Enrichment Effects on Object-in-Place Task Performance in the Adolescent Rat are Due to Task Novelty and Subject Age

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Abstract

Adolescence is a period of rapid physical growth, correlated with increased exploration and novelty-seeking behaviors that can be potentially risky. These behaviors can be mediated by processes such as environmental enrichment (EE), which consists of repeated exposure to novelty and physical exercise beyond that available in standard lab housing. Prior research indicates EE has a significant mitigating effect on novelty-seeking behaviors in the adolescent rat during early adolescence (pnd 36), but not late adolescence (pnd 50), in an Object-in-Place (OiP) task. Prior research also indicates there are significant sex differences at pnd 36 but not pnd 50 in OiP testing. This study elucidates whether task novelty or subject age at time of testing produced these observed differences by testing OiP once in late adolescence. The current study analyzed the CA1, DG, and CA3 regions of the hippocampus to determine processing of novelty following the OiP task, and analyzed the BLA and LA regions of the amygdala to determine potential fear-learning during the task. Behavioral results found that the main effect of enrichment was age-dependent, whereas observed sex differences were task-novelty dependent. Neural analysis indicated that the CA1 and LA were significantly less active in EE subjects, but no other region was statistically different between groups. EE was found to have a beneficial impact on subjects, but that this impact was stronger in early adolescence.

Keywords: novelty preference, basolateral amygdala, lateral amygdala, hippocampal formation

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Across species, adolescence can be characterized by a sharp increase in physical and neural development that can, in turn, significantly alter behavior (Stansfield & Kristen, 2005). This rapid development can lead to behavioral changes such as an increase in exploratory behaviors and other changes in how the subject interacts with its environment (Lynn & Brown 2009). This period of development can be impacted significantly by exposure to an enhanced environment and a wider variety of stimuli (such as new scenery, objects, and conspecifics) that can alter both brain and behavior (Hüttenrauch, Salinas & Wirths, 2016; Spear 2000). This study examines the relationship between early and late adolescence, enrichment, and exploratory behaviors through an object-in-place (OiP) in the adolescent rat.

The current study is an elaboration on prior work that observed the differences in an OiP task in early and late adolescence. Cobb (2015) observed the impact of enrichment on OiP task performance in early adolescence, an effect that did not persist during late adolescence. The results found in her work were elaborated on by testing the animals exclusively in late adolescence, since subject maturation over time or participation in the OiP task itself may have impacted the differing results found at the two times of testing. Additionally, an in-depth microscopic analysis was conducted on the brain tissue gathered in the earlier work to confirm and clarify the earlier preliminary microscopy results by re-analyzing a greater component of the hippocampal formation as well as analyzing the amygdala for the first time. By analyzing the brain tissue, the relationship between the behavioral results of the OiP task after two times of testing and the activity in correlate neural regions can be elucidated.

Adolescence

Adolescence in rats begins after weaning at postnatal day (pnd) 21, with mid adolescence at pnd 33 and late adolescence at pnd 47, and lasts until early adulthood at pnd 60 (Lynn & Brown, 2009; Tirelli, Laviola, & Adrinani, 2003). Adolescence is characterized by rapid behavioral and neural development that often features a sharp increase in risk-taking and sensation-seeking behaviors, such as drug experimentation in human adolescents (Stansfield & Kristen, 2005). In rat models, novelty-seeking and riskier behaviors include exploration of novel objects and unfamiliar environments, with decreased latency between exposure and exploration (Lynn & Brown, 2009; Stansfield & Kristen, 2005; Hendershott et al., 2016). Novelty-seeking can be deemed risky as the rat does not have prior experience with the object or environment and cannot know what danger the unfamiliar thing poses. Adolescence is also marked as a period in which the presence of reward is more salient and pleasurable, as seen by increased intake of drug and drug-seeking behaviors in adolescent rodents as compared to their adult counterparts (Hammerslag & Gulley, 2016). The sharp increase of drug-seeking behavior is seen in adolescent rodents even without the signal that the drug is available (Hammerslag & Gully, 2016). Increased reward-seeking demonstrated with drug intake is not reflected in studies that train rodents with food; adult rodents respond more keenly to food as a reward than adolescent rodents (Hammerslag & Gully, 2016). These differing responses to drug and food as reward clearly mark the preference for reward as a risk in adolescent development.

In open-field studies, exploratory behaviors directed to novel, possibly riskier areas of the field increase across adolescence (Lynn & Brown, 2009). Observed increases in exploratory and risk-taking behavior in adolescence may be due to complex brain development occurring in adolescence (Stansfield & Kristen, 2005; Spear, 2000). Additional developments in the rat's

physical stature may also contribute to the increased levels of exploratory and locomotive behavior, as the rat is more physically capable of engaging in exploratory behaviors in adolescence (Lynn & Brown 2009). Behaviorally, adolescent rats have been found to spend as much as twice the amount of time with novel objects in a familiar environment than their adult counterparts, and approach the novel object faster (Stansfield & Kristen, 2005). Though this behavior is not inherently risky, by exhibiting increased exploratory tendencies the rat is exposing itself to greater potential threat within a novel environment.

Differences in exploratory behavior within adolescence is also subject to sex differences. In the Elevated Plus Maze task, female rats and female mice spend more time on the open, riskier arms than their male counterparts (Lynn & Brown, 2009; Hendershott et al., 2016). This tendency toward risk is decreased in mice exposed to environmental enrichment (Hendershott et al., 2016). Additionally, dispersal patterns rats exhibit when leaving the natal area behaviorally begins during adolescence and is male-driven (Calhoun, 1963). Since females tend to stay closer to the natal-burrow system, whereas males disperse further, this may offer some explanation for behavioral sex-differences (Lynn & Brown, 2009). In the current study as well as Cobb's research (2015), subjects were tested on the same pnd regardless of sex. This is a potential ground for sex differences within results, since female subjects are faster to mature than male subjects (Willing, Drzewiecki, Cuenod, Cortes, & Juraska, 2016). Since level of maturation is sex-dependent, and dispersal patterns from the home nest is also sex-dependent, the OiP task is inherently prone to sex differences in how female and male subjects of different maturation rates interact with their environment (Lynn & Brown, 2009; Willing et al., 2016).

Enrichment

In rodent models, environmental enrichment (EE) can include repeated exposure to an enhanced environment often featuring unfamiliar objects, obstacles to navigate, and novel conspecifics. This experience can alter brain and behavior, particularly in adolescents (Hüttenrauch, Salinas & Wirths, 2016; Spear 2000). For adolescent rats, performing a novel task to measure behavior can enhance novelty-seeking and be enriching. EE has been shown to increase spatial exploration and learning in mice, and rats exposed to EE exhibited increased exploratory tendencies and greater brain function (Hendershott et al., 2016). EE in mice showed significant improvements in long term spatial memory (Hüttenrauch, Salinas & Wirths, 2016). In open field tasks, EE mice exhibit less anxious behavior than controls, seen by spending a greater proportion of time in the center of the field (Hüttenrauch, Salinas & Wirths, 2016). Beyond behavioral differences, EE has been shown to be both protective and provide improvements for symptoms associated with a rodent model of Alzheimer's disease and a rodent model of mild cognitive impairment; EE has also shown marked improvements in situations of prenatal injury in rodents (Kentner et al., 2016; Hüttenrauch, Salinas & Wirths, 2016).

