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How does environmental enrichment reduce repetitive motor behaviors? Neuronal activation and dendritic morphology in the indirect basal ganglia pathway of a mouse model

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Abstract

Repetitive motor behaviors are observed in many neurodevelopmental and neurological disorders (e.g. autism spectrum disorders, Tourette syndrome, fronto-temporal dementia). Despite their clinical importance, the neurobiology underlying these highly stereotyped, apparently functionless behaviors is poorly understood. Identification of mechanisms that mediate the development of repetitive behaviors will aid in the discovery of new therapeutic targets and treatment development. Using a deer mouse model, we have shown that decreased indirect basal ganglia pathway activity is associated with high levels of repetitive behavior. Environmental enrichment (EE) markedly attenuates the development of such aberrant behaviors in mice, although mechanisms driving this effect are unknown. We hypothesized that EE would reduce repetitive motor behaviors by increasing indirect basal ganglia pathway function. We assessed neuronal activation and dendritic spine density in basal ganglia of adult deer mice reared in EE and standard housing. Significant increases in neuronal activation and dendritic spine densities were observed only in the subthalamic nucleus (STN) and globus pallidus (GP), and only for those mice that exhibited an EE-induced decrease in repetitive motor behavior. As the STN and GP lie within the indirect pathway, these data suggest that EE-induced attenuation of repetitive motor behaviors is associated with increased functional activation of the indirect basal ganglia pathway. These results are consistent with our other findings highlighting the importance of the indirect pathway in mediating repetitive motor behaviors.

Keywords

Animal models; basal ganglia circuitry; subthalamic nucleus; cytochrome oxidase; Golgi-Cox histochemistry; deer mice

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1. Introduction

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

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

Environmental enrichment (EE) in rodents includes increased social and spatial density as well as exposure to novelty and complexity, usually in the form of toys, tunnels, and nesting material. Increased opportunity for exercise (e.g. a running wheel) is also a mainstay of EE. Well-studied in the context of learning and memory, EE increases neurogenesis and synaptogenesis (van Praag et al., 2000) as well as cortical thickness, dendritic spine density and length, synaptic plasticity, and resistance to disease (Kolb & Wishaw, 1998; Nithianantharajah & Hannan, 2006). A range of behavioral domains are also affected by EE including cognitive, social, and emotional functioning as well as aberrant behaviors (Morley-Fletcher et al., 2003; Branchi, 2006). Attenuation of repetitive behavior is a robust behavioral effect of EE, seen not only in deer mice, but across most captive species (Lewis, 2004; Mason et al., 2007). To date, however, the mechanisms underlying this effect are

largely unknown. Previous work in deer mice suggested that EE-induced attenuation of repetitive motor behaviors was associated with increased neuronal activation and dendritic spine density in basal ganglia (Turner et al., 2002; 2003). Importantly, EE effects on neurobiological outcomes were found only for those mice exhibiting EE-induced attenuation of repetitive behavior.

Although this earlier work implicated the basal ganglia, it did not address selective alterations of specific basal ganglia pathways. The basal ganglia include the striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra (SN). The striatum is the main input structure of the basal ganglia and receives projections from the sensory-motor and associative cortical areas, and projects to nuclei via the direct or indirect pathways of the basal ganglia. These projections converge in the output nuclei, the SN, relay to the thalamus and then back to the cortex to complete the loop. The monosynaptic direct pathway projects from striatum to SN pars reticulata (SNR), whereas the indirect pathway projects from striatum to GP which in turn projects to STN before converging on SNR (Schmitt et al., 2014). Appropriate selection, activation and suppression of movement are dependent on the coordination of the direct and indirect basal ganglia pathways, which classically were thought to function in an antagonistic fashion (Alexander et al., 1986). More recent evidence reveals a dynamic interplay between the basal ganglia pathways with concomitant activity during action sequence initiation but differential encoding of action sequences (Jin et al., 2014), and increased complexity of neuronal cell populations within a given region (Wall et al., 2013; Antal et al., 2014; Macpherson et al., 2014). Direct pathway neurons facilitate selection of relevant motor programs whereas the indirect pathway functions to suppress competing motor programs. In addition, a direct connection between the cortex and STN, known as the hyperdirect pathway, although largely understudied, is thought to modulate response inhibition in situations of conflict (Jahfari et al., 2011; Jahanshahi, 2013). An imbalance in the direct and indirect basal ganglia pathways has been implicated in the dysregulation of cortico-striato-thalamo-cortical circuitry associated with both hyperkinetic and hypokinetic movement disorders (Graybiel, 2000).

