Environmental Enrichment's Effects on Exploration and Response to Novelty in Adolescent Rats

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Abstract

Adolescence is a critical period in development for both neural and behavioral change that is characterized by risk-taking, exploration, and novelty-seeking. However, these behavioral and neural changes may be influenced by experiences such as environmental enrichment (EE). In this study, the effects of EE on exploration and response to novelty was examined in rats at two different points in adolescence (PND 36 and PND 50). An Object in Place (OiP) task, which combines aspects of both field and object exploration upon initial exposure to the environment and at 15 and 60 min delays, was utilized to assess novel-object preference, novel-object location preference, and time and movement within the risky interior of a field. After behavioral testing was completed, activation of brain regions relevant to exploration and novelty response, the lateral amygdala (LA) and basolateral amygdala (BLA), was assessed. At PND 36, EE rats exhibited increased risk-taking among several behavioral measures when compared to controls. However, at PND 50, EE rats exhibited increased habituation or no difference among those same behavioral measures when compared to controls. While no significant difference was found in neural activation in the LA between EE rats and controls, EE rats showed significantly greater activation in the BLA than controls. These results highlight the significance of the impact of EE during adolescence and that significant behavioral changes may occur in conjunction with EE from early to late adolescence.

Effects of Environmental Enrichment on Exploration and Response to Novelty in Adolescent Rats

Adolescence is a developmental period that is characterized by a variety of behavioral changes in many mammals. The behaviors typifying adolescence are oftentimes considered to be more high-risk than behaviors that typify other developmental periods. Previous studies (Simpson & Kelly, 2011) have suggested that environmental enrichment, repeated interaction with conspecifics and novel objects and locations, may moderate these high-risk behaviors. Similarities across mammalian brain structure and their corresponding functional significance allow tentative conclusions to be drawn about human behavior from an animal model. For example, comparisons can be made between high-risk, sensation-seeking behaviors in humans, such as reckless driving, and high-risk, novelty-seeking behaviors in rodents, such as uninhibited exploration of novel objects and spaces and spending time in open, vulnerable positions in an environment. In this study, adolescent rats were exposed to an enriched environment, then tested using the Object in Place task, which combines object and open field exploration at both early and late adolescence. The purpose of this study was to utilize an animal model to determine how environmental enrichment impacts exploration, response to novelty, and the lateral and basolateral amygdala, a related brain structure, during adolescence.

Adolescence

Adolescence is a developmental period of hormonal, physiological, and behavioral changes for many mammalian species. These behavioral changes include increased risk-taking, exploration, sensation-seeking, and response to novelty. In humans, adolescence is often characterized by poor decision-making that leaves adolescents vulnerable to engagement in risky, dangerous behaviors, including substance abuse, reckless driving, and unprotected sex

(Ernst, Pine, & Hardin, 2005; Steinberg, 2007). These risky behaviors have been found to be largely unaffected by interventions aimed at changing knowledge, attitudes, and beliefs due to the fact that adolescents are no worse at understanding risks and vulnerability to risks than adults (Steinberg, 2007). While interventions may improve adolescents' understanding of risks, the risky behaviors themselves do not change (Steinberg, 2007). This is largely due to the tendency of adolescents to be worse than adults at skills related to decision-making and risk-taking, such as impulse control, emotional regulation, delay of gratification, and resistance to peer influence (Steinberg, 2007). These deficits are based in regions of the brain that act as cognitive controls, which do not fully develop until adulthood (Blakemore & Choudhury, 2006; Ernst et al., 2005; Steinberg, 2007). While limbic regions of the brain, which control basic emotions, are remodeled during adolescence due to the hormonal changes taking place during puberty, the regions of cognitive control, such as the cortex, are underdeveloped (Blakemore & Choudhury, 2006; Ernst et al., 2005; Steinberg, 2007). This disparity between cortical and limbic development is largely responsible for the behavioral changes observed during adolescence.

Analogues of these behavioral changes that characterize adolescence are also observed in rats. Both human and rodent adolescents have been found to exhibit more impulsive choices, which describes a preference for smaller rewards over a shorter period of delay versus larger rewards over a longer period of delay (Sturman & Moghaddam, 2011). These similarities are also observed in other facets of human and animal adolescent behavior. For example, adolescent rats exhibit a significantly shorter latency period when approaching novel objects than adult rats (Stansfield & Kirstein, 2006). Subsequently, adolescent rats exhibit greater time exploring novel objects than adult rats, but also decreased frequency of

contact with novel objects after a delay compared to adult rats (Stansfield & Kirstein, 2006). This suggests that, while adolescent rats show increased response to novelty, they also habituate to novelty faster than adult rats.

While adolescents tend to take more risks during exploration than juvenile or adult rats, there is also research to suggest that risk-taking behaviors increase across the adolescent development period (Lynn & Brown, 2009; Macrì, Adriani, Chiarotti, & Laviola, 2002). Macrì et al. (2002) found that adolescent rats spent more time in the open areas of a plus-maze and entered the open areas more times and more quickly than juvenile and adult rats. These adolescent behavioral tendencies exemplify increased novelty-seeking. Additionally, they are indicative of both increased drive to explore and decreased anxiety towards novel, potentially dangerous environments (Macrì et al., 2002). While these novelty-seeking behaviors are adaptive, in that adolescent rats must leave their home nest and seek new territories, food sources, and mates to survive and reproduce, these novelty-seeking behaviors are also maladaptive in that they leave adolescents vulnerable to predators and other dangers (Lynn & Brown, 2009; Macrì et al., 2002).