Environmental enrichment can be used to model increased exercise in humans, as the components of EE can reflect those of a cognitively and physically healthy lifestyle in humans (Hüttenrauch et al., 2016; Hüttenrauch, Salinas & Wirths, 2016). This comparison is based on similarities between rodent performance in EE paradigms as compared to studies on the effects of exercise on adult humans, including older adults and twins, such as a reduced rate of symptoms and morbidity associated with Alzheimer's Disease and mild cognitive impairment (Hüttenrauch et al., 2016; Hüttenrauch, Salinas & Wirths, 2016; Iso-Markku, Waller, Kujala, & Kaprio, 2015; Weuve et al., 2004). EE can also be used to model a cognitively and physically

healthy lifestyle since both EE rodents and physically active humans when compared to non-EE and sedentary controls exhibit increased hippocampal volume, which is correlated with greater spatial memory (Erickson et al., 2011; Hüttenrauch et al., 2016; Hüttenrauch, Salinas & Wirths, 2016). Zhu et al. (2009) found that EE rats exhibited greater dendritic spine volume than controls in two hippocampal regions, and this increased hippocampal plasticity was correlated with an increase in exploratory behavior. In a study that observed the relationship between EE and fear-learning through a foot-shock paradigm, EE rats had less activity in the amygdala (Mosaferi, Babri, Mohaddes, Khamnei, & Mesgari 2015). The reduced amygdala activity could indicate a more efficient coping mechanism for stress and fear related events, since the amygdala is related to fear experiences and formation of fear memory in the brain (Mosaferi et al., 2015).

By regularly exposing adolescent rats to EE, the relationship between adolescence, EE, and the characteristic novelty-seeking behaviors can be explored. Previous studies have indicated that repetitive exposure to EE can moderate the novelty-exploration characteristic of adolescence (Simpson & Kelly, 2011). Prior work has shown that when exposed to EE, performance in Novel Object Preference (NOP) and Novel Place Preference (NPP) tasks demonstrate decreased novelty seeking in adolescent but not adult rats (Cobb & Zrull, 2014). This study builds on prior research by utilizing an Object in Place (OiP) task, which combines the object and location preferences a rat might exhibit when engaging in exploratory behavior of a field (Barker & Warburton, 2009). The task places the rat in an open field containing four objects, then after a delay returns the rat to the field where two of the objects were swapped, then observes preferences by calculating the proportion of time spent attending to the two swapped objects as opposed to the two stationary ones (Barker & Warburton, 2009). By examining the differences in

proportion of time with swapped objects and stationary objects in EE rats and non-enriched controls, the preference for novelty within an environment can be elucidated.

Behavior Study

This study builds on prior research conducted on enriched adolescent rats as compared to non-enriched controls in an OiP task in both mid and late adolescence (Cobb 2015). In Cobb's study, rats were enriched throughout adolescence and exposed to an OiP task on both pnd 36 and pnd 50, to assess the impact of enrichment on novelty seeking during the complex developmental changes during adolescence (Cobb 2015). Cobb found that at pnd 36, enriched males spent significantly increased time attending to the swapped objects in the field than non-enriched controls, whereas enriched females spent significantly less time exploring the swapped objects than non-enriched controls (Cobb 2015). However, at pnd 50, this effect was not observed, and there were no significant behavioral differences between enriched rats and controls (Cobb 2015). The current study seeks to find a more complete understanding of Cobb's work by conducting an additional experiment where the rats are tested exclusively in late adolescence. Because the rats in the earlier study were tested during both mid and late adolescence, it is unclear whether the observed differences at the two times of testing are due to the age of the animal when tested, or the novel exposure to the task that can obviously only occur once. By exposing the rats to enrichment throughout adolescence in the same way as the original study, but only testing during late adolescence, this study seeks to determine whether the impact of enrichment is due to the age of the animal or the novelty of the task.

Observing OiP task performance exclusively at late adolescence is especially of interest to determine the maximum utility of enrichment. EE increases spatial exploration and learning and develops increased exploratory tendencies, and significant improvements in long term spatial

memory (Hendershott et al., 2016; Hüttenrauch, Salinas & Wirths, 2016). Spending time in an enriched environment gives ample opportunity to explore, adapt to, and interact with novel environments, objects, and locations (Forgays & Forgays, 1952; Lynn & Brown, 2009). As such, it is possible that the frequent exposure to EE itself in conjunction with improved spatial memory and exploratory tendencies leads subjects to interact differently with the task upon the second time of testing. Additionally, Ali, Wilson and Murphy (2009) suggest that EE has a visible impact within multiple circuits of the brain after a single exposure, and the subjects in Cobb's study were exposed to EE throughout adolescence which would compound these potential effects (Cobb 2015). If the observed differences were due to task novelty, it would suggest that the EE animals would engage differently in the task during each exposure to it, since the task itself becomes less novel, and that EE is equally beneficial to rodents at any age. If the differences in effect observed in Cobb's study were due to the subject age, it would implicate that exposure to an enriched environment is most useful to younger subjects, as seen by Mora-Gallegos and colleagues (2015) when young, enriched rats outperformed all other conditions by showing progressive improvement in a radial-maze task. When observing the level of response to an environment, level of exploration in a novel scenario was moderated by age differences, suggesting that the reaction to novelty may differ over time in the subject regardless of the influence of EE (Wooters et al., 2006). By testing subjects that have been consistently exposed to EE exclusively at late adolescence, it will be possible to determine which of these two scenarios is more likely in the adolescent rat.

Hypothesis 1 anticipates that the outcome of the task at pnd 49 will reflect that of those on pnd 36 in Cobb's study. Enriched subjects are constantly exposed to novelty, and this exposure moderates how they react in novel situations. Since the subjects at pnd 50 in Cobb's

study have participated in the task previously, the task itself becomes less novel, and due to the familiarity at second time of testing, the subjects will perform differently. Therefore, it is expected that the subjects will react similarly to the task at the first time of testing in both studies.

Brain Study

Adolescence in rats is a complex time of developmental changes within the brain (Hüttenrauch, Salinas & Wirths, 2016; Spear 2000). These widespread structural changes that occur throughout the brain are correlated with novelty-seeking behaviors (Hüttenrauch, Salinas & Wirths, 2016; Spear 2000). Enrichment has not only shown to have beneficial impacts on behavior, but has been shown to modulate brain changes that are correlated with these behavioral differences (Ashokan, Hegde & Mitra, 2016; Hüttenrauch, Salinas & Wirths, 2016; Schuch et al., 2016; Zhu et al., 2009). In studies that exposed adolescent rodents to EE across adolescence, EE mice possessed an increase in total hippocampal volume compared to controls, which implies that increases in cognitive function and performance are correlated with changes in brain structure in EE animals (Hüttenrauch, Salinas & Wirths, 2016; Zhu et al., 2009; Zhu et al., 2009). Repetitive exposure to EE during adolescence in rodents as also been shown to decrease the effects of potential brain damage compared to non-EE controls (Schuch et al., 2016). In the basolateral amygdala, EE animals have shown to have fewer markers of plasticity and less neural activation after performing tasks that potentially elicit fear than age-matched controls (Artz, 2016; Mosaferi, Babri, Mohaddes, Khamnei, & Mesgari, 2015). As such, it is of interest to observe the neural differences in the hippocampal formation and amygdala between EE and non-EE controls.

