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The development of repetitive motor behaviors in deer mice: Effects of environmental enrichment, repeated testing, and differential mediation by indirect basal ganglia pathway activation

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Abstract

Little is known about the mechanisms mediating the development of repetitive behaviors in human or animals. Deer mice reared with environmental enrichment (EE) exhibit fewer repetitive behaviors and greater indirect basal ganglia pathway activation as adults than those reared in standard cages. The developmental progression of these behavioral and neural circuitry changes has not been characterized. We assessed the development of repetitive behavior in deer mice using both a longitudinal and cohort design. Repeated testing negated the expected effect of EE, but cohort analyses showed that progression of repetitive behavior was arrested after one week of EE and differed significantly from controls after 3 weeks. Moreover, EE reductions in repetitive behavior were associated with increasing activation of indirect pathway nuclei in males across adolescence, but not females. These findings provide the first assessment of developmental trajectories within EE and support indirect pathway mediation of repetitive behavior in male deer mice.

Keywords

animal models; *Peromyscus maniculatus*; environmental enrichment; repeated testing; subthalamic nucleus

Repetitive motor behaviors are prevalent across neuropsychiatric, neurological, and neurodevelopmental disorders, including autism spectrum disorders (ASD), for which they are diagnostic (Lewis & Bodfish, 1998). These rigid and rhythmical patterns of movement that seem functionless are also associated with early experiential deprivation, such as congenital blindness and impoverished rearing environments (Fazzi, Lanners, Danova, Ferarri-Ginevra, Gheza, Luparia, Balottin, Lanzi, 1999; Rutter, Anderson-Wood, Beckett, Bredenkamp, Castle, Groothues, Kreppner, Keaveney, Lord, O'Connor, English Romanian Adoptees Study Team, 1999). Early in life, repetitive motor behaviors are also performed by

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typically developing children (Thelen, 1979, 1981; Evans, Leckman, Carter, Reznick, Henshaw, King, Pauls, 1997; Kim & Lord, 2010). In spite of their clinical prominence, relatively little is known about the mechanisms underlying repetitive motor behaviors or how they develop across time. Understanding the neurobiological mechanisms mediating normative versus pathological progression of repetitive motor behaviors will promote the development of appropriately timed therapeutic interventions.

Characterization of repetitive behavior development has focused mostly on children with ASD (e.g. Esbensen, Seltzer, Lam, Bodfish, 2009; Militeri, Bravaccio, Falco, Fico, Palermo, 2002). Repetitive motor behaviors are one of the first behavioral manifestations of ASD, and identifying reliable differences in the developmental trajectories of repetitive behavior in young children is an important tool in early identification and intervention (Barber, Wetherby, Chambers, 2012; Kim & Lord, 2010; Kim & Lord 2012a, 2012b; Sacey, Bennett, Zwaigenbaum, 2015; Watt, Wetherby, Barber, Morgan, 2008; Wolff, Botteron, Dager, Elison, Estes, Gu, Hazlett, Pandey, Paterson, Schultz, Zwaigenbaum, Piven, & The IBIS Network, 2014). Kim and Lord (2010) differentiated children (ages 8–56 months) with ASD from those with nonspectrum disorder and typically developing children using the prevalence and severity of early repetitive motor behavior development across time. Assessments of repetitive behaviors at 2, 3, 5, and 9 years of age revealed increased prevalence and severity of repetitive motor behaviors in children with ASD compared to children with developmental delays. Within the group with ASD, however, trajectory analysis revealed considerable heterogeneity in the development of repetitive motor behaviors over time (Richler, Heurta, Bishop, Lord, 2010). More recently, differences in frequency and intensity, but not topography of repetitive motor behaviors were detected in children with ASD versus typically developing children as early as 9–12 months of age (Wolff et al., 2014).