Research has indicated that adolescent development is influenced by corresponding changes in neural function in areas of the brain involved in motivation, emotional response, and decision-making, such as the amygdala (Ernst et al., 2005; Lynn & Brown, 2009). These changes have been implicated in increased exploration and locomotor activity, even in potentially risky and aversive environments (Lynn & Brown, 2009). Increased risk-taking has been associated with reduced gray matter in the brain compared to adults, impulsive choice has been associated with augmentation of white matter during adolescence, sensation-seeking has been associated with neurogenesis, and increased novelty preference has been

associated with heightened receptor expression (Sturman & Moghaddam, 2011). However, these changes in neurochemistry during adolescence may be moderated by influences of the environment on the brain.

Environmental Enrichment

Environmental enrichment (EE) can be defined as exposure to physical and social stimulation that is greater than what would be experienced in a typical home environment. EE has been shown to have a variety of effects on animal behavior, neurochemistry, and neuroplasticity. EE has been shown to have especially strong and enduring effects on both neurochemistry and resulting behavior when subjects are exposed to the enriched environment early on in development, including during adolescence (Forgays & Forgays, 1951; Hymovitch, 1952; Simpson & Kelly, 2011).

The extent of the effects of EE may also vary depending on the type and length of time of EE to which the animals are exposed (Simpson & Kelly, 2011). Common enrichment objects include platforms, plastic toys, ropes, ramps, and tubes (Leggio et al., 2005; Magalhaes et al., 2006; Segovia, Del Arco, De Blas, Garrido, & Mora, 2008; Simpson & Kelly, 2011). Will et al. (1986) found improvements in maze learning among rats with hippocampal lesions when they were exposed to cages with as few as three enrichment objects. The length of time and number of exposures may also vary from a single, hour-long EE exposure to continuous exposure (Ali, Wilson, & Murphy, 2009; Simpson & Kelly, 2011). Ali et al. (2009) found that a single, hour-long EE exposure stimulated neural activation in areas of the brain including the claustrum, infralimbic cortex, hippocampus, hypothalamus, and amygdala. However, intermittent, one- to two-hour-long daily exposure for approximately one to four weeks is a common EE protocol (Pinaud, Penner, Robertson,

& Currie, 2001; Simpson & Kelly, 2011; Will, Rosenzweig, Bennett, Hebert, & Morimoto, 1977; Will et al., 1986).

EE has a profound impact on animal behavior. In adult rats, early EE experience increases habituation to novelty, decreases locomotor activity, and increases novel-object preference during novel-object recognition tasks (Zimmerman, Stauffacher, Langhans, & Würbel, 2001). In open field tests, EE rats move less and exhibit increased habituation to the novel environment than controls (Gould, Dao, & Kovacsics, 2009). When compared to control rats, enriched rats tend to exhibit improved performance on complex problem-solving tasks, improved memory, and lower anxiety (Forgays & Forgays, 1951; Hymovitch, 1952; Sztainberg, Kuperman, Tsoory, Lebow, & Chen, 2010). However, there is some conflicting evidence in how EE affects the exploration of novel objects and environments in adult rats. Simpson and Kelly (2011) assert that most of the research points to adult EE rats exploring novel objects and environments less than controls, while Renner and Rosenzweig (1987) found that EE rats interacted more with objects in a free exploration situation than controls. This contradiction in the existing literature could potentially be explained by differences in the type and extent of EE. Additionally, certain tasks may lead to a tendency among EE rats to exhibit the effects of habituation to novelty, while other tasks may lead to a tendency towards novelty recognition and preference. Further, the effects of EE on exploration in adult rats are likely to be different than the effects on explorations in adolescent rats.

Forgays and Forgays (1951) found that when adult rats had been kept in a free environment at a young age, they performed better at a maze task requiring problem solving than adult rats that had been kept in a caged environment at a young age. Additionally, adult rats kept in more complex free environments at a young age were better at problem solving

the maze than rats kept in a simpler free environment (Forgays & Forgays, 1951). Hymovitch (1952) found support for these findings in a study that was very similar methodologically, with the exception of the fact that rats were tested in late adolescence instead of in adulthood. These behavioral differences between EE and non-EE rats may be even more profound in adolescents, who are already experiencing a period of increased behavioral changes and neuroplasticity when compared to older rats.

These behavioral changes in exploration and novelty preference associated with EE could likely be attributed to the changes in the brain caused by EE. Matsumori et al. (2006) found that EE facilitated the process of neurogenesis in the dentate gyrus of the hippocampus, a region related to memory, after an ischemic stroke in adult rats. When Matsumori et al. (2006) compared EE rats to control rats at approximately 5 days post-ischemia, EE rats had approximately four times as many cells in the dentate gyrus. In fact, EE has been found to promote neurogenesis, cell differentiation, plasticity, dendritic growth, cortical thickness, and the formation of synapses (Simpson & Kelly, 2011). Okuda et al. (2009) found that EE stimulates the generation of progenitor cells and their differentiation in the amygdala of mice, which may account for the anxiolytic effects typically observed in the behaviors of EE animals. Sztainberg et al. (2010) found a similar effect in their study of the effects of EE on anxiety and the amygdala. According to Sztainberg et al. (2010), the anxiolytic effect of EE on rats is mediated by a decrease in corticotropin-releasing factor receptors in the limbic system, and, in this case, the basolateral amygdala (BLA) specifically. These effects of EE on neurogenesis and cell differentiation in the amygdala are likely to impact the functionality of different regions of the amygdala and corresponding behaviors.

