathway.

In the present study, we conducted two sets of experiments to assess the function of the indirect pathway in the expression of stereotypy. First, we assessed the neuronal metabolic capacity of STN as an index of indirect pathway activity using cytochrome oxidase (CO) histochemistry in adult deer mice (Experiment 1a). We repeated the same analysis in animals which were either housed under standard laboratory or environmentally enriched conditions (Experiment 1b). CO activity reflects oxidative metabolic capacity of neurons and has been shown to be directly related to neuronal functional activity and has been used to identify neuronal pathways activated by experience (Sakata et al., 2005). Moreover, unlike 2-DG or c-Fos, CO histochemistry reflects sustained alterations in neuronal activity. In addition, Hevner and Wong-Riley

(1989) have shown that the optical density of histochemically labeled brain sections closely correlates with the amount of CO in CNS tissue.

Second, we pharmacologically manipulated striatopallidal neuronal activity via adenosine A_{2A} receptors which are highly enriched in the striatum and selectively expressed in these neurons (Jarvis and Williams, 1989; Fink et al., 1992; Schiffmann et al., 2007). Karcz-Kubicha et al. (2003; 2006) reported that administration of an A_{2A} receptor agonist alone failed to induce c-Fos in the striatum, whereas co-administration of the same A_{2A} receptor agonist plus an A_1 receptor agonist resulted in significant increase in striatal c-Fos expression as well as enkephalin expression. Importantly, this increased expression was observed in striatopallidal but not striatonigral neurons. Thus, we assessed the effect of an A_{2A} receptor agonist alone (Experiment 2a) as well as the combination of an A_{2A} receptor agonist plus an A_1 receptor agonist (Experiment 2b, c, and d) on the attenuation of the expression of stereotypy.

General Methods

Subjects

All deer mice (Peromyscus maniculatus) were obtained from the breeding colony maintained in our laboratory, and kept on a 16:8-h light/dark cycle with lights off at 10:00 AM. Mice were weaned at 21 days of age. Rodent chow and water were available *ad libitum*. The room was maintained at 20-25^{o}C and 50-70% humidity. When housed in standard laboratory cages, deer mice exhibit stereotyped behaviors in the form of hindlimb vertical jumping or backward somersaulting with considerable between animal variability. All procedures were performed in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Florida Institutional Animal Care and Use Committee.

Stereotypy Assessment

Rates of spontaneous stereotypy were assessed using a modified automated photocell detection apparatus (Columbus Instruments). The session consisted of the eight hours of the dark cycle. Mice were individually placed in testing cages (22 x 28 x 25 cm) made of Plexiglas and habituated for at least one hour prior to the beginning of the dark cycle. Food and water were provided. Stereotypy counts, which equated to the number of vertical jumps or backward somersaults a deer mouse performed during that 8 hours, were automatically scored for each mouse. Photobeams were positioned (13.5 cm above the floor) to be interrupted by the vertical motion of jumping and somersaulting but not by rearing. All sessions were digitally video-recorded for identification of behavioral topographies and accuracy of the automated counters. Each animal received a stereotypy score that represented the average stereotypy frequency per hour.

Data Analysis

Differences in CO activity between high- and low-stereotypy mice and between enriched and standard caged mice were assessed by t-test with CO differences in STN being the primary comparison of interest. The association between the frequency of spontaneous stereotypies and the CO activity were analyzed by Pearson correlation (Experiment 1). Behavioral responses to pharmacological manipulations were analyzed by ANOVA and t-test (Experiment 2). All tests were two-tailed and effects were considered significant when $p<0.05$.

Specific Methods and Results

Experiment 1

Experiment 1a: Neuronal Activation of STN in High- and Low-Stereotypy Mice

Methods

Twenty-one adult mice (>8 weeks post-weaning; 15 males and 6 females) exhibiting repetitive vertical jumping were used. All mice were group-caged (5-6 mice per cage) from weaning in standard rodent cages (48 x 27 x 15 cm). Each mouse was assessed for stereotypy as described previously and separated into two groups (high and low stereotypy) based on a median split of stereotypy scores. Following behavioral assessment, mice were killed and their brains were quickly removed and frozen by immersing them in cold 2-methylbutane. They were stored at -80°C until cryostat sectioning. The sections were cut sagittally at 20µm at -20°C starting approximately 2.7 mm lateral to the midline and collected every 100µm for both hemispheres. Sections were mounted on pretreated slides (Superfrost plus, Fisher), and stored in the -80°C freezer until assayed.

The CO spectrophotometric analysis and staining assay was carried out according to the Gonzalez-Lima protocol (1998). Standards were cut at five thicknesses (20, 30, 40, 50, and 60µm) and assayed with other brain sections. The CO staining was quantified by densitometric analysis using ImagePro (Media Cybernetics), and standards were used to convert optical density values into enzymatic activity values (µmol/min/g). Multiple optical density readings were made per animal for STN as well as for other areas comprising cortico-basal ganglia circuitry (motor cortex, striatum, substantia nigra pars reticulata (SNpr), substantia nigra pars compacta (SNpc)) and negative controls (hippocampus, somatosensory cortex). Dorsal and ventral aspects of

the striatum were defined by horizontal bisection into approximately equivalent halves, whereas medial versus lateral aspects were defined by approximately <2.0mm or >2.0mm from the midline respectively. Under our experimental conditions, the staining intensity was highly correlated to the thickness of the standard sections (r=0.95).

Results

Baseline stereotypy scores varied from 137.9 to 3992.8 counts per hour with a median score of 652.0. The average score was 394.1 for low-stereotypy animals (n=11) and 1393.0 for high-stereotypy animals (n=10). CO enzymatic activity within selected areas is summarized in Table 2-1. Significant differences were found in STN (t(19)=2.74, p=0.01) where low-stereotypy mice showed higher CO activity compared to high-stereotypy mice. Similar differences were found in the ventromedial striatum (t(19)=2.21, p=0.04) and SNpr (t(19)=2.25, p=0.04). In addition, individual levels of stereotypy were negatively correlated with CO activities in STN (r=-0.50, p=0.02) (Figure 2-1) and SNpr (r=-0.53, p=0.01). When the highest stereotypy score was excluded based on its value being more than 2 SDs greater than the mean, the correlations with CO were no longer significant (STN: r=-0.41, p=0.07; SNpr: r=-0.41, p=0.07). In addition, without this outlying score, the differences in the ventromedial striatum and SNpr between high and low stereotypy mice were no longer statistically significant (t(18)=1.96, p=0.07; t(18)=1.91, p=0.07 respectively).

Experiment 1b: Effects of Enriched Environment on Stereotypy and Neuronal Activation in STN

Methods

Twenty-three mice (14 males and 9 females) were group-caged (5-6 mice per cage) in standard rodent cages (48 x 27 x 15 cm) at weaning for 30 days, and then

tested for baseline levels of stereotypy as described in the previous section. After testing, animals were randomly assigned to one of two housing conditions: standard rodent cages (SC) (n=12) or environmental enrichment (EE) (n=11) (Figure 2-2). The EE housing consisted of large dog kennels (122 x 81 x 89 cm) with two extra levels of floors constructed of galvanized wire mesh and connected by ramps of the same material. Bedding, a running wheel, shelters, and various other objects were placed in each kennel. In addition to *ad libitum* food and water, one oz. of Cockatiel vita seed was scattered throughout the kennel three times each week to encourage foraging behavior. A running wheel remained undisturbed in the kennel, but other objects were removed and replaced with clean novel objects on a weekly basis. The SC housing took place in the same rodent cages described above except that each cage contained 2-3 mice. They received *ad libitum* food and water as well as Cockatiel vita seed placed at one corner on the same schedule as the EE housing.

Animals were kept in their respective housing conditions for 30 days, during which time handling was kept to a minimum. Each mouse was tested again for stereotypy at the end of the EE or SC housing. Their brains were collected for the CO assessment as described previously.

Results

Mean baseline stereotypy scores assessed 30 days post-weaning were virtually identical for the animals assigned to SC versus EE ($t(21)=0.05$, $p=0.96$; see Figure 2-3). The subsequent 30 days of environmental enrichment significantly attenuated the development of stereotypy, whereas rates of stereotypy continued to increase in SC animals ($t(21)=-3.68$, $p<0.01$). CO enzymatic activity within selected brain regions is summarized in Table 2-2. Significant differences were found in STN ($t(21)=3.57$,

$p<0.01$) as well as SNpc ($t(21)=4.16$, $p<0.01$) and SNpr ($t(21)=3.74$, $p<0.01$). In these nuclei, EE mice had higher CO activities compared to SC mice.

Experiment 2:

Experiment 2a: Effects of CGS21680 on Stereotyped Behavior

Methods

Thirty-nine male mice (>6 weeks post weaning) were randomly assigned to one of four groups and were administered an acute subcutaneous injection of vehicle (n=13), 0.02mg/kg (n=9), 0.03mg/kg (n=9), or 0.05mg/kg (n=8) of 2-p-(2-carboxyethyl)phenethylamino-50-N-ethylcarboxamidoadenosine (CGS21680) (Sigma) dissolved in 5% dimethylsulfoxide (DSMO) in a volume of 10ml/kg of body weight. Drug injections were given two hours before the end of the dark cycle, a time period during which deer mice show higher rates of spontaneous stereotypy (unpublished data). The behavioral response to the drug was assessed starting immediately after drug administration.

Results

Administration of CGS21680 did not significantly alter the frequency of stereotyped behaviors for the 1hr post-injection period at doses up to 0.05mg/kg ($F(3,35)=1.45$, $p=0.25$). A preliminary study with a small number of animals indicated that CGS21680 at the 0.1mg/kg level had a non-selective effect on stereotypy as this dose suppressed motor activity in general. Similar effects were also seen during the 30min post-injection period.

Experiment 2b: Effects of Co-Administration of CGS21680 and CPA on Stereotyped Behavior

Methods

Eighteen male mice (6 to 8 weeks post-weaning) were administered acute subcutaneous injections of a combination of CGS21680 and the selective adenosine A_1 receptor agonist N6-cyclopentyladenosine (CPA) (Sigma) dissolved in 5% DSMO. Each mouse received each drug combination (0.03/0.03; 0.05/0.05; and 0.1/0.1mg/kg) and vehicle only, serving as its own control. Each injection was administered one-week apart using successively higher doses and randomizing the order of drug and vehicle. For Experiment 2 (b, c, and d), we employed new testing cages (22 x 28 x 31 cm) made of Plexiglas to replace the old testing cages (22 x 28 x 25 cm).

Results

For all the drug combinations tested (n=18), there was no difference in stereotypy between vehicle and drug groups for the 1hr pre-injection period at 0.03/0.03mg/kg ($t(17)=3.28$, $p<0.01$), 0.05/0.05mg/kg ($t(17)=-0.60$, $p=0.56$), and 0.1/0.1mg/kg ($t(17)=-0.32$, $p=0.75$). For the 1hr post-injection period, drug groups showed significantly lower rates of stereotypy at 0.03/0.03mg/kg ($t(17)=3.97$, $p<0.01$), 0.05/0.05mg/kg ($t(17)=5.48$, $p<0.001$), and 0.1/0.1mg/kg ($t(17)=10.09$, $p<0.001$) when compared to their respective vehicle conditions (Figure 2-4). Similar drug effects were also seen during the 30min post-injection period.

To characterize this result further, we used the total pre-injection stereotypy score to categorize mice into high- and low-stereotypy groups based on a median split (n=9 each), and we assessed whether rates of baseline stereotypy predicted the magnitude of the drug response. At the 30min period, there was no difference from pre-injection

baseline in high-stereotypy mice ($t(8)=0.97$, $p=0.36$) whereas the reduction from baseline for low-stereotypy mice approached significance ($t(8)=2.10$, $p=0.07$). This was also seen for the 1hr period in high-stereotypy mice ($t(8)=1.00$, $p=0.35$) and in low-stereotypy mice ($t(8)=1.91$, $p=0.09$). The percentage change from baseline also showed that 0.03/0.03mg/kg of CGS21680/CPA was more effective in low-stereotypy mice (63.9% versus 3.5% for vehicle) than in high-stereotypy mice (12.4% reduction versus 0.6% for vehicle). No differential response was seen in high versus low baseline stereotypy mice at 0.05/0.05mg/kg and 0.1/0.1mg/kg.

Experiment 2c: Effects of CGS21680 and CPA in Drug-Naïve Animals

Methods

An independent group of 10 male mice (6 to 8 weeks post-weaning) was used to provide a partial replication of the results described in the prior experiment. In this case, we assessed the effects of 0.05/0.05mg/kg versus vehicle in drug-naïve animals using a cross-over design.

Results

Consistent with what we found previously, there was no difference in the frequency of stereotypy between the drug and vehicle conditions for the 1hr pre-injection periods ($t(9)=0.49$, $p=0.64$). Drug-treated animals, however, exhibited significantly less stereotypy for the 1hr post-injection periods compared to vehicle ($t(9)=3.40$, $p=0.01$) (Figure 2-5). Thus the effect of the CGS21680 and CPA dose combination was replicated in drug-naïve mice. The similar drug effects were also seen during the 30min post-injection period. To determine that the effects of CGS21680/CPA were not solely due to CPA alone, we tested the effect of CPA at 0.05mg/kg using an independent group of animals (n=12) in a cross-over design. Administration of CPA

alone at 0.05mg/kg induced non-selective motor suppression, rendering about a third of animals akinetic.

Experiment 2d: Effects of CGS21680 and CPA on Locomotor Behavior

Methods

To assess how selective the effect of the drug combination was on stereotyped behaviors, the animals from Experiment 2c received an additional administration of vehicle and the CGS21680 and CPA combination (0.05/0.05 and 0.1/0.1mg/kg) in a cross-over design. Their post-injection locomotor activity was tracked for 1 hr using Ethovision (Noldus). One control animal for the 0.05/0.05mg/kg group was excluded from the analysis due to missing data (n=9).

Results

There was a significant difference in distance traveled (cm) for the duration of the 30min post-injection period ($t(8)=2.70$, $p=0.03$) but not for the 1hr post-injection period ($t(8)=1.88$, $p=0.10$) between vehicle and 0.05/0.05mg/kg groups (Table 2-3A). There was significant reduction in distance traveled for the 30min post-injection period ($t(9)= 4.85$, $p=0.001$) and the 1hr post-injection period between vehicle and 0.1/0.1mg/kg groups ($t(9)= 4.58$, $p=0.001$) (Table 2-3B). There was no difference between 0.05/0.05mg/kg and 0.1/0.1mg/kg groups, however, at either time point. Locomotor activity was significantly correlated with the frequency of stereotypy for the 1hr post-injection period in vehicle-treated mice ($r=0.89$, $p<0.001$) but not in drug-treated mice ($r=0.51$, $p=0.13$) at 0.05/0.05mg/kg.

Discussion

The present experiments were designed to test the hypothesis that the expression of spontaneous stereotypy in deer mice is linked to decreased activity of the indirect

basal ganglia pathway. This hypothesis was based on our previous finding that level of stereotypy in deer mice was negatively correlated with the striatal expression of the neuropeptide enkephalin, whereas no relationship was found between stereotypy and the expression of dynorphin (Presti and Lewis, 2005). In support of this hypothesis, CO activity in STN was significantly lower in high-stereotypy mice compared to low-stereotypy mice (Experiment 1a) and in SC mice compared to EE mice (Experiment 1b).

Contrary to our expectation, no difference in CO activity between high- and low-stereotypy mice was found in the dorsolateral striatum, which is typically considered the sensorimotor area of the striatum. The medial aspect of the dorsal striatum, however, showed lower CO activity in high- compared to low-stereotypy mice (Experiment 1a), although a similar difference in the dorsomedial striatum was not found between SC and EE mice (Experiment 1b). The dorsomedial striatum has been implicated in the mediation of behavioral flexibility (Ragozzino et al., 2009). We have previously shown that high rates of stereotyped jumping in deer mice were associated with perseverative behavior in a reversal learning task (Tanimura et al., 2008), further supporting alterations of the dorsomedial striatum in stereotypic deer mice.

In addition, we also found significantly higher CO activity in SNpc and SNpr in EE versus SC mice (Experiment 1b). For SNpc, this effect is likely due to the monosynaptic excitatory STN-SNpc projection in rodents and non-human primates widely reported in the literature (Hammond et al., 1978; Kita and Kitai, 1987; Chergui et al., 1994; Shimo and Wichmann, 2009). This difference, however, was not found between high- and low-stereotypy mice (Experiment 1a). For SNpr, increased CO staining was associated with

lower stereotypy in both Experiments 1a and 1b. These differences are consistent with higher glutamatergic activation from STN.

If high rates of spontaneous stereotypy are associated with decreases in indirect pathway activation, then stimulation of A_{2A} receptors, which are expressed on striatopallidal neurons and activate $G_{s/olf}$ proteins upon stimulation, should attenuate stereotypy. When administered alone, the selective adenosine A_{2A} receptor agonist CGS21680 failed to reduce stereotypy up to 0.05mg/kg (Experiment 2a). In a preliminary study, 0.1mg/kg of CGS21680 non-selectively reduced motor activity, rendering some mice akinetic. The addition of CPA to CGS21680, however, selectively attenuated stereotypy in a dose-dependent manner without adverse suppression of general motor activity (Experiment 2b, c, and d) even at the 0.1 mg/kg dose. Although the lowest dose of CGS21680/CPA significantly attenuated stereotypy compared to vehicle controls, a subsequent analysis suggested that it was not effective in mice exhibiting higher rates of baseline stereotypy. Higher doses of CGS21680/CPA were required to attenuate stereotypy in this group. These pharmacological results indicate that higher doses of the drug combination may be required to drive the activity of the indirect pathway to levels associated with low stereotypy.

The relative efficacy of the combined stimulation of A_{2A} and A_1 receptors compared to A_{2A} alone may be explained by the results reported by Karcz-Kubicha et al. (2003; 2006). In this work, administration of an A_1 or A_{2A} receptor agonist alone did not induce striatal c-Fos expression. Stimulation of both receptor subtypes, however, did induce striatal c-Fos expression and in a selective fashion with activation seen in striatopallidal, but not striatonigral, neurons. This combined treatment of A_1 and A_{2A} receptor agonists

also increased striatal enkephalin expression. It should be noted, however, that these effects were seen at higher doses (0.5 mg/kg CGS21680 and 0.3 mg/kg CPA) than were used in the present experiments. Similarly, administration of an A_{2A} receptor antagonist alone produced either no effect or only a slight decrease in c-Fos and *preproenkepahlin* expression in the striatum (Pinna et al., 1997; Svenningsson et al., 1998; Aoyama et al., 2000; Aoyama et al., 2002; Karcz-Kubicha et al., 2003; Lundblad et al., 2003; Wardas et al., 2003). This is consistent with our unpublished data showing that administration of the A_{2A} receptor antagonist SCH58261 failed to induce or exacerbate repetitive behavior.

The mechanisms to account for these effects likely involve functional antagonistic interactions of A_{2A} and D_2 receptors on adenynyl cyclase that regulate the cAMP-PKA signaling pathway in striatopallidal neurons. Tonic inhibition of D_2 receptors attenuates the ability of an A_{2A} receptor agonist to stimulate this signaling pathway and its downstream effects on c-Fos and *preproenkephalin*. Moreover, A_{2A} receptor agonist administration has been reported to increase striatal dopamine release, possibly indirectly through A_{2A} receptors on presynaptic glutamate terminals (Golebioqska and Zyewska, 1997; Karcz-Kubicha et al., 2003; Quarta et al., 2004). Conversely, A_1 receptor agonist administration has been reported to decrease striatal dopamine release (Okada et al., 1996; Quarta et al., 2004) through A_1 receptors on presynaptic dopaminergic terminals where upon stimulation, release of dopamine is directly inhibited via activation of $G_{i/o}$ protein (Karcz-Kubicha et al., 2003; Yabuuchi et al., 2006; Borycz et al., 2007). Stimulation of presynaptic A_1 receptors by CPA allows A_{2A} receptors on striatopallidal neurons to overcome tonic inhibition by D_2 receptors to activate the cAMP-

PKA pathway. Moreover, the effect of the A_1 and A_{2A} receptor heteromeric complex on glutamate release from corticostriatal neurons is dependent on the local concentration of adenosine (Ciruela et al., 2006), and so the drug effects reported here may also be due, at least in part, to alterations in glutamate release.

The biochemical and pharmacological findings presented here provide additional, important support for the role of the indirect pathway in mediating repetitive behavior in our model. These findings are consistent with both clinical and animal studies that have found a link between repetitive behavior and the indirect basal ganglia pathway. For example, the uncontrolled motor movements characteristic of Huntington's disease are attributed to the differential degeneration of striatopallidal neurons (Deng et al., 2004; Starr et al., 2008). Deep brain stimulation (DBS) applied to STN reduced the severity of symptoms in previously treatment refractory OCD patients (Burdick et al., 2009). Similarly, DBS of STN and GPe has been found to be effective in ameliorating the L-DOPA induced tardive dyskinesia observed in Parkinson's disease (e.g., Kleiner-Fisman et al., 2006). In animal models, Grabli et al. (2004) have reported that stereotyped behavior (e.g., licking and biting of fingers) was induced in monkeys when the GABA antagonist bicuculline was microinjected into the limbic aspect of GPe, which was reduced by DBS applied to STN without affecting a control motor task (Baup et al., 2008). Winter et al. (2008c) have shown that rats that sustained ibotenic acid lesions to STN exhibited an increase in compulsive lever pressing in the signal attenuation model of OCD. This same research group has also shown that bilateral high frequency stimulation of STN as well as pharmacological inactivation of STN reduced quinpirole-

induced compulsive checking in rats (Winter et al., 2008a; Winter et al., 2008b; Klavir et al., 2009).

In addition to identifying pathophysiological changes associated with repetitive behavior, our pharmacological findings also point to novel targets for development of drug therapies to treat repetitive behavior in clinical disorders such as autism. Currently, the two drug classes used to treat such behaviors in neurodevelopmental disorders include atypical antipsychotics and selective serotonin re-uptake inhibitors (SSRIs). A recent multi-site study of the SSRI citalopram provided no evidence for its efficacy in treating repetitive behavior in autism (King et al., 2009). There is limited evidence for the utility of atypical antipsychotics in treating repetitive behavior. Risperidone has recently been FDA approved for use in autism to treat irritability with no approval sought for repetitive behavior. Thus, there is a pressing need for the development of effective drug treatments, based on an understanding of the specific pathophysiological mechanisms of repetitive behaviors.