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

In the present study, we conducted two experiments to assess the function of the indirect pathway in the EE-induced attenuation of the development of repetitive motor behaviors.

We hypothesized that such attenuation is associated with increased neuronal activation of the indirect basal ganglia pathway, an outcome mediated by increased dendritic spine density. In Study 1, we compared neuronal metabolic activation of basal ganglia nuclei in adult deer mice reared in EE to mice reared in standard housing. Neuronal activation is tightly coupled with oxidative energy metabolism, and can be indexed using cytochrome oxidase (CO) histochemistry (Gonzalez-Lima & Cada, 1998). We measured CO as an indicator of long-term neuronal metabolic activity in the dorsal lateral striatum (DLS), GP, STN, SNR, SNC, motor cortex and CA1 region of the hippocampus (HPC). In Study 2, we compared dendritic morphology in basal ganglia nuclei of adult deer mice reared in EE or standard laboratory cages. The dendritic surface receives over 95% of the synapses on a neuron, aided by specialized protrusions (i.e. spines) that act as the basic functional unit of integration for neuronal circuits. Dendritic spines mediate fast excitatory transmission, are heterogeneous in morphology, and modifiable by experience and activity (Markham & Greenough, 2004). Experience-dependent dendritic plasticity is a sensitive index for inferring synapse number and strength, and dendritic remodeling can change the functional properties of a neuron (Harris & Kater, 1994; Kolb & Wishaw, 1998). A number of earlier studies investigating morphological plasticity have found increased dendritic spine density following EE exposure in a variety of brain regions important in the processing of environmental stimuli (e.g. Comery et al., 1995; 1996; Juraska et al., 1985, 1989; reviewed by Markham & Greenough, 2004). More recently, dendritic remodeling as a consequence of EE has been related to tasks of learning and memory (Farrell et al., 2015) and pathology of Huntington's and Parkinson's diseases (Spires et al., 2004; Murmu et al., 2013; Kim et al., 2013). To our knowledge, however, only our own work has examined dendritic morphological differences in the basal ganglia as a function of EE-induced attenuation of repetitive motor behaviors (Turner et al., 2003).

2. Materials and Methods

2.1. Animals

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

2.2. Enriched housing conditions

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

2.3. Standard housing conditions

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

2.4 Assessment of repetitive motor behaviors

At six weeks post-weaning, mice were removed from their housing conditions and placed into individual test cages ($28 \times 22 \times 25$ cm) for measurement of repetitive motor behaviors. In deer mice, these behaviors take the form of vertical hindlimb jumping and backward somersaulting. Both topographies involve vertical activity and so can be automatically quantified using photobeam arrays (Columbus Instruments) positioned such that when the animal jumps the photobeam is interrupted and a count is recorded (Labview software, National Instruments; see Tanimura et al., 2010, 2011; Muehlmann et al., 2015). At 13.5 cm above the floor, the photobeams are positioned high enough to avoid being broken by drinking, rearing, or any other behavior that does not include all four paws leaving the ground. Random sampling of the videos (Geovision software) recorded during each test session insured the accuracy of the automated counts.