Hippocampal formation. When considering the OiP task, the hippocampus is of particular interest. In the OiP task, preference for an item or place within a field can only be established with prior recognition, which is by necessity developed based on an integrated understanding of cues within an environment. The integration of cues for both the objects themselves as well as their location within the environment is processed within the hippocampus (Barker & Warburton, 2011). The hippocampal formation is made up of several distinct units that all play complex roles in formation and processing of environments, such that it is essential in tasks such as OiP. These units include the *cornu ammonis* 1 (CA1), *cornu ammonis* 3 (CA3), and dentate gyrus (DG).

The CA1 functions as the efferent information pathway from the hippocampus, but also receives afferent cortical signals from the CA3 and DG, such that the CA1 contains both past and current information on the current state (VanElzakker et al., 2008). As such, the CA1 is critical for the formation of memories at the first exposure to an environment, as well as detecting potential novelty within that environment and integrating those sources of information together (Cheng & Frank, 2008; VanElzakker et al., 2008). Because the activity of the CA1 is critical to the detection and processing of environmental and novel cues within a task, examining activity within the CA1 should show how the novel information from tasks such as the OiP is being processed and how that processing differs between EE and non-enriched subjects (VanElzakker et al., 2008).

The DG functions as the input zone of the hippocampus, and processes signals before delivering it to the CA1 (VanElzakker et al., 2008). Function within the DG is strongly related to an animal's ability to detect differences between two contexts, even if those differences are minor (Clemenson, Deng, & Gage, 2015). This ability to separate differences is implicated in the

process of separating patterns such that the animal is capable of distinguishing two similar events as separate but familiar (Clemenson et al., 2015). As such, the DG is highly implicated in the detection of novelty within an environment, since it is responsible for distinguishing similar patterns (Clemenson et al., 2015; VanElzakker et al., 2008). In the presence of novelty, the DG has been shown to exhibit greater activation since this process of pattern separation is key in novel contexts (Clemenson et al., 2015; Nitz & McNaughton, 2004). Though the CA3 is also implicated in pattern separation, the CA3 is not as sensitive to minute changes that the DG is capable of detecting (Clemenson et al., 2015).

Prior research in the field indicates a divergent response to novelty in CA1 and DG regions, where CA1 activation decreases in the presence of novelty whereas DG activation increases (Nitz & McNaughton, 2004). Since rodents show a decrease in CA1 activation when exposed to novelty, Cobb hypothesized that subjects adapted to novelty (such as those exposed to EE) would exhibit increased CA1 activation compared to non-EE controls and that EE subjects would have a decreased activation in DG regions as compared to controls (Cobb 2015). Cobb (2015) observed both DG and CA1 regions, and found, contrary to her hypotheses, that CA1 exhibited decreased neural activation following exposure to EE as compared to non-enriched controls, and no significant differences in DG activation were observed between groups. Since this result was opposite to that established in the literature, and the counts of this tissue were preliminary, it was of interest in this study to re-observe the data obtained in Cobb's work to confirm or clarify her result.

In addition to the CA1 and DG regions, CA3 has also been shown to be used in location and object-memory tasks (Beer, Chwiesko, & Sauvage 2014). Activation of the CA3 is correlated preferentially with spatial tasks as opposed to non-spatial ones and is of interest in

spontaneous, non-emotional tasks that rely on location in space, such as the OiP task of interest (Beer, Chwiesko, & Sauvage 2014). CA3 is additionally implicated in the formation of second or minute-long short-term memories for reliant on spatial information and episodic memory (Kesner 2007). The CA3 is also important for tasks that involve multiple trials and the formation of arbitrary connections between spatial observations, such as those necessary for the OiP task (Kesner 2007). Finally, the CA3 is involved with automatic associations that allow for pattern completion based on short-term memory retrieval (Kesner 2007). Therefore, in addition to re-analyzing the CA1 and DG, it was of interest to observe a wider scope of the hippocampus by including the CA3 region to further clarify the impact of EE on the HF.

Amygdala. In addition to the hippocampal formation (HF), the amygdala is of great interest in studying the relationship between novelty-seeking and adolescence in enriched versus non-enriched subjects. The amygdala is critical for the processing and development of emotional memories, particularly for fearful memories and fear-based learning (Wellman et al., 2017). As such, the amygdala plays a crucial role in processing fear, a process which is developed by adolescence. This process is particularly of interest in this study, as the impact of emotional fear-based memories can significantly impact the exploration of novel environments and stimuli. Since enrichment can increase plasticity in neural areas even after they are developed (Okuda et al., 2009; Pinaud et al, 2001), exploring the relationship between the amygdala and exploratory behavior in the OiP task is of interest. Repeated exposure to EE has shown a decrease in markers of plasticity (e.g. dendritic spine volume) in the amygdala after participation in a fear-based task, suggesting that EE subjects have increased fear coping mechanisms as compared to non-EE controls (Ashokan et al., 2016; Mosaferi et al., 2015). Since being placed in an unknown area surrounded by novel, displaced objects can be potentially fear-inducing, it is of interest to

explore the relationship between neural activation in the amygdala to participation in the OiP task in EE and non-EE controls.

The basolateral amygdala (BLA) serves as the main input zone within the amygdala, and it processes and relays inputs such as contextual and sensory inputs on to the central nucleus of the amygdala (CeA) (Wellman et al., 2017). The BLA integrates convergent information from multiple sources, and processes memories with context allowing for emotional memories to form (Wellman et al., 2017). The role of the BLA in emotional memory formation is such that should it be inhibited in a subject then exposed to a fearful stimulus, the fear memory will not form (Miserendino, Sananes, Melia, & Davis 1990). Since the BLA is the convergent site of processing in the amygdala, the according activity (similar to that of CA1 in the HF) should demonstrate processing of the novel environment occurring during the OiP task.

An additional portion of the amygdala of interest is the lateral amygdala (LA). Where the BLA functions as a site of convergence to form the initial fear-based memory, the LA functions to coordinate prior memories with new, unconditioned stimuli (Repa et al., 2001). In this way, the LA can be considered responsible for the beginning of long-term memories associated with emotional stimuli. Similarly to BLA, the role of LA in the OiP task is of interest. Whereas the BLA would suggest that the subject is interacting with its environment and forming emotional connections to stimuli, activity in the LA would suggest that the subject is forming long-term emotional memories about the stimuli.

Hypothesis 2 of this study is that brain regions in the HF will see an increase in activation in EE rats as compared to non-enriched controls, as the HF is a center of convergence of inputs regarding stimuli of the environment, and non-EE rats are less accustomed to the exposure of such novel inputs. In contrast, Hypothesis 3 states that the LA and BLA will show decreased

activation as compared to controls, as the increased adaption to novelty in EE rats will lead to a lessened reaction to potentially fearful stimuli, e.g. Ashokan et al. (2016); Mosaferi et al. (2015).

Method

Behavior Study

Experimental design. Novelty-seeking behavior was observed through use of an OiP task, which placed subjects in an open field and allowed for preferences of novelty of both objects within the field and location to be observed. The study utilized a split plot factorial design, which compared enriched adolescent male (n=6) and female (n=6) rats that were tested and re-tested after a delay. Non-enriched controls were tested at the same age and intervals as their enriched counterparts. After completing the task at the second time of testing, all rats were sacrificed and brain tissue was extracted and processed for c-*fos* activation to use as a correlate of neural activity.