The present finding suggests that a combination of an A_{2A} receptor agonist and an A_1 receptor agonist to drive indirect pathway activity reduced repetitive behaviors selectively, whereas either drug alone induced no or non-selective effect on behaviors respectively. Although cardiovascular relaxation induced by adenosine receptor agonists could be a possible drawback (e.g., Tabrizchi and Bedi, 2001; Ponnoth et al., 2009), these findings suggest drug development efforts that may be potentially useful in treating repetitive behaviors in neurodevelopmental disorders.

Table 2-1. CO activity (µmol/min/g) of STN and the other brain areas in high- and low-stereotypy adult deer mice. Values expressed are group means with SEM in parentheses.

	Low Stereotypy	High Stereotypy	*P*-value
Subthalamic Nucleus	64.60 (1.42)	59.01 (1.46)	0.01
Motor Cortex	48.01 (0.88)	47.84 (1.12)	0.89
Somatosensory Cortex	49.64 (0.74)	49.67 (1.25)	0.98
Hippocampus	44.12 (1.12)	42.46 (1.79)	0.80
Caudate/Putamen			
Dorsolateral Striatum	48.48 (0.81)	47.87 (1.42)	0.71
Dorsomedial Striatum	49.30 (1.02)	46.76 (1.12)	0.10
Ventrolateral Striatum	49.43 (0.74)	48.25 (1.08)	0.37
Ventromedial Striatum	50.28 (1.15)	46.96 (0.98)	0.04
Nucleus Accumbens	50.85 (1.12)	48.08 (1.02)	0.09
Substantia Nigra pars Compacta	45.10 (1.66)	42.22 (0.91)	0.16
Substantia Nigra pars Reticulata	50.72 (1.66)	46.25 (1.05)	0.04

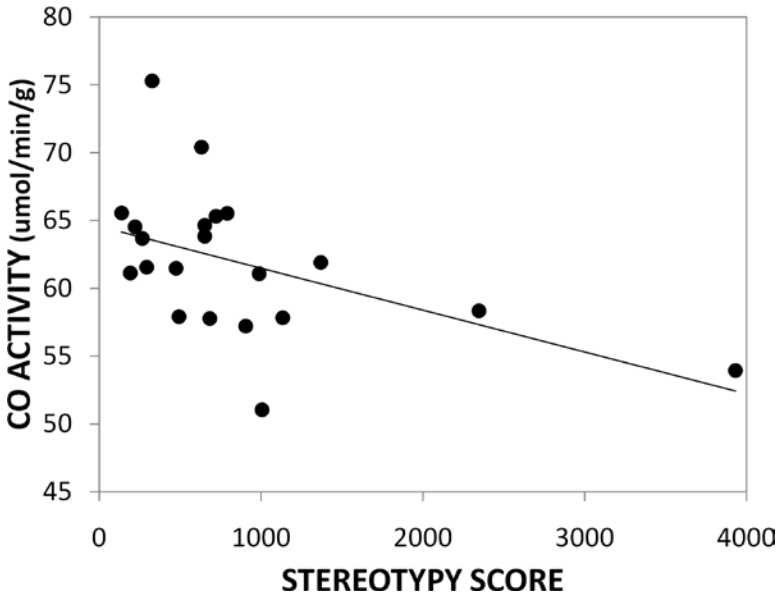


Figure 2-1. Correlation between the frequency of stereotypy and CO activity in STN of adult deer mice.

Table 2-2. CO activity (µmol/min/g) of STN and the other brain areas in deer mice housed in the SC and EE conditions. Values expressed are group means with SEM in parentheses.

	SC	EE	*P*-value
Subthalamic Nucleus	64.23 (0.71)	68.97 (1.15)	<0.01
Motor Cortex	51.36 (0.58)	50.62 (0.64)	0.40
Somatosensory Cortex	51.36 (0.68)	51.19 (0.68)	0.86
Hippocampus	42.73 (1.12)	44.83 (0.81)	0.15
Caudate/Putamen			
Dorsolateral Striatum	52.31 (0.91)	52.92 (0.85)	0.61
Dorsomedial Striatum	51.23 (1.05)	53.39 (1.05)	0.16
Ventrolateral Striatum	52.65 (0.81)	52.78 (0.68)	0.89
Ventromedial Striatum	50.01 (1.12)	52.21 (1.02)	0.16
Nucleus Accumbens	50.62 (1.29)	52.28 (0.91)	0.31
Substantia Nigra pars Compacta	43.10 (0.88)	47.30 (0.44)	<0.01
Substantia Nigra pars Reticulata	47.27 (0.78)	51.19 (0.71)	<0.01

A

B

Figure 2-2. Housing conditions. A) environmentally enriched (EE) condition with a larger space, a running wheel, shelters, habitrails, and novel objects; B) standard caged (SC) condition.

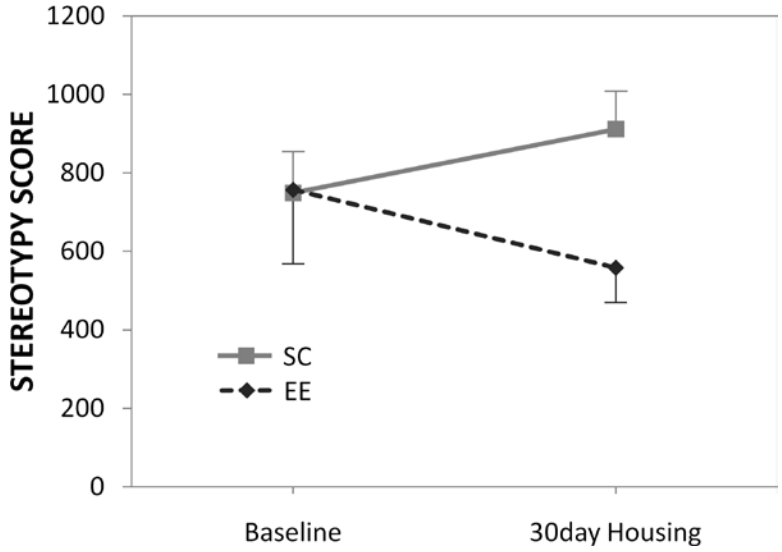


Figure 2-3. The effect of 30-day EE housing on the stereotypy scores (mean ± SEM).

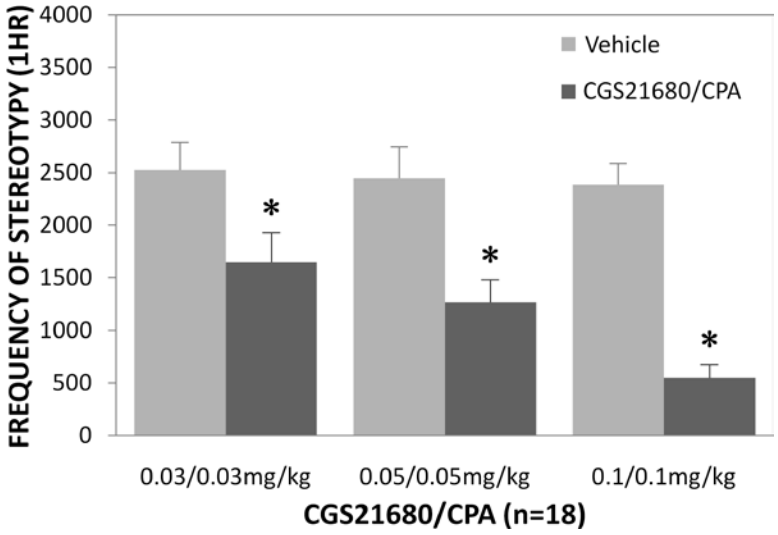


Figure 2-4. The effects of CGS21680/CPA on stereotypy. The frequency of stereotypy for the 1hr post-injection period (mean + SEM). * represents statistical significance at $p<0.05$ as compared to vehicle group.

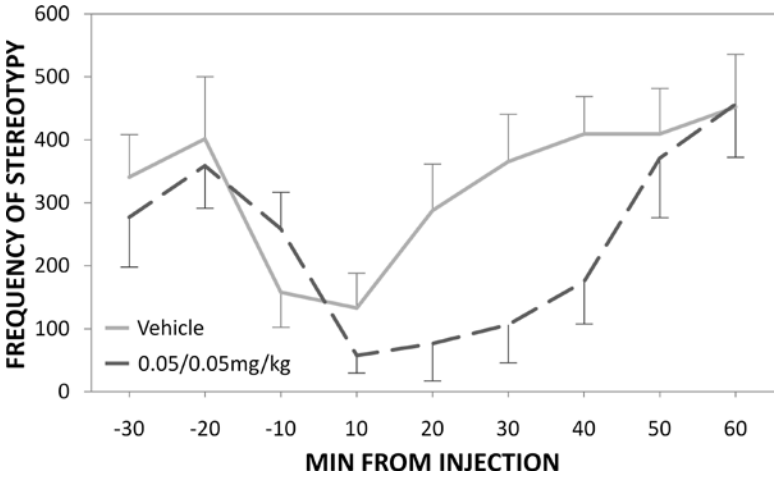


Figure 2-5. The time-course showing the efficacy of CGS21680/CPA in drug-naïve animals (0.05/0.05mg/kg). Zero at the time of drug or vehicle injection (mean ± SEM).

Table 2-3. Distance traveled immediately after vehicle or CGS21680/CPA administration (cm). A) 0.05/0.05mg/kg and B) 0.1/0.1mg/kg. Values expressed are group means with SEM in parentheses. * represents statistical significance at $p<0.01$ as compared to vehicle control group.

A

	Vehicle	0.05/0.05mg/kg CGS21680/CPA
30min post-injection	5801.8 (1026.8)	3479.7 (685.6)*
1hr post-injection	11554.7 (1730.3)	8622.6 (1594.6)

B

	Vehicle	0.1/0.1mg/kg CGS21680/CPA
30min post-injection	6484.8 (722.9)	3520.4 (486.3)*
1hr post-injection	14227.6 (1612.5)	6649.6 (927.4)*

CHAPTER 3
DEVELOPMENTAL TRAJECTORIES AND TEMPORAL ORGANIZATION OF REPETITIVE BEHAVIORS

Introduction

Restricted Repetition of a behavior or set of behaviors that appears to serve no obvious purpose has long been considered an important dimension of psychopathology. These behaviors variously termed stereotypies, compulsions, and rituals are seen in a variety of neuropsychiatric and neurodevelopmental disorders (Frith and Done, 1990; Ridley, 1994; Lewis and Bodfish, 1998). For example, in autism, for which repetitive behavior is diagnostic (American Psychiatric Association, 2000), affected individuals exhibit stereotyped motor movements, compulsions, rituals and routines, an insistence on sameness, and circumscribed interests (Lewis and Bodfish, 1998). These same repetitive behaviors are also a common feature of a number of neurodevelopmental disorders some of which have an identifiable genetic defect (e.g., Rett, Fragile X, Prader-Willi syndromes) (Symons et al., 1999; Carter et al., 2009; Moss et al., 2009). Repetitive behaviors are also part of the clinical phenotype of obsessive-compulsive disorder, Tourette syndrome and frontotemporal and Alzheimer's dementias (Muller et al., 1997; Cath et al., 2001; Mateen and Josephs, 2009).

Various forms of aberrant repetitive behavior also occur in a significant percentage of individuals without cognitive impairment or neurological or psychiatric disorder (Castellanos et al., 1996; Rafaeli-Mor et al., 1999; Singer, 2009). At the same time, there is ample documentation that repetitive motor behavior, compulsions, and rituals are features of normative development as well (e.g., Thelen, 1980; Evans et al., 1997). For example, throughout early childhood, children engage in a number of stereotyped

motor acts (e.g., swaying, rocking, flapping) and later display a variety of compulsive and ritualistic behaviors (e.g., insistence on certain clothing or foods, bedtime rituals).

Despite the importance of repetitive behavior in a variety of clinical disorders and its ubiquity in typical development, little is known about how such behaviors develop. As Symons et al. (2005) have pointed out, very few investigations have addressed the developmental course of repetitive behaviors in children at risk for disorders such as autism. Esbensen et al. (2009) examined a wide range of ages (2-62 years of age) in individuals with autism using a modified cross-sectional design. They reported that repetitive behaviors decreased across age regardless of subtype of repetitive behavior. In a recent study of children with autism and developmental delay, Richler et al. (2010) assessed the development of restricted repetitive behaviors longitudinally at 2, 3, 5, and 9 years of age. They measured the two major subtypes of repetitive behaviors (repetitive sensorimotor behavior and insistence on sameness) and reported decreased sensory-motor repetitive behaviors and increased insistence on sameness behaviors with increasing age. Importantly, using a group based trajectory modeling procedure, they found three sub-groups with qualitatively distinct developmental trajectories. For repetitive sensory-motor behaviors, approximately half the individuals showed a gradual decrease whereas about one-fourth exhibited consistently low levels and one-fourth consistently high levels. For insistence on sameness behaviors, about 70% of individuals showed an increase across ages whereas the other two groups showed persistently low (13%) or moderate (15%) levels across development. Despite these studies, the lack of knowledge on the development of repetitive behavior seriously impairs the ability of clinicians to design efficacious early intervention and prevention

strategies. Similarly, relatively little is known about the developmental trajectories of normative repetitive behavior. A notable exception is the work of Thelen (1979) who showed that specific rhythmical stereotyped movements in infants had a clear onset, peak, and decline coincident with emerging voluntary motor control. In large measure, then, the factors that influence the persistence or decline of repetitive behaviors with age remain unidentified.

In addition to development, the temporal structure of repetitive behavior has received scant attention despite the fact that stereotypy is generally considered to be characterized by temporal regularity rather than variability or complexity. In addition as Newell et al. (2009) have pointed out, the temporal dynamics of repetitive behavior may be more informative and robust than the more typically used mean frequency or duration measures. Early work by Lewis et al. (1981) identified ultradian rhythms in the stereotyped motor behavior of individuals with developmental disabilities. In one of the few additional efforts to characterize the temporal dynamics of repetitive behavior, Peterson and Leckman (1998) analyzed the distribution of tics in Tourette syndrome patients. These investigators showed that tic intervals were not statistically independent and that the tic time series reflected a fractal, deterministic and possibly chaotic process. In a intriguing set of studies, Newell, Bodfish and colleagues have shown increased regularity or reduced dimensionality in postural stability (Bodfish et al., 2001), sitting (Newell and Bodfish, 2007) and spontaneous blinking (Lee et al., 2010) of individuals with neurodevelopmental disorders who engage in stereotyped behavior versus matched controls. Finally, as far as we are aware, there has been no attempt to

address the intriguing question of how changes in temporal dynamics interact with developmental trajectory.

Appropriate animal models could provide a wealth of information about how repetitive behaviors develop. This would seem particularly true for rodents given their abbreviated developmental period. In addition, the ability to extensively monitor laboratory animals provides the opportunity for assessing temporal dynamics and, further, addressing the important question of how temporal structure systematically changes with developmental trajectory.

Stereotyped motor behaviors can be induced in animals following a variety of perturbations including early social deprivation, environmental restriction, exposure to specific pharmacological agents, and specific CNS insults including genetic mutations (see Lewis et al., 2007 for a review). Few attempts have been made in any of these models, however, to investigate development and/or temporal organization. In rodents, cage stereotypies in ICR mice (Würbel and Stauffacher, 1996) and bank voles (Odberg et al., 1987) and stereotypic digging in gerbils (Wiedenmeyer, 1997) have been reported to develop by 30 days of age. No developmental trajectories were provided with these reports, however.

Our laboratory has employed a deer mouse (*Peromyscus maniculatus*) model of aberrant repetitive behavior (e.g., Powell et al., 1999). When housed under standard laboratory conditions, deer mice exhibit excessive repetitive vertical jumping and/or backward somersaulting. These behaviors occur at a high rate, persist across much of the life of the animal and appear relatively early in development, sometimes as early as weaning. These behaviors do not require social isolation, specific cues or contexts,

pharmacological agents, or specific CNS insult for induction. Consistent with stereotypy in deer mice having its origins in environmental restriction, housing deer mice in larger, more complex environments (environmental enrichment) following weaning markedly attenuates the development and expression of these behaviors (Powell et al., 1999; Hadley et al., 2006). An early assessment of the developmental trajectory of stereotypies in deer mice (Powell et al., 1999) indicated that stereotypies increased in frequency and reached adult levels at about two months after weaning at which point the frequency plateaued.

In our previous assessments of adult deer mice, we consistently observe a high degree of variability in the expression of repetitive behavior. In addition, we have consistently observed that a small proportion of deer mice, despite being reared in standard laboratory cages, never develop appreciable levels of stereotypy. Conversely, anecdotal observations suggest that stereotyped behavior is exhibited as early as weaning in some animals. Thus, in the present paper, we have sought to track the development of stereotypy in deer mice and determine whether multiple trajectories best characterize our sample.

Newell (1996) has suggested that key to understanding the emergence of stereotypies is the change in dynamics and the loss of behavioral adaptability. Thus, we also sought to characterize the temporal organization of repetitive behavior in deer mice. We hypothesized that repetitive behavior would become more predictable or regular over development. The interaction between temporal structure and developmental trajectory has not previously been examined and so we have sought to examine the interplay between temporal dynamics and developmental trajectory.

General Methods

Subjects

All deer mice (*Peromyscus maniculatus*) were obtained from the breeding colony maintained in our laboratory, and kept on a 16:8-h light/dark cycle with lights off at 10:00 AM. Rodent chow and water were available *ad libitum*. The room was maintained at 20-25^{o}C and 50-70% humidity. Mice sharing the same weaning date were group-caged (5-6 mice/cage) at weaning (PND21) in standard rodent cages (48 x 27 x 15 cm) and they remained in the same cage group throughout the experiment. All procedures were performed in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Florida Institutional Animal Care and Use Committee.

Stereotypy Assessment

Rates of spontaneous stereotypy (hindlimb vertical jumping or backward somersaulting) were assessed using a modified automated photocell detection apparatus (Columbus Instruments). Photocells were placed such that vertical activity (jumping and somersaulting) resulted in beam breaks (stereotypy counts) whereas rearing did not. Each occurrence of a detected photocell interruption was registered with a real time value using Labview (National Instruments). This allows us to construct a time series of the intervals between individual consecutive stereotyped responses. The session consisted of the eight hours of the dark cycle. Mice were individually placed in testing cages (22 x 28 x 25 cm) made of Plexiglas and habituated for at least one hour prior to the beginning of the dark cycle. Food and water were provided. All sessions were digitally video-recorded for identification of behavioral topographies and accuracy of the automated counters.

Experiment 1: Characterization of the Development of Spontaneous Stereotypy

Methods and Data Analysis

Fifty-two mice (female: n=23; male: n=29) were randomly selected from the colony and tested for their rates of stereotypy as described previously. Starting at one week post-weaning (PND26-28), the frequency of stereotypy for one complete dark cycle was measured weekly for at least eight consecutive weeks.

Group-based trajectory modeling: In order to determine if there were qualitatively different developmental trajectories of repetitive behavior, we subjected the data from Experiment 1 to a group-based trajectory modeling procedure. We chose the Trajectory Procedure (Proc Traj), which is designed to run on the SAS platform (Jones et al., 2001; Jones and Nagin, 2007). Proc Traj is a specialized mixture model that estimates the trajectories of multiple groups within the population. This provides an important advantage over conventional regression or growth curve procedures that model only one mean within the population. Proc Traj is a particularly useful strategy for our purposes where the number and structure of the trajectories are largely unknown. This group-based trajectory modeling allowed for the calculation of the probability of membership in each discrete trajectory for every subject. The number of discrete developmental trajectories that best accounted for individual data was determined by comparing the absolute value of the Bayesian Information Criterion (BIC) between different models, where smaller values indicate a better fit (see Jones et al., 2001). There was considerable between-animal variability as well as week-to-week variability in the frequency of stereotyped responses. Hence, we performed a log transformation of the stereotypy counts; a commonly used procedure to stabilize the variance of data related to growth.

Results

From Figure 3-1, it is apparent that stereotyped activity reached asymptotic levels by around 6 weeks post-weaning. This was also confirmed by a polynomial fitted to the whole data set. When the log (10 based) counts *y* was fit to the time *t* (in weeks) for all the data, the following equation

$$y = -1.95 + 0.78t - 0.063t^2,$$

had the best fit versus any other order polynomials. Based on this model, stereotyped behavior was judged to reach a plateau at t=6.22 weeks, with the mid-point of development being at about t=3.5 weeks.

Using Proc Traj, the developmental trajectories were best classified into three groups based on the Bayesian Information Criterion (BIC) as shown in Figure 3-2. There are two curves for each cluster, one from the estimated mean and one from the smoothed 4th order polynomial. The first trajectory (Traj 1, the bottom two curves) represents a small percentage of subjects (11.2%) and reflects what we have known for some time, namely, a small group of mice that did not develop high levels of repetitive behavior despite being reared in standard cages. The second group, (Traj 2; 41.1% of mice, the middle curves), show a gradual progression from low levels of stereotypy at week 1 post-weaning to high levels by week 6 post-weaning. The third group, (Traj 3; 47.7% of mice), is noteworthy in their already high levels of stereotypy by week 1 post-weaning. Interestingly, despite a different developmental course, the Traj 2 and 3 groups end up at about the same level of repetitive behavior by 8 weeks post-weaning (Figure 3-2).

In this sample of 52 mice, only 9 exhibited backward somersaulting. Of these 9 mice (5 male, 4 female) 5 were grouped in Traj 2 whereas 4 were grouped in Traj 3. Interestingly, for 8 of the 9, somersaulting replaced jumping, typically at approximately 4 weeks post-weaning. Additionally, Traj 2 had an equal number of male and female mice whereas Traj 3 was 44% male. Traj 1 had more males than females but the total number of mice in this trajectory was small.

Experiment 2: Temporal Organization of Spontaneous Stereotypy for Different Trajectory Groups

Methods and Data Analysis

Temporal organization was assessed for all three trajectory groups using all stereotypic responses counted during a single dark cycle at week 1, 3.5 and 6 post-weaning. We choose week 1, (PND26-28), week 6 (PND62-64) and the middle point week 3.5 (PND43-45) as the times to measure stereotyped responding based on the analysis described for Figure 3-1. The distributions of stereotypy counts by hour across the dark cycle at each developmental period are depicted in Figure 3-3.

Based on the trajectory information obtained from Experiment 1, mice unambiguously categorized as belonging to the Traj 1 (n=13), 2 (n=9), or 3 (n=7) groups were used for further analyses of temporal organization of behavior during each session. The frequency of stereotyped responses was used to determine developmental trajectories and trajectory groups in Experiment 1. Frequency is only the first (mean) order of the stereotypy pattern, however. Frequency does not provide any information about the temporal dynamics or complexity of the repetitive behaviors. Although a variety of models could be applied to the data to assess temporal organization, we

chose to examine the second order property of stereotyped responses, i.e., the difference between consecutive inter-response intervals.