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

Animal models are useful for studying underlying mechanisms of repetitive motor behaviors as they are inducible via CNS insult (e.g. genetic modifications to Shank3, MECP-2), pharmacology (e.g. amphetamines) and restricted environments (Lewis, Tanimura, Lee,

Bodfish, 2007; Bechard & Lewis, 2012). Across species confined to captivity, repetitive motor behaviors are a prominent feature (Mason, Clubb, Latham, Vickery, 2007). Rodent models are an important tool for investigating neurobehavioral developmental trajectories as they have relatively short periods of development. Well-characterized in deer mice (*Peromyscus maniculatus*) reared in standard cages, repetitive hind limb jumping emerges in early development, often before weaning, and increases across adolescence to peak levels by day 56 of age (Muehlmann, Bliznyuk, Duerr, Lewis, 2015). Adult repetitive motor behaviors in deer mice manifest as both hind limb jumping and backward somersaulting.

Evidence from both humans and animal models suggest impairments in cortico-basal ganglia circuitry in the development of repetitive behaviors. The basal ganglia include the striatum, globus pallidus (GP), subthalamic nucleus (STN), and substantia nigra (SN). These nuclei coordinate to control movement facilitation, suppression, and refinement via direct and indirect basal ganglia pathways. In the direct pathway, the striatum receives glutamatergic input from the cortex and sends GABAergic monosynaptic projections directly to the output nucleus, the pars reticulata of the SN (SNR). In the indirect pathway, the striatum sends GABAergic projections to the GP (external GP, in primates), which relays to the STN, before converging on the SNR. A third largely understudied projection, the hyperdirect pathway, directly connects the motor cortex with the STN and modulates inhibition of responses in conflict situations (Jahfari, Waldorp, van den Wildenberg, Scholte, Ridderinkhof, Forstmann, 2011; Jahanshahi, 2013). An imbalance in the dynamic interplay of these pathways has been implicated in circuitry dysregulation associated with both hyperkinetic and hypokinetic movement disorders (Graybiel, 2000).

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

Restricted or confined environments give rise to repetitive behavior whereas the attenuation of repetitive behaviors by environmental enrichment (EE) is a robust and well-documented effect (e.g. Lewis 2004; Mason et al., 2007). Enriching the rearing environments of deer mice attenuated repetitive motor behaviors in adulthood and was associated with increased neuronal activation and dendritic spine densities in nuclei lying within the indirect basal ganglia pathway (Tanimura et al., 2010, Bechard et al., 2016). The development of repetitive motor behaviors within an enriched environment has not been characterized previously,

however, and there are no studies on the neurobiological correlates of repetitive behavior development within an enriched environment. Within a given environment, repetitive motor behavior development in deer mice is quite heterogeneous (e.g. Tanimura et al., 2010; Muehlmann et al., 2015; Bechard et al., 2016). For example, rearing deer mice with EE attenuates adult repetitive motor behaviors in most, not all, deer mice, but does not completely eliminate them (Turner, Yang, Lewis, 2002; Turner, Lewis, King, 2003; Lewis, 2004, Lewis et al., 2007; Tanimura et al., 2010; Bechard et al., 2016). This heterogeneity both models the clinical presentation and provides an opportunity to investigate neurobiological associations of between and within group behavioral differences (Turner et al., 2002; 2003; Bechard et al., 2016). By including multiple assessments across time, we can address the question of how associated neurobiological mechanisms change with the progression of repetitive motor behaviors.