Lateral and Basolateral Amygdala

Along with the amygdala, both the medial prefrontal cortex (mPFC) and periaqueductal gray (PAG) function as the fear and anxiety circuit of the brain. When a potentially dangerous stimulus is presented to an animal, the amygdala responds by sending signals to the PAG, which then initiates the appropriate fight, flight, or freeze response (Chan et al., 2011). However, the activity of the amygdala may be modulated by the mPFC. While the infralimbic subdivision of the mPFC inhibits fear responses by decreasing signals sent to the PAG by the amygdala, the prelimbic subdivision of the mPFC intensifies fear responses by increasing signals sent to the PAG by the amygdala (Chan et al., 2011). This relationship between the amygdala, which is part of the limbic system and is fully developed by adolescence, and the mPFC, which is part of the brain's system of cognitive control, and, therefore, does not fully develop until adulthood, help to explain the novelty-seeking and potentially dangerous exploratory and risk-taking behaviors that characterize the period of adolescence (Blakemore & Choudhury, 2006; Ernst et al., 2005). Examination of the amygdala in relation to EE and its effects on behavior is especially significant. EE promotes plasticity in many regions of the brain, even in regions that have already developed (Okuda et al., 2009; Pinaud et al., 2001), and, as the amygdala has already developed by the onset of adolescence, the influence of EE on amygdala plasticity and corresponding behaviors is of interest.

The amygdala is involved in important aspects of survival and serves as the epicenter of emotional memory, which holds significant implications for the role of the amygdala in exploration and response to novelty (Phelps & Anderson, 1997). As the epicenter of emotional memory, the amygdala allows individuals to remember single-exposure fear events and affects how individuals respond to new and potentially dangerous stimuli during

exploration, serving an important adaptive role in evolution. Additionally, the amygdala plays a large role in both the acquisition of and expression of emotional memories, influencing both the storage of information about arousing stimuli and reactions to future arousing stimuli (Phelps & Anderson, 1997).

An area of the amygdala considered essential specifically for its role in fear learning is the BLA. The plasticity of the BLA is essential for associations to be made between stimuli and fear responses (Ponnusamy, Poulos, & Fanselow, 2007). For example, when the BLA is made reversibly inactive prior to fear conditioning, the formation of a fear response to the stimulus cannot be acquired (Miserendino, Sananes, Melia, & Davis, 1990). Further, inactivation of the BLA during testing of a conditioned fear response eliminates any expression of fear towards the conditioned stimulus (Helmstetter & Bellgowan, 1994). The ability to acquire and express fear responses to dangerous stimuli is particularly important for adolescent rats, who are likely to be exploring novel environments and learning about new dangers.

The lateral amygdala (LA) may function as an area involved in the storage of emotional information (Schafe, Doyere, & LeDoux, 2005). Repa et al. (2001) found that the LA is a particularly important site of convergence between conditioned and unconditioned fear stimuli, allowing for the initiation and storage of long-term memories related to those stimuli. The role of the amygdala in responding to and remembering fearful, potentially dangerous situations impacts how individuals react to novel, potentially dangerous stimuli (Phelps & Anderson, 1997). Further, the effects of EE on neurogenesis and cell differentiation in the amygdala, as seen in the research by Okuda et al. (2009), may modify the relationship between the amygdala and responses to novel, potentially fear-provoking

stimuli. This relationship between EE and neurogenesis and cell differentiation in the amygdala holds functional significance for research on adolescents because, while the mPFC is still developing, the amygdala develops early on, and changes to the amygdala during adolescence may modify the maturation of the pathway between amygdala and mPFC.

A common method for identifying activated neurons in different parts of the brain is by staining for c-fos protein (Ali et al., 2009; Bullitt, 1990; Loebrich & Nedivi, 2009; Pinaud et al., 2001; Sagar, Sharp, & Curran, 1988). C-fos proteins are expressed in neurons following depolarization of the cell after neural activation (Bullitt, 1990; Loebrich & Nedivi, 2009; Sagar et al., 1988). Previous studies have found that rodents exposed to EE may exhibit increased neural activation when compared to controls, as evidenced by increased c-fos expression (Ali et al., 2009; Pinaud et al., 2001).

Exploration and Response to Novelty

Exploration, which can be defined as active investigation and locomotion that allows an animal to gain information about a novel environment, has played a critical role in evolution and individuals' abilities to survive in any given environment (Lynn & Brown, 2009). While exploration is adaptive in its potential for finding an improved environment, novel environments also pose a threat in the potential for unknown danger, such as the presence of predators. Therefore, exploration and interaction with novel stimuli are associated with higher levels of anxiety and greater risk-taking behaviors (Macrì et al., 2002). Greater tendency towards exploratory behaviors is indicative of a greater tendency towards novelty-seeking. However, exploratory behavior, especially during adolescence, potentially allows individuals to learn, disperse to new and improved territories, and develop the skills necessary for survival (Lynn & Brown, 2009).

Despite these general trends in exploration and response to novelty across all rats, there are some sex differences in how and how much male and female rats explore novel environments. Lynn and Brown (2009) found that locomotor activity and time spent in aversive areas of a novel environment increased across adolescence for female rats, but not for male rats. This suggests that there may be sex differences in the underlying neuroendocrine systems of male and female rats, affecting fear, anxiety, response to novelty, motivation to explore, willingness to take risk, and memory of the environment (Lynn & Brown, 2009). These sex differences in exploration and novelty-seeking are important to consider when examining the impact of EE on such behaviors and related brain structures, such as the amygdala, during the adolescent period, when these behaviors are already likely to be changing.

Open Field Tests (OFT) were developed as a measure of exploration, habituation and response to novel environments, and general activity and emotionality (Simpson & Kelly, 2011). An OFT consists of a large, open platform surrounded by a barrier. In an OFT, rats tend to display a phenomenon known as thigmotaxis, in which they prefer to explore the perimeter of a novel environment (Simpson & Kelly, 2011). This phenomenon is considered to be due to the underground burrows and enclosed structures in which rodents have evolved to live (Gould et al., 2009). Time spent in the interior of the field is typically associated with lower levels of anxiety and increased exploratory drive, since the interior of the field is a riskier area in which to be than the periphery (Gould et al., 2009; Simpson & Kelly, 2011). These low levels of anxiety and harm avoidance are frequently associated with increased risk-taking, as seen in adolescent rats (Macrì et al., 2002).