Thus, let $x_1, x_2, \ldots, x_t, \ldots$ be the intervals between successive stereotyped responses. The second order property may be measured by $D_t=x_t-x_{t-1}$, or the change or difference between adjacent inter-response intervals. The magnitude of D_t is difficult to interpret, however, because it is affected by the relative duration of the time between stereotyped responses or behaviors. Consequently, it is greatly affected by the frequency of stereotypy. Thus, we chose to "normalize" D_t by the magnitude of the durations of x_t and x_{t-1}, or

$$\delta_t = \frac{D_t}{x_t + x_{t-1}} = \frac{x_t - x_{t-1}}{x_t + x_{t-1}} \qquad (1)$$

Actually, the numerator of (1) is similar to the standard deviation and its denominator is similar to the mean. Thus (1) is related to the coefficient of variation which has been used to measure the regularity of cluster formation (see Cressie, 1991, p.590). It is easy to see that δ_t is unit free and $-1 \leq \delta_t \leq 1$.

From our plots of successive differences, it was clear that in adult animals, the data points in the scatter plot formed a characteristic pattern resembling the letter Y (see Figure 3-4). We thus measured the temporal pattern of stereotyped behavior relative to its conformity to the Y-shape pattern by defining the Y-score of a point (x, y) in the scatter diagram as

$\phi = \min(d_1, d_2, d_3)$, where
$d_1 = |x|$, if $y \geq 0$; $d_2 = |y|$, if $x \leq 0$; and
$d_3 = (x + y)/\sqrt{2}$, if $x > 0$ and $y < 0$.

We were interested in changes in Y-scores as a function of different development stages and whether there were systematic differences in Y scores across the three developmental trajectories. Thus, we assessed the two factors of trajectory group (3 levels; Traj 1, 2, and 3) and developmental period (3 levels, weeks 1, 3.5, and 6). To consider possible non-stationarity within the same developmental period, Y-scores were estimated three times in each developmental period by dividing the time series into three consecutive intervals. SAS PROC MIX was used for the data analysis. The mice were considered as a random factor.

Results

Y plots: The extent to which Y plots are useful in representing the relative temporal organization of repetitive behavior can be illustrated by Figure 3-5. In this example, if all individual stereotyped responses were equally spaced in time, all the points would cluster at the 0,0 coordinate (Panel A). If all stereotyped responses were clustered in bouts and the inter-bout intervals were all equivalent and if all stereotyped responses within a bout were equally spaced, the plot would be made up of 4 points at the following coordinates: (-x,0); (0,+y); (0,0); and (+x,-y) (Panel B). Finally, if the distribution of stereotyped responses was temporally unorganized, the plot would reflect points made up of randomly distributed coordinates (Panel C).

Figure 3-6A and B depicts representative Y plots from three mice representing each of the three developmental trajectories and, for each individual mouse, each of the three developmental time points. All the plots in Figure 3-6A are based on the same

frequency of stereotyped responses (n=850). As these plots indicate, at week 1 post-weaning, Traj 3 shows a much lower value of Y-score and a clear Y-shaped plot. Little difference can be seen between Traj 1 and 2 with little evidence for a Y-shaped pattern. At 3.5 weeks post-weaning, the Y-score has dropped for the Traj 2 mouse consistent with a now clear Y-shaped pattern of coordinates. Little change can be seen in Traj 1 and 3 with the former remaining poorly organized or more random and Traj 3 maintaining a high level of organization. At 6 weeks post-weaning the Traj 2 mouse has the lowest of its three d scores and exhibits a clear Y-shaped pattern. Little change is observed in the Traj 1 and 3 mice. In contrast to these plots, Figure 3-7 is based on a simulated random inter-response interval. As a Poisson process, frequency is irrelevant, because δ_t is invariant to it.

Thus, for Traj 3 mice, the relation between δ_t (x-axis) and δ_{t+1} (y-axis) forms a Y-shaped pattern at each of the three developmental time periods. For Traj 2 mice, this relation resembles a random pattern early in development and a clear Y shaped pattern later in development. For Traj 1 mice, this relation shows only little evidence of a Y shaped pattern and does not systematically change over development.

The Y-scores of the patterns in Figure 3-5 are given in each panel. They are the averages of φ from all the data pairs. If all the jumps have the same arrival time, the Y-score is 0 and the smaller the Y-score, the more regular are the stereotyped responses. A random pattern has the highest Y-score which is equivalent to 0.371. Table 3-1 summarizes the mean Y-scores for animals at different developmental periods within each trajectory group. The numbers in the parentheses are standard errors for the mean estimation. The significance among the factors is given in the Analysis of

Variance (ANOVA) table (Table 3-2). From this table, it is obvious that the Y-score is strongly affected by the interaction between trajectory and developmental period as well as by each factor independently.

Figure 3-8 gives the multiple comparisons of the Y-score for each trajectory group-developmental period combination. Any two values circumscribed within the same parallelogram are not statistically distinguishable based on our analyses. The overall significance level was set at 0.05 after Bonferroni correction. Thus, the criterion for statistical significance for any pair was set at 0.05/36 or 0.0014 as there are 36 pairs in the comparison. From this figure, we can see that for the Traj 2 group, their repetitive behavior becomes increasingly organized or regular across the three developmental periods. For the Traj 1 and 3 groups, however, no significant changes in Y-scores are observed over the developmental periods.

Within a developmental period, the repetitive behavior of Traj 3 mice was found to be significantly more organized or regular at week 1 post-weaning than the repetitive behavior of Traj 1 or 2 mice which did not differ and which approached random. At 3.5 weeks post-weaning, the organization of repetitive behavior did not differ between Traj 2 and 3 mice and was significantly more organized or regular than the behavior of Traj 1 mice. Finally, by 6 weeks post-weaning, the temporal organization of repetitive behavior in the Traj 2 and 3 groups was virtually identical whereas Traj 1 mice remained relatively unorganized in the distribution of their stereotyped responses over time.

The similarity of the conclusions from Figure 3-2 based on frequency and Figure 3-8 based on the Y-score makes one suspect the Y-score and frequency provide similar or redundant information. The data showed the opposite, however. Figure 3-9 depicts

the relation between Y-score and mean inter-response interval at each of the three sections in which stereotyped behavior over the dark cycle was divided. Recall that each dark cycle was divided into three sections to assess stationarity. Since the frequency of stereotypy varies between mice, the normalized inter-response interval using 1 for the third developmental period (6 weeks post-weaning) for each mouse is used for comparison. The R^2 for Y scores and frequency was 0.11, and if the inter-response intervals were not normalized for the individual mouse, the R^2 was only 0.03. Thus, the Y score contains a substantial amount of new information beyond frequency.

Discussion

In the present paper, we sought to characterize the ontogeny of stereotypy in deer mice and determine whether these animals follow discrete trajectories of development. We also sought to characterize the temporal organization of repetitive behavior in deer mice. We hypothesized that repetitive behavior would become more predictable or regular over development. We then sought to examine temporal organization within the context of different trajectories of development. The interaction between temporal structure and developmental trajectory has not previously been examined and so we sought to explore the interplay between these fundamental behavioral processes.

Our findings indicate that, as a group, deer mice develop adult levels of repetitive behavior by about 6 weeks post-weaning. This is in substantial agreement with our earlier report despite the less sophisticated data acquisition and analysis methods used in that study (Powell et al., 1999). Given the considerable heterogeneity associated with the group curve depicted in Figure 3-1, however, we employed a group based trajectory modeling procedure (Proc Traj) which yielded three distinct trajectories as the optimal solution. These groups consisted of animals that showed a monotonic increase in the

frequency of stereotypy (Traj 2) across the six week period, a group that expressed uniformly low levels of stereotypy across development (Traj 1) and the last group which exhibited high levels of stereotypy by week 1 post-weaning with relatively little increase observed subsequently (Traj 3). Identification of the Traj 1 group is consistent with our prior observations of typically having a small number of animals that, despite being reared in standard cages, do not ever develop appreciable levels of stereotypy. The Traj 3 group is also consistent with our previous anecdotal observations that some of our animals displayed stereotypy as early as weaning. Thus, a drawback of the current study is lack of inclusion of animals younger than one week post-weaning. Future experiments will determine if the developmental trajectory of Traj 3 mice is qualitatively different when such observations are added.

Abnormal repetitive behaviors are commonly displayed in animals housed in restricted (e.g., zoo, farm, laboratory) environments. Indeed, repetitive behaviors are the most common category of abnormal behavior observed in confined animals (Würbel, 2001). Despite this, there has been little attention paid to the development of these behaviors. Proc Traj allowed us to examine the heterogeneity of stereotypy trajectories and identify differences that would not have been seen using conventional cross-sectional or longitudinal analyses. Although this group-based trajectory modeling has been used in human developmental studies including autism (e.g., Anderson et al., 2009; Richler et al., 2010), it has not been used in animal studies or applied to the problem of repetitive behavior in animals. Identification of discrete trajectories provides a number of opportunities for further investigation. For example, we can now pursue identification of variables that might predict trajectory membership. Of particular

importance would be investigating potential mechanisms that protect Traj 2 mice from developing high levels of repetitive behavior despite being reared in standard cages. Mapping trajectories will also allow us to pursue identification of neurobiological mechanisms that play a role in the differential timing of the expression of stereotypy. Finally, we can now examine various experiential and biological manipulations for their impact on altering the development of repetitive behavior. Such studies would have important implications for treatment efforts in neurodevelopmental disorders such as autism.

Stereotyped behavior is typically thought of as behavior characterized by regularity or relative lack of variability. Longer time series of stereotyped behavior do show systematic variability, however (e.g., Lewis et al., 1981). In the present study, we showed a temporal distribution of stereotyped behavior across the 8 hour dark cycle with a decrease in frequency over the first two hours of the dark cycle and a prominent increase in frequency over the last 2-3 hours of the dark cycle. Interestingly, when animals were sorted by trajectory group, this pattern could be seen at all three developmental time points in the Traj 1 and 3 groups but was slow to develop in the Traj 2 group.

Similar results were obtained when the data were subjected to a statistical model that assessed the relative organization of stereotyped behavior. This analysis showed that the stereotypy of Traj 2 mice became significantly more organized or less random across development, whereas Traj 1 and 3 mice showed no significant change in temporal organization of stereotypy across development. We also found trajectory group differences in temporal organization across developmental periods. The

stereotyped behavior of Traj 1 and 2 mice was significantly less organized (more random) at week 1 post-weaning than Traj 3 mice. At post-weaning weeks 3.5 and 6 the repetitive behavior of Traj 1 mice remained largely unorganized whereas Traj 2 and 3 mice were significantly more organized. The absence of increased temporal organization in the behavior of Traj 1 mice is noteworthy in that it shows that despite five weeks of engaging in the same behavior many times per day, its expression didn't become more fixed or regular. Whether the failure to become more organized is important in the failure of these animals to develop appreciable levels of stereotypy is a matter of conjecture.

The changes across development and among trajectories were seen not only statistically but also graphically in the successive difference or Y plots. These plots are instructive in that they depict the degree to which repetitive behavior is organized into bouts and the degree to which individual stereotyped responses are organized within bouts. For example, in the Traj 2 mice tested at week 1 post-weaning, there is little evidence of stereotyped responses being clustered in bouts and little evidence for regularity in individual stereotyped responses within a bout. That organization clearly is in evidence by 3.5 weeks post-weaning, however. The Traj 3 group, conversely, showed considerably more structure, in terms of responses being clustered in bouts and exhibiting regularity of responding within a bout, at week 1 post-weaning than observed in Traj 2 mice. It has been known for some time that repetitive behavior tends to occur in bouts but little, if any, attention has been given to individual variability in such structure or the developmental timing of such structure.

Plotting successive differences to generate plots of the temporal structure of repetitive behavior is simple and straightforward. Differencing removes slower, larger amplitude trends in the time series and highlights briefer fluctuations rather than the mean trends (Williams, 1997). We generated one index or coefficient of organization from the plot which was nearly independent of frequency. Similar diagrams expressing the first order relation, i.e., the quintiles of x_t and x_{t+1} called the "first return map" have been used to describe the temporal dynamics of tics (Peterson and Leckman, 1998). The use of quintiles is similar to normalization in our δ_t. However, this approach did not yield a pattern as simple as the pattern observed with Y-scores. When a two-dimensional pattern is complex, it is difficult to summarize the diagram using formal statistical tests. The other methods, such as ARIMA and spectral analysis, require model identification for each time series. We found that for our own data even within the same time series, lack of stationarity happens quite often. In this respect, Y-scores were more stable than the frequency. The coefficients of variation for Y-score and frequency within the same epoch for the same mouse were 0.15 and 0.67 respectively.

A simple graphical and statistical method to examine temporal organization of repetitive behavior provides a very useful tool for subsequent investigations. Behavior is often characterized or quantified in terms of frequency and duration and not typically in terms of temporal structure. Assessing temporal organization provides information about complexity, variability, and flexibility (Asher et al., 2009). Thus, temporal organization may prove to be a more useful variable than frequency or duration in terms of how resistant the behavior may be to intervention. The strikingly predictable or regular dynamics found in the repetitive behavior of most adult deer mice is in sharp

contrast with the complexity, variability and relative unpredictability that characterizes adaptive, functional behavior. In the “dynamical disease” model of Glass and Mackey (1988), medical illness (e.g. cardiovascular disease) can be characterized by periodic or predictable dynamics in the relevant physiological systems, whereas healthy systems are complex and variable. Indeed, within the field of dynamical diseases, the most repetitive and predictable dynamics are associated with the most severe pathological conditions (e.g., Cheyne-Stokes breathing), a phenomenon referred to as the ‘law of stereotypy’ (Goldberger, 1997). Abnormal repetitive behavior may be viewed from this perspective as representing a loss or lack of complexity. Loss of complexity or variability in behavior would seem to reflect adverse neuroadaptations in the CNS that occur over development (Hadders-Algra, 2008). Thus, it may be that developmental trajectory and temporal organization may provide more powerful correlates of neurobiological variables than frequency or duration of behavior. In addition, the methods used in the current study may be useful tools in the assessment and treatment of repetitive behavior in clinical populations such as autism and related neurodevelopmental disorders.

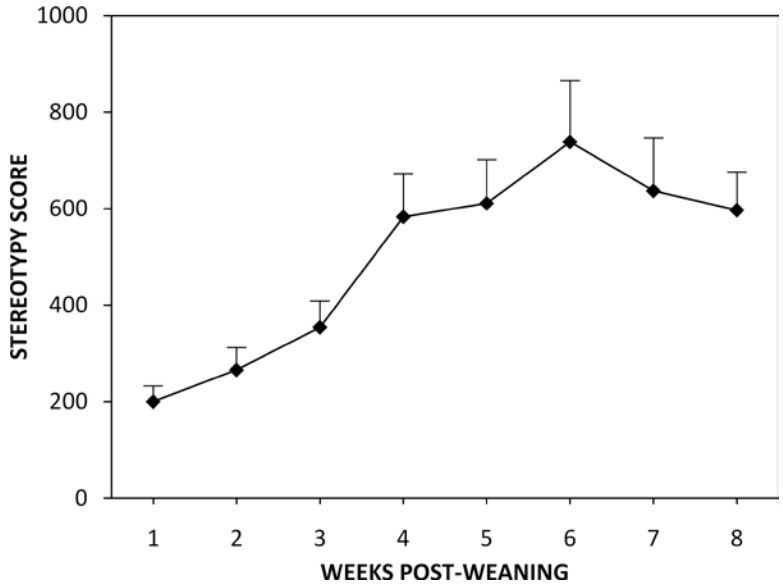


Figure 3-1. The development of stereotypy post-weaning (mean frequency per hour + SEM).

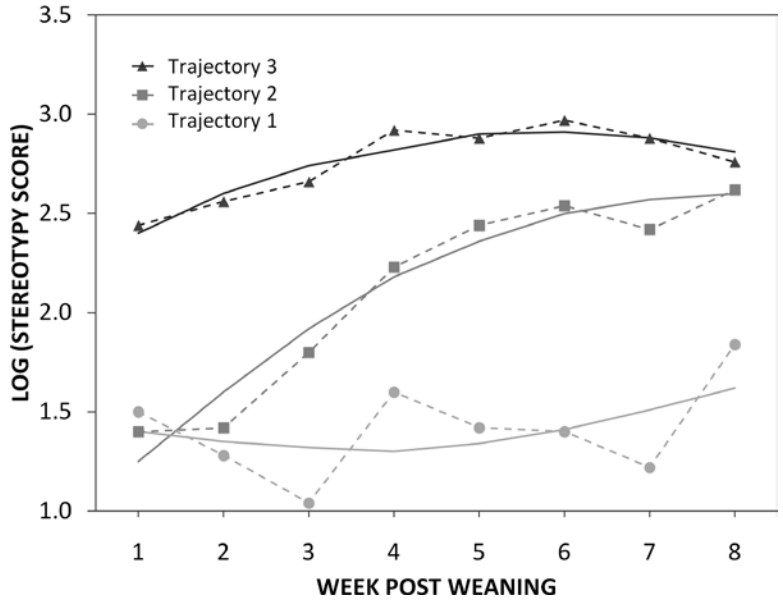


Figure 3-2. Three distinct developmental trajectories yielded by SAS Proc Traj (log of the mean frequency per hour; raw data and smoothed curves).

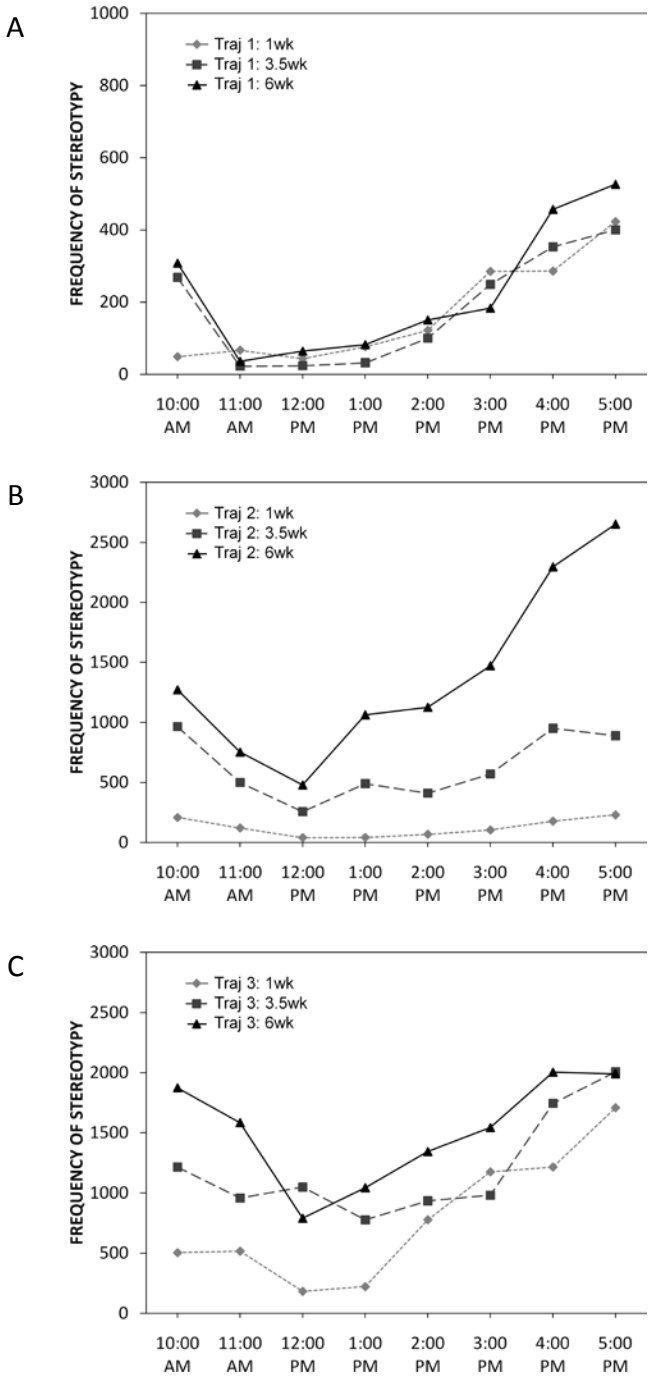


Figure 3-3. Repetitive behavior during the 8-hr dark cycle. Panel A: Traj 1 at each of three developmental periods (1, 3.5, 6 weeks post-weaning); Panel B: Traj 2 at each of the same three developmental periods; Panel C: Traj 3 at each of the same three developmental periods.

Table 3-1. Mean Y-scores for animals at three developmental periods within each trajectory group (mean ± SEM).

		Traj 1	Traj 2	Traj 3
Developmental Stage 1	(1wk)	0.252 (0.012)	0.260 (0.013)	0.116 (0.014)
Developmental Stage 2	(3.5wk)	0.240 (0.012)	0.166 (0.012)	0.123 (0.014)
Developmental Stage 3	(6wk)	0.218 (0.011)	0.132 (0.012)	0.127 (0.014)

Table 3-2. ANOVA Table from SAS PROC MIXED with Y-score as the response variable.

Effect	Num DF	Dem DF	F	*p*-value
Trajectories	2	139	27.02	<0.0001
Developmental Stages	2	139	27.65	<0.0001
Interaction	4	139	15.89	<0.0001

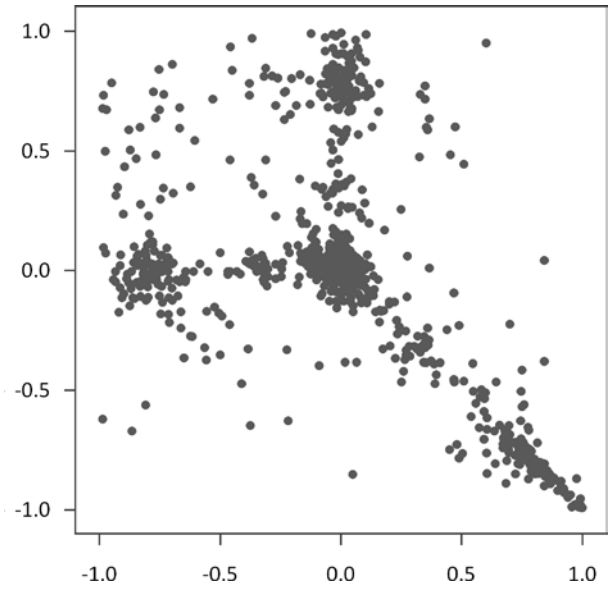
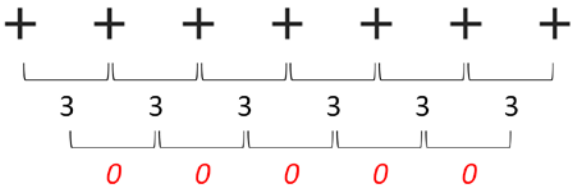
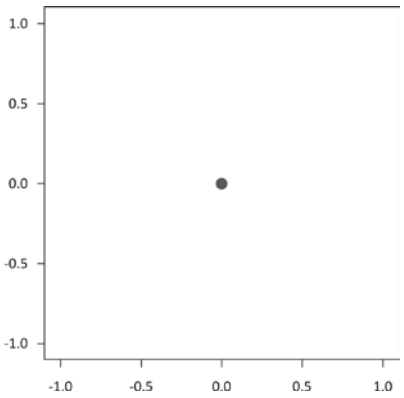


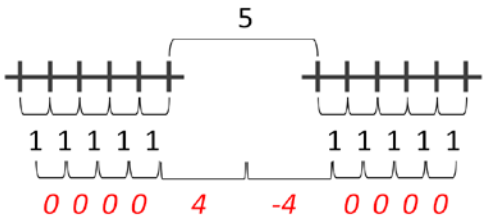
Figure 3-4. Typical Y-plot of successive differences seen in an adult deer mouse. Plot contains n=850 behavioral responses.