An important goal of this study was to characterize the development of repetitive motor behaviors in deer mice reared within an enriched environment. Our overall hypothesis was that EE housing would attenuate the development of repetitive motor behaviors, an outcome mediated by increased functioning of the indirect basal ganglia pathway. In Study 1, we sought to characterize repetitive motor behaviors within an enriched environment across development. We hypothesized that rearing mice in EE cages would generate mice with different developmental trajectories of repetitive motor behavior and associated changes in indirect basal ganglia pathway activation. This longitudinal design and statistical approach was used previously in standard reared deer mice to identify three developmental trajectory groups and associated differences in neuronal activation (Muehlmann et al., 2015; Tanimura et al., 2010, 2011). Within the enriched environment, however, this longitudinal approach employing repeated testing of individual mice was unsuccessful in attenuating repetitive motor behavior development. Therefore, in Study 2, we employed a cohort design to characterize repetitive behavior development in deer mice reared in enriched and standard laboratory cages and associated neurobiological differences in basal ganglia function at key developmental time points. We hypothesized that, compared to standard reared mice, EE reared mice would develop lower frequencies of repetitive motor behaviors with age, and corresponding increased levels of activation in indirect pathway nuclei as indicated by cytochrome oxidase (CO) histochemistry. To our knowledge, we are the first to characterize the development of repetitive motor behavior within an enriched environment and associate neurobiological changes that mediate their progression versus attenuation.

Methods

Animals

All procedures were approved by the University of Florida's Institutional Animal Care and Use Committee and performed according to the NIH Guide for the Care and Use of Laboratory Animals. Deer mice were bred and housed at the University of Florida in one room maintained at a 16:8 light: dark cycle, 70–75 F and 50–70% humidity. Home cages were furnished with SaniChip bedding, Teklad rodent chow, water, and Nestlet squares for nest construction. Offspring of monogamous breeding pairs were weaned at day 21 and litters split to ensure siblings were housed both in EE and standard housing conditions. In

Study 1, we tested N=59 subjects; n=40 mice were reared in EE housing and n=19 mice were reared in standard housing. In Study 2, we tested N=216 subjects; n=93 were reared in EE housing and n=123 mice were reared in standard housing. Cages were comprised of same-sex, similar age mice (± 1 day) grouped together at weaning and tested at the same age. The day after testing, brains were obtained by anesthetizing mice with isoflurane followed by rapid decapitation.

Enriched housing conditions

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

Standard housing (SH) conditions

Standard laboratory caging ($29 \times 18 \times 13$ cm; n=3–4) reliably induces high levels of repetitive motor behaviors in the majority of deer mice (Powell et al., 1999; Tanimura et al., 2010; Muehlmann et al., 2015). To control for any nutritional differences without promoting foraging, a small amount (~0.25 oz) of bird seed was deliberately placed into the corner of the standard cages each week. Food, water, nestlets and bedding were refreshed every two weeks.

Assessment of repetitive motor behaviors

Mice were removed from their home environments and placed into individual testing chambers ($28 \times 22 \times 25$ cm) at least 30 minutes prior to lights off (10:00 am) for assessment of repetitive motor behaviors. Each assessment lasted for the duration of one entire dark cycle: 10:00–18:00 (8 h total), during which food and water were always available. Deer mice display two topographies of repetitive motor behaviors: vertical jumping, which emerges early in development (by weaning age) and backward somersaulting (emerges later in development). Both topographies involve vertical activity that can be quantified automatically by counting the number of interruptions to photo beam arrays (Columbus Instruments, Columbus, OH, USA) positioned high enough so that normal activity (e.g. rearing, feeding, drinking, ambulation) does not interfere with them. Simultaneous video recordings of each test chamber and each test session insured the accuracy of the automated counts. For each mouse, the total number of repetitive behaviors summed over the entire 8 h test session was calculated and used in subsequent analyses.

The longitudinal design employed in Study 1 involved assessing repetitive motor behaviors repeatedly in the same individuals at 22, 25, 28, 35, 42, 49, 56 and 63 days of age. After each assessment, subjects were returned to their home environments. After completion of the day 63 assessment, mice were anaesthetized with isoflurane and sacrificed via rapid decapitation, and brains were harvested for use in neurobiological assays. The cohort design

employed in Study 2 tested for repetitive behaviors at corresponding developmental time points: 22, 28, 35, 42, 49, 56, and 63 days of age. In this study, individuals were assessed once at a randomly determined developmental time point and then sacrificed for neurobiological assessment. In both studies, same-aged mice reared in standard cages were tested at the same time as EE-reared mice.