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

2.3 Study 1: CO histochemistry

CO is an integral transmembrane protein of the inner mitochondrial membrane that catalyzes the transfer of electrons during the process of generating ATP, and is therefore directly related to neuronal functional activity (Wong-Riley et al. 1998). CO activity is a measurement of long-term (days to weeks) neuronal metabolic activity (Sakata et al., 2005) and has been successfully used to detect alterations in basal ganglia in rodent models of

dystonia, ataxia and repetitive motor behaviors (Nobrega et al., 1998; Jacquelin et al., 2013; Turner et al., 2002; Tanimura et al., 2010). The CO staining assay was executed according to the protocol of Gonzalez-Lima and Cada (1998) and has been used previously in our lab to find differences in basal ganglia neuronal activity between deer mice with high and low levels of repetitive motor behaviors (e.g. Turner et al., 2002; Tanimura et al., 2010; Tanimura et al., 2011). Briefly, the morning after behavioral testing, brains were harvested and immediately snap-frozen in cold 2-methylbutane and stored in a -80°C freezer. Brains were sectioned on a cryostat (-20°C) into $20\text{ }\mu\text{m}$ sagittal sections. We collected tissue from both hemispheres beginning at $\sim 1\text{ mm}$ lateral to the midline, positioning them onto microscope slides (Superfrost Plus, FisherBrand), such that adjacent sections were $100\text{ }\mu\text{m}$ apart. Homogenized brain tissue from non-subject deer mice was used to make standards that were snap-frozen and sectioned at increasing thicknesses (10, 20, 30, 40, 50, $60\text{ }\mu\text{m}$) to ensure linearity of optical density measurements. Slides were stained and cover slipped with Permount.

2.4 Study 1: Quantification of CO histochemistry

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

2.5 Study 2: Golgi-Cox histochemistry

Golgi-Cox histochemistry was used to assess dendritic morphology in key basal ganglia nuclei (dorsal lateral striatum (DLS), STN, GP, and SNR). At 4 pm on the day following behavioral testing, mice were anesthetized with isoflourane and sacrificed by decapitation. Golgi-Cox histochemistry was completed according to FD Rapid GolgiStain Kit guidelines (FD NeuroTechnologies, Inc.). In brief, brains were removed and placed into the first solution for Golgi-Cox staining. Time from sacrifice to immersion was less than one minute. Brains were impregnated in solution for 2 weeks at room temperature in a dark environment. After this time, the brains were sliced in a 30% sucrose solution into $200\text{ }\mu\text{m}$ coronal sections using a Vibratome, and allowed to dry at room temperature without exposure to light. The slides were then stained and coverslipped with Permount.

2.4 Study 2: Quantification of Golgi-Cox histochemistry

Linear dendritic spine density was quantified using ImagePro (Media Cybernetics) under $40\times$ magnification (Zeiss microscope with Leica DFC camera) by an observer blind to housing condition and sex. Within a given brain region, spines along unobstructed dendritic segments $>15\text{ }\mu\text{m}$ were marked on graphic overlays on live digital video images during continuous manual focus adjustment. Any protrusion from the main cylindrical column of the dendrite was counted as a spine. After labeling all the spines along a sample, the approximate dendritic cylinder centerline was traced for return of calibrated length by the

software. Dendrites were measured starting beyond the first bifurcation point and at least 50 μm from the soma. It has been shown that enrichment effects on dendritic morphology appear at distances of 50 μm or greater from the cell body (Leggio et al., 2005) and beyond the second order (Spires et al., 2004). In addition, we sought to complement the Turner et al. (2003) findings on first order dendrites. The GP has aspiny and spiny neurons (Kita & Kitai, 1994), so for each mouse, both types were selected for measurement. Within each region of interest, 10–12 dendrites were measured ensuring samples represented both hemispheres and multiple tissue sections. Spine densities were calculated as the total number of spines divided by the total length of segments in a given brain region, such that each animal had one density measurement per brain region then used in subsequent analyses. The SNR region of one enriched mouse and the GP region of one enriched mouse were not analyzable due to processing error.