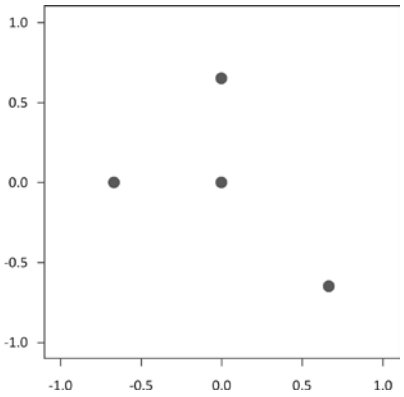
A


3 3 3 3 3 3
0 0 0 0 0

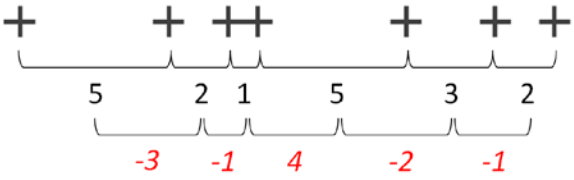

1.0
0.5
0.0
-0.5
-1.0
-1.0 -0.5 0.0 0.5 1.0

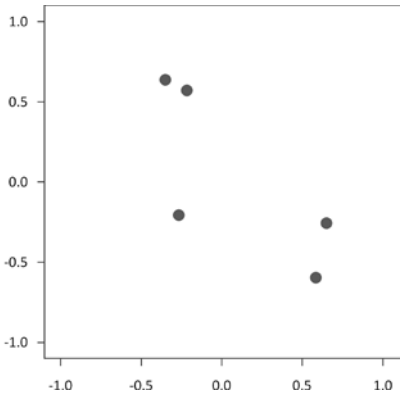
B


5
1 1 1 1 1 1 1 1 1 1
0 0 0 0 4 -4 0 0 0 0


1.0
0.5
0.0
-0.5
-1.0
-1.0 -0.5 0.0 0.5 1.0

C


5 2 1 5 3 2
-3 -1 4 -2 -1


1.0
0.5
0.0
-0.5
-1.0
-1.0 -0.5 0.0 0.5 1.0

D

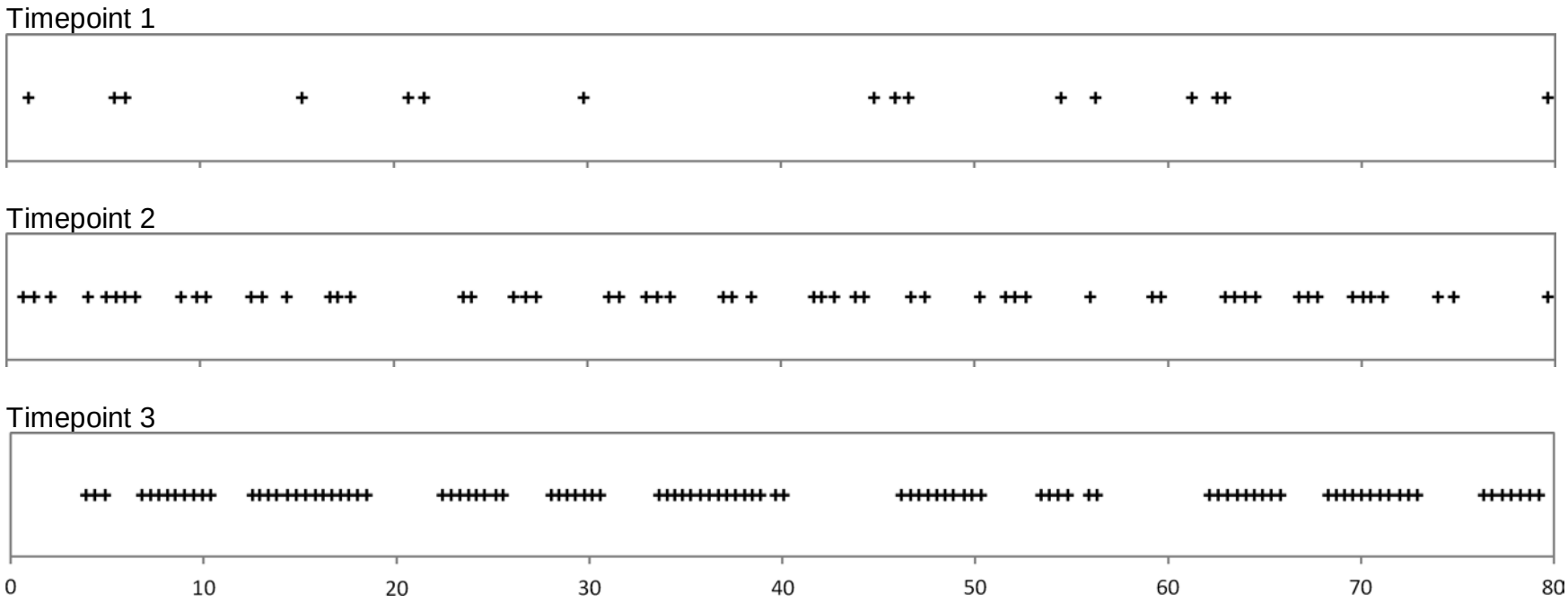
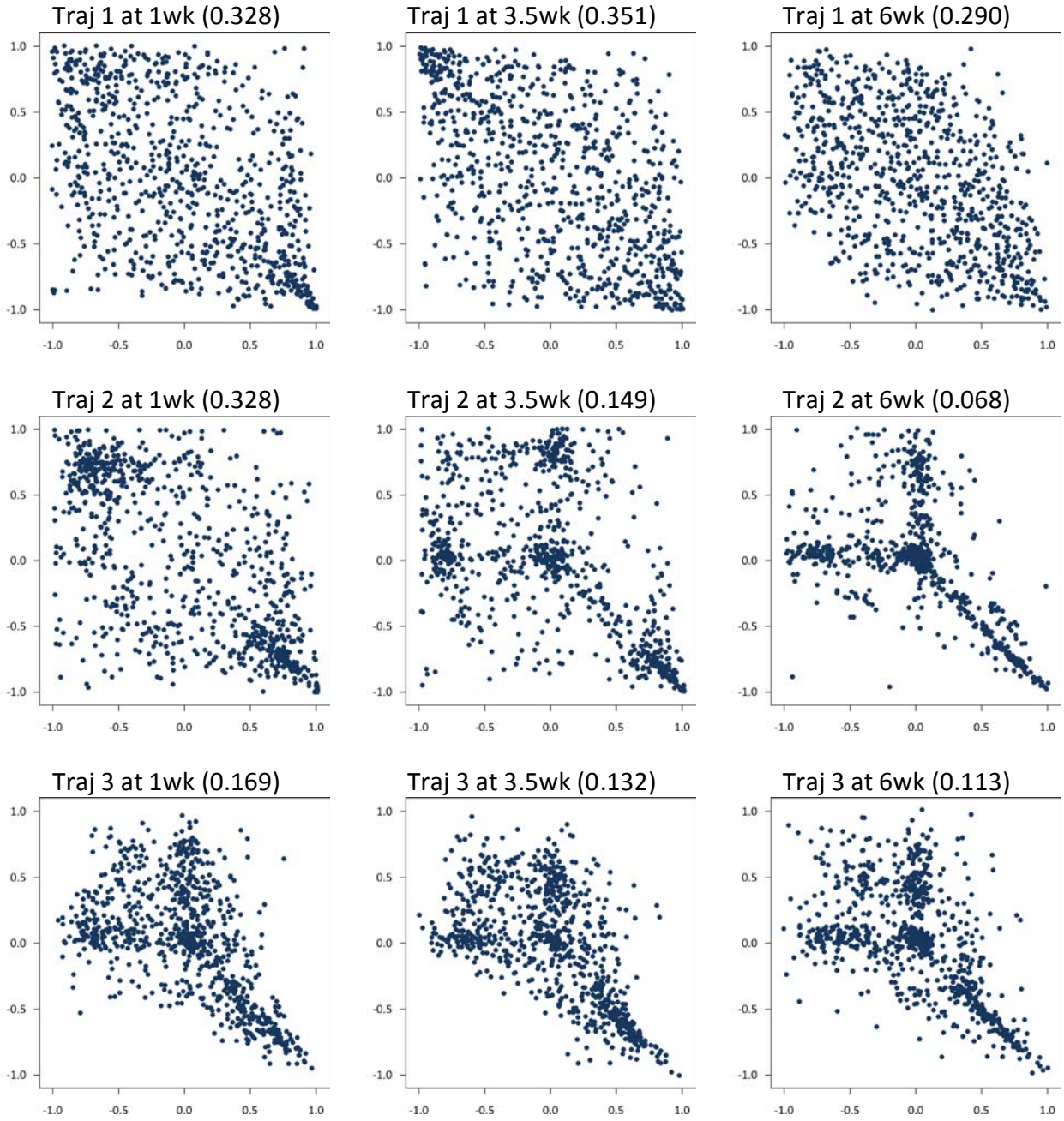


Figure 3-5. Model successive difference plots. A: behavioral responses equally spaced in time yield points clustered at the (0,0) coordinate; B: behavioral responses organized in bouts yield 4 points; C: unorganized behavioral responses yield randomly distributed coordinates. Their normalized (δ_t , δ_{t+1}) are given in the right diagrams; D: representative temporal organization of behavior in a Traj 2 mouse at 1, 3.5, and 6 weeks post-weaning. Each figure depicts 80sec of the dark cycle. + represents one vertical jump.

A


Traj 1 at 1wk (0.328)
Traj 1 at 3.5wk (0.351)
Traj 1 at 6wk (0.290)
Traj 2 at 1wk (0.328)
Traj 2 at 3.5wk (0.149)
Traj 2 at 6wk (0.068)
Traj 3 at 1wk (0.169)
Traj 3 at 3.5wk (0.132)
Traj 3 at 6wk (0.113)
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0

B

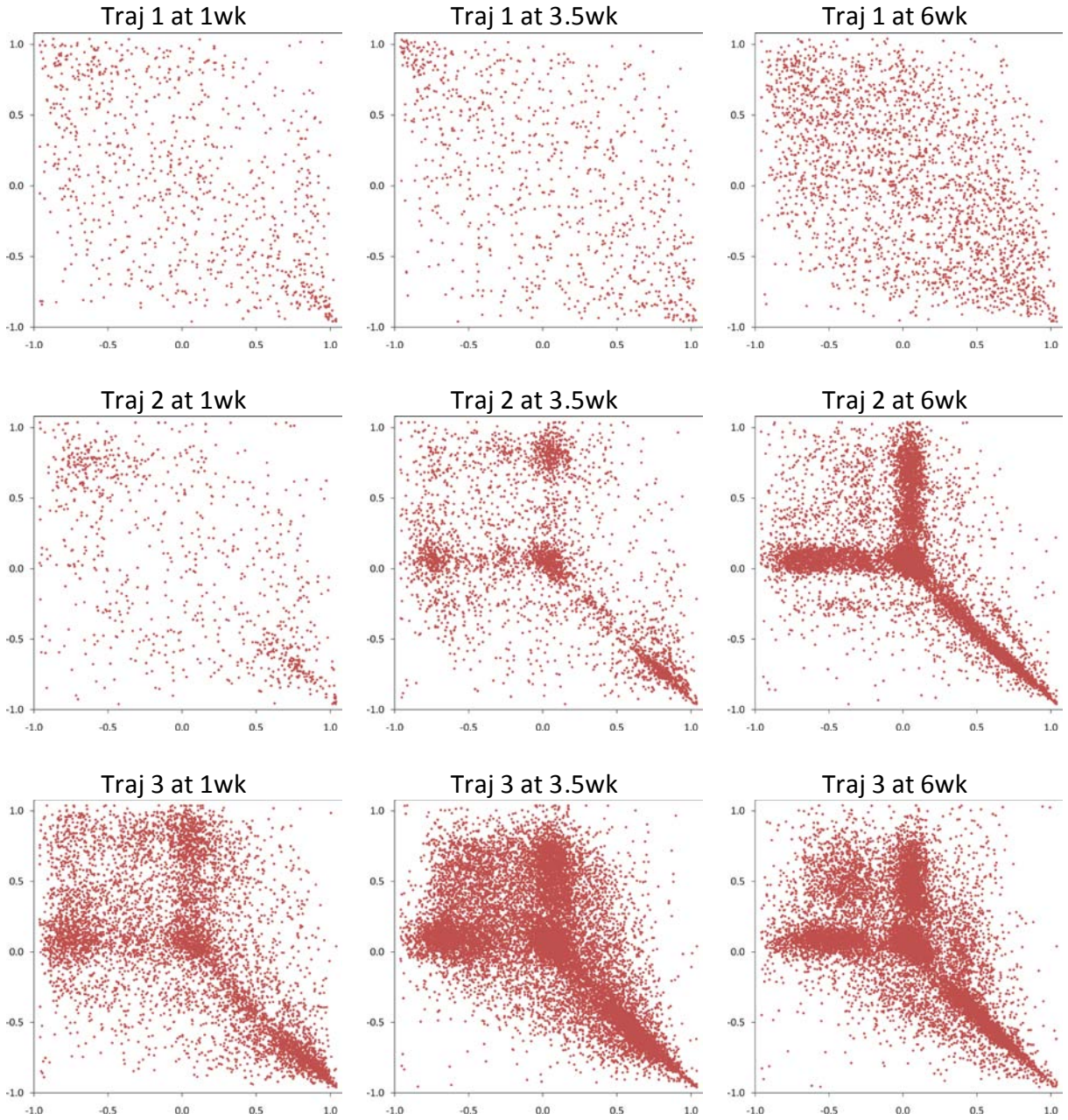


Figure 3-6. Y-plots from three mice representing each of the three developmental trajectories at three developmental time-points. The top three panels are from a Traj 1 mouse, middle panels from a Traj 2 mouse and bottom from a Traj 3 mouse. The values in the parentheses are the Y-scores. A: Each plot contains n=850 behavioral responses; B: Each plot contains all the behavioral responses.

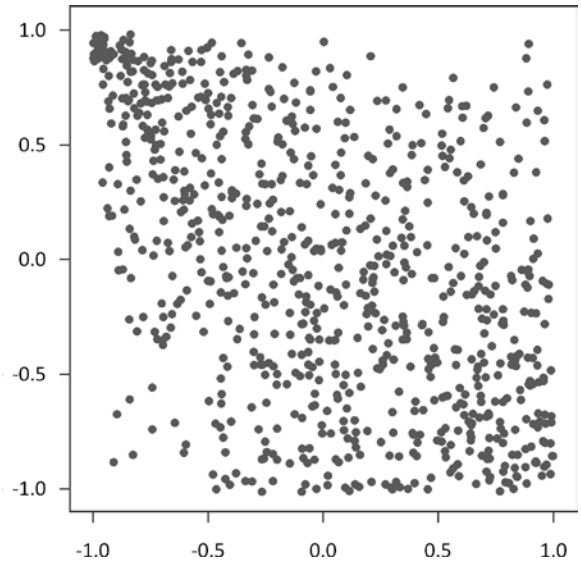


Figure 3-7. A simulated random inter-jump interval plot containing n=850 behavioral responses.Y-score equivalent to 0.371.

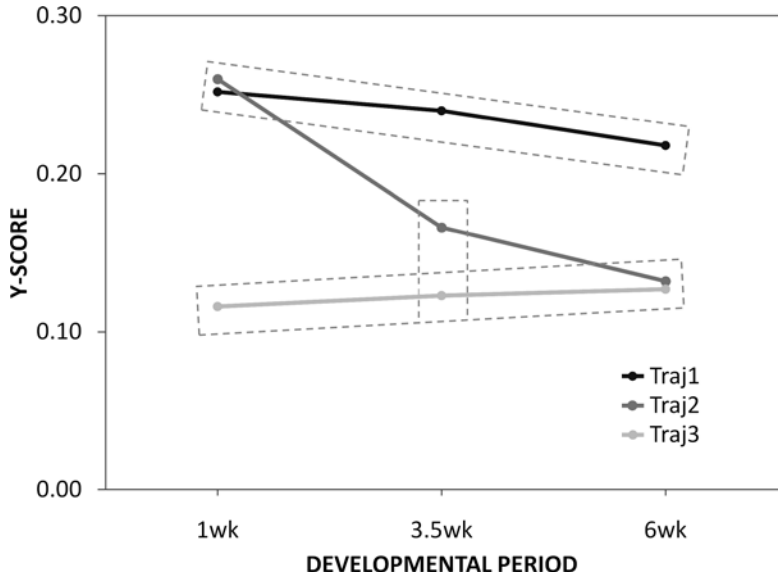


Figure 3-8. Y-scores for each trajectory group at each developmental period. Values circumscribed within the same parallelogram are not statistically different.

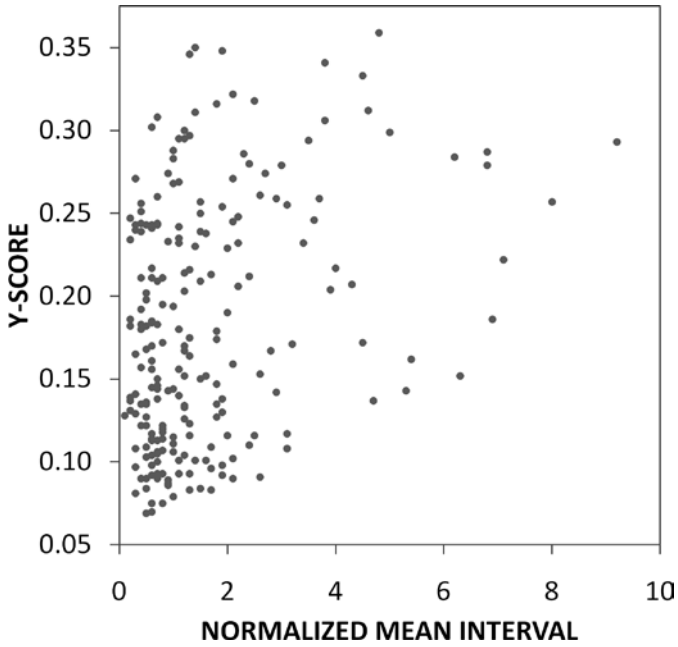


Figure 3-9. Scatter-plot depicting the association of Y-scores and mean inter-stereotyped response intervals. The mean intervals were normalized using an inter-response interval of 1 for the third developmental period.

CHAPTER 4
INDIRECT BASAL GANGLIA PATHWAY MEDIATION OF THE DEVELOPMENT OF REPETITIVE BEHAVIORS

Introduction

In Chapter 2, we have shown that decreased indirect basal ganglia pathway activity was linked to the expression of repetitive behaviors, and these behaviors were selectively attenuated by co-administration of adenosine A_{2A} and A_1 receptor agonists in adult deer mice. To further investigate the mediation of repetitive behavior by indirect pathway activity, we assessed its role in the development of repetitive behaviors. Based on the overall trajectory of repetitive behavior described in Chapter 3, we selected three developmental time-points (1, 3.5, and 6 weeks post-weaning) to assess indirect pathway activity in each of the three developmental trajectory groups. As in Chapter 2, activity of the indirect pathway was indexed by metabolic capacity of STN using cytochrome oxidase (CO) histochemistry (Experiment 1). We hypothesized that the CO activity in STN would decrease from 1 week to 6 weeks post-weaning. In addition, we hypothesized that the three distinct developmental trajectory groups would exhibit differential patterns of CO activity in STN.

Studies of repeated intermittent psychostimulant administration have suggested that differential activation of striatal compartments may also play an important role in the genesis of repetitive behaviors. Striosomes, which comprise approximately 15 percent of the striatum, are anatomically and neurochemically distinguished from the extrastriosomal matrix. Medium spiny neurons (MSN) in the striosomes receive inputs primarily from the limbic cortices (e.g., anterior cingulate cortex, orbitofrontal cortex, and basolateral amygdala), whereas MSNs in the extrastriosomal matrix receives afferents primarily from the sensorimotor cortices. MSNs in the matrix comprise the direct and

indirect pathways of the basal ganglia, whereas MSNs in the striosomes make up a pathway, distinct from the direct and indirect pathways, which sends GABAergic projections to the substantia nigra pars compacta (SNpc) (Figure 4-2). These striosomal neurons are thought to directly regulate the nigrostriatal dopaminergic neuronal activity.

Several studies have addressed functional differences in MSNs within the striosomes versus matrix. Brown et al. (2002) showed that animals' neutral/normal behaviors (e.g., voluntary movements, occasional grooming, tactile stimulation) were associated with relatively higher activity of neurons in the matrix. White and Hiroi (1998), conversely, found that intracranial self-stimulation was maximized when the electrodes were implanted in or around the striosomes. Canales and Graybiel (2000b) reported that chronic intermittent administration of psychostimulants induced preferential activation of striosomes over extrastriosomal matrix. More specifically the degree of stereotyped behavior due to psychostimulant-induced behavioral sensitization was positively correlated with the ratio of c-Fos expression in the striosome versus matrix. These findings collectively suggest that the striosomes mediate reinforcement and the matrix mediates naturalistic species-typical behaviors, and disruptions of balanced activity between these compartments may be associated with expression of invariant functionless repetitive behaviors.

More recently structural changes of striosomes were found in clinical populations with specific neurological disorders and in animal models of those disorders. YAC128 mice, a model of Huntington's disease which expresses approximately 128 CAG repeats showed a smaller striosome volume as well as more neuronal loss in the striosomes as compared to wild-type mice (Lawhorn et al., 2008). Moreover their motor

deficits on the rotarod and balance beam were inversely correlated with neuronal loss in striosomes. Similar structural changes were found in a clinical population, where mood dysfunction and cognitive symptoms of Huntington's disease, among the triad (motor, mood, and cognitive symptoms), was correlated with $GABA_A$ receptor loss in the striosomes compared to healthy individuals (Tippett et al., 2007).