Subjects in this study were 24 young Long-Evans rats, split evenly between sexes such that there were 12 males and 12 females, provided by the Arts and Sciences Animal Facility at Appalachian State University. The rats were housed in standard laboratory setting in plastic cages in triplets in a room controlled for both humidity and temperature that featured alternating 12-h light and 12-h dark lighting. Food and water were supplied *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Appalachian State University (Protocol #15-02, M. C. Zrull, PI).

Environmental enrichment. Enrichment consisted of 18 1.5-h sessions, where EE subjects (n=12) were all placed in the enrichment cage (see Figure 1). The cage had wire mesh walls and door, as well as wire mesh ramps between three different platforms that allowed the rats to climb and explore freely. The cage also featured numerous toys, such as plastic tubes

hanging at various lengths from the roof of the cage, a mirror, a plastic tube to climb through, and various squishy objects of different sizes, shapes, and colors. Since all the enriched subjects were placed in a cage according to their sex, the enrichment experience was also socially enriching as the subject got to interact with conspecifics of their own age and gender. The enrichment cage had an alternating layout of toys that was differentiated between each enrichment experience on a rotating basis between four different toy set-ups (see Figure 1). Non-enriched rats were handled twice during each enrichment session, but were returned to their home cages rather than being placed into the enrichment cage, to control for handling.

Object-In-Place. The OiP task takes place on a 1-m^2 field that features four objects equally dispersed to each quarter of the field. The field is surrounded by a curtain that contained visual cues. The room where the task occurred was sound-proofed, and the task was projected via video camera to a screen in the adjacent room for monitoring such that the experimenters did not interfere with the subject's perception of the environment. Each subject performed the OiP task exclusively at pnd 49. At the start of the first trial in the task, the rat is placed in the center of the field and allowed to explore freely. The total time spent attending to each object is monitored, and the subject must have spent a certain amount of time with the objects that were to be swapped to be included in data analysis. After the first trial and a 15-min delay interval, the subject was returned to the field where the objects in the top two quadrants were swapped and the free interaction with objects was again monitored. After the second trial and a 60-min delay from the first trial, the subjects were returned to the field for a final trial where the objects in the bottom two quadrants of the field were swapped (see Figure 2). Proportion of time spent with the stationary objects vs the swapped objects were calculated, producing the variable of interest. All

object contact times were measured by multiple research assistants, and each trial was videotaped.

Brain Study

Histology procedures from Cobb (2015). The tissue utilized in this study was from the prior work that tested rats at both pnd 36 and pnd 50, and followed the same histology procedure typically used in this lab. Following the final trial of the OiP task on pnd 50 in Cobb's study, subjects were placed in a quiet and dark environment for 1-h to 2-h then injected with a lethal dose of sodium pentobarbital (100 mg/kg, ip). Subjects were placed into a housing cage and observed until corneal and tail reflexes ceased to occur, at which point they were perfused intracardially with phosphate-buffered saline, then 4% paraformaldehyde in 10 mM phosphate buffer. After perfusion, the brain was removed from the subject and post-fixed in a 10% sucrose, 4% paraformaldehyde in phosphate buffer for 5 days after which it was transferred to phosphate buffer with 0.05% sodium azide for storage.

One hemisphere of each brain was cut into 50 µm sagittal sections, and sections were processed to visualize c-*fos* expression using floating-section immunohistochemistry, as c-*fos* was the proxy for neural activation for this data. Sections were rinsed in phosphate-buffered saline (PBS, 2 x 5 min), then placed in 1% hydrogen peroxide for 15 min. Sections were then rinsed in PBS again (2 x 5 min) and placed in 15% goat serum with 0.25% Triton-X for 60 min. Following the blocking and rinsing steps, the floating sections were then placed in anti-c-*fos* made in rabbit (Santa Cruz, SC-52) for 40-h. After the 40-h period, sections were again rinsed in PBS (6 x 10 min) and placed in biotinylated goat anti-rabbit secondary antibody (Vector) for 60 min, after which they were again rinsed in PBS (3 x 10 min). Sections were then placed in a peroxidase-labeled avidin-biotin complex for 1-h (Vector) and rinsed in PBS (2 x 10 min). The

sections were then exposed to an enzyme substrate (VIP, Vector) for 2 to 3 min. After completing the floating-section immunohistochemistry the brain sections were then mounted on gel-coated slides and air dried, then dehydrated in ethanol, cleared with toluene, and cover-slipped with Permount (Fisher). Sections that were not put through c-Fos immunohistochemistry were Nissl stained to observe brain structure.

Microscopy and cell counts. Analysis of each brain section was completed through use of a Nikon Eclipse microscope, and a PixeLink digital camera. The HF was recounted with the additional analysis of CA3, and the BLA and LA were counted for the first time. Since the tissue from Cobb's (2015) study was an initial and preliminary observation of the histology from the brains collected in the earlier study, it was of interest to observe the tissue collected in greater detail with inclusion of the additional brain regions of interest. Additionally, Cobb (2015) observed total neural activation of each section observed, and the current study observed exclusively darkly activated cells to ensure the activity observed was present and correlated with the OiP task participation.

The BLA, LA, CA1, DG, and CA3 regions of the brain were located through use of a rat brain atlas (Pellegrino, Pellegrino, & Cushman, 1969). Microscopy identified c-*fos*(+) neuron densities to determine level of neural activation in each brain region of interest (see Figure 3). Through microscopy brain sections were scanned using a Plan 4 objective to find the brain region to count, then sections were counted using a Plan 10 objective. A counting frame was placed over the screen, where the brain region was projected on a Plan 10 objective with a 1024 x 768 pixel image, and the counting frame was then used to count each c-*fos*(+) cell within the frame of either light, medium, or dark activation, which provided a basis for how much protein was present. The counting frame for BLA included six sampling boxes 200 µm x 200 µm in size,

LA included four such boxes, CA1, CA2, CA3, and DG all used three 200 µm x 200 µm boxes. Only neurons labeled medium or dark were used for analysis, since these cells expressed the most appropriate levels of c-*fos* to be used as a proxy neural activity. Sections were then averaged to produce one value for each brain observed. Observed activation was compared between enriched and non-enriched controls, as well as between sexes.

Results

Behavior Study

Cobb's (2015) Behavioral Results. The proportion of time EE and non-EE subjects spend attending to swapped objects as compared to stationary objects was of interest. There was a significant interaction between EE and trial at pnd 36, but not at pnd 50 (see Table 2). After Trial 2 on pnd 36, EE animals spent roughly half of task interacting with swapped objects as opposed to the stationary ones (M = 0.53, SD = 0.10), whereas non-EE subjects paid greater attention to the swapped objects during Trial 2 (M = 0.71, SD = 0.15), $t(27) = 2.92$, $p = .007$. There was not a significant effect of EE on trial at pnd 50 ($p = .1084$). At pnd 36, there was a significant interaction between EE and sex. At pnd 36 EE male subjects spent a greater proportion of time attending to the swapped objects (M= 0.57, SD = 0.14) than non-EE males (M = 0.49, SD = 0.32) who split their time evenly between swapped and stationary objects, but this difference was not statistically significant $t(27) = 1.30$, $p = .2089$. Opposite to the males, non-EE females (M= 0.58, SD= 0.19) spent more time attending to the swapped objects than EE females (M= 0.53, SD= 0.11) but this difference was slight and not significant $t(27) = 0.798$, $p = 0.432$.