2.5 Statistical analysis

A General Linear Model (SPSS v21, SPSS Inc, Chicago, USA) with housing, sex and the interaction of these factors was used to assess behavioral and neurobiological differences. If no interaction or main effect of sex was seen, we ran a revised model without sex as a factor. Neurobiological correlates of behavior were assessed using a Pearson's correlation (SPSS v21, SPSS Inc, Chicago, USA). Subsequently, to identify neurobiological effects of EE specific to repetitive behavior, for each analysis, we conducted a secondary analysis of the data comparing only those mice that exhibited the typical EE-induced attenuation of repetitive behavior and mice that developed high levels of repetitive behavior as a consequence of standard housing (SH). The rationale for a secondary analysis of the data derived from earlier deer mouse studies (Powell et al., 1999, 2000; Turner et al., 2002, 2003; Turner & Lewis, 2002; Tanimura et al., 2010, 2010a; Muehlmann et al., 2015) which consistently demonstrated a subpopulation of animals that are atypical in their development of repetitive motor behaviors, given their rearing environment (i.e. enriched mice that develop high levels of repetitive motor behaviors, and standard caged mice that do not develop high levels of repetitive motor behaviors). This secondary analysis of subjects based on repetitive behavior scores was necessary to test our hypothesis that EE-induced attenuation of repetitive behavior is associated with increased functioning of the indirect basal ganglia pathway. This allowed us to interpret our results in the context of attenuation of repetitive behavior rather than potentially non-selective effects of exposure to EE. Individuals that do not show attenuation of repetitive behavior in response to EE would not be predicted to show increased functioning of the mechanisms hypothesized to mediate repetitive behaviors. To differentiate these subpopulations, we used selection criteria previously established in Turner et al., 2002; 2003, and fit our group criteria to best match the previous studies in terms of frequency ($\text{EE} < 4000 \text{ total jumps} > \text{SH}$) and proportion of atypical individuals (~30%). The frequencies of repetitive motor behaviors and results from secondary analyses are depicted using these stratified group delineations (i.e. EE low repetitive behavior (RB), EE high RB, SH high RB and SH low RB). Raw mean values from both the full and selected data sets are presented in summary tables. In all analyses, Levene's test of equality of error variances and the Kolmogorov-Smirnova test of normality were used to ensure model assumptions were met.

3. Results

3.1 Study 1: EE reduced repetitive motor behaviors

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

3.2 Study 1: EE-induced attenuation of repetitive motor behaviors increased neuronal metabolic activity in the indirect basal ganglia pathway

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

Secondary analysis comparing the mice that demonstrated EE-induced attenuation of repetitive behavior and the mice that developed high levels of repetitive motor behaviors induced by standard housing altered our results only in indirect pathway nuclei. In the STN, a main effect of housing was found with EE increasing STN CO activity ($F(1,16)=5.40$, $p=0.034$; see Fig. 4), with the main effect of sex ($F(1,16)=11.7$, $p=0.003$) and housing by sex interaction ($F(1,16)=6.94$, $p=0.018$) remaining. In the GP, secondary analysis based on repetitive behavior scores showed a significant main effect of housing, with EE increasing neuronal activation ($F(1,16)=5.46$, $p=0.033$; see Fig. 5) and the significant effect of sex being maintained (females < males: $F(1,16)=6.99$, $p=0.018$). Mice that developed high frequencies of repetitive motor behaviors in spite of enriched housing (i.e. subgroup: EE high RB) showed neuronal activation values in the GP that were quantitatively similar to those of standard caged mice with high frequencies of repetitive motor behaviors (i.e.

subgroup: SH high RB; see Fig. 5). Table 1 provides a descriptive summary of raw mean CO optical density values for EE vs standard housed mice.