Loss of voluntary movements in Parkinson's disease, caused by degeneration of nigral dopamine neurons, is alleviated by L-DOPA. Repeated administration with this compound, however, frequently results in dyskenisias, which are characterized by involuntary motor movements, which typically are orofacial. In a rat model of Parkinson's disease, this L-DOPA-induced motor abnormality was associated with down-regulation and up-regulation of matrix-enriched CalDAG-GEFI and striosome-enriched CalDAG-GEFII respectively, two striatal regulators of the kinase signal transduction cascade (Crittenden et al., 2009). Conversely, dopa-responsive dystonia, which likely results from striatal dopamine deficiency, was associated with greater loss of tyrosine hydroxylase labeled nigrostriatal neurons terminating in the striosomes versus matrix (Sato et al., 2008). This differential loss was particularly evident during the post-natal period when their behavioral symptoms emerged.

Based on these findings, the activity of striosomal neurons has been of interest to elucidate the mechanism of movement disorders. Because high rates of stereotyped behaviors in deer mice likely compete with naturalistic behaviors, we hypothesized that a relative increase of striosomal activity largely due to decreased matrix activity would be linked to the development of stereotypy. Thus, we first, identified striosomes by labeling selected sections with the mu opioid receptor-1 (MOR1) antibody. We then

assessed the intensity of CO staining in adjacent sections on which the outlined striosomal areas were superimposed (Experiment 2). This allowed us to calculate CO activity in the two striatal compartments. These experiments were done using deer mice tissue collected at three developmental time-points from Experiment 1. In addition, we assessed the striosomal volume and striosomal neuronal counts in an independent group of deer mice at the same three developmental time-points described previously (Experiment 3).

General Methods

Subjects

All deer mice (*Peromyscus maniculatus*) were obtained from the breeding colony maintained in our laboratory, and kept on a 16:8-h light/dark cycle with lights off at 10:00 AM. Rodent chow and water were available *ad libitum*. The room was maintained at 20-25^{o}C and 50-70% humidity. Mice sharing the same weaning date were group-caged (5-6 mice/cage) at weaning (PND21) in standard rodent cages (48 x 27 x 15 cm) and they remained in the same cage group throughout the experiment. All procedures were performed in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Florida Institutional Animal Care and Use Committee.

Stereotypy Assessment

Rates of spontaneous stereotypy (hindlimb vertical jumping or backward somersaulting) were assessed using a modified automated photocell detection apparatus (Columbus Instruments). The session consisted of the eight hours of the dark cycle. Mice were individually placed in testing cages (22 x 28 x 25 cm) made of Plexiglas and habituated for at least one hour prior to the beginning of the dark cycle.

Food and water were provided. All sessions were digitally video-recorded for identification of behavioral topographies and accuracy of the automated counters.

Specific Methods and Results

Experiment 1: STN Activation and the Development of Stereotypy

Methods

Deer mice available in our colony were tested for stereotypy as described in the next section at 1, 3.5, and 6 weeks post-weaning; three time-points selected based on the developmental trajectory information described in Chapter 3. Following each behavioral testing, a third of the animals were randomly selected. For the animals selected at 3.5 and 6 weeks post-weaning, their developmental trajectories were subjectively determined based on the stereotypy score at each developmental time-points. The animals from the three time-points, which clearly showed the representative trajectory (Traj 1, 2, or 3) were selected, and their brains were collected to conduct CO histochemistry (n=8, n=13, and n=25 respectively).

CO histochemistry

The CO spectrophotometric analysis and staining assay was carried out according to the Gonzalez-Lima protocol (1998). Standards were cut at five thicknesses (20, 30, 40, 50, and 60µm) and assayed with other brain sections. The CO staining was quantified by densitometric analysis using ImagePro (Media Cybernetics), and standards were used to convert optical density values into enzymatic activity values (µmol/min/g). Multiple optical density readings were made per animal for STN as well as for other areas comprising cortico-basal ganglia circuitry (motor cortex, striatum, substantia nigra pars reticulata (SNpr), substantia nigra pars compacta (SNpc)) and negative controls (hippocampus, somatosensory cortex). Dorsal and ventral aspects of

the striatum were defined by horizontal bisection into approximately equivalent halves, whereas medial versus lateral aspects were defined by approximately <2.0mm or >2.0mm from the midline respectively. Under our experimental conditions, the staining intensity was highly correlated to the thickness of the standard sections (r=0.95).

Data analysis

Differences in CO activity among three developmental time-points were assessed by one-way ANOVA with CO differences in STN being the primary comparison of interest. In addition, at 3.5 and 6 weeks post-weaning, differences in CO activity among three trajectories were assessed by one-way ANOVA. The association between the frequency of stereotypies and CO activity were analyzed by Pearson correlation. All tests were two-tailed and effects were considered significant when $p<0.05$.

Results

Average stereotypy scores from each time-point are shown in Figure 4-1. CO enzymatic activity within selected areas from each developmental time-point is summarized in Table 4-1. Contrary to our hypothesis, no significant difference was found in STN across development ($F(2,38)=1.87$, $p=0.17$) despite the increase in stereotypy scores. Significant differences were found, however, in the ventromedial striatum ($F(2,38)=3.39$, $p=0.04$) and SNpc ($F(2,38)=9.00$, $p=0.001$) where CO activity increased in the ventromedial striatum and decreased in SNpc over development. The post-hoc analysis shows that the significant differences were found between 1 week and 6 weeks post-weaning for the ventromedial striatum, and between 1 week and 6 weeks as well as 3.5 and 6 weeks post-weaning for SNpc.

Based on the stereotypy scores obtained at 3.5 and 6 weeks post-weaning, animals were divided into low- and high-stereotypy groups using a median split. At 3.5

weeks post-weaning, there was no difference in STN ($t(6)=0.33$, $p=0.76$), whereas at 6 weeks post-weaning, the high-stereotypy group showed significantly lower CO staining in STN ($t(23)=5.45$, $p<0.001$). In addition, at 6 weeks post-weaning significant differences were also found in the dorsomedial striatum ($t(23)=2.07$, $p=0.05$), ventrolateral striatum ($t(22)=2.21$, $p=0.04$), ventromedial striatum ($t(23)=2.73$, $p=0.01$), nucleus accumbens ($t(23)=3.75$, $p=0.01$), and SNpc ($t(23)=3.28$, $p<0.01$).

Furthermore, the animals from the 3.5 and 6 weeks post-weaning groups were categorized into one of the three developmental trajectories (Traj 1, 2, and 3) based on Chapter 3 (n=4,n=5, and n=4 for 3.5 weeks post-weaning; n=8,n=12, and n=5 for 6 weeks post-weaning respectively). The CO enzymatic activity within selected areas in three trajectory groups at 6 weeks post-weaning is summarized in Table 4-2. Although there was no difference in STN across development, a significant difference was found at 6 weeks post-weaning where Traj 2 and Traj 3 mice showed lower CO activity in STN compared to Traj 1 mice ($F(2,22)=14.21$, $p<0.001$). Statistical differences were also found among three trajectory groups in the nucleus accumbens ($F(2,22)=6.85$, $p=0.01$), ventromedial striatum ($F(2,22)=3.56$, $p=0.05$), and SNpc ($F(2,22)=7.54$, $p<0.01$). In all these areas, Traj 1 mice showed higher CO activity compared to Traj 2 and Traj 3 mice. Moreover, stereotypy score at 6 weeks post-weaning were negatively correlated with STN ($r=-0.68$, $p<0.001$), the nucleus accumbens ($r=-0.50$, $p=0.01$), and SNpc ($r=-0.68$, $p<0.001$) (Table 4-3).

Table 4-1. CO activity (µmol/min/g) of STN and the other brain areas in deer mice at three developmental time-points. Values expressed are group means with SEM in parentheses.

	1wk	3.5wk	6wk	*P*-value
Subthalamic Nucleus	66.27 (1.58)	67.09 (1.06)	63.97 (0.96)	0.17
Motor Cortex	48.81 (1.29)	49.66 (0.73)	49.07 (0.55)	0.82
Somatosensory Cortex	49.07 (0.98)	50.48 (0.65)	50.33 (0.59)	0.50
Hippocampus	40.79 (0.58)	41.75 (0.68)	40.62 (0.52)	0.49
Caudate/Putamen				
Dorsolateral Striatum	45.77 (1.48)	46.93 (1.10)	48.91 (0.71)	0.09
Dorsomedial Striatum	47.16 (0.97)	49.20 (0.80)	49.85 (0.56)	0.06
Ventrolateral Striatum	47.14 (1.60)	47.70 (0.81)	49.12 (0.68)	0.32
Ventromedial Striatum	45.97 (1.08)	48.20 (0.89)	48.88 (0.55)	0.04
Nucleus Accumbens	49.48 (1.50)	51.44 (0.88)	51.64 (0.73)	0.34
Substantia Nigra pars Compacta	49.16 (0.59)	48.85 (0.76)	44.47 (0.79)	0.001
Substantia Nigra pars Reticulata	52.90 (0.61)	53.93 (0.93)	49.61 (0.96)	0.02

Table 4-2. CO activity (µmol/min/g) of STN and the other brain areas in three developmental trajectories at 6 weeks post-weaning. Values expressed are group means with SEM in parentheses.

	Traj 1	Traj 2	Traj 3	*P*-value
Subthalamic Nucleus	68.85 (1.18)	61.54 (0.99)	61.56 (0.85)	<0.001
Motor Cortex	50.97 (1.22)	48.22 (0.67)	48.81 (0.85)	0.10
Somatosensory Cortex	51.92 (1.05)	48.94 (0.67)	51.26 (1.08)	0.05
Hippocampus	40.02 (1.13)	40.83 (0.61)	40.12 (0.50)	0.82
Caudate/Putamen				
Dorsolateral Striatum	51.28 (1.19)	47.38 (0.65)	49.80 (2.12)	0.08
Dorsomedial Striatum	51.37 (0.94)	49.21 (0.73)	48.82 (0.96)	0.15
Ventrolateral Striatum	51.59 (1.07)	47.72 (0.70)	49.43 (1.95)	0.07
Ventromedial Striatum	50.73 (0.89)	47.99 (0.66)	47.92 (0.88)	0.05
Nucleus Accumbens	54.85 (1.34)	50.30 (0.69)	49.69 (0.62)	0.005
Substantia Nigra pars Compacta	48.31 (1.05)	43.87 (1.01)	40.82 (0.47)	0.003
Substantia Nigra pars Reticulata	52.52 (1.19)	49.77 (1.42)	45.36 (1.14)	0.04

Table 4-3. Correlation between stereotypy score and CO activity (µmol/min/g) in three developmental trajectories at 6 weeks post-weaning.

	r	*P*-value
Subthalamic Nucleus	-0.671	<0.001
Motor Cortex	-0.236	0.24
Somatosensory Cortex	-0.158	0.43
Hippocampus	0.055	0.79
Caudate/Putamen		
Dorsolateral Striatum	-0.246	0.24
Dorsomedial Striatum	-0.173	0.39
Ventrolateral Striatum	-0.281	0.17
Ventromedial Striatum	-0.246	0.22
Nucleus Accumbens	-0.505	0.007
Substantia Nigra pars Compacta	-0.725	<0.001
Substantia Nigra pars Reticulata	-0.536	<0.01

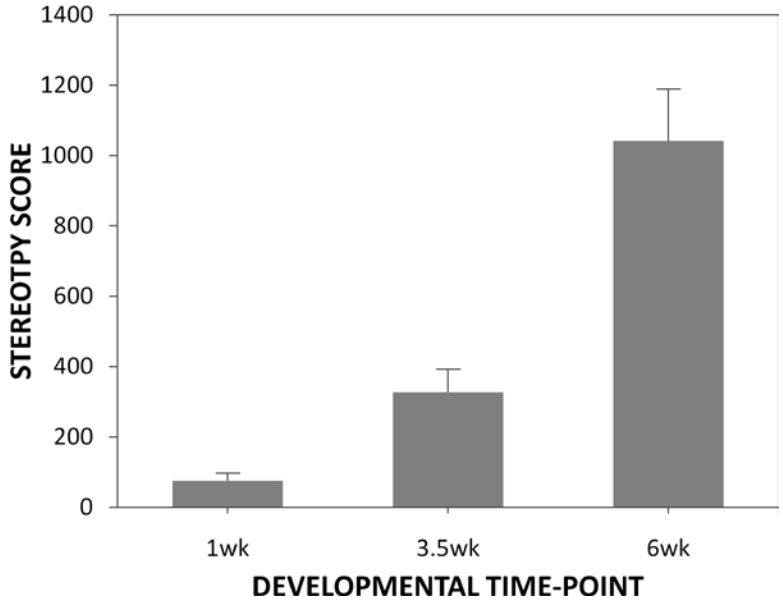


Figure 4-1. The development of stereotypy assessed at three developmental time-points. Each bar represents separate sets of animals sacrificed at the respective time-points (mean + SEM).

Experiment 2: Neuronal Activation of Striosomes versus Matrix and the Development of Stereotypy

Methods

The brain sections adjacent to CO-stained sections from Experiment 1 were used to label striosomes. There were 7, 8, and 24 mice sacrificed at 1, 3.5, and 6 weeks post-weaning respectively, and mice at 6 weeks post-weaning were categorized into Traj 1, 2, or 3 (n=8, n=11, n=5 respectively). Two mice, which were originally in the analysis for CO histochemistry (Experiment 1), were dropped due to the unsuccessful MOR1-staining on the adjacent sections.

Immunohistochemistry

The tissue was fixed on slides with 4% paraformaldehyde, rinsed 3x with 0.01M PBS, incubated for 3% hydrogen peroxide, rinsed 3x with PBS, incubated for 5% normal goat serum for 30min, and incubated for mu-opioid receptor 1 antibody (MOR1, Abcam) at a dilution of 1:1500 in PBS with 0.2% TritonX-100 and 0.1% sodium azide overnight at 4°C. The sections were rinsed 3x with PBS, incubated with biotynylated secondary antibody (goat anti-rabbit, Millipore) at a dilution of 1:300 in PBS with TritonX-100 for 2hrs, rinsed 3x with PBS, incubated with avidin-biotin-peroxidase complex (Sigma) at a dilution of 1:300 in PBS with TritonX-100 for 2hrs, and rinsed 3x with PBS. The sections were visualized by 3,3-diaminobenzidene (Sigma) with nickel intensification, dehydrated in successive ethanol, cleaned in xylene, and coverslipped.

Image analysis

The optical density readings for striosome and matrix were made by superimposing the MOR1-stained adjacent sections onto the CO-stained sections described in Experiment 1 (Figure 4-3). The CO staining was quantified by

densitometric analysis using ImagePro (Media Cybernetics), and standards were used to convert optical density values into enzymatic activity values (µmol/min/g). Multiple optical density readings were made per animal. Striosomal volume was also calculated using ImagePro.

Statistical analysis

CO activity and striosomal volume among three developmental time-points and trajectories were assessed by one-way ANOVA. Effects were considered significant when $p<0.05$.

Results

There were significant developmental effects on CO enzymatic activity such that metabolic activity in the matrix increased as animals developed ($F(2,36)=3.66$, $p=0.04$) whereas no statistically significant developmental change was observed in striosomes ($F(2, 36)=2.43$, $p=0.10$) (Table 4-4). There was no significant difference across development in the ratio or index of striosome to matrix predominance (ISMP) ($F(2,36)=0.68$, $p=0.51$). At 6 weeks post-weaning, there was no difference among the three trajectories in striosome ($F(2,21)=0.64$, $p=0.54$), matrix ($F(2,21)=0.98$, $p=0.41$), or ISMP ($F(2,21)=2.28$, $p=0.13$) measures (Table 4-5). Analysis of low- versus high-stereotypy mice based on median split of their stereotypy scores also showed no statistical difference in striosome ($t(22)=-0.59$, $p=0.56$), matrix ($t(22)=-1.35$, $p=0.19$), and ISMP ($t(22)=2.00$, $p=0.06$) CO activity. There was a general increase in striosomal volume over development ($F(2,39)=15.73$, $p<0.001$), although no association with rates of stereotypy was found at 6 weeks post-weaning ($F(2,39)=0.25$, $p=0.78$). The relative striosome volume, the overall striatal volume, and stereotypy were also not associated with stereotypy at 6 weeks post-weaning.

Experiment 3: Striosomal Neuronal Counts and the Development of Stereotypy

Methods

Deer mice available in our colony were tested for stereotypy as described above at 1, 3.5, and 6 weeks post-weaning. Following stereotypy assessment at each time-point, animals were randomly assigned to developmental time point (n=3, n=3, and n=3 respectively) and their brains were perfused with 4% paraformaldehyde, post-fixed, cryoprotected with PBS with 30% sucrose, and stored at -80°C until assayed.

Immunohistochemistry

The brains were cut sagittally at 30µm and sections acquired beginning at approximately 2.7 mm lateral to the midline (see Experiment 2). Free-floating sections were incubated with 0.01M sodium citrate buffer at 50°C for 20min and room temperature for 20min for better antigen detection. Sections were washed in PBS, incubated in 5% normal goat serum for 30min, and incubated with anti-NeuN antibody (Millipore) in PBS with TritonX-100 and sodium azide at a dilution of 1:500 at 4°C overnight. The sections were rinsed 3x with PBS, incubated with goat anti-mouse secondary antibody (Millipore) at a dilution of 1:1000, rinsed 3x with PBS, incubated with avidin biotin complex at a dilution of 1:1000, and rinsed 3x. The neurons were visualized by 3,3-diaminobenzidene (Sigma) to yield brown color.

The same sections were incubated with MOR1 antibody (Abcam) in PBS with TritonX-100 and sodium azide at the dilution of 1:1500 at 4% for 48hrs. The sections were rinsed 3x with PBS, incubated with goat anti-rabbit secondary antibody (Millipore) in PSB with TritonX-100 at a dilution of 1:1000, rinsed 3x with PBS, incubated with avidin-biotin-peroxidase complex in PBS with TritonX-100 at a dilution of 1:1000, and

rinsed 3x. The striosomes were visualized by 3,3-diaminobenzidene with nickel intensification to yield black color (Figure 4-4).

Image analysis

The neuronal cell counts within well-identified striosomes were made using ImagePro (Media Cybernetics), and average cell counts (cells/μm^2) were calculate.

Results

No difference in striosomal neuronal counts across development ($F(2,6)=0.53$, $p=0.62$). The striosomal neuronal counts among trajectory groups at 6 weeks pos-weaning cannot be reported here due to the small number of animals.

Discussion

The present experiments tested the hypothesis that the development of spontaneous stereotypy in deer mice is linked to gradual decrease in activity of the indirect basal ganglia pathway based on the findings in Chapter 2. In contrast to our hypothesis, CO activity in STN did not differ across development. When CO activity in STN at 6 weeks post-weaning was assessed by trajectory group, however, the difference was apparent; animals in Traj 2 and 3 showed significantly lower level of CO activity in STN as compared to animals in Traj 1. Thus this finding suggests that the development of stereotypy is linked to the gradual decrease of the indirect pathway activity.

The finding in Experiment 1 compliments our previous findings that lower CO activity in STN was negatively correlated with the rates of stereotypy in adult mice (<8 weeks post-weaning) (Chapter 2). These results with the finding by Presti and Lewis (2005) collectively suggest that the development and expression of stereotypy in deer mice is linked to decreased activity of the indirect basal-ganglia pathway. Consistent

with our finding in Chapter 2, other regions such as the ventromedial striatum, SNpc, and SNpr showed higher CO activity in Traj 1 animals as compared to Traj 2 and 3 animals at 6 weeks post-weaning. There was general decrease in CO activity over development in SNpc and SNpr, although the CO activity of the ventromedial striatum increased from 1 week to 6 weeks post-weaning. The decreased activity of the nucleus accumbens was also noted at 6 weeks post-weaning, but no difference was found across development.

The medial striatum has been implicated in the mediation of behavioral flexibility (Ragozzino et al., 2009), and we have previously shown that high rates of stereotypy in deer mice were associated with perseverative behaviors in a reversal learning task (Tanimura et al., 2008). These data support the differential metabolic activity of the medial striatum in deer mice of different developmental trajectory. The decreased activity in SNpc and SNpr is likely secondary to the altered activity of STN via monosynaptic glutamatergic inputs to respective areas. Similarly there were negative correlations between STN, SNpc, SNpr, and nucleus accumbens CO activity and rates of stereotypy at 6 weeks post-weaning. The CO activity of the medial striatum, which was found significantly different among trajectory groups, however, was not significantly correlated with rates of stereotypy.

Experiment 1 utilized the novel approach to test the interaction between neurobiological mechanisms and developmental trajectory groups. The group-based trajectory modeling described in Chapter 3 allowed us to categorize animals into three groups based on their longitudinal behavioral data (1, 3.5, and 6 weeks post-weaning), and assessed the developmental changes of CO activity in STN. There is a problem,

however, where developmental trajectories of animals cannot be determined using the trajectory modeling prior to the 6 weeks post-weaning time-point.

For example, the inability to recruit more animals at 3.5 weeks post-weaning was largely because trajectories can only be identified subjectively at the mid-point of the development. No statistical difference was seen in STN CO activity at 3.5 weeks post-weaning period between high versus low-stereotypy animals. The data from this time-point is particularly important to distinguish two trajectory groups (Traj 2 and 3), both of which result in high rates of stereotypy at 6 weeks post-weaning. Specifically, all the differences found among trajectory groups were between Traj 1 versus Traj 2 and 3. Traj 2 and Traj 3 cannot be behaviorally distinguished unless the stereotypy data from 1 and 3.5 weeks post-weaning are analyzed. Further investigation, particularly of earlier developmental time-points (e.g., 3.5 weeks post-weaning), may dissociate between these trajectory groups and CO activity.

This non-significant difference of STN activity among three trajectory groups at 3.5 weeks post-weaning could be due to a relatively small number of animals in each trajectory group or lack of much variability in stereotypy counts among the animals. Conversely, one can also argue that the relatively late decrease in STN activity reflects a long-term neuroadaptation to the expression of repetitive behavior. In either case, better identification and categorization of animals in trajectory groups at the early developmental time-point will be necessary to advance our knowledge further on the underlying mechanism linked to the development of stereotypy.