Current Study Behavioral Results. Hypothesis 1, which stated that the subject's task performance at pnd 49 in the current study would match that of the rats at pnd 36 in Cobb (2015) was partially supported. There was not a significant main effect of EE in this study at pnd 49

($F(1,22) = 3.06$, $p = .0954$). There was an interaction between EE and sex (see Table 1). EE male spent significantly greater proportion of time with the swapped objects ($M = 0.64$, $SD = 0.11$) than non-EE male controls ($M = 0.50$, $SD = 0.11$) $t(22) = 3.46$, $p = .0018$. At pnd 49 non-EE females (M= 0.58, SD= 0.12) also spent slightly more time than EE females (M= 0.56, SD= 0.14) which was also not statistically significant $t(22)= .4948$, $p = .6247$.

Brain Study

Tissue counts indicated that Hypothesis 2, that the HF of EE subjects will see an increased activation compared to non-EE subjects was not supported (see Table 3). The CA1 in EE rats ($M= 25.9$, $SD= 19.5$) exhibited significantly less c-Fos activation than that of non-EE controls ($M= 37.6$, $SD= 14.4$), $t(31) = 2.67$, $p = .005$. There was also less activation in the DG of EE subjects ($M= 22.6$, $SD= 19.6$) than controls ($M= 34.7$, $SD= 20.4$) but this difference is not statistically significant $t(31) = 1.18$, $p = .1216$. There was no significant difference between EE and non-EE subjects in CA3 activation $t(31) = .745$, $p = .2318$.

Hypothesis 3, that the LA and BLA will have less activation in EE brains than non-EE brains, was partially supported (see Table 4). There were no statistically significant differences in BLA c-Fos activation between groups $t(31) = 0.527$, $p = .3012$, however, the LA activation observed in EE brains (M=14.0, SD= 33.8) was significantly higher than that observed in the brain tissue of non-EE controls (M= 6.9, SD= 10.2) $t(11) = 3.06$, $p = .00109$.

Discussion

This study sought to clarify why an effect of enrichment is observed at early adolescence but not late adolescent during an OiP task by exposing the subjects to the task exclusively in late adolescence. By comparing the data from subjects who had participated in the OiP task once in

late adolescence to those who participated in the task in both early and late adolescence, the study seeks to determine whether the observed differences are due to task novelty or subject age.

Hypothesis 1 anticipated that since enrichment allows the subject to adapt to novelty within an environment in a variety of forms, and the task itself may provide a source of enrichment, the subjects will perform similarly in the first exposure to the task regardless of subject age at time of testing. This hypothesis was partially supported. The significant effect of enrichment alone, seen at early adolescence, was not seen at pnd 49 of the current study. This is in accordance with literature that EE has a more profound impact in younger subjects than older subjects when compared in a learning and memory task (Mora-Gallegos et al., 2015). However, the interaction between sex and EE seen at pnd 36 in Cobb's study was seen clearly at pnd 49 in the current study. At both pnds, the enriched males spent more time with the swapped objects than controls, whereas enriched females spent slightly less time than non-enriched controls (see Figure 4). This EE by sex interaction was not seen on pnd 50 and second time of testing in the original study.

The main effect of enrichment did not persist into late adolescence. At Trial 2 in early adolescence, the enriched rats spent about half of their time with the novel, potentially risky objects, as opposed to the non-EE controls that exhibited a clear fixation on the novel objects (see Table 2). This behavior is beneficial to the subjects, as it prevents them from engaging with novelty for novelty's sake, shielding subjects from engaging with a potentially detrimental component of the environment simply because it is unfamiliar. This beneficial feature of enrichment was not observed in either of the older subject groups, indicating enrichment is more protective at a younger age.

Since males and females mature at different rates across adolescence, it may be that the different way each sex approaches the swapped objects are largely driven by differences in maturity between sexes (Lynn & Brown, 2009; Hendershott et al., 2016; Willing et al., 2016). Prior work has demonstrated unenriched females spend more time with the potentially riskier stimuli than unenriched males; the tendency toward risk in female rats and mice has been shown to be mitigated by enrichment (Lynn & Brown 2009). In both cases with a significant sex by enrichment interaction, female subjects followed this exact pattern. In contrast, the enriched males spent significantly more time attending the novel, potentially riskier objects than their unenriched counterparts. This could be due to males at this age being inherently less mature than the female subjects, as well as the natal nest dispersal instincts that are typically driven by male rats (Calhoun ,1963; Willing et al., 2016). Since these differences were not maintained upon second time of testing, and were present at first time of testing in late adolescence, enrichment moderated this interaction. It is additionally possible that the differing maturity level and dispersal patterns were not as significant influences on behavior upon the second exposure to the field and task.

Additionally, this study analyzed brain tissue from subjects that had performed the OiP task twice, once during early adolescence and once during late adolescence. By observing potential differences in brain activation in regions related to the task of interest, the relationship between OiP task performance in EE and non-EE controls and relevant neural regions can be quantified.

Hypothesis 2, which anticipated that the HF regions would exhibit increased activation in EE subjects as compared to controls, was not supported. When compared to the preliminary data on the CA1 and DG regions of the HF collected by Cobb (2015), the data obtained in this

study fully confirmed her initial observations. The CA1 was significantly less active in EE subjects as compared to controls, even when accounting for exclusively highly activated cells (See Figure 5). Since the CA1 is the output zone of the HF, it would follow that by becoming adapted to novelty the enriched rats develop a more efficient processing system for cues about the environment around them, which is supported by literature that indicates EE resulting in an increased volume and dendritic branching within the HF (Hüttenrauch, Salinas & Wirths, 2016; Zhu et al., 2009). Though prior work has established that rodents have decreased CA1 activation in the presence of novelty, by being enriched it is possible that environmental cues are being processed more efficiently or elsewhere in the HF than in non-EE controls.

As seen in Cobb's preliminary counts, the DG, the input zone of the hippocampus, was not significantly different between groups when counting exclusively darkly stained cells (see Figure 5). The CA3 was also negligibly different between groups (see Figure 5). This may be that the nature of initial environmental processing and observing of differences within the environment is the same regardless of adaption to novelty, and that the nature of spatial awareness and object recognition are essentially the same regardless of how adapted the subject is to novelty. It is an area of interest for potential future work to observe the HF in enriched rats in a potential variety of tasks that allow the subject to engage with novelty. This would provide clarification on why the activation in these subjects is so contrary to the established literature on the processing of novelty within these brain regions.

Hypothesis 3, that the BLA and LA of enriched brains would be less activated in the EE brain tissue than that of controls, was partially supported. The LA of EE brains exhibited on average a 50.7% reduction in activation compared to non-EE controls (see Figure 6). This is reflective of the literature in the field that indicates EE subjects possessing more efficient coping

mechanisms for anxiety-inducing tasks, and how EE subjects recover more rapidly after being subjected to a fear-inducing event (Ashokan et al., 2016; Mosaferi et al., 2015). Since a more active amygdala is indicative of a more fearful subject, and an unknown environment is understandably more fear-inducing in a subject unaccustomed to novelty, it follows that the enriched rats becoming accustomed to novelty allows them to be less frightened by it.