The Object in Place (OiP) task was developed as a way to assess recognition memory in rats. However, at its core, the OiP task examines object and location preference, as well as familiarity and novelty preference (Barker & Warburton, 2009). During the initial trial of an OiP task, rats are familiarized with four different objects. The locations of two of the objects are then switched. Rats that spend more time with the newly located objects express a novel location preference. Additionally, the OiP task functions as a modified OFT, where the only difference in layout between the OiP task and a traditional OFT is the addition of the four objects to the field. This allows for simultaneous assessment of both open field exploratory behaviors and novelty and familiarity preferences during an OiP task.

The Current Study

Adolescence is a period of increased response to novelty and exploration (Lynn & Brown, 2009; Stansfield & Kirstein, 2005). However, EE is likely to affect these adolescent risk-taking behaviors in an interaction mediated by brain regions that include the LA and BLA (Okuda et al., 2009; Sztainberg et al., 2010). In this study, this interaction was examined using an OiP task featuring aspects of an OFT. The purpose of this study was to determine the effects of EE on exploration, novelty preference, and risk-taking at two different time points in adolescence, as well as to evaluate any potential underlying differences between EE and control rats in BLA and LA structure that may influence task performance.

Based on the existing body of research, it was posited that EE adolescent rats would exhibit differences in novelty preference and risk-taking behaviors when compared to the controls during exploration of a novel environment. Hypothesis 1 predicted that EE rats would spend less time with the objects than non-EE rats overall, based on evidence that EE

promotes habituation to novel environments (Zimmerman et al., 2001). Due to these same effects of EE on habituation, it was expected that EE rats would spend less time with the newly located objects than the controls (Hypothesis 2).

In addition to differences between experimental groups in the exploration of the objects, it was posited that there would also be differences in field exploration. Due to the anxiolytic effects of EE on the amygdala previously mentioned (Sztainberg et al., 2010), it was hypothesized that the EE rats would spend more time overall in the riskier interior of the field, and travel through more squares in the interior than the controls, due to these same anxiolytic effects on the amygdala (Hypothesis 3). Due to the maturation of the brain across adolescence and the increased amount of time spent in an enriched environment for the EE rats, Hypothesis 4 predicted that there would be a difference in behaviors relating to both object and field exploration from PND 36 to PND 50.

Because these behavioral differences between EE and non-EE rats and between younger and older adolescent rats are largely based on changes in brain activity, a difference in cell activation in the LA and BLA between EE and control rats was hypothesized. Based on research indicating that EE promotes neurogenesis (Matsumori et al., 2006; Okuda et al., 2009; Simpson & Kelly, 2011), it was predicted that EE rats would have greater activation in both the BLA and LA than controls (Hypothesis 5).

Materials and Methods

Subjects

The subjects were 16 male and 16 female Long-Evans rats born in the Arts and Sciences Animal Facility at Appalachian State University. After weaning, subjects were housed in groups of four in plastic shoebox cages. Each home cage contained a layer of

shredded bedding and subjects had access to food and water ad libitum. Subjects were housed in a temperature- and humidity-controlled room with a 12-hour on/12-hour off light and dark cycle. The housing room remained closed and relatively quiet with the exception of light traffic due to animal care and maintenance and the retrieval of subjects for experimentation. All care and use of rats in this study was approved by the Institutional Animal Care and Use Committee at Appalachian State University (#15-02, M. C. Zrull, PI, approved August 14, 2014, see Appendix).

Environmental Enrichment

Eight male and eight female rats were exposed in same-sex groups to EE cages as a part of the experimental condition (see Figure 1). The subjects were exposed to the EE cages 10 times from PND 21 to PND 36, then nine more times from PND 37 to PND 49. Each exposure to the EE cage lasted for 90 min. EE cages consisted of hardware cloth and a wooden frame measuring 45.7 x 48.3 x 78.7 cm (w x d x h) with four platforms at various heights throughout the cage. The platforms were connected by ramps, with the lowest platform connected to the floor of the cage via steps. Various objects, including hair curlers, balls, and PVC pipes were placed around the cage or hung from the ceiling of the cage during EE sessions. An alternating sequence of different objects and arrangements of objects was used for the EE sessions.

A total of 16 age-matched control rats, eight male and eight female, experienced only non-enriched, home cages. To control for handling of the EE subjects by experimenters during transfer from home cages to EE cages, control subjects were picked up and handled twice for about 10 seconds each time in accordance with each instance of EE for the experimental group.

OiP Task

At PND 36 and at PND 50, an OiP task was performed. At each age, a three-trial version of the task was performed with each trial lasting 3 min. The delay after the first trial was 15 min and the delay after the second trial was 60 min. The task took place in an open field measuring 1 m x by 1 m that was marked with an 8 x 8 grid. A curtain fixed with different visible markers surrounded the field to provide a point of reference for the subjects. Four different objects were fixed to the field as shown in Figure 2. After Trial 1, the locations of the bottom two objects were switched, with the locations of the top two objects being switched after Trial 2. During each trial, time in contact with each object was recorded. All trials were videotaped for reliability analysis and further behavioral coding.

Field Exploration

Exploration of the field was coded using tapes of the OiP task. The number of perimeter squares moved, defined by the outermost squares in the gridded field, and the number of interior squares moved were counted for all 3 min. of all trials. Additionally, the time spent along the perimeter and time spent in the interior were timed, allowing for a calculation of the rate of movement in the field.