Experiments 2 and 3 examined the association between the striosomal pathway and the development and expression of stereotypy in deer mice. The study by Canales

and Graybiel (2000) suggests that the degree of repetitive behavior expression induced by intermittent psychostimulant administration is best predicted by the relative increase and decrease in activity of striosomes and matrix respectively. In addition if the development of stereotypy is associated with lack of experience in impoverished environment, which limit animals' naturalistic species-typical behaviors, literature suggests that matrix activity would be decreased (e.g., Brown et al., 2002). Contrary to our hypothesis, the present study showed that the striosomal metabolic activity, volume, and cell counts were not associated with stereotypy in deer mice. The statistical difference found in matrix across development seem to be largely due to striatal CO activity differences described in Experiment 1, not likely due to the differential activation of striosome versus matrix. Moreover no statistical differences of striosomal and matrix CO activity were found in association with stereotypy. It needs to be noted that MOR1-labled striosomes were superimposed onto the CO-stained striatal sections in this study, assuming that striosome structures are preserved in the adjacent sections. Although we were conservative and avoided small striosomes on the sections, which likely disappear on the adjacent sections, we cannot be absolutely certain that the area selected on CO-stained sections were otherwise MOR1-labeled striosomes.

Our hypotheses came from the finding by Canales and Graybiel (2000) that the preferential activation of striosome versus matrix by chronic administration of psychostimulants (apomorphine, amphetamine, and cocaine) which induce repetitive behaviors in rats. In this study, stereotyped behavioral response to the acute psychostimulant challenge to assess behavioral sensitization was best predicted by ISMP. If spontaneous stereotypy in deer mice is also mediated by the same preferential

activation of striosome, metabolic capacity shown by CO histochemistry should be differentially detected in the striosomes and matrix. Our present data, however, suggest it is unlikely that a preferential increase in striosomal activation is linked to repetitive behaviors in deer mice. Conversely, if spontaneous stereotypy is due to the preferential activation of the striosomes, the manipulations to drive this mechanism by chronic administration of psychostimulants should exacerbate the expression and development of stereotypy in deer mice. This hypothesis was tested in the experiments described in Chapter 5.

In addition, based on the findings in Chapter 3 and 4, we attempted in subsequent studies (Chapters 5 and 6) to alter the developmental trajectory of repetitive behavior by administration in the early post-weaning period of specific pharmacological agents. In Chapter 5, we employed repeated administration of amphetamine which was hypothesized to induce neuroadaptations likely to augment the development and expression of stereotypy. In Chapter 6, we employed the same drug cocktail used in Chapter 4 to test the hypothesis that early drug treatment would result in long-term attenuation of the development and expression of stereotypy.

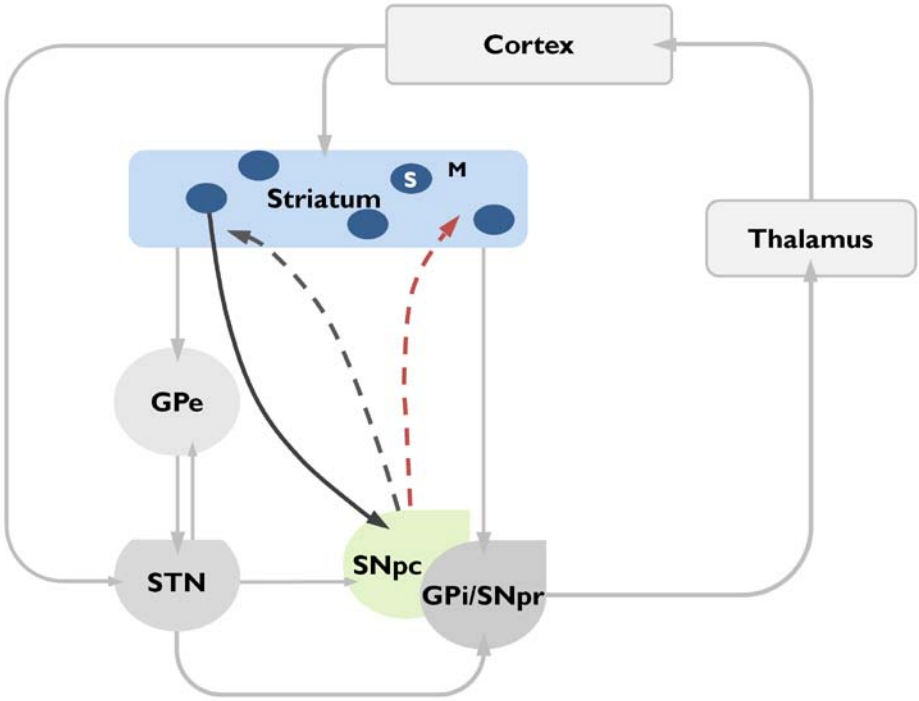


Figure 4-2. Schematics of the striosomal pathway. Striosomes (S: shown in dark blue as compared to Matrix shown in light blue) comprise approximately 15% of the striatum. Medium spiny neurons located in striosomes project directly to SNpc, modulating the dopaminergic neurons projecting back to the striosome. These dopaminergic projections act positively on D_1 dopamine receptors (shown in dotted red line) and negatively on D_2 dopamine receptors (shown in dotted black line).

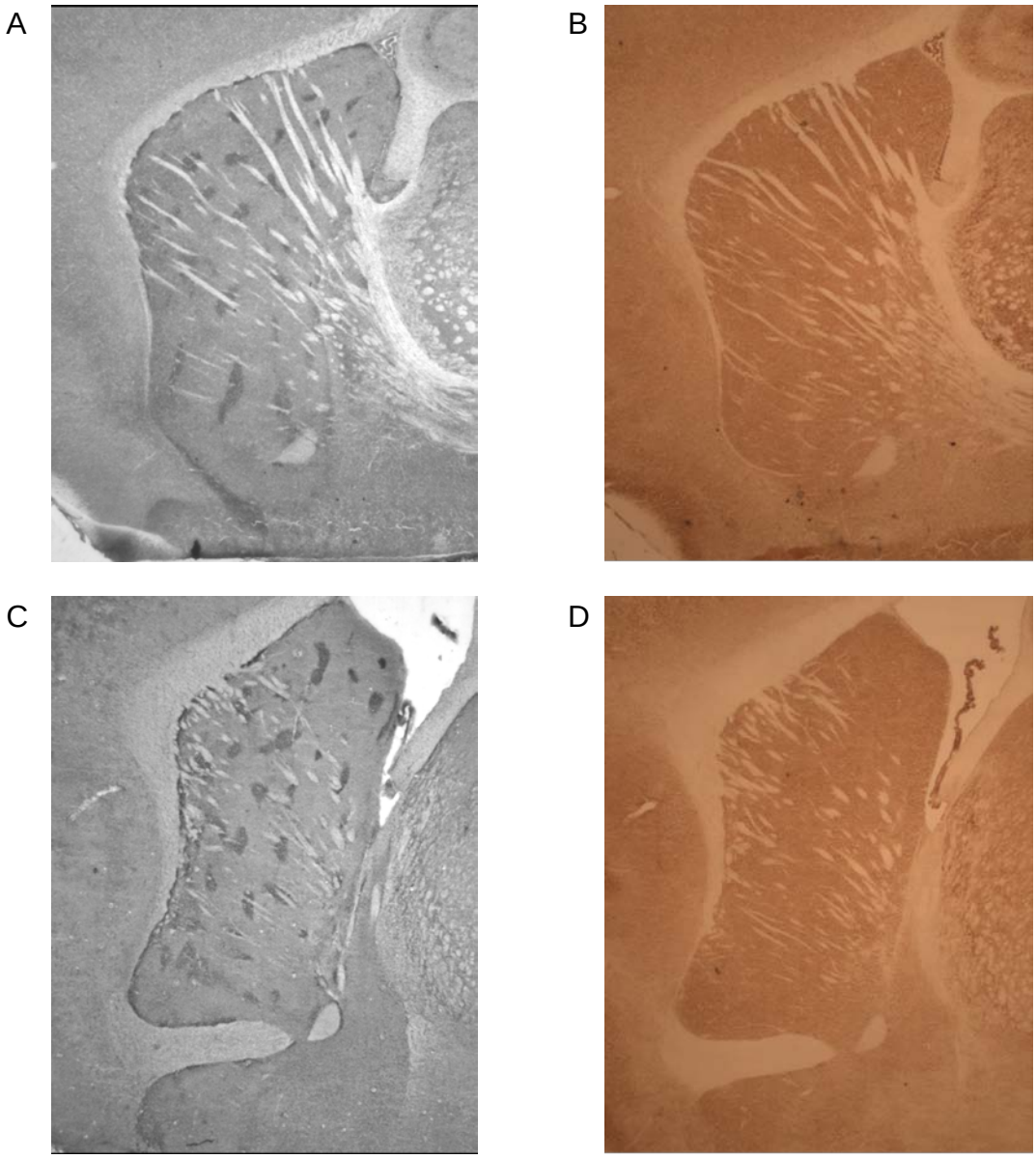


Figure 4-3. MOR1 stained sections (A,C) and CO-stained adjacent sections (B,D). MOR1-stained sections were superimposed onto the adjacent CO-stained sections to measure striosome and matrix optical density.

Table 4-4. CO activity (µmol/min/g) of striosome and matrix in deer mice at three developmental time-points (mean ± SEM in parentheses).

	1wk	3.5wk	6wk	*P*-value
Striosome	51.75 (1.61)	52.99 (0.99)	54.66 (0.64)	0.10
Matrix	50.60 (2.45)	52.41 (1.01)	53.34 (0.72)	0.04
ISMP	0.9837 (0.0157)	0.9744 (0.0070)	0.9706 (0.0456)	0.51

Table 4-5. CO activity (µmol/min/g) of striosome and matrix in three developmental trajectories at 6 weeks post-weaning (mean ± SEM in parentheses).

	Traj 1	Traj 2	Traj 3	*P*-value
Striosome	55.04 (1.09)	53.91 (0.68)	55.72 (2.19)	0.54
Matrix	56.02 (1.15)	55.76 (0.74)	58.14 (2.22)	0.41
ISMP	0.9828 (0.0063)	0.9672 (0.0074)	0.9584 (0.0046)	0.13

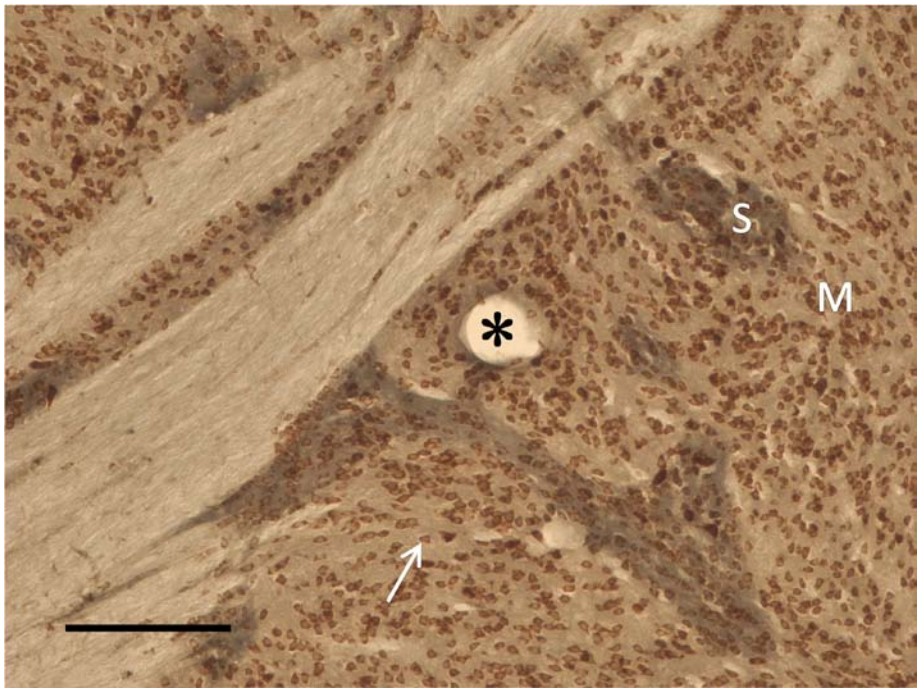


Figure 4-4. Photomicrograph of striatal tissue immunostained for striosomes and neurons. Striosomes (S) are shown in blue-black surrounded by matrix (M). NeuN labeled neurons are indicated with arrows. A blood vessel is indicated with *. Scale bar =50µm.

CHAPTER 5
AMPHETAMINE-INDUCED SENSITIZATION AND REPETITIVE BEHAVIOR

Introduction

Stereotyped behavior typically refers to motor responses of unknown function or purpose that are performed repetitively in a nearly identical manner such that the behavior often appears aberrant or abnormal (Sprague and Newell, 1996; Mason and Rushen, 2006). Stereotypies and related repetitive behaviors are diagnostic for autism spectrum disorders and represent a common component of other developmental, genetic, and neuropsychiatric disorders (Lewis and Bodfish, 1998; Bodfish et al., 2000). Although these restricted, repetitive behaviors have been linked to alterations in cortico-basal ganglia circuitry (Lewis et al., 2007), an understanding of specific mechanisms of action that give rise to the development and expression of stereotypies and related repetitive behaviors in clinical populations remains elusive.

Animal models of aberrant repetitive behavior in neurodevelopmental disorders generally fall into three classes: repetitive behavior associated with targeted insults to the CNS (e.g., gene deletion); repetitive behavior induced by pharmacological agents; and repetitive behavior associated with restricted environments and experience (see Lewis et al., 2007 for a review). Studies of drug-induced (e.g., amphetamine, cocaine) stereotyped behavior have made the largest contribution by far to our knowledge of the neurobiological basis of repetitive motor behaviors. These studies have highlighted the importance of cortical-basal ganglia circuitry and the neurotransmitter dopamine, particularly in models of psychostimulant-induced stereotypy.

Repeated, intermittent psychostimulant (e.g., amphetamine, cocaine) administration has been shown by a number of groups to be associated with the

potentiated expression of locomotion and stereotypy (Robinson and Berridge, 2003; Vezina, 2004). This outcome reflects behavioral sensitization, a process by which repeated psychostimulant administration results in a progressive increase in the efficacy of a psychostimulant drug. Intermittent psychostimulant administration has also been shown to sensitize animals to the effects of environmental stressors. Reciprocally, repeated intermittent exposure to stressors can sensitize animals to the effects of psychostimulants. Thus, stressors and psychostimulants exhibit cross-sensitization (Antelman et al., 1980; Nikulina et al., 2004).

Sensitization may have particular relevance for understanding processes such as the escalation of drug use to drug craving and abuse (Robinson and Berridge, 2003), the transition from goal-directed to habitual responding (Graybiel, 2008), and the development of L-DOPA-induced dyskinesias in Parkinson's disease (Cenci et al., 1999). Sensitization has been associated with long-lasting functional changes in cortico-striatal circuitry and can thus be viewed as a model of pathological neuroadaptation. For example, preferential activation of striatal striosomes or patches has been reported in sensitized animals at much lower doses of amphetamine than those required to induce this effect in non-sensitized animals (Vanderschuren et al., 2002). As mentioned previously, preferential activation of striosomes versus matrix has been shown to be highly correlated with the occurrence of drug-induced stereotyped behavior (Canales and Graybiel, 2000b). Repeated exposure to psychostimulants also induces alterations that progress from ventral to dorsal areas of the striatum which likely mediates augmented stereotypy. Such findings are certainly consistent with evidence for dopamine modulation of synaptic plasticity in cortico-striatal pathways.

Cabib (2006) has hypothesized that spontaneous stereotypy associated with environmental restriction may be mediated by the same neurobiological mechanisms that give rise to stress-induced or drug-induced sensitization. Such a hypothesis is linked to the notion that confined or restricted environments that give rise to stereotypy are inherently stressful and that stress alters dopamine neurotransmission and, like psychostimulants, can result in neuronal/behavioral sensitization. This sensitization hypothesis of stereotypy would thus predict that any stimulus or experience capable of activating mesoaccumbens dopamine should also be able to promote stereotypy.

The purpose of this study was to determine if exposure to repeated doses of amphetamine, known to induce neuronal/behavioral sensitization, would significantly alter the expression and development of spontaneous repetitive behavior exhibited by deer mice (*Peromyscus maniculatus*). Both male and female deer mice develop high rates of persistent, spontaneously emitted stereotypy consisting of repetitive vertical jumping and backward somersaulting when housed under standard laboratory conditions (Powell et al., 2000; Presti et al., 2002; Hadley et al., 2006). Support for the importance of environmental restriction in generating stereotypy in deer mice comes from our studies of the attenuation of such behavior by environmental enrichment (see Lewis et al., 2006 for review).

Four experiments were performed to assess whether neuronal/behavioral sensitization might play a role in the expression or development of spontaneous or non-drug induced stereotypies in deer mice. These experiments included examining the effects of context-independent sensitization on adult animals reared in either conventional (Experiment 1) or enriched (Experiment 2) housing. In addition, younger

animals reared in conventional housing were also used to examine either context-independent or context-dependent sensitization (Experiment 3 and 4 respectively).

General Methods

Subjects

All deer mice (*Peromyscus maniculatus*) were obtained from the breeding colony maintained in our laboratory, and maintained on a 16:8-h light/dark cycle with lights off at 10:00 AM. Rodent chow and water were available *ad libitum*. The room was maintained at 20-25^{o}C and 50-70% humidity. Mice were weaned at 21 days of age. All procedures were performed in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Florida Institutional Animal Care and Use Committee.

Stereotypy Assessment

Rates of spontaneous stereotypy (hindlimb vertical jumping and backward somersaulting) were assessed using a modified automated photocell detection apparatus obtained from Columbus Instruments (Columbus, OH). The session consisted of the eight hours of the dark cycle. The testing protocol involved removing mice from their home cages and placing them singly in testing cages (22 x 25 x 28 cm) made of Plexiglas. The mice were left undisturbed for two to three hours for habituation and recovery from the stress of handling prior to the beginning of the dark cycle. Food and water were provided. All sessions were digitally video-recorded for further identification of behavioral topographies and accuracy of the automated counters. Each animal received a stereotypy score that represented the average stereotypy frequency per hour.

Amphetamine Administration and Behavioral Assessment

Injections of either saline or d-amphetamine (Sigma) were administered in the light cycle and were spaced approximately 10 hours apart (9:30 AM and 7:30 PM) over a seven-day period. After a seven day drug-free period, all mice received a single injection of 2.5 mg/kg of d-amphetamine given during the light cycle at approximately 7:30 PM. The behavioral response to this pharmacological challenge was assessed immediately following drug administration. This was done by video-recording individual mice in the test chambers described previously for one hour following the acute amphetamine challenge. The frequencies of rearing, locomotion, and stereotypies were recorded using either video, Ethovision (Noldus, Netherlands, for Experiment 4), and/or the automated photocell detection apparatus described previously. Seven days after the acute amphetamine challenge, all mice were assessed for stereotypy levels using the method described in a previous section.

Data Analysis

For all four experiments, the effects of a seven-day regimen of amphetamine on the frequency of spontaneous stereotypies were assessed using a two-factor (dose and time) repeated measures analysis of variance (ANOVA). For all four experiments, group differences in behavioral responses recorded over one hour following acute amphetamine challenge were analyzed by two-tailed t-tests. A one-factor analysis of covariance (ANCOVA) was also used in Experiment 2 to account for differences in ages between the two groups. In addition, the association between baseline stereotypy scores and the motor response to acute amphetamine challenge was analyzed by Pearson correlations. Effects were considered significant when $p<0.05$.

Specific Methods and Results

Experiment 1: Context-Independent Sensitization in Conventionally Housed Adult Mice

Methods

Forty-six male mice (63-105 days of age) were group-caged (4-5 mice/cage) from weaning in standard rodent cages (48 x 27 x 15 cm) before and during the experimental procedures. All mice were tested for baseline levels of stereotypy as described in the previous section. These animals were then randomly assigned to one of three groups (n=12) and were administered two doses of saline, 2.5, or 5.0 mg/kg of d-amphetamine subcutaneously for seven consecutive days.

Results

Mice in the two amphetamine pre-treatment groups exhibited higher levels of motor activity following drug challenge compared to saline pre-treatment controls (Table 5-1). Mice pre-treated with 2.5 mg/kg amphetamine exhibited significantly higher levels of rearing one-hour post-injection ($t(22)=-2.09$, $p<0.05$) and higher frequency of locomotor activity (mid-line crosses of the test chamber) for the first 30 min post-injection ($t(22)=-2.11$, $p=0.04$) compared to mice treated with saline. For mice pre-treated with the 5.0 mg/kg amphetamine, significantly higher levels of locomotor activity were seen for both one-hour ($t(22)=-3.08$, $p<0.01$) and the first 30-min post injection ($t(22)=-2.21$, $p=0.04$), but no significant increase in the frequency of rearing ($p=0.09$ for one hour, $p=0.17$ for 30 min). No significant differences were seen between the 2.5 and 5.0 mg/kg pre-treatment groups on any of the measures. Drug challenge was also not associated with any group differences in stereotyped vertical jumping ($p=0.85$ for one hour, $p=0.75$ for 30 min). With regard to spontaneous stereotypy, a two-factor ANOVA

indicated no main effect of treatment (saline, 2.5, or 5.0 mg/kg amphetamine) ($F(2,33)=1.19$, $p=0.32$) or time (baseline, post-testing) ($F(1,33)=1.67$, $p=0.21$) nor was there a treatment-by-time interaction ($F(2,33)=0.41$, $p=0.67$) (Figure 5-1).

Experiment 2: Context-Independent Sensitization in Environmentally Enriched Adult Mice

The relatively high levels of spontaneous stereotyped behavior typical of adult deer mice may well reflect long-term neuroadaptations, perhaps attenuating subsequent neuroplasticity in these animals. If this is the case, neuronal sensitization using a psychostimulant may not have an appreciable effect on spontaneous stereotypy. To test this potential outcome, Experiment 2 employed adult animals that had been reared in larger, more complex environments (environmental enrichment) which we have shown repeatedly attenuates the development of stereotypy (see Lewis et al., 2006).

Methods

Twenty-two deer mice (46-123 days of age; 11 female and 11 male) were group-caged (5-6 same sex mice/cage) in large dog kennels (122 x 81 x 89 cm) from weaning (PND21). This environmentally complex housing consisted of two extra levels of floors constructed of galvanized wire mesh and connected by ramps of the same material. Bedding, a running wheel, shelters, and various other objects were placed in each kennel. In addition to *ad libitum* food and water, one oz. of Cockatiel vita seed was scattered throughout the kennel three times each week to encourage foraging behavior. A running wheel remained undisturbed in the kennel, but other objects were removed and replaced with clean novel objects on a weekly basis. Mice were not handled or disturbed prior to the baseline stereotypy testing.