Cytochrome Oxidase (CO) histochemistry

CO activity reflects the oxidative metabolic capacity of neurons due to its functional role in the process of generating ATP (Wong-Riley, Nie, Hevner, Liu, 1998). CO activity is a measurement of long-term (days to weeks) neuronal metabolic activity (Sakata, Crews, Gonzalez-Lima, 2005), and has previously been used to detect differences in activation of basal ganglia as a function of repetitive motor behaviors (Turner et al., 2002; Tanimura et al., 2010, 2011; Bechard et al., 2016). The optical density dependent variable is a metric for the amount of light that passes through the tissue stained for CO. Thus, darker staining will have a higher optical density value indicating more CO activity. The CO staining protocol (Gonzalez-Lima and Cada, 1998) was performed on brains that were snap-frozen in 2-methylbutane and stored in a -80°C freezer. Sagittal sections (20 μm) sliced on a cryostat (-20°C) were collected from both hemispheres at 1–2 mm lateral to the midline and mounted onto microscope slides (Superfrost Plus, FisherBrand, Fisher Scientific, USA) with 6 sections on one slide. These adjacent sections were collected 100 μm apart. Standards were made from homogenized brain tissue of non-subject deer mice and were included in each assay to ensure linearity of optical density measurements. Slides were stained and cover slipped with Permount.

Quantification of CO histochemistry

Optical density measurements were taken (ImagePro software, Media Cybernetics) from the basal ganglia regions of interest: dorsal lateral striatum (DLS), GP, STN, and SNR, as well as the CA1 region of the hippocampus (HPC) and motor cortex. The HPC was chosen for analysis to assess for general enrichment effects on activation, as this region is heavily implicated in experience-dependent plasticity (Nithianantharajah and Hannan, 2006). The motor cortex was selected as an additional reference region. For each brain region, neuronal metabolic activation values were calculated by averaging the optical density measurements across multiple adjacent sections.

Statistical analyses

In Study 1, a Repeated Measures General Linear Model (SPSS v23, SPSS Inc. Chicago, IL, USA) with age, housing, and sex as factors was used to assess differences in repetitive behavior development. The individual mouse was treated as a subject-specific random effect. When our model violated Mauchly's Test of Sphericity ($p < 0.05$), which assesses whether the variances of the differences between groups are equal, we report the corrected degrees of freedom using Greenhouse-Geisser estimates of sphericity, which adjusts the F-ratio to reduce the likelihood of committing a Type I error. Post-hoc comparisons were assessed using Bonferroni's test.

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

A General Linear Model with age, housing, and sex as factors was used to assess neurobiological differences in the STN, GP, SNR, DLS, HPC and motor cortex. We assessed neuronal activation at two key developmental time points: day 42 (EE: n=12, SH: n=17), the first age at which significant differences in repetitive behavior frequencies due to housing were apparent; and day 63 (EE: n=13, SH: n=20), the age at which asymptotic levels of repetitive behavior frequencies were reached. To aid the interpretation of three-way interactions, we further analyzed the neurobiological data of males (n=35) and females (n=27) in separate analyses, assessing each data set using a General Linear Model with age and housing as factors. For all analyses, Levene's test of equality of error variances and the Kolmogorov-Smirnov test of normality were employed to assure model assumptions were met, and transformations of the data were performed where necessary. We also conducted Pearson Correlation analyses (SPSS) to assess significant relationships between repetitive behavior frequencies and activation of each brain region (STN, GP, SNR, DLS, HPC and motor cortex) separately for each sex, age, and housing group. The SNR of 2 mice and the GP of 1 mouse were not analyzable due to processing errors.

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

Results

Study 1: Developmental trajectories of repetitive motor behaviors within an enriched environment (repeated testing)

In the longitudinal study where individual mice were repeatedly tested, the frequencies of repetitive motor behaviors increased with age ($F(3,171)=45.6$, $p<0.001$; see Fig. 1), but did not differ due to rearing environment or sex.