Histology and Microscopy

After the final trial at PND 50, each subject was placed in an empty plastic shoebox cage in a quiet and dark room for anywhere from 60 to 120 min to allow for depolarization of the neurons and expression of c-fos protein. After this quiet and dark period, subjects were anesthetized, checked for tail reflex, then intracardially perfused using phosphate buffered saline (PBS) followed by phosphate buffered 4% paraformaldehyde. Brains were removed, fixed in sucrose-paraformaldehyde, and stored at 4 °C until they were cut into 50 μm sagittal

sections. Sections were then rinsed with PBS (2 x 5 min), incubated in 0.5% hydrogen peroxide in water for 15 min, rinsed with PBS again (2 x 5 min), incubated in goat serum and for 60 min, and then placed in primary c-fos antibody SC Cruz 52 made in rabbit (1:1500, Santa Cruz) until the next day. The sections were then rinsed in PBS (6 x 10 min) and incubated in a secondary antibody made from goat (1:400, Vector Labs) for 60 min. The sections were rinsed in PBS again (3 x 10 min), incubated ABC in for 60 min, rinsed in PBS (2 x 10 min, Vector Labs), allowed to react in VIP (Vector Labs), and placed in distilled water for at least 10 min. Some sections were counterstained for all neural cells. During this procedure, thionin was used to stain for Nissl bodies. The sections were then mounted on slides in preparation for microscopy.

After the staining and mounting process was completed, the Nissl sections were used in conjunction with an atlas of structures of the rat brain (Pellegrino, Pellegrino, & Cushman, 1969) to identify the locations of the BLA and LA in the sections stained for c-fos. A Nikon microscope attached to a PixelLink (Ottawa, ON) digital camera was utilized during microscopy. A frame was placed over the 1024 x 768 pixel image at the Plan 10 objective, allowing for consistency in the area of cell-density counting. Three sections for both BLA and LA in each animal were counted, with the exception of sections that were somehow obscured or irregularly stained. Data was collected on the number of darkly-, medium-, and lightly-stained cells in a fixed area of both the BLA and LA. For analysis purposes, only dark cells, which indicated the greatest extent of neural activation, were included in the final data set.

Results

Object Exploration

There was a significant interaction found between delay, EE, and PND for time exploring all objects ($F(2, 27) = 8.48$, $p < .01$). During Trial 1 at PND 36, EE rats spent 33.8% less time exploring all of the objects than the non-EE rats (see Table 1). However, EE rats spent more time exploring the objects during Trials 2 (+38.9%) and 3 (+53.4%) than the non-EE rats (see Table 1). These results indicate that Hypothesis 1, which predicted that EE rats would spend less time with the objects than non-EE rats, was only partially supported at PND 36. At PND 50, EE and non-EE rats spent approximately the same amount of time with the objects during Trial 1 (see Table 1). However, EE rats spent less time with the objects during Trials 2 (-22.9%) and 3 (-33.9%) than the non-EE rats (see Table 1). These results show that, once again, Hypothesis 1 was only partially supported at PND 50.

A significant interaction was found between EE and delay for the proportion of time spent with newly located objects in Trials 2 and 3 ($F(1, 28) = 17.61$, $p < .001$). Non-EE rats spent a significantly greater proportion of time with the switched objects *(*$M = 0.73$, $SD = 0.22$*)* than EE rats ($M = 0.56$, $SD = 0.16$) during Trial 2. However, this pattern reversed during Trial 3, with non-EE rats spending a significantly smaller proportion of time with the switched objects ($M = 0.34$, $SD = 0.26$) than EE rats ($M = 0.47$, $SD = 0.22$). These results partially support Hypothesis 2, which predicted that EE rats would spend less time with the newly located objects than the controls did.

Field Exploration

A significant interaction was found between EE and PND for the time spent in the interior squares ($F(1, 27) = 10.94$, $p < .01$). At PND 36, EE rats spent a significantly greater amount of time in the interior of the field during Trials 1 (+73.6%), 2 (+52.6%), and 3 (+23.3%) than non-EE rats (see Table 2). These results support Hypothesis 3, which

predicted that EE rats would spend more time in the interior environment and move through more squares in the interior environment than non-EE rats. There was a significant effect of delay on time spent in the interior ($F(2, 27) = 9.57$, $p < .01$), where time spent in the interior increased after Trial 1 for both EE (+25.1%) and non-EE rats (+58.1%) at PND 36 (see Table 2). At PND 50, similar results were found to PND 36, where time spent in the interior increased after the first delay for both EE (+33.0%) and non-EE rats (+78.3%; see Table 2).

Movement through squares in the interior followed a similar pattern as time spent in the interior. There was a significant interaction between EE, PND, and number of interior squares entered ($F(1, 27) = 4.76$, $p < .05$). At PND 36, EE rats traveled through a significantly greater number of squares during Trials 1 (+83.4%), 2 (+38.9%), and 3 (+58.2%) than non-EE rats (see Table 3). These results support Hypothesis 3, which predicted that EE rats would spend more time in the interior environment and move through more squares in the interior environment than non-EE rats. Squares traveled in the interior increased after Trial 1 for both EE (+37.8%) and non-EE rats (89.1+%; see Table 3). However, these results did not hold up at PND 50, as there was a significant interaction between PND, delay, and number of interior squares entered ($F(2, 27) = 4.53$, $p < .05$). The rats at PND 50 moved 46.2% less than at PND 36 (see Table 3). Further, the difference between EE and non-EE rats was significantly diminished at PND 50 (see Table 3).