The BLA activation observed was not statistically different between subjects (see Figure 6). This result was counter-intuitive, considering how drastic the observed differences within the LA were, and potentially warrants a review of the tissue counted within this region. The BLA is the site of convergence of contextual information about an environment and of the emotion that environment potentially induces, and when the BLA is inhibited, fear-based memories do not form (Miserendino, Sananes, Melia, & Davis 1990; Wellman et al., 2017). Since the LA is highly activated during the formation of long-term memories about an environment and the BLA is necessary to form the initial emotional cues, it does not follow that these two regions would have such divergent responses in EE subjects compared to controls (Repa et al., 2001). This finding is also discordant with prior studies that have shown EE subjects to have lessened activation within the BLA than controls (Artz, 2016; Ashokan et al., 2016; Mosaferi et al., 2015). Since the BLA is the site of initial observation and relation of contextual cues within the environment to the emotions those cues incite, it is possible that the EE subjects and non-EE controls engage with the environment initially in the same way. Therefore, the formation of long-term memories as processed within the LA is the true region of adaption to novelty within the environment.

Enrichment has an observable impact on the behavior and neural correlate regions of adolescent rodents, though this impact is more powerful in early adolescence. Additionally,

repeated exposure to a task employing novelty within an open field is enriching, and as such behavior is altered in each additional exposure to the task. Though regions correlated with initial processing of novelty and familiarity within objects and the environment are statistically the same between enriched and unenriched subjects, the final output of this processing is significantly more efficient in EE subjects than in non-EE controls. Finally, regions associated with long-term fear-based learning are significantly less active in EE subjects compared to controls, though initial cue observation and potential emotional connections are similar between groups, indicating a more efficient coping mechanism in EE subjects as compared to controls. Therefore, EE provides adaption to environmental novelty that is ultimately beneficial in subjects during both early and late adolescence, but is more beneficial in early adolescence.

These findings are potentially limited by the cell counts in this work being performed by undergraduates undergoing training while analyzing this tissue. The protocol for how to count in such a way that limits subjective influence on analysis was updated immediately prior to the analysis of the BLA. Considering the unusual result obtained from the data analyzed during this transition period, the BLA data in this study could benefit from further analysis. Additionally, after completion of data coding in the OiP task two research assistants indicated they were unclear on whether to code when the subject was attending to an object or physically near it. Though this discrepancy would not largely impact the results found, it may have potentially biased the results.

Future studies include the analysis of neural tissue obtained from the subjects tested exclusively in late adolescence, to compare the differences in neural activation in those who had undergone OiP testing one versus two times. It would be useful to establish a further understanding of the current results by testing a group exclusively during early adolescence and

sacrificing them after the first completion of the task, to understand neural activation differences in EE and non-EE groups throughout adolescence. Since this study indicates enrichment alters behavior at any age but is most impactful in early adolescence, it would be of interest to perform multiple incidents of testing to see how behavior and neural activity differ across adolescence, and to observe conclusively when the impact of enrichment has the most significant influence on behavior.

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Table 1.

Proportion of Time (seconds) Spent with the Swapped Objects in Male vs Female Subjects

	pnd	Group	Sex	*N*	*M*	*SD*
Cobb (2015)	36*	EE	F	15	0.53	0.11
			M	16	0.57	0.14
		No-EE	F	16	0.58	0.19
			M	16	0.49	0.32
	50	EE	F	16	0.46	0.22
			M	14	0.51	0.32
		No-EE	F	16	0.58	0.31
			M	15	0.48	0.33
Current Study	49**	EE	F	12	0.56	0.14
			M	12	0.64	0.11
		No-EE	F	12	0.58	0.12
			M	12	0.50	0.11

Note. The significant differences is indicated (*$p < .05$, **$p < .02$). Abbreviations: post-natal day, pnd; environmental enrichment, EE; Non-enriched controls, No-EE.

Table 2.

Proportion of Time (seconds) Spent with Swapped Objects in EE vs Control subjects

	Pnd	Trial	Group	*N*	*M*	*SD*
Cobb (2015)	36*	2	EE	16	0.53	0.11
			No-EE	16	0.71	0.15
		3	EE	15	0.57	0.14
			No-EE	16	0.34	0.23
	50	2	EE	15	0.59	0.22
			No-EE	15	0.75	0.21
		3	EE	15	0.37	0.27
			No-EE	16	0.32	0.26
Current Study	49	2	EE	12	0.58	0.12
			No-EE	12	0.46	0.09
		3	EE	12	0.63	0.14
			No-EE	12	0.61	0.10

Note. The significant EE by trial effect is indicated (*$p < .0002$). Abbreviations: post-natal day, pnd; environmental enrichment, EE; Non-enriched controls, No-EE.

Table 3.

Mean Neurons Expressing c-Fos Protein in the Hippocampus Regions for Enriched and Non-Enriched Subjects.

	CA1			DG			CA3		
Group	*N*	*M*	*SD*	*N*	*M*	*SD*	*N*	*M*	*SD*
EE	12	25.9*	19.5	9	22.6	19.6	12	25.0	17.7
No-EE	9	37.6*	14.4	9	34.7	20.4	9	30.7	13.8

Note. The significant difference is indicated (*$p < .05$). Abbreviations: Environmental enrichment, EE; Non-enriched control, No-EE; *cornu ammonis* 1, CA1; dentate gyrus, DG; *cornu ammonis* 2, CA2; *cornu ammonis* 3, CA3.

Table 4.

Mean Neurons Expressing c-Fos Protein in the Amygdala for Enriched and Non-Enriched Subjects.

	BLA			LA		
Group	*N*	*M*	*SD*	*N*	*M*	*SD*
EE	11	9.7	6.7	9	6.9*	10.2
No-EE	10	11.7	6.8	8	14.0*	33.8

Note. The significant difference is indicated (*$p < .011$). Abbreviations: basolateral amygdala, BLA; lateral amygdala, LA.

A. B.

Figure 1. The female enrichment cage assembled on two different set ups, as seen in (A) and (B). Each female subject was loaded into this cage for 1.5-hr daily, and toys were swapped out between each 1.5-hr session. The assortment of toys seen above as well as the novel conspecifics allowed subjects to freely interact both socially and with the environment provided.

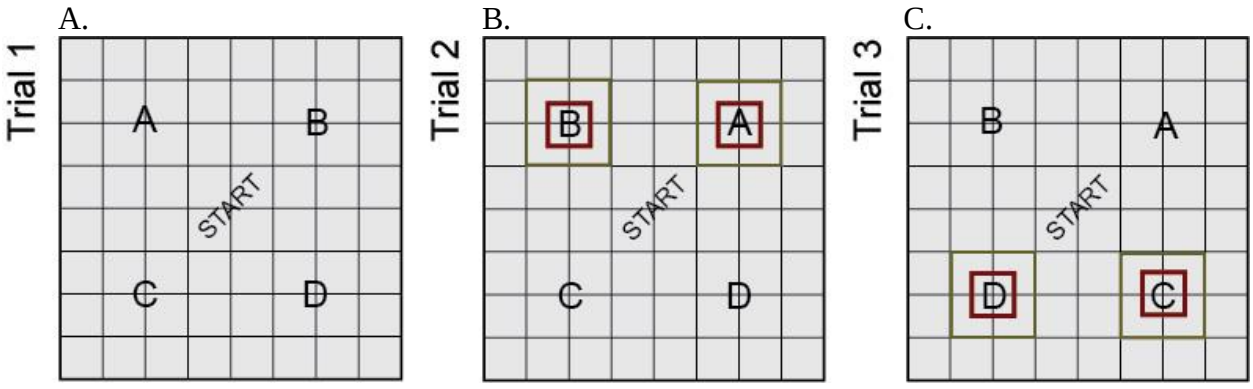


Figure 2. The positions of objects during each trial of the Object in Place task. In Trial 1 (A) subjects interact with objects and explore the field freely to acclimate to the objects and field used in the task. During Trial 2 (B) two objects were switched, and attention to the swapped objects vs the stationary ones was monitored. In the final trial (C) a second swap was performed in the remaining two objects.