All mice were tested for baseline levels of stereotypy as described in the previous section. They were then caged in standard rodent cages (5-6 mice/cage) with one shelter to maintain some complexity in the environment. This housing change was necessitated in order to minimize the effect of handling at the time of drug administration. These animals were then randomly assigned to one of two groups (n=11) and were administered two doses of either saline or 5.0 mg/kg of d-amphetamine subcutaneously for seven consecutive days as in Experiment 1. The subsequent challenge and behavioral assessment procedures were the same as described in Experiment 1.

Results

Mice in the amphetamine pre-treatment group exhibited higher levels of rearing one-hour post-challenge compared to saline pre-treatment controls ($t(20)=-2.58$, $p=0.02$) (Table 5-2), although no significant differences in the frequency of locomotor activity were observed in response to drug challenge ($p=0.25$ for one hour, $p=0.46$ for 30 min). A one-factor ANCOVA confirmed these group effects when differences in age were removed from the model. Drug challenge was also not associated with any increase in stereotyped vertical jumping ($p=0.32$ for one hour, $p=0.29$ for 30 min).

With regard to spontaneous stereotypy, a two-factor ANOVA indicated a main effect of time (baseline, post-testing; $F(1,20)=18.03$, $p<0.001$), but no main effect of treatment (saline, 5.0 mg/kg amphetamine) ($F(1,20)=0.09$, $p=0.76$) and no treatment-by-time interaction ($F(1,20)=2.02$, $p=0.17$) (Figure 5-2). Spontaneous stereotypy in both groups increased after amphetamine administration. This might be due to the change in housing condition from the large kennels to the smaller laboratory cages with shelters.

Experiment 3: Context-Independent Sensitization in Conventionally Housed Young Mice

This experiment used conventional housing but was conducted with younger animals at an age that we have shown predates the expression of asymptotic adult levels of stereotypy. Use of younger animals was hypothesized to increase the potential effect of intermittent amphetamine on neuroadaptations in cortico-basal ganglia circuitry.

Methods

Twenty-four male deer mice (30 days of age) were group-caged (2-3 mice/cage) in standard rodent cages before and during the experimental procedures. All mice were tested for baseline levels of stereotypy as described previously. These animals were then randomly assigned to one of two groups and were administered either saline (n=12) or 2.5 mg/kg d-amphetamine (n=11) using the identical protocol described in Experiment 1. In addition, on a subsequent occasion approximately 12-14 days later, all mice were assessed for a second time to determine the effects of amphetamine sensitization at an age (approximately PND60) when rates of spontaneous stereotypy in deer mice reach asymptote (unpublished observations).

Results

Mice in the amphetamine pre-treatment group exhibited higher levels of both rearing ($t(21)=-2.71$, $p=0.01$) and locomotor activity ($t(21)=-2.20$, $p=0.04$) for the first 30-min post-challenge compared to saline pre-treatment controls (Table 5-3). Drug challenge was also associated with an increase in stereotyped vertical jumping which was significant for the one-hour post-injection data ($t(21)=-2.33$, $p=0.03$).With regard to spontaneous stereotypy, a two-factor ANOVA indicated no main effect of treatment

(saline, 2.5 mg/kg amphetamine) ($F(1,21)=1.14$, $p=0.30$) but a significant effect of time (baseline, post-testing, follow-up testing; $F(2,42)=19.66$, $p<0.001$) (Figure 5-3) as expected from our previous observations in younger mice. There was no treatment-by-time interaction ($F(2,42)=0.85$, $p=0.44$).

Experiment 4: Context-Dependent Sensitization in Young Mice

The behavioral expression of sensitization has been shown to be influenced by the environmental context in which it has been established. Thus, animals repeatedly injected with the drug in a consistent and distinct environment exhibit an increased behavioral reactivity to the drug compared to animals receiving the same drug pre-treatment outside of the test apparatus. Thus, Experiment 4 used younger, conventionally housed animals but employed a context-dependent sensitization protocol.

Methods

Twenty-one male deer mice (30 days of age) were group-housed in standard rodent cages before and during the experimental procedures. All mice were tested for baseline levels of stereotypy as described previously. Mice were randomly assigned to either the saline (n=11) or 2.5 mg/kg of d-amphetamine (n=10) group. The same protocol for injections was followed as described in Experiment 3 (twice per day for 7 days) with one important difference. After each injection, the mice were immediately placed singly in testing cages for one hour rather than being returned to their home cage.

Following a seven day drug free period, all mice received an acute challenge of 2.5 mg/kg d-amphetamine in the same testing environment as previously used for the prior injections of amphetamine or saline. Behavioral responses to acute amphetamine

were recorded for one hour and included the frequency of rearing, locomotion (total distance travelled, cm), and stereotypy. Data on total distance travelled was acquired using the EthoVision system (Noldus, Wageningen, Netherlands) which is a fully automated video tracking system. Rearing and stereotypy were assessed using methods as described in the previous experiments. Spontaneous stereotypy was then assessed again a week following acute amphetamine challenge when the animals were approximately PND60.

Results

Amphetamine pre-treated mice exhibited significantly greater locomotor activity (distance travelled) for one-hour post-injection ($t(19)=-2.48$, $p=0.02$) and for the first 30 min post-injection ($t(19)=-2.26$, $p=0.04$), but no significant increase was found in the frequency of rearing ($p=0.11$ for one hour, $p=0.17$ for 30 min) compared to saline pre-treated mice (Table 5-4). Drug challenge was not associated with an increase in stereotyped vertical jumping ($p=0.08$ for one hour, $p=0.07$ for 30 min). With regard to spontaneous stereotypy, no main effect of treatment (saline, 2.5 mg/kg amphetamine) ($F(1,19)=0.18$, $p=0.67$) was observed but there was a significant effect of time (baseline, post-testing, follow-up testing; $F(2,38)=23.9$, $p<0.001$) (Figure 5-4). There was no treatment-by-time interaction ($F(2,38)=0.07$, $p=0.93$).

Spontaneous stereotypy and response to amphetamine challenge

We also examined the association between baseline levels of stereotypy and response to the acute challenge of amphetamine. No systematic relationship was found between baseline levels of stereotypy and drug challenge induced motor activity (rearing plus locomotion) in adult, conventionally housed mice ($r=0.15$, $p=0.38$) (Experiment 1) (Figure 5-5). Similarly, no association was found in adult,

environmentally enriched mice (r=-0.24, p=0.28) (Experiment 2) and younger mice (r=-0.13, p=0.55; r<0.01, p=0.98) (Experiments 3 and 4 respectively). No systematic association was seen between baseline levels of stereotypy and response to acute amphetamine in amphetamine pre-treated mice from all four experiments. In addition, this was also the case when only saline pre-treated control mice were used.

Discussion

In the present study, we sought to determine if we could behaviorally sensitize deer mice using amphetamine and whether such sensitization would significantly impact the expression or development of spontaneous (non-drug related) stereotypy in these mice. Experiment 1 employed context-independent sensitization in adult mice. Amphetamine pre-treated mice (both doses) showed significant increases in motor activity relative to saline controls following drug challenge. Despite behavioral evidence for sensitization, amphetamine pre-treated mice were not different from saline controls in their expression of spontaneous stereotypy. Environmentally enriched adult animals exhibited less evidence of sensitization, with amphetamine pre-treatment resulting in a significant increase in only rearing at one hour following drug challenge. As in Experiment 1, no group differences in spontaneous stereotypy were found. The last two experiments with younger mice demonstrated significant behavioral sensitization, although significant context-independent sensitization (Experiment 3) was seen only at the 30 min, whereas context-dependent sensitization (Experiment 4) was seen at the 60 min time point following challenge. In any case, sensitization did not result in any difference in the expression and development of spontaneous stereotypy.

The present results show that repeated, intermittent psychostimulant exposure can successfully induce behavioral sensitization in the genus *Peromyscus*. In addition,

significant behavioral sensitization can be induced in deer mice using either a context-independent or a context-dependent protocol. This finding is somewhat at odds with previous reports demonstrating that repeated psychostimulant administration in the testing context produces a much more robust locomotor sensitization (Cabib, 1993; Badiani et al., 1995; Anagnostaras and Robinson, 1996; Anagnostaras et al., 2002; Mattson et al., 2008). Behavioral sensitization was least apparent under context-independent conditions in adult environmentally enriched deer mice. The relative absence of amphetamine-induced sensitization in environmentally enriched mice is consistent with a previous report showing that enrichment had a neuroprotective effect with regard to amphetamine-induced sensitization (Bardo et al., 1995). In addition, context-dependent sensitization appeared to have a more robust effect than context-independent sensitization in younger mice, given the large increase in locomotor activity observed at 30 and 60 min.

Locomotion and rearing were used as indices of sensitized motor activity. Interestingly, vertical jumping following drug-challenge was typically not significantly increased except in younger amphetamine pre-treated mice tested in context-independent conditions (Experiment 3). In our previous work, neither systemically nor intrastriatally administered apomorphine increased spontaneous cage stereotypies in deer mice acutely, although other repetitive behaviors (e.g., stereotyped sniffing) were observed (Presti et al., 2002; Presti et al., 2004). We have similar unpublished observations with systemic amphetamine in drug-naive deer mice. The present results indicate that a psychostimulant can increase environmentally related stereotypies in drug-sensitized animals. Despite clear evidence of behavioral sensitization in

Experiments 1, 3, and 4, no differences were found in levels of spontaneous stereotypy tested one-week post challenge in mice exposed to amphetamine pre-treatment when compared to controls. The only systematic differences in spontaneous stereotypy were observed in younger mice (Experiments 3 and 4) and these differences were associated with developmental age.

Neuronal sensitization has been advanced as a mechanism responsible, at least in part, for the development of environmentally related stereotypies (Dantzer, 1986; Cabib, 2006). Little evidence has been available to evaluate such an assertion, however. Cabib and Bonaventura (1997) examined the ability of food restriction to induce stereotypy and to induce sensitization to psychostimulants in two inbred mouse strains. Food restriction was found to induce cage stereotypies in drug-naive mice as well as behavioral sensitization to amphetamine. These effects were observed in DBA but not C57BL/6 mice, however. These investigators concluded that the parallel strain-dependent susceptibility to cage stereotypy and behavioral sensitization provides evidence for a common neurobiological mechanism. Although these findings provide some indirect support for a common mechanism, few other data are available. The present findings, as far as we are aware, are the first effort to assess directly the effects of neuronal/behavioral sensitization on non-drug induced stereotypy. As we have indicated, sensitized animals did not express spontaneous stereotypies at a higher rate than non-sensitized controls. Moreover, if spontaneous stereotypy is a consequence of neuronal/behavioral sensitization, then mice exhibiting high levels of such repetitive behavior should exhibit a potentiated response to drug challenge. The level of baseline stereotypy, however, did not predict response to acute amphetamine in either pre-

treatment group. We should hasten to add, though, that a number of theorists have advanced a pre-eminent role for stress, and stress-induced sensitization, in the genesis of stereotypy. Although, there is evidence to support the cross-sensitization of stress and psychostimulants (Conversi et al., 2008), we only employed stimulant-induced sensitization. Future experiments should employ a stress-induced sensitization model to examine further the relationship of stereotypy and sensitization.

Neuronal/behavioral sensitization is associated with long-lasting changes in brain function in striatum and nucleus accumbens as well as prefrontal cortex (Vanderschuren and Kalivas, 2000; Ehrlich, et al., 2002; Robinson and Berridge, 2003; Mattson et al., 2008). For example, behavioral sensitization results in the potentiation of dopamine efflux in the nucleus accumbens by a number of different drugs (Robinson and Berridge, 2000). In addition, sensitization results in D1 dopamine receptor supersensitivity in the ventral striatum, altered gene expression in the caudate nucleus (Canales and Graybiel, 2000a) and alterations in striatal glutamate signaling (Vanderschuren and Kalivas, 2000). Sensitization is also associated with morphological changes including persistent alterations in dendritic length and branching in the cortex and the striatum (Robinson and Berridge, 2000). The potentiated stereotyped behavior that is observed after chronic, intermittent amphetamine or cocaine is strongly predicted, across several species, by a preferential activation of striosomal striatal cells (Canales and Graybiel, 2000b). These and many other changes that have been documented reflect a process of pathological neuroadaptation in cortical-basal ganglia circuitry.

Our previous studies (see Lewis et al., 2007 for a review) have highlighted the importance of this circuitry in the development and expression of spontaneous stereotypy in deer mice. Thus, one important question is whether sensitization-related neuroadaptations in cortical-basal ganglia circuitry are similar to those neuroadaptations that underlie spontaneous or environmentally linked stereotypy. Our results lead to the conclusion that stereotypy associated with environmental restriction and behavioral sensitization do not appear to share common mechanisms. As indicated earlier, drugs that induce stereotypies do not always enhance an animal's spontaneous cage-induced stereotypies and often elicit stereotypies that are quite different in form. Moreover, sensitized animals did not display greater levels of spontaneous stereotypy. In addition, greater levels of stereotypy did not predict an augmented (sensitized) response to a psychostimulant in drug-naive animals. Taken together, it can be concluded that the hypothesis that spontaneous stereotyped behavior, at least in deer mice, reflects neuronal/behavioral sensitization is not supported by the current findings. Whether sensitization-related neural mechanisms may play a role in aberrant repetitive behavior observed in neurodevelopmental disorder like autism remains an open question.

Table 5-1. Effects of saline or amphetamine (2.5 or 5.0 mg/kg) pre-treatment on the motor response to a subsequent acute challenge of amphetamine in adult, conventionally housed mice. Values expressed are group means with SEM in parentheses, n=12 per group. * represents statistical significance at $p<0.05$ as compared to saline group.

	Rearing		Locomotion	
	30 min	60 min	30 min	60 min
Saline	139.9 (28.0)	236.5 (51.8)	67.6 (15.0)	129.3 (28.1)
2.5 mg/kg amphetamine	219.7 (27.4)	505.9 (117.9)*	113.5 (14.4)*	326.3 (105.9)
5.0 mg/kg amphetamine	205.8 (36.9)	397.4 (74.3)	128.8 (23.2)*	382.2 (77.0)*

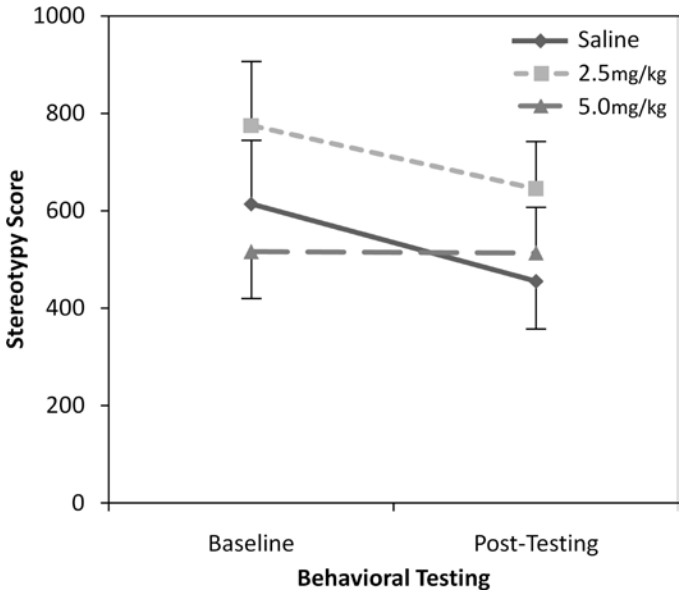


Figure 5-1. Rates of spontaneous stereotypy before and after amphetamine administration in adult, conventionally housed mice (mean ± SEM).

Table 5-2. Effects of saline or amphetamine (5.0 mg/kg) pre-treatment on the motor response induced by a subsequent acute challenge of amphetamine in adult, environmentally enriched mice. Values expressed are group means with SEM in parentheses, n=11 per group. * represents statistical significance at $p<0.05$ as compared to saline group.

	Rearing		Locomotion	
	30 min	60 min	30 min	60 min
Saline	107.1 (19.0)	153.6 (32.5)	66.3 (11.6)	99.5 (24.6)
2.5 mg/kg amphetamine	171.4 (26.9)	300.5 (46.8)*	79.0 (12.4)	143.7 (28.1)

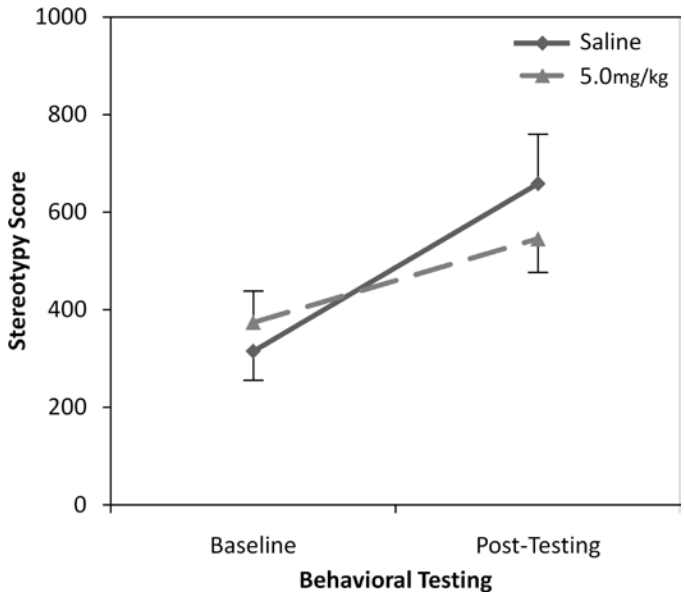


Figure 5-2. Rates of spontaneous stereotypy before and after amphetamine administration in adult, environmentally enriched mice (mean ± SEM).

Table 5-3. Effects of saline or amphetamine (2.5 mg/kg) pre-treatment on the motor response induced by a subsequent acute, context-independent challenge of amphetamine in younger, conventionally housed mice. Values expressed are group means with SEM in parentheses, n=12/11. * represents statistical significance at $p<0.05$ as compared to saline group.

	Rearing		Locomotion	
	30 min	60 min	30 min	60 min
Saline	118.3 (24.5)	272.0 (87.2)	63.4 (13.0)	170.3 (68.0)
2.5 mg/kg amphetamine	220.0 (28.6)*	422.9 (97.1)	101.3 (11.1)*	230.3 (46.9)

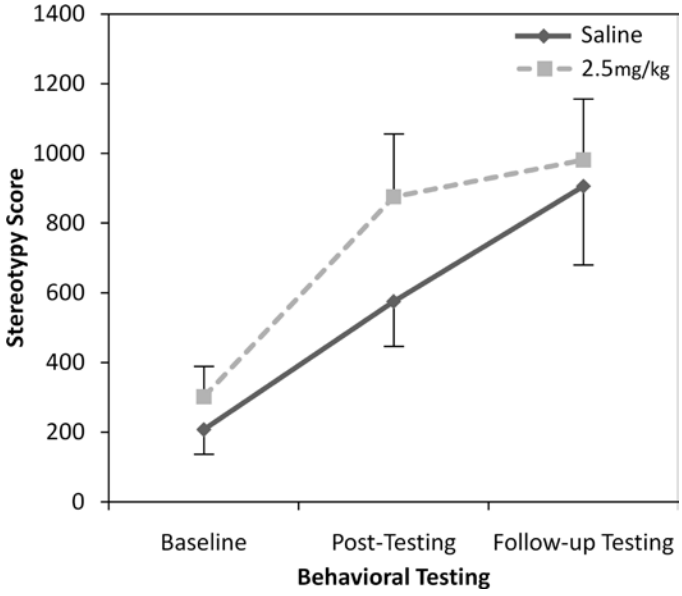


Figure 5-3. Rates of spontaneous stereotypy before and after context-independent amphetamine administration in younger, conventionally housed mice (mean ± SEM).

Table 5-4. Effects of saline or amphetamine (2.5 mg/kg) pre-treatment on the motor response induced by a subsequent acute, context-dependent challenge of amphetamine in younger, conventionally housed mice. Locomotion is expressed as total distance traveled (cm). Values expressed are group means with SEM in parentheses, n=11/10. * represents statistical significance at $p<0.05$ as compared to saline group.

	Rearing		Locomotion	
	30 min	60 min	30 min	60 min
Saline	60.5 (16.3)	64.7 (18.3)	2399.2 (431.4)	3471.0 (562.1)
2.5 mg/kg amphetamine	107.5 (29.3)	150.2 (49.5)	5180.6 (1206.2)*	7917.8 (1781.8)*

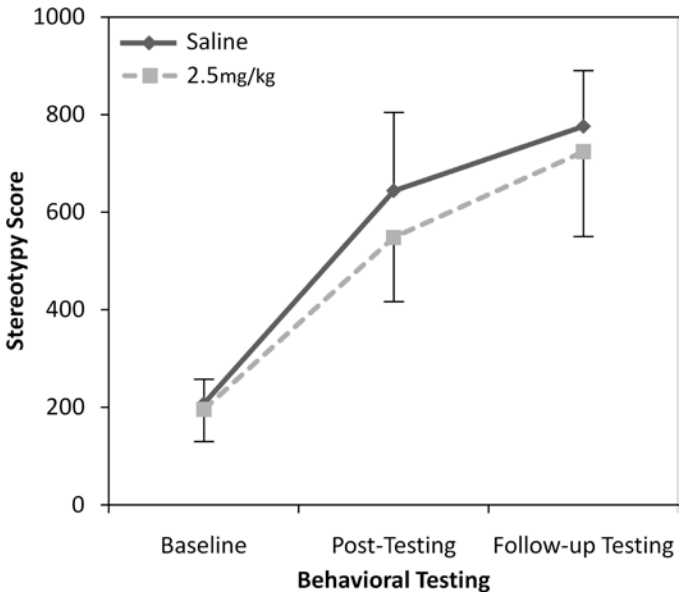


Figure 5-4. Rates of spontaneous stereotypy before and after context-dependent amphetamine administration in younger, conventionally housed mice (mean ± SEM).

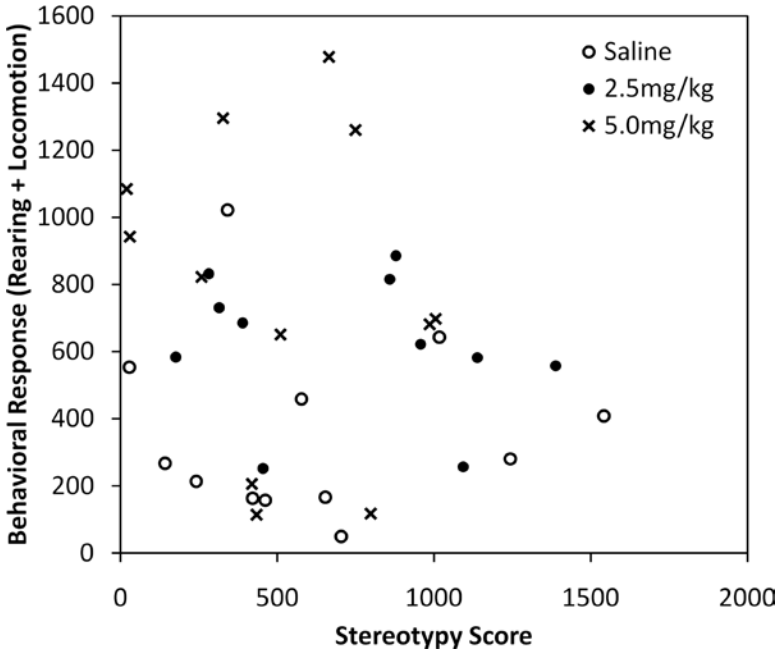


Figure 5-5. The association between baseline spontaneous stereotypy and the behavioral (rearing plus locomotion) response to an acute amphetamine challenge in adult conventionally housed mice (Experiment 1).