Study 2: Development of repetitive motor behaviors in EE and standard housing (single test)

Figure 2 shows the mean total frequencies of repetitive motor behaviors across development for deer mice reared in standard and enriched environments and assessed at only one time point. To meet model assumptions, a square root transformation was applied to the behavioral data. Compared to standard housing, EE housing inhibited the development of repetitive motor behaviors (age*housing: $F(6,188)=4.36$, $p<0.001$; see Fig. 2). A main effect of housing ($F(1,188)=40.4$, $p<0.001$) and age ($F(6, 188)=15.9$, $p<0.001$) were also found. Post-hoc analysis for age indicated that days 28 and 35 ($p=0.59$), and 42, 49, and 56 (all $p>0.05$) were not different from each other, whereas all other days showed significant differences in repetitive behavior frequencies (all $p<0.05$).

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

Study 2: Neuronal activation of the indirect basal ganglia pathway across development

Assessment of neuronal activation in the STN at days 42 and 63 resulted in a significant three-way interaction of sex, age, and housing ($F(1,54)=14.8$, $p<0.001$; see Fig. 3a). A significant interaction between housing and age ($F(1,54)=8.0$, $p=0.006$) and a main effect of sex, indicating females had overall higher levels of CO ($F(1,54)=5.9$, $p=0.018$), were also found. In the SNR, this same pattern of a significant sex, age, and housing interaction ($F(1,52)=8.0$, $p=0.007$; see Fig. 3b) and age by housing interaction ($F(1,52)=5.5$, $p=0.022$) resulted, with no main effect of sex in this brain region. The neuronal activation patterns in the GP, DLS, and HPC also resulted in significant three-way interactions between sex, age, and housing (GP: $F(1,53)=10.5$, $p=0.002$; DLS: $F(1,54)=5.7$, $p=0.02$; HPC: $F(1,54)=4.9$, $p=0.03$; see Fig. 3), and a significant effect of age in the DLS ($F(1,54)=5.2$, $p=0.026$); no other significant interactions or main effects were found. In the motor cortex, there were no significant differences in activation.

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

were no significant differences in neuronal activation in any region (i.e. the STN, SNR, GP, DLS, HPC and motor cortex; all $p > 0.05$). Pearson correlation analyses revealed no significant correlations between repetitive behavior frequencies and activation in any brain region of any subgroup (all $p > 0.05$).

Effects of repeated testing on repetitive motor behavior development

Within the EE condition, the effect of age for single versus multiple tested mice was highly significant ($p = 0.002$); see Fig. 4a). Within the SH condition, this effect was again significant ($p = 0.037$); see Fig. 4b), although less pronounced. Thus, repeated testing significantly increased repetitive motor behavior development within the enriched environment and, to a much lesser extent, in the standard cage environment.

Discussion

In two studies, we characterized the development of repetitive behavior within an enriched environment and associated developmental changes in indirect basal ganglia pathway functioning. We hypothesized that rearing mice in EE housing would attenuate the progression of repetitive motor behavior development and align with developmental increases in indirect pathway nuclei activation. In Study 1, we characterized the development of repetitive motor behaviors within an enriched environment by repeatedly testing subjects from weaning into adulthood. As rearing within an enriched environment typically has a robust attenuating effect on repetitive motor behavior development (Lewis, 2004; Mason et al., 2007), the result from the longitudinal approach was unexpected, in that we were unsuccessful in generating the expected overall EE-induced attenuated frequencies of repetitive behavior (see Turner et al., 2002, 2003; Bechard et al., 2016) or an asymptotic low trajectory (Tanimura et al., 2010; Muehlmann et al., 2015).