Hypothesis 4, which predicted that there would be a difference in behaviors relating to both object and field exploration from PND 36 to PND 50, was supported in some way for all measures of object and field exploration. As previously described, a difference in trends was found for time EE and non-EE rats spent exploring objects between PND 36 and PND 50 (see Table 1). Additionally, a significant interaction between PND and delay was found

for the time spent with newly located objects ($F(1, 28) = 7.26$, $p < .01$). Rats at PND 36 spent similar proportions of time with the switched objects *(*$M = 0.62$, $SD = 0.16$*)* as rats at PND 50 ($M = 0.67$, $SD = 0.22$) during Trial 2. However, during Trial 3, rats at PND 36 spent a significantly greater proportion of time with the switched objects ($M = 0.46$, $SD = 0.22$) than rats at PND 50 ($M = 0.34$, $SD = 0.26$). Hypothesis 4 was also supported in measures of field exploration. Differences in time spent in the interior were not as prominent between EE and non-EE rats at PND 50 as they were at PND 36 (see Table 2). Further, there was a significant interaction between PND and delay for the number of interior squares entered ($F(2, 27) = 4.53$, $p < .05$). The rats at PND 50 moved 46.2% less than at PND 36 (see Table 3). Additionally, the difference between EE and non-EE rats was significantly diminished at PND 50 (see Table 3).

Histology

Hypothesis 5, which predicted that EE rats would have greater activation in both the BLA and LA than controls, was not supported by the results. There was no significant difference between LA activation in EE rats and controls, $t(43) = 0.19$, $p < .8502$ (see Table 4). However, there was a significant difference between EE animals and controls in activation of the BLA, $t(52) = 2.08$, $p < .05$, with EE rats exhibiting significantly less activation (see Table 4).

Discussion

The current study examined how EE may influence both object and field exploration at two separate points in adolescence, in addition to evaluating neural activation in the LA and BLA. Hypothesis 1 predicted that EE rats would spend less time overall with the objects than non-EE rats, due to the tendency of EE to increase habituation to novelty. This

hypothesis was only partially supported in that EE rats spent more time with the objects than non-EE rats at PND 36 during Trials 2 and 3 and the same amount of time with the objects during Trial 1 at PND 50. These results suggest that, at a younger age, EE may actually lead to increased exploration and risk-taking due to the anxiolytic effects of EE. However, at an older adolescent age, EE may lead to increased habituation, which is consistent with the majority of research findings on adult rats (Simpson & Kelly, 2011; Zimmerman et al., 2001).

Hypothesis 2 predicted that EE rats would spend less time with the newly located objects than the controls did due to habituation. This hypothesis was supported during Trials 1 and 2 at both ages, which suggests that EE promotes adaptation to a rearranged environment within the first 60 min of exposure. However, during Trial 3, the pattern reversed and EE rats spent significantly more time with the newly-located objects than the non-EE rats did. This latent novelty preference among EE adolescents is consistent with the patterns of exploration and novelty preference typically observed in adult non-EE rats (Lynn & Brown, 2009; Macrì et al., 2002; Stansfield & Kirstein, 2006), but inconsistent with patterns of novel-object recognition typically observed in adult EE rats (Zimmerman et al., 2001). As previously indicated, this disparity in findings concerning novelty preference in the existing body of literature may have resulted from differences in EE type and extent, differences in task type, or differential effects of EE on adolescent and adult rats.

Hypothesis 3 predicted that EE rats would spend more time in the interior environment and move through more squares in the interior environment than non-EE rats due to the anxiolytic effects of EE. This hypothesis was supported during all three trials at PND 36. At PND 50, however, the difference between EE and non-EE rats was diminished.

These results resemble the findings concerning total object exploration time, and suggest that EE may have anxiolytic effects during an earlier point in adolescence, but lead to habituation effects later in adolescence when compared to the increased exploration observed at PND 36. While the EE rats and controls showed similar field exploration tendencies at PND 50, the trend among EE rats toward increased adaptation to novel environments with age supports the habituation effects observed in adult EE rats compared to adult controls (Simpson & Kelly, 2011; Zimmerman et al., 2001).

Hypothesis 4 predicted that there would be a difference in behaviors relating to both object and field exploration from PND 36 to PND 50. This hypothesis was supported in some manner for all measures of object and field exploration. Rats at PND 50 tended to display habituation behaviors more consistent with those of adult EE rats (Gould et al., 2009; Simpson & Kelly, 2011; Zimmerman et al., 2001), with the exception of the tendency of adult EE rats to display novel-object-location preference during novel-object-location recognition tasks. However, it is important to note that the effects of EE exposure and age cannot be disentangled in this study, as rats at PND 50 were exposed to a greater number of EE sessions than rats at PND 36. An examination of exploration and novelty preference among rats at different ages in which the extent of EE exposure is controlled for could further elucidate the differential effects of EE based on age.

Hypothesis 5 predicted that EE rats would have greater activation in both the BLA and LA than controls. This hypothesis was not supported for BLA or LA. EE rats actually exhibited less activation in the BLA than controls, which is consistent with the behavioral effects of habituation observed at PND 50. Further, there was no significant difference in activation in the LA between EE rats and controls. The lack of support for this hypothesis

may potentially be explained by the differing functions of the LA and BLA. The BLA is largely responsible for making the initial connections between novel stimuli and the resulting emotional response, meaning it is likely to be activated when any novel stimuli is introduced. The LA, however, is more closely related to long-term storage of emotional information about previously novel stimuli. Therefore, it is plausible that the LA may not be activated to the extent that the BLA is activated in a task such as the OiP task. The OiP task is not as likely to elicit a salient emotional response that would activate long-term storage of information relevant for survival. However, a task involving fear conditioning, which does elicit a salient emotional response relevant to survival and long-term storage, would likely increase activation of both the LA and BLA in EE rats when compared to controls.

While there was a lack of a significant difference between EE rats and controls in activation of the LA, results concerning activation of the BLA are inconsistent with previous research related to differential activation of the BLA in adult EE rats and controls (Ali et al., 2009; Okuda et al., 2009; Sztainberg et al., 2010). However, it should be noted that the histological data in this study is based on neural activation at PND 50 and may not be representative of neural activity at PND 36, which is especially relevant considering the behavioral differences at the two ages.