CHAPTER 6
EFFECTS OF ADMINISTRATION OF ADENOSINE RECEPTOR AGONISTS ON THE DEVELOPMENT OF REPETITIVE BEHAVIOR

Introduction

Restricted repetitive behaviors are diagnostic for autism and a common feature of several neurodevelopmental disorders (e.g., Tourette's syndrome, Rett's syndrome, intellectual disability). Despite its significance in clinical populations, the etiology and pathophysiology of these behaviors remain unclear. Moreover, no effective biological treatments for repetitive behaviors are available. Several clinical studies as well as animal studies have highlighted perturbation of cortico-basal ganglia circuitry associated with the expression of repetitive behaviors (e.g., Graybiel, 2000; Hollander et al., 2005).

Among several categories of models available (Lewis et al., 2007), our lab has adopted a deer mouse (*Peromyscus maniculatus*) model of restricted repetitive behaviors, which are associated with environmental restriction. These mice spontaneously develop high rates of repetitive jumping and backward somersaulting under standard laboratory caging conditions, which can be consistently attenuated by exposing animals to enriched (more complex) environments (e.g., Turner et al., 2002; Hadley et al., 2006). We have previously shown that the development of repetitive behaviors in deer mice was associated with decreased indirect basal-ganglia pathway activity (Chapter 2, 4). In addition, increasing indirect pathway activity by stimulating striatopallidal indirect pathway neurons by acute administration of A_{2A} and A_1 adenosine receptor agonists attenuated the expression of repetitive behaviors in adult mice dose-dependently.

Based on our findings in Chapter 2, in this preliminary study a combination of A_{2A} and A_1 receptor agonists was given subchronically during the early post-weaning

period. Rates of stereotypy in deer mice gradually increase and reach asymptote at 6.22 weeks post-weaning (Chapter 3). If drugs were given during this period when animals are susceptible to environmental restriction, which results in the development of stereotypy, they could have long-term effects on the trajectory of such behaviors.

Methods

Subjects

Twenty-two male and female deer mice (30 days of age; female=13, male=9) were group-housed in standard rodent cages (48 x 27 x 15 cm) before and during the experimental procedures. They were kept on a 16:8-h light/dark cycle with lights off at 10AM. Rodent chow and water were available *ad libitum*. All mice were tested for baseline levels of stereotypy as described previously. Mice were randomly assigned to either the vehicle group (n=10) or the drug group (n=12), which received a combination of 2-p-(2-carboxyethyl)phenethylamino-50-N-ethylcarboxamidoadenosine (CGS21680; 0.03mg/kg) and N6-cyclopentyladenosine (CPA; 0.03mg/kg) dissolved in saline with 5% DSMO. Mice received injections twice per day (9AM and 7PM, before and after the active dark cycle respectively) for seven days starting at PND32-33. Rates of stereotypy were tested again five days after the last injection as well as at 4 weeks and 6 weeks post-weaning.

Stereotypy Assessment

Rates of spontaneous stereotypy were assessed using a modified automated photocell detection apparatus (Columbus Instruments). The session consisted of the eight hours of the dark cycle. Mice were individually placed in testing cages (22 x 28 x 31 cm) made of Plexiglas and habituated for at least one hour prior to the beginning of the dark cycle. Food and water were provided. Stereotypy counts were automatically

scored for each mouse. Photobeams were positioned (13.5cm above the floor) to be interrupted by the vertical motion of jumping and somersaulting but not by rearing. All sessions were digitally video-recorded for identification of behavioral topographies and accuracy of the automated counters. Each animal received a stereotypy frequency score, which represented the average stereotypy frequency per hour, and a Y-score, which indexed the degree of behavioral organization (see Chapter 3).

Data Analysis

The effects of a seven-day regimen of CGS21680/CPA on spontaneous stereotypies were assessed using a two-factor (time and drug) repeated measures analysis of variance (ANOVA). Effects were considered significant when $p<0.05$.

Results

The baseline stereotypy score was 626 and 627 for vehicle and drug groups respectively. Two-way ANOVA with repeated measures showed that there was a significant effect of time ($F(3,60)=23.46$, $p<0.001$), although no main effect was found for drug ($F(1,20)=0.96$, $p=0.34$) or the time x drug interaction ($F(1,20)=1.74$, $p=0.20$) on the frequency of stereotypy (Figure 6-1). Significant effects of time and drug were found when Y-scores were used as the dependent measure ($F(3,60)=12.62$, $p<0.01$; $F(1,20)=5.40$, $p=0.03$) (Figure 6-2). Post hoc pair-wise comparisons showed that the differences between the vehicle and drug groups were found at 5 days post-injection (PND44) ($t(20)=-2.45$, $p=0.02$) and 4 weeks post-weaning (PND54) ($t(20)=-2.99$, $p<0.01$). No significant drug by time interaction was found ($F(3,60)=0.21$, $p=0.89$).

Discussion

In Chapter 2, we showed that acute administration of adenosine A_{2A} and A_1 receptor agonists selectively reduced the expression of stereotypy in adult mice. In the

present experiment, we administered this drug combination subchronically during the period when environmental restriction results in repetitive behaviors and associated neurobiological changes (Chapter 3 and 4). Our results showed that exposure to a combination of A_{2A} and A_1 receptor agonists for seven days during the early post-weaning period had long-lasting effects on the developmental trajectory of stereotypy after the termination of drug administration. Importantly, the main effect of drug was found in Y-scores during the immediate post-injection periods (5 days post-injection and 4 weeks post-weaning), but not in the frequency of stereotypy counts. This suggests that Y-score, a measure of behavioral dynamics, may be a better and more sensitive index of drug effects.

It needs to be noted that because baseline stereotypy was assessed at around 30 days of age, which was a few days before the drug administration was initiated, it was not possible to classify animals by developmental trajectory group. The data from Chapter 3 showed that mice categorized in Traj 1 and Traj 3 showed little change in frequency or temporal organization of stereotypy across development. Thus the animals, which likely benefit most from the drug effects, are Traj 2 animals. If this is the case, the present study likely underestimated the effects of adenosine receptor agonists on repetitive behavior. Because we cannot predict developmental trajectories of animals at 1 week post-weaning, we cannot reliably screen and recruit only the Traj 2 animals. Future studies could employ low-stereotypy animals (based on median split) at 1 week post-weaning as an attempt to eliminate Traj 3 mice.

The observed neuroprotective effect of adenosine receptor agonists is likely due to long-term changes in gene expression in striatopallidal neurons (Karcz-Kubicha et al.,

2003; 2006). As the studies of Karcz-Kubicha et al. (2003; 2006) did not involve repeated administration of an A_{2A}/A_1 receptor agonist combination, the mechanism by which this drug combination attenuated the negative neuroadaptive changes leading to the development of stereotypy is still obscure. Lack of striatopallidal neuronal activation during this period is speculated to be linked to abnormal experience-dependent structural and functional neuronal development, which results in the development of stereotypy, however.

The alteration of A_{2A} receptors is one of the earliest neurochemical features occurring in patients of Huntington's disease (Glass et al., 2000). Administration of CGS21680 and the A_{2A} receptor antagonist MSX-3 in an animal model of Huntington's disease was shown to attenuate and exacerbate involuntary motor symptoms respectively (Blum et al., 2003; Chou et al., 2005). Although CGS21680 seems to have neuroprotective effect, its administration increased degeneration of medium spiny neurons through increased glutamate transmission by presynaptic A_{2A} receptors located on corticostriatal nerve terminals. Conversely, an *in vitro* study using striatal neurons suggested a neurotrophic role for postsynaptic A_{2A} receptors on striatopallidal neurons by showing that activation of PKA in these neurons protected against neurodegeneration by the neurotoxin 3NP (Blum et al., 2003). Thus, this biphasic neurotoxic and neuroprotective effect of A_{2A} receptor ligands seems to depend on either the presynaptic or postsynaptic site of drug's action respectively. Yet, the role of A_{2A} receptors in pathophysiology of neurodegenerative disorders and the biphasic properties of the receptors still remain largely unclear (for review see Fredholm, 1997).

Chronic administration of CGS21680 counteracted the development of L-DOPA-induced dyskinesias when co-administration of L-DOPA and CGS21680 was initiated immediately after a 6-hydroxydopamine lesion in rats (Agnati et al., 2004). The absence of dyskinesias in drug-treated rats was attributed to higher striatal tyrosine hydroxylase immunoreactivity compared to vehicle-treated rats. This suggests the nigrostriatal dopaminergic cells were neuroprotected from the neurotoxin by A_{2A} receptor stimulation, which consequently blocked the development of L-DOPA induced dyskinesia. No systematic study has been done in deer mice to assess possible neuronal loss in cortico-basal ganglia circuitry linked to stereotypy, nor is there evidence suggesting dysfunctional dopamine transmission. Yet A_{2A} receptor stimulation, and hence striatopallidal neuronal activation, did protect deer mice from perturbations which results in the expression of stereotypy by unknown mechanisms. It is obvious that explanation of this phenomenon requires more systematic pharmacological plus neurobiological studies assessing specific changes induced by chronic A_{2A} receptor administration.

The drug classes that are currently used to treat restricted, repetitive behaviors in neurodevelopmental disorders include atypical antipsychotics and selective serotonin re-uptake inhibitors (SSRIs). The SSRI citalopram, however, was recently shown to be ineffective in attenuating such behaviors in autism (King et al., 2009). Similarly, the atypical antipsychotic drug risperidone has been recently approved by FDA for use in autism for irritability, but its effect on restricted repetitive behavior is questionable. Using deer mice, we showed that neither the SSRI fluoxetine nor the atypical antipsychotic drugs risperidone and olanzapine in adult deer mice by means of daily injection and/or

osmoic minipump (unpublished data) selectively reduced the expression of stereotypy. These results plus evidence from clinical studies suggest the necessity of the development of pharmacoptherapy based on the neurobiological alterations associated with these abnormal behaviors. If the present findings are generalizable to clinical populations, adenosine A_{2A} and A_1 receptor may serve as potential therapeutic targets for the treatment of restricted, repetitive behaviors in neurodevelopmental disorders.

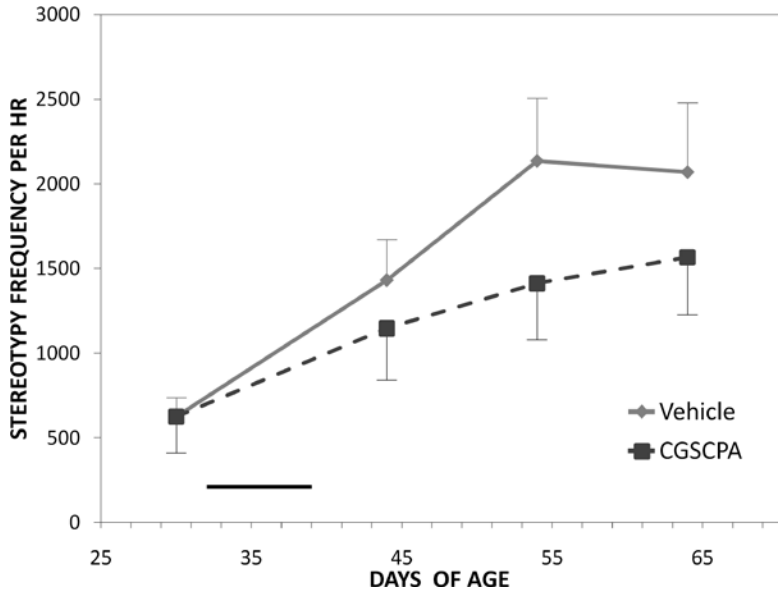


Figure 6-1. Change in developmental trajectory of stereotypy frequency after chronic administration of CGS21680 and CPA (0.03/0.03mg/kg) as compared to vehicle control (mean ± SEM). Bar represents the period of daily drug administration.

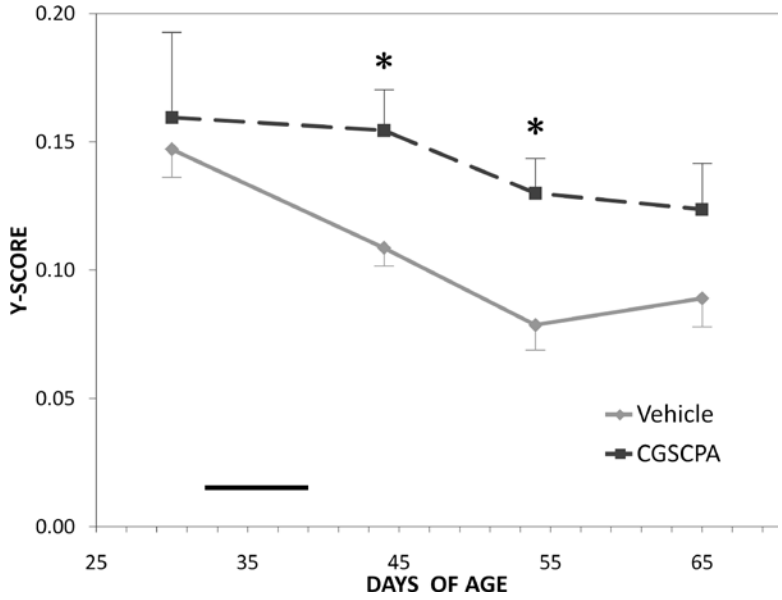


Figure 6-2. Change in developmental organization of behaviors after chronic administration of CGS21680 and CPA (0.03/0.03mg/kg) as compared to vehicle control (mean ± SEM). Bar represents the period of daily drug administration. * represents statistical significance at $p<0.05$ as compared to vehicle group.

CHAPTER 7
GENERAL DISCUSSION

The overall purpose of this series of studies was to investigate the development of repetitive behaviors in deer mice. These behaviors associated with environmental restriction model the repetitive behaviors characteristic of autism and related neurodevelopmental disorders. The repetitive behaviors of deer mice (vertical jumping, backward somersaulting) are discrete and salient, occur spontaneously and at high frequencies, and exhibit considerable individual variability. These features made them ideal target behaviors to study developmental trajectories and change in temporal dynamics across development. A detailed analysis of the development of repetitive behavior in deer mice would then allow assessment of developmental changes in brain circuitry that mediate the expression of repetitive behavior. With this as the overall goal, we started by establishing alterations in cortico-basal ganglia circuitry in adult animals. We then focused on ways to characterize repetitive behavior in ways that would capture heterogeneity in developmental trajectory and developmental changes in the organization of stereotyped behavior. With this as a foundation, we were then able to examine correlated developmental changes in key basal ganglia pathways. In addition, we could then test the effects of inducing alterations in cortical-basal ganglia circuitry during development and assess the effects of these neuroadaptations on developmental trajectory.

Summary of Results

The findings presented in Chapter 2 extended our previous finding that the expression of stereotypy was correlated with the reduced activity in the indirect basal ganglia pathway in adult mice. To confirm this alteration in indirect pathway activity,

pharmacological experiments designed to increase indirect pathway activity were found to attenuate the expression of stereotypy dose-dependently. Moreover, a higher dose of the drug combination used appeared to be required to normalize this pathway's activity to the level of low-stereotypy animals.

In Chapter 3, we extended work reported in our first deer mouse publication (Powell et al., 1998) by providing a more detailed characterization of the development of repetitive behavior. Using a group-based trajectory modeling analysis, we demonstrated three distinct developmental trajectories. In addition, we presented a novel approach to analyze the temporal dynamics of repetitive behaviors and examined such temporal organization in the context of discrete developmental trajectories. These findings suggested that Traj 2 mice change their behavioral temporal organization dramatically from 1 week to 6 weeks post-weaning, whereas Traj 1 and 3 mice show little change in temporal organization across development.

Building on the findings reported in Chapter 3 describing developmental trajectories of behavior, we investigated developmental changes in the indirect basal ganglia pathway using the same histochemical method described in Chapter 2. We found that changes in indirect pathway activity were dependent on developmental trajectory. Specifically, CO staining was decreased in Traj 2 and Traj 3 animals compared to Traj 1 animals by 6 weeks post-weaning. Conversely, the analysis of neuronal activation in striatal striosomes versus matrix showed no preferential striosomal activation across development regardless of developmental trajectory group.

The development of stereotyped behaviors can be induced by repeated intermittent psychostimulant administration (behavioral sensitization), which among

other effects alters the balance in activation between the striosomal and matrix compartments of the dorsal striatum. Repeated, intermittent administration of amphetamine did not change the development and expression of spontaneous stereotypy (repetitive jumping and backward somersaulting) in deer mice, however. These results extend our previous pharmacological studies (e.g., Presti et al., 2004) that the mechanisms mediating the development of spontaneous stereotypy are likely different from the neuroadaptive changes induced by psychostimulant administration. These results also complement findings presented in Chapter 4 showing that alteration of striosome versus matrix activity was not associated with the development of spontaneous stereotypy in deer mice.

Lastly, based on the findings from Chapters 2 and 3, we employed subchronic administration of the same drug combination used in Chapter 2 using younger animals. The results suggested long-term changes in the developmental trajectory of stereotypy following termination of drug exposure (Chapter 6). This preliminary finding suggests that this pharmacological manipulation during the early post-weaning period may induce neuroprotective adaptations, which resulted in the attenuation of developing high rates of spontaneous stereotypy.

Conclusions

The results briefly reviewed in the previous section, when taken collectively, indicate that the development of high rates of stereotypy that are temporally organized in a highly regular or fixed pattern was linked to decreased indirect basal ganglia pathway activity. Moreover, a drug cocktail designed to increase striatopallidal or indirect pathway neuron activity selectively reduced the development and expression of

such behaviors. These findings not only support the importance of the indirect pathway but provide potentially important and novel therapeutic targets.

Despite restricted, repetitive behaviors being common in several neurodevelopmental disorders and neurological disorders, clinical behavioral observations have not provided a wealth of information about the development and organization of these behaviors. Moreover, the etiology and specific pathophysiology of repetitive behavior disorders are largely unknown. This dearth of information seriously impairs the development of effective treatment programs. The work presented here utilized novel approaches to characterize repetitive behaviors, methods which can certainly be applied to clinical populations. Ideally, therapeutic manipulations should be designed to reduce the frequency as well as increase the complexity (less regularity) of behaviors in affected individuals. In addition, effective pharmacotherapy to treat these behaviors should be developed based on the empirical understanding of neurobiological mechanisms associated with these behaviors.

Future Directions

A great deal more work needs to be done to elucidate the neurobiological changes that mediate the development of repetitive behavior. One important step to extend the present work would be to examine behavioral and neurobiological changes that occur in the first week post-weaning. In addition, characterization of behavioral and neurobiological changes during the pre-weaning period that are linked to negative adaptation to the restricted environment and predict future development of stereotypy need to be characterized.

In the present studies, we did not assess the activity of the direct pathway. In contrast to STN in the indirect pathway, the direct pathway doesn't involve any key brain

regions that are wholly part of the direct pathway (Figure 1-1). The only available evidence that we have for involvement of direct pathway in the stereotypy in deer mice comes from Presti and Lewis (2005) who showed that striatal dynorphin content was not associated with the level of stereotypy.

To further confirm the importance of the indirect pathway in the development of stereotypy in deer mice, assessments of specific striatal cell populations could be conducted. Striatal medium spiny neurons comprising the direct pathway (striatonigral neurons) and the indirect pathway (striatopallidal neurons) are intermingled, but can be differentially labeled. These labeled cells can then be sorted using techniques such as fluorescence activated cell sorting (FACS). Assessment of these homogeneous neuronal populations will provide more direct evidence for the importance of the indirect pathway in stereotypy. One possible drawback to such a technique is that the *Peromyscus* genome has not yet been fully sequenced. Thus gene expression or proteomics based methods could be difficult.

The findings presented in Chapters 3 and 6 indicate that Y-scores might serve as a more sensitive index of interventions designed to affect repetitive behavior than frequency. Moreover, Y scores may be more highly correlated with neurobiological measures than frequency. For example, as shown in Chapter 6, the significant difference between vehicle and drug treatment was found only in Y-scores but not in the frequency counts of stereotypy. Thus Y-score analyses should be employed in our future studies, examining the effect of any manipulations designed to exacerbate or attenuate stereotypy and studies correlating neurobiological measures and behavior.

Although our studies have provided strong evidence for the role of the indirect pathway in repetitive behavior, we have no information as to what specific mechanisms may be mediating reduced indirect pathway activity. Thus, future studies should build on the findings reported in Chapters 3 and 4 and examine specific molecular alterations that may be mediating changes in indirect pathway activity. Various transcription factors may be promising candidates in this regard.

Lastly, the work reported in Chapter 6 would seem to be particularly important to extend as this work may have the most direct translational value. It represents an early intervention that may have long term neuroprotective effects. The findings of Chapter 6 are preliminary, however, and require systematic replication with an evaluation of the long-term neurobiological changes wrought by such an intervention. Progress in the areas outlined in this section coupled with the current findings will provide valuable information about the pathophysiology and potentially efficacious treatment of aberrant repetitive behavior. It is hoped that such work will translate to better ways to treat and improve the lives individuals with autism and related neurodevelopmental disorders.

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BIOGRAPHICAL SKETCH

The candidate was born in Tokyo Japan to Nobuo and Reiko Tanimura. She has an older sister, Chie. She completed high school at Meguro Seibi Gakuen in Tokyo. She moved to California in 1998 and received her Bachelor of Arts in Psychology from California State University, Sacramento in May 2002 with university honors, Phi Kappa Phi, and *cum laude*. During her undergraduate career, she worked as a research assistant for Drs. Arnold Golub and Jeffery Calton where she found her passion in behavioral neuroscience. After working for a short period of time in Tokyo, she joined the research laboratory of Dr. Mark H. Lewis at University of Florida and began her graduate education in August 2003. She received her Master of Science from behavioral neuroscience program at Department of Psychology in 2006. She completed her PhD training in May 2010. Shortly following that she will start postdoctoral training in Dr. Suzanne Haber's laboratory at University of Rochester.