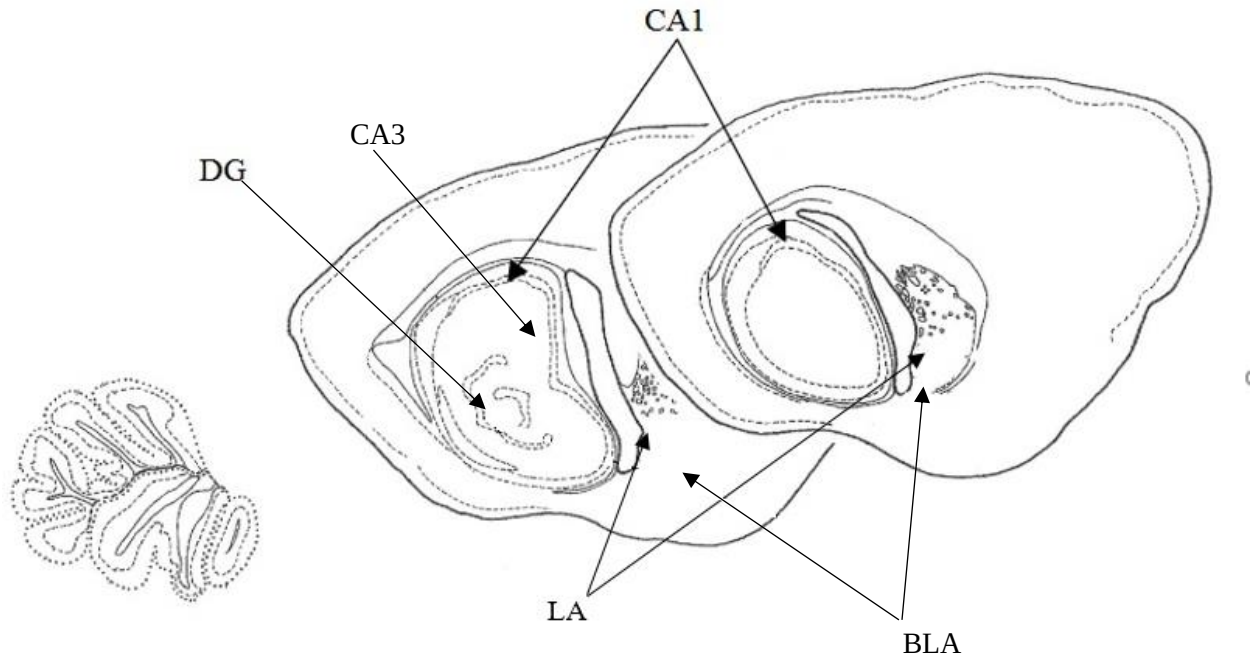
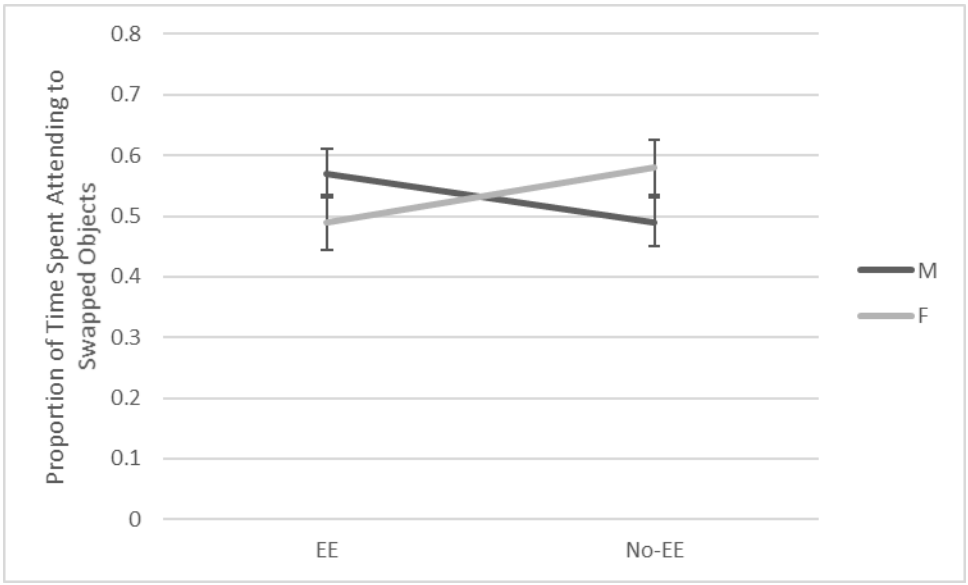


Figure 3. Locations of sampled regions of CA1, CA3, DG, LA, and BLA as indicated by arrows adapted from the atlas of the rat brain published by Pellegrino & Cushman (1967).

A.



B.

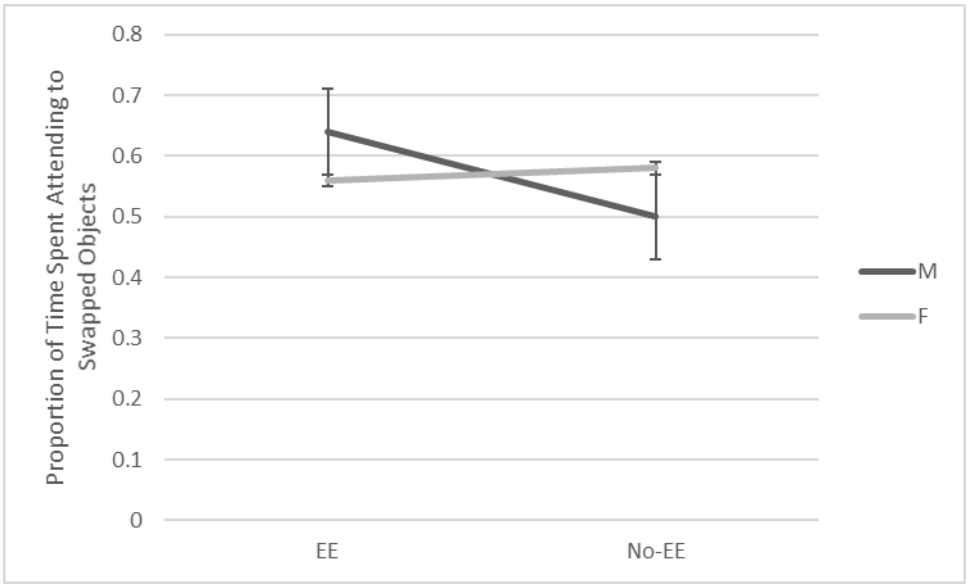


Figure 4. The interaction of EE and sex on the proportion of time spent with swapped objects on pnd 36 in Cobb's data (A) and pnd 49 in the current study (B). On both pnds EE males spent more time with swapped objects than non-EE controls, and females spent less time with swapped objects than non-EE controls. This interaction was not statistically significant at second time of testing.