With the exception of the results concerning latent novel-object recognition, both the behavioral and neural results of this study seem to indicate that older adolescents exhibit similar responses to EE as adult rats. At PND 50, EE rats exhibited both greater habituation to novel objects and less neural activation in the BLA. These results contrast with those of Ali et al. (2009), who found that EE promotes activation in the amygdala. However, the habituation to novel objects exhibited by older rats in this study is similar to prior research

indicating that EE promotes habituation to novel environments (Gould et al., 2009; Simpson & Kelly, 2011; Zimmerman et al., 2001). Further, EE may have vastly different behavioral effects on younger adolescents. While the increased exploration observed in the younger EE adolescents is consistent with some of the research on the effects of EE observed in adults (Renner & Rosenzweig, 1987), these results indicate that the anxiolytic effect of EE may promote increased response to novelty, as opposed to increased habituation to novelty, in younger adolescents.

While a conservative approach must always be taken when applying animal models to humans, these results do hold significance for potential interventions for reducing risk-taking behaviors among human adolescents, especially considering the ineffectiveness of many current interventions (Steinberg, 2007). Strategies involving carefully structured EE may be taken to attenuate certain risky behaviors at different stages within adolescence due to the different effects of EE at different ages. EE may promote latency and greater caution towards approaching novel objects at all ages, in opposition to the impulsivity typically observed in adolescents (Steinberg, 2007). While EE may induce greater exploration overall in younger adolescents, that exploration may be exhibited in a more cautious manner. For older adolescents, EE interventions may promote greater habituation to novel objects, which is supported by evidence of decreased BLA activation. Because EE includes both psychosocial elements and may accelerate the development of self-regulatory skills, it could plausibly provide adolescent individuals with the ability to be more cautious in their impulses towards risky behaviors (Steinberg, 2007). Effectiveness of EE as an intervention is further supported by evidence suggesting that the brain is especially sensitive to environmental cues during adolescence due to synaptic reorganization that typically occurs during this period

(Blakemore & Choudhury, 2006). Overall, these results indicate that EE plays a critical role in both early and late adolescence, although its effects may differ between these ages and between adolescence and adulthood.

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Table 1

Total Time with Objects on Postnatal Day (PND) 36 and 50 OiP Sessions

			PND 36	PND 50
Delay	Group	*N*	*M* (*SD*)	*M* (*SD*)
0 min	EE	16	38.3 (13.1)	24.0 (11.1)
	No EE	16	53.9 (15.3)	26.4 (20.0)
15 min	EE	16	42.4 (10.1)	30.4 (16.5)
	No EE	16	28.6 (16.3)	38.3 (22.4)
60 min	EE	16	46.3 (18.0)	22.8 (15.3)
	No EE	16	26.8 (13.8)	32.1 (24.4)

Table 2

Time Spent in the Interior on Postnatal Day (PND) 36 and 50 OiP Sessions

			PND 36	PND 50
Delay	Group	*N*	*M* (*SD*)	*M* (*SD*)
0 min	EE	16	57.8 (21.2)	42.4 (23.8)
	No EE	16	26.7 (11.4)	35.5 (27.4)
15 min	EE	16	72.3 (20.0)	56.4 (35.5)
	No EE	16	42.2 (33.5)	63.3 (43.0)
60 min	EE	16	73.4 (27.8)	52.1 (34.7)
	No EE	16	58.1 (24.7)	52.2 (36.8)

Table 3

Interior Squares Traveled on Postnatal (PND) 36 and 50 OiP Sessions

			PND 36	PND 50
Delay	Group	*N*	*M* (*SD*)	*M* (*SD*)
0 min	EE	16	51.3 (19.8)	43.6 (25.5)
	No EE	16	21.1 (11.4)	20.4 (22.4)
15 min	EE	16	70.7 (25.5)	39.2 (29.0)
	No EE	16	39.9 (29.3)	28.1 (17.2)
60 min	EE	16	68.1 (26.8)	35.0 (25.7)
	No EE	16	37.4 (25.2)	25.5 (19.1)

Table 4

Activated Neurons in the Amygdala Regions

	BLA		LA	
Group	*N*	*M* (*SD*)	*N*	*M* (*SD*)
EE	9	4.9* (6.4)	6	2.8 (1.4)
No EE	9	8.0* (4.4)	9	2.9 (1.9)

Note. The significant difference has been indicated (*$p < .05$).

Figure 1. The photos show two of the different object arrangements in the enrichment cage.