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

Rearing effects on behavior result from a complex interaction among prior handling, social experience, and test conditions (Holson, Scallet, Ali, Turner, 1991). Many EE paradigms employ brief repeated handling sessions, as this typically generates mice with less anxiety-like behaviors (e.g. Ábrahám and Kovács, 2000; Río-Álamos, Oliveras, Cañete, Blázquez, Martínez-Membrives, Tobeña, Fernández-Teruel, 2015). Deer mice, however, are an outbred

wild stock, and contrary to *Mus musculus*, appear to resist habituation to handling. In deer mice from both enriched and standard environments, repeated testing exacerbated repetitive motor behavior development, although this effect was more pronounced in the enriched mice. The evidence for a systematic effect of repeated testing in the SH condition was less apparent. As the single testing involved separate groups of mice, it could be something anomalous at days 49 and 56 in the SH group, as by day 63, the two testing conditions converge. These exploratory analyses further showed that the effect of repeated testing on repetitive motor behavior development does not become apparent until later in development (SH: at 49 days of age; EE: at 42 days of age), suggesting that at least four tests are required to influence behavior. The relationship between repeated testing, stress, and development of repetitive motor behaviors and the differential effects of housing condition will need further investigation. To this end, conducting observations of repetitive motor behaviors within the home cages of EE mice may be another potential approach, although protracted individual assessment would be very challenging.

Due to the abolition of our typically robust enrichment effect by use of a longitudinal design, in Study 2 we employed a cohort design to assess the development of repetitive motor behaviors within an enriched environment. With this single-test approach to assessing behavior, the repetitive motor behavior development within an enriched environment was significantly attenuated. A one-week period of exposure to the enriched environment starting at weaning was sufficient to arrest further progression of repetitive motor behaviors. Differences between EE and standard reared mice were apparent three weeks after mice were introduced to their environments. This aligns with other studies investigating enrichment effects on behavior and structural brain plasticity (Young, Lawlor, Leone, Dragunow, During, 1999; Scholz, Allemang-Grand, Dazai, Lerch, 2015). Within both rearing environments, repetitive motor behaviors significantly increased with age for both enriched and standard reared mice, but significant differences due to housing were apparent by the beginning of adolescence (i.e. day 42). At this age, the slopes of repetitive motor behavior development diverge based on housing condition, with repetitive behavior frequencies of standard mice continuing to increase compared to plateaued frequencies of enriched mice. Follow-up analyses confirm that within EE housing, the progression of repetitive behavior development was complete by day 28, in this study, less than 1 week after being introduced into the enriched environment. In contrast, mean frequencies of repetitive motor behaviors in standard caged mice rose in a step-wise fashion across adolescence. This novel finding suggests that, rather than reducing already established repetitive motor behaviors, rearing in EE prevents the progression of repetitive motor behavior development. Interestingly, mean frequencies following 1 week of post-weaning EE exposure were no different than those following 6 weeks of post-weaning EE exposure.

In line with our hypothesis, we found increased neuronal activation of indirect pathway nuclei, particularly in the STN, in mice reared with EE that corresponded to lower frequencies of repetitive behavior. Results were dependent on sex, however. In enriched males, CO activity in the STN, GP, SNR and DLS increased across adolescence and levels of repetitive motor behaviors remained low. In standard housed males, CO activity in the STN, GP, and SNR decreased (DLS levels did not change) across adolescence and levels of repetitive behavior increased. However, there was no clear relationship between CO activity

and repetitive behavior development in female mice. As a consequence of CO activity in female mice showing minimal changes with age, several significant three-way interactions resulted. Only the motor cortex was unaffected by the interaction of housing, sex, and age. Conducting separate analyses by sex further clarified the influence of environment on the activation of nuclei across development. Males reared in EE housing show similar or slightly lower activation of basal ganglia nuclei in adolescence than standard reared males, yet higher activation in adulthood. As housing differences in repetitive behavior were already apparent at day 42, our data suggest that increased performance of the aberrant behavior preceded changes in neuronal activation as indicated by CO histochemistry. Outside of the basal ganglia nuclei, there were no differences in activation due to age or housing. No regional, developmental or housing differences in neuronal activation were found in females. Although secondary analyses separated by sex indicated that CO differences between EE and standard males were not significant outside of the basal ganglia, the initial significant three-way interaction in the HPC suggests a potential generalized effect of EE to increase neuronal activation. A generalized effect of EE was not unanticipated, given the wide-ranging therapeutic effects of EE on CNS development (Hannan, 2014). Notwithstanding, the STN and GP lie within the indirect pathway, and therefore these data continue to support an important role for the functioning of the indirect basal ganglia pathway in the development of repetitive motor behaviors.