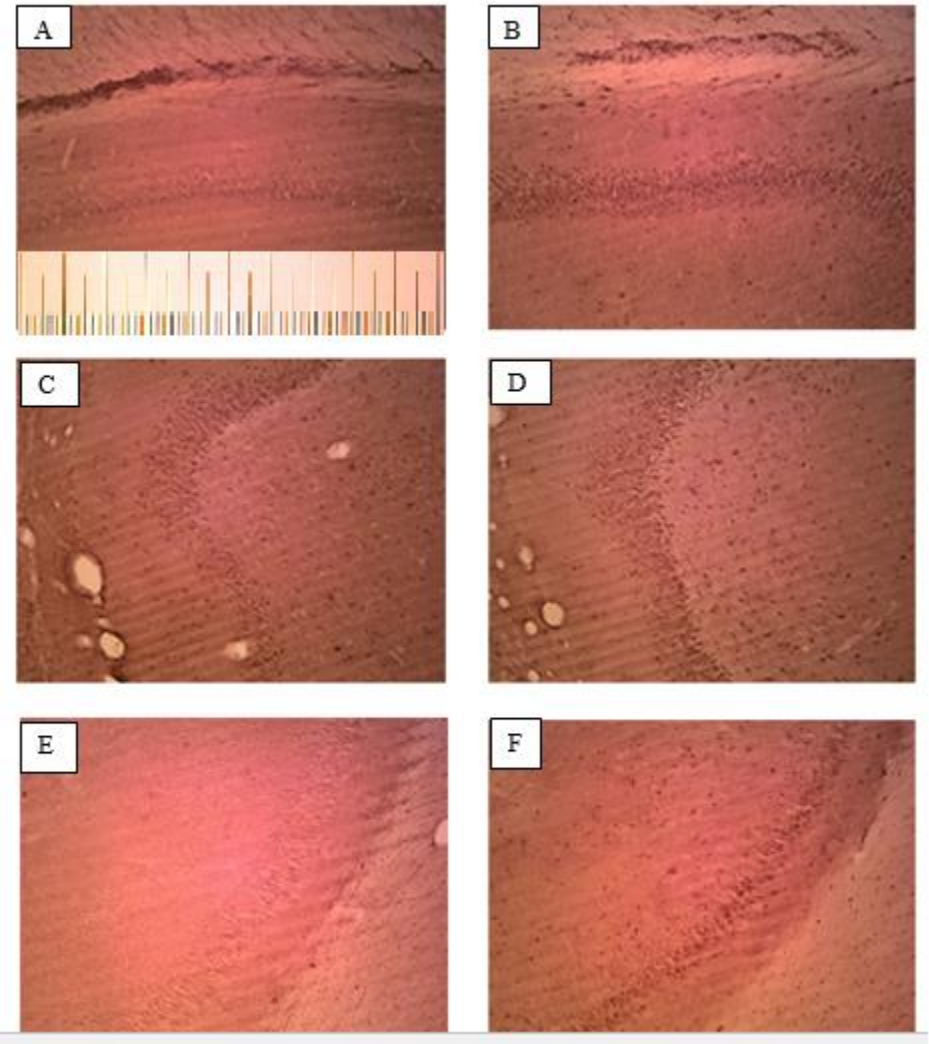


Figure 5. Microscopic images of 3 hippocampus regions. (A) The CA1 region of the hippocampus in EE animals. (B) The CA1 region of the hippocampus in non-EE animals. EE animals showed a 33.2% reduction compared to controls. (C) The DG region of the hippocampus in EE animals. (D) The DG region of the hippocampus in non-EE animals. (E) The CA3 region of the hippocampus in EE animals. (F) The CA3 region of the hippocampus in non-EE animals. There were no statistically significant differences in the DG or CA3 regions. Each image is 1000 µm as indicated by the scale (A).

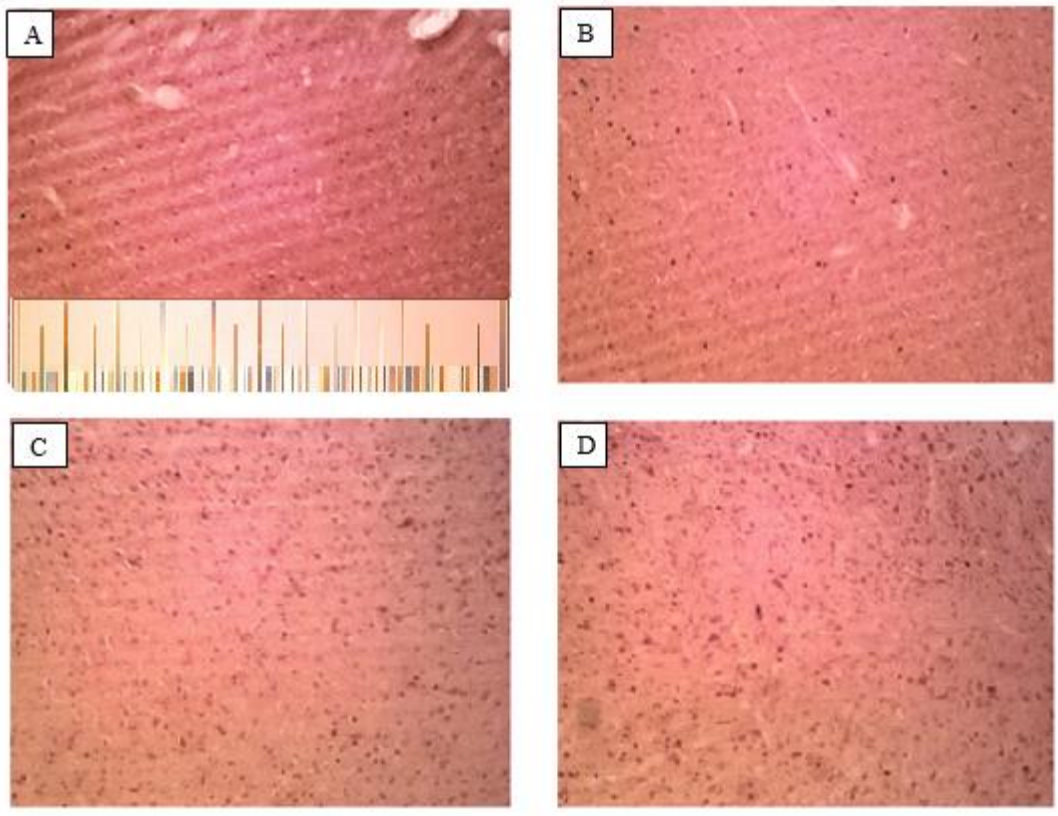


Figure 6. Microscopic images of two regions of the amygdala. (A) The basolateral amygdala in EE animals. (B) The basolateral amygdala in non-EE animals. There were no statistically significant differences between subjects in this reason. (C) The lateral amygdala in EE animals. (D) The lateral amygdala in non-EE control animals. There was a 50.7% reduction of activity in EE animals as opposed to non-EE controls in the lateral amygdala. Each image is 1000 μm as indicated by the scale (A).