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

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

4. Discussion

As reported in numerous other captive species, rearing deer mice with EE significantly reduced adult levels of repetitive motor behavior. In Study 1, EE-induced attenuation of repetitive motor behavior was associated with increased neuronal activity in the indirect basal ganglia pathway nuclei of the STN and GP. In the STN and motor cortex, the effects of EE were influenced by sex, such that EE males showed greater increases in CO activity in these regions compared with the other groups. It is notable that males had significantly greater levels of CO in all of the nuclei measured except the SNR. Although not all were statistically significant, on average, EE mice had more CO activity than standard housed mice in all seven of the regions measured. In Study 2, a significant increase in dendritic spine density was seen only in the STN, and only when comparing mice that exhibited EE-induced attenuation of repetitive behavior to standard housed mice with high levels of repetitive behavior typical for that restricted environment. We did not see increased dendritic spine densities in the GP. Although connections with the STN bring some glutamate into the GP that promotes spine density, the influx of GABA from the striatum

promotes the competitive selection of spines (Hayama et al., 2013; see discussion below). Analyzing only mice that showed EE-induced attenuation of repetitive behavior and those that developed high levels of repetitive behavior typical of standard caging did not affect the GP spine density results. The current findings suggest that attenuation of repetitive motor behavior by EE is linked to increased neuronal activity and dendritic spine density in the indirect pathway, and is specific to those animals that are behaviorally responsive to EE relative to animals that develop high rates of repetitive motor behavior. These data support our overarching hypothesis that functional activation of the indirect basal ganglia pathway mediates repetitive behaviors.

Rearing animals in EE attenuated the development of repetitive motor behaviors in 70% of mice. Rearing animals in standard conditions generated repetitive motor behaviors in 75% of mice. These percentages are similar to previous findings from our lab that a small proportion of deer mice are atypical for their housing condition (Powell et al., 2000; Turner & Lewis, 2003; Turner et al., 2003). Why these individuals do not show environment typical levels of repetitive behavior is not known and will require further investigation. Perhaps, mice that do not show EE-induced attenuation of repetitive behavior are less exploratory and interact less with enrichment objects. To this end, future work may wish to include observations of mice interacting with enrichment devices and conspecifics in their home environments.

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

The present findings extend this work on EE effects on basal ganglia, to address selective effects on direct and indirect basal ganglia pathways. We add to a small study conducted by Tanimura et al. (2010) who found increased neuronal activity in the STN, as well as SN (reticulata and compacta) of EE vs standard housed mice. The present study extended the developmental period of EE exposure, now beginning at the time of weaning and lasting for 6 weeks, and included the GP as another indirect pathway nucleus. As in Tanimura et al. (2010), we found increased activity in the STN as a consequence of EE-induced attenuation of repetitive motor behavior. Moreover, we saw increased CO staining in the GP, another indirect pathway nucleus. These findings provide additional evidence for hypofunctioning of the indirect basal ganglia pathway in repetitive motor behavior development.

A similar pattern emerged following our assessment of dendritic spine densities. Significant main effects due to EE were observed only in the STN, and only when comparing mice that exhibited behavior typical of their housing conditions. Thus, neurobiological results for

indirect pathway nuclei seem specific to a repetitive motor behavior phenotype and not a global effect of EE. Moreover, differences were found only in brain regions that are implicated in repetitive motor behaviors. We did not see EE-induced differences in neuronal activation or dendritic spine densities in any nuclei outside of the indirect pathway.

We are the first to report an effect of sex on CO activity levels in association with repetitive motor behaviors. This effect of sex was unexpected given that no differences were found in the repetitive behavior of males and females, and no differences in CO activity levels due to sex have been shown previously in the deer mice. Sex differences in striatal CO activity levels following pharmacological intervention have been reported, however (Jones et al., 2008). Consistent with our prior work in deer mice, in Study 2 we found that males and females did not differ in their behavioral response to housing condition or spine densities in any of the brain regions analyzed (Turner et al., 2003). This result may be specific to deer mice as many studies using other species have found sex differences in dendritic morphology in response to environment (rat: Juraska et al., 1985, 1989; human: Jacobs et al., 1993). Moreover, our sample size was small and it is therefore possible that we failed to detect an effect of sex on dendritic spine density.