The current results replicate our recent finding that EE-induced attenuation of repetitive motor behaviors is associated with increased functioning of the indirect basal ganglia pathway in adult male, but not female, deer mice (Bechard et al., 2016). Within the standard environment, they also support Tanimura et al. (2011) findings of reduced activation in the STN and SNR with increasing age (developmental time points: days 28, 46, and 63) and repetitive behavior development, although these were statistical trends. Changes in activation occurred mostly between days 46 and 63, and so neuronal activation changes in the indirect pathway were proposed to lag behind rather than lead repetitive motor behavior development (Tanimura et al., 2011). Our results support this, and also that standard reared male mice show decreased activity in the STN and SNR, and very little change in activity of the DLS and motor cortex, between adolescence and adulthood, a developmental time period in which repetitive motor behavior significantly increased. Unfortunately, no investigation of sex effects was available for comparison. Future investigations of other brain regions shown to be important in stereotypy (e.g., nucleus accumbens, frontal cortex; McBride and Hemmings, 2005; Phillips, Choleris, Ervin, Fureix, Harper, Reynolds, Niel, Mason, 2016; Güldenpfennig, Wet Wolmarans, du Preez, Stein, Harvey, 2011) might yield differences in female mice.

Neuronal activation differences in basal ganglia nuclei of males and females have been reported previously. For example, differences in CO activity in the striatum of males and females have been reported following caffeine administration (Jones, Jing, Stonehouse, Stevens, Edelman, 2008), and in the frontal cortex regions of preweanlings following mother-infant separation and early handling (Spivey, Padilla, Sumake, Gonzalez-Lima, 2011). CO histochemistry may be interacting with female hormonal phases. In support of this, differences in the specific activation of CO based on phase of estrus cycle were found in mouse ovarian cells (Chapman, Waterhouse, Michael, 1992) and brown adipose tissue of

rats (Puerta, Rocha, González-Covaleda, McBennett, Andrews, 1998). Since we did not evaluate the phase of estrus cycle in females, the variations in CO activity may have masked any effects of age and housing, a hypothesis now needing direct testing.

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

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Figure 1.

Longitudinal study: The effect of housing on repetitive motor behavior development in repeatedly tested deer mice reared in enriched (EE: n=40) or standard housed (SH: n=19) environments. Error bars are $\pm$ SEM.

Figure 2.

Cohort study: Mean total frequencies of repetitive motor behaviors across development for mice reared in enriched (EE: n=93) and standard housed (SH: n=123) environments. Error bars are $\pm$ SEM.

Figure 3.

The effect of housing on neuronal activation at two developmental time points for deer mice reared in enriched (EE: males n=11, females n=14) and standard housed (SH: males n=16, females n=21) environments in a) the subthalamic nucleus (STN), b) the substantia nigra pars reticulata (SNR), c) the globus pallidus (GP), d) the dorsal lateral striatum (DLS), e) motor cortex (M1), and f) hippocampus (HPC). Error bars are $\pm$ SEM.

Figure 4.

The effect of repeated testing on repetitive motor behavior development for deer mice reared in (A) enriched (repeatedly tested EE: $n=40$, single tested EE: $n=93$) and (B) standard environments (repeatedly tested SH: $n=19$, single tested SH: $n=123$). Data values are back-transformed mean square root repetitive behavior frequencies and so no error bars are depicted.