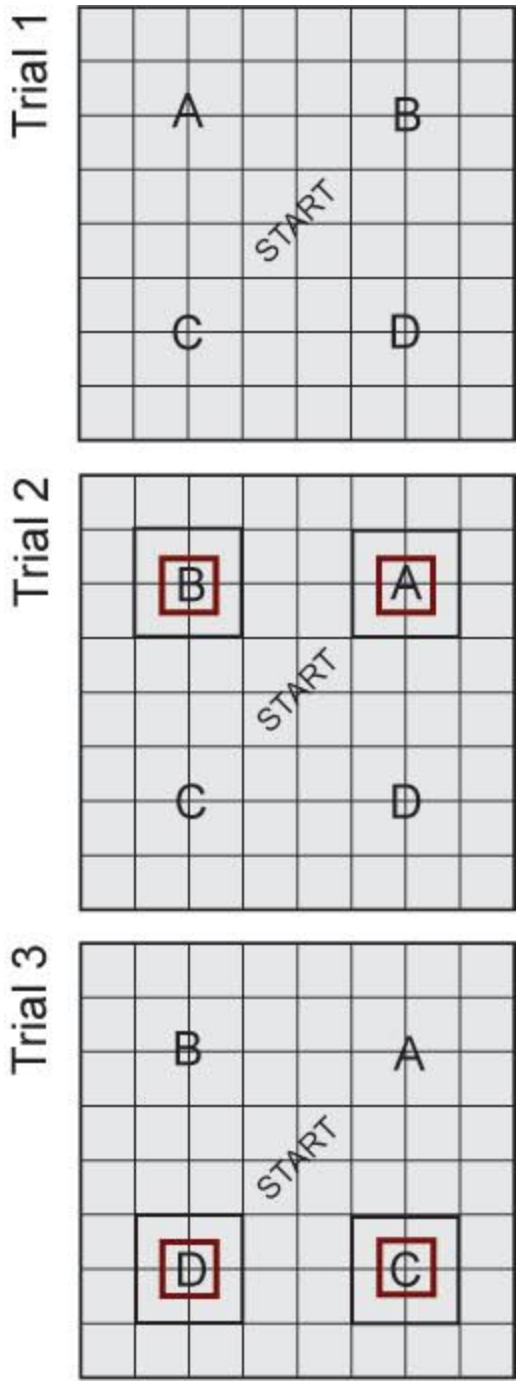

Figure 2. This figure depicts the object placement during all 3 trials of the OiP task. The boxed letters identify the newly-located objects in Trials 2 and 3. Trial 1 occurred after a 15 min delay. Trial 3 occurred after a 60 min delay.

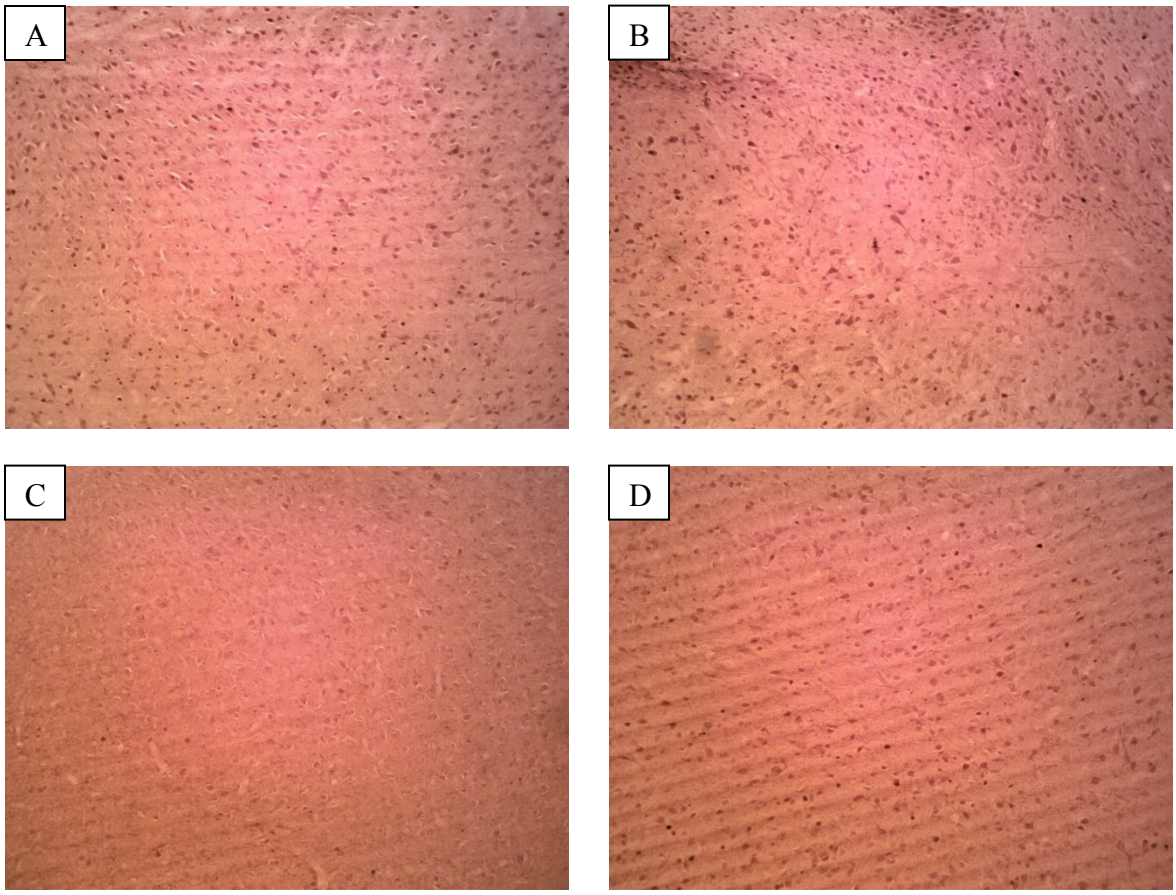

Figure 6. (A) Lateral amygdala in the enriched animals. (B) Lateral amygdala in the control animals. No significant difference was found between enriched and control animals in this region. (C) Basolateral amygdala in the enriched animals. (D) Basolateral amygdala in the control animals. Control animals exhibited significantly more activation in the BLA than enriched animals.

Appendix

TO:	Dr. Mark Zrull Department of Psychology
FROM:	Dr. Ted Zerucha, Chair Institutional Animal Care and Use Committee
DATE:	August 14, 2014
SUBJECT:	Institutional Animal Care and Use Committee Request for Animal Subjects Research
REFERENCE:	*Environmental enrichment, object placement preference, social preference, and associated evoked neural activity in adolescent rats*
	IACUC Reference #15-02
	Initial Approval Date – August 14, 2014 **End of Approval Period – August 13, 2017**

The above referenced protocol has been approved by the IACUC for a period of three years.
A list the individuals cleared for research activities with live, vertebrate animals will be sent in a separate email.

Best wishes with your research.

TZ/rst