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

Predictions for the relationship between neuronal activation and dendritic spine densities are challenging, as this relationship has not been fully elucidated. CO activity is known to reflect contributions of both inhibitory and excitatory inputs, although it is most tightly coupled to the energy demands of mitochondria located in postsynaptic dendritic compartments (Wong-Riley & Welt 1980; Wong-Riley, 1989; Kelly et al., 2010), whereas dendritic spine densities may be more reflective of glutamatergic activity at presynaptic inputs (McKinney, 2010). Further complicating the relationship between CO activity levels and spine densities, a regulatory role for GABA in local dendritic calcium signaling has been implicated in the shrinkage and competitive elimination of dendritic spines, independent of the generation of action potentials (Hayama et al., 2013). It is noteworthy that elimination of dendritic spines is not necessarily associated with loss of synaptic function between neurons, as morphological remodeling can maintain signal activity (Hasbani et al., 2001; Haws et al., 2014).

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

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

A study by Woo and Leon (2013) and their recent follow up study (Woo et al., 2015) applied a direct translation from the EE literature on rodents, focusing on components of novelty and complexity, to children with an ASD diagnosis. Results demonstrated that EE in the form of parent-imposed daily exposure to multiple sensorimotor stimuli (e.g. olfactory, tactile, thermal, motor, balance, auditory, and cognitive tasks), which ensured complexity of stimuli (e.g. combinations of odorant and tactile stimulation) and novelty (new enrichment activities introduced at regular intervals) successfully improved ASD symptoms for many children in terms of overall severity and cognitive performance (Woo & Leon, 2013, Woo et al., 2015). No independent assessment was made, however, on the effects of their intervention on repetitive behaviors. This research has pioneered EE as a clinical approach for treatment of ASD, and illustrates the potential value of parallel clinical and animal studies in the development of new therapeutics. A better understanding of the mechanisms mediating experience-dependent plasticity will promote the use of EE in conjunction with standard pharmacotherapies.

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Highlights

- Environmental enrichment reduced repetitive motor behaviors in a deer mouse model
- The attenuation of repetitive motor behaviors by environmental enrichment was associated with increased activation of nuclei within the indirect basal ganglia pathway
- The attenuation of repetitive motor behaviors by environmental enrichment was associated with increased dendritic spine densities in the subthalamic nucleus

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

Fig 2.

Study 1. The effect of housing on the repetitive motor behaviors of adult deer mice.

Compared to mice reared in standard housing (SH: n=13), rearing deer mice with environmental enrichment (EE: n=13) significantly reduced the total frequency of adult repetitive motor behaviors occurring over an 8 h dark cycle ($F(1,24)=11.5$, $p=0.002$).

Subgroups based on frequency of repetitive motor behaviors (RB): EE low RB (n=10), EE high RB (n=3), SH high RB (n=10), SH low RB (n=3). Data bar shows mean $\pm$ SEM

Fig 3.

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

Fig. 4.

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

Fig 5.

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

Fig 6.

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

Fig 7.

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

Table 1

Mean (SD) values for CO optical density in a given region.

	EE, n=13	EE, n=10	SH, n=13	SH, n=10
TN	0.176 (0.037)	0.181 (0.041)	0.162 (0.023)	0.161 (0.019)
P	0.069 (0.016)	0.074 (0.013)	0.060 (0.016)	0.058 (0.015)
LS	0.130 (0.034)	0.132 (0.039)	0.119 (0.014)	0.118 (0.012)
NR	0.112 (0.016)	0.114 (0.018)	0.110 (0.014)	0.110 (0.014)
NC	0.095 (0.016)	0.096 (0.018)	0.089 (0.016)	0.089 (0.011)
I	0.147 (0.025)	0.149 (0.027)	0.139 (0.010)	0.140 (0.012)
PC	0.068 (0.016)	0.069 (0.017)	0.064 (0.007)	0.063 (0.008)

Table 2

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

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