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The effect of an enriched environment and cognitive maturity on long-term reactivation of object recognition memory in male Sprague-Dawley rats.

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Running head: NOR AND EE

The effect of an enriched environment and cognitive maturity on long-term reactivation
of object recognition memory in male Sprague-Dawley rats.

A thesis presented by
Lisette Roman
to the Department of Psychology
in partial fulfillment of the requirements
for the degree of
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Abstract

This study examined the effect of environment and cognitive maturity on long-term reactivation of recognition memory in rats. Forty male Sprague-Dawley rats were housed in either an enriched environment or standard conditions. Animals were tested in the Novel Object Recognition (NOR) task as either adults or periadolescents. Results showed exposure to an enriched environment significantly increased the ability of animals to discriminate between a novel and a familiar object. Results also revealed that the impact of environmental enrichment was significant in adults but not in periadolescents. Immunohistochemical staining for the immediate-early gene products c-Fos and zif268 was performed to examine the involvement of prefrontal cortex, hippocampus, and perirhinal cortex in the NOR task. Levels of c-Fos in the prefrontal cortex were significantly higher in adults. Significant inconsistencies were found among levels of both IEG products in the brains of periadolescents. The findings in the present study support the need for prefrontal cortical maturation in rats in order to discriminate between novel and familiar after a long delay.

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The effect of an enriched environment and cognitive maturity on long-term reactivation of object recognition memory in male Sprague-Dawley rats.

On December 2nd, 2009, a human brain was sliced into more than 2,400 sections, broadcasted live on the Internet, and attracted more than 400,000 hits over the 53-hour long process (Holden, 2010). This was the brain of Henry Molaison, better known as H. M., the now-famous neurological patient known for suffering profound amnesia (Winters, Saksida, & Bussey, 2010). The brain of H. M. will be made into a digitized brain atlas and is the first brain to be entered into an unprecedented online human brain library at the University of California, San Diego (Miller, 2009). Available to scientists and students alike, the neuroanatomical map of the brain of H. M. will undoubtedly lead to better understanding of human declarative memory. Thanks to such breakthroughs, research into memory disorders has advanced significantly in the past decade, so much so that a new category has been proposed for memory-related disorders in the *Diagnostic and Statistical Manual – Fifth Edition*; whereas currently memory disorders fall under the category ‘Delirium, Dementia, Amnesic, and Other Cognitive Disorders’, the new edition due out May 2013 may use the category *Neurocognitive Disorders* (“Proposed revisions,” 2010).

Human brains are not frequently available for such invasive research purposes, therefore animal research has been paramount in the effort to better understand human declarative memory. As stated earlier, H. M. suffered from amnesia, specifically organic amnesia from irreversible brain damage sustained during an operation meant to help control his epilepsy (Squire, 2009). Amnesia is defined as a disorder in which a selective impairment in memory occurs but other cognitive functions, including both short term

and procedural memory, are preserved. Amnesic patients typically have grave difficulty learning new facts and an inability to form memories of events occurring from the time of the brain damage, referred to as anterograde amnesia (Tulving, Haymond, & Macdonald, 1991). These impairments involve semantic and episodic memory, respectively, and both memory types collectively form what is known as declarative memory.

Declarative memory describes our ability to report knowledge that one is aware of possessing (Schacter, 1987). This definition is problematic for animal research; humans most commonly “report” facts verbally whereas animals have no such verbal ability. Animal research addresses this issue by eliciting behavioral (non-verbal) cues from subjects to assess declarative memory, such as lever presses, eye movements, and choices made in mazes or other test apparatuses.

Animal Models of Declarative Memory

The first animal model of human amnesia was published in 1978 (Mishkin). Mortimer Mishkin sought to induce retrograde amnesia (characteristic of the human amnesic disorder) in non-human primates. Previously, studies had elicited profound spatial memory impairment in primates via ablation of the hippocampus, but substantial non-spatial impairment had been yet to be achieved. Using the delayed non-matching to sample task, Mishkin assessed the animals’ non-spatial memory impairment after lesioning of the hippocampus and amygdala. Whereas subjects in this study with either hippocampal lesions or with amygdaloid lesions did not have significantly impaired memory, subjects with both areas lesioned were significantly impaired. This study was important to establish an animal model of the human amnesic syndrome and for its

implication of an area outside the hippocampal system in memory; Mishkin highlighted the idea that specific types of memory may be served by specific circuitry.

By employing the delayed nonmatching to sample task in his 1978 study, Mishkin evaluated one example of declarative memory: recognition. Recognition memory can be defined as the ability to identify a previously encountered element as having been encountered before, and this memory type is often viewed as an aspect of episodic memory (Dere, Kat-Teke, Huston, & De Souza Silva, 2006). Certainly no rat model exists that translates into a comprehensive model of human episodic memory (Ennaceur, 2010); it may not be feasible in the rat at all if there exists a conscious experience of “time travel” involved in episodic memory unique to the higher-level human memory system (Tulving, 1972). While this conscious aspect of episodic memory may render modeling impossible in a single animal model, it has been proposed that recognition memory is a subcomponent of episodic memory, along with temporal order memory and spatial memory (Dere *et al*, 2006; Winters, Saksida, & Bussey, 2008). Therefore, the development of rodent models of recognition memory is necessary for insight into the more complex human episodic memory processes.

Common Object Recognition Memory Tasks Used with the Rat

Although animal models used in object recognition memory research include several different animals, the scope of this paper will be limited to study of the rat. Furthermore, although recognition memory may be assessed via all the sensory modalities (auditory, olfactory, gustatory, tactual, and visual), the scope of this paper will be limited to visual object recognition memory. Contrary to past assumptions about the rat as a poor model for object recognition memory due to a weak visual system, the ability of the rat to

visually discriminate between even complex objects has more recently been empirically supported (Zoccolan, Oertelt, DiCarlo, & Cox, 2009), and there is now a rich body of literature examining visual object recognition in the rat (Aggleton et al., 2005, 2010; Albasser et al., 2009, 2010, 2011; Barker & Warburton, 2011; de Bruin et al., 2011; Ennaceur, Neave, & Aggleton, 1997; Ennaceur et al., 1988, 1996, 1998, 2010; Frye, Duffy, & Walf, 2007; Hannesson, Howland, & Phillips, 2004; Inagaki, Gautreaux, Luine, 2010; Luine, Jacome, & MacLusky, 2003;).

Object recognition memory is most commonly assessed in rodents using two tasks: the delayed non-matching to sample task (DNMS) and the novel object recognition task (NOR) (Squire, 2007). A significant difference between the DNMS task and the NOR task is that the DNMS is reward-based (Ennaceur & Delacour, 1988). For this reason, among several others, the NOR task is often regarded as a stronger assessment of recognition memory than is the DNMS task. The NOR task was first developed by Ennaceur in 1988, and like the DNMS task for rats, has been slightly modified since its introduction to research. Most notably, a Y-shaped apparatus is becoming increasingly popular, whereas the original design calls for an open or quadrangular arena (Winters, Saksida, & Bussey, 2008). Winters and colleagues first introduced the Y-shaped design to eliminate the influence of spatial-contextual factors from the room housing the task apparatus; it is possible that features of the room may be visible to the rat from certain spots within the rectangular apparatus, thus the researchers sought to eliminate this possibility (2004). Although the Y-shaped design may eliminate external stimuli that can influence performance on the task, the problematic arena cited in Winters and colleagues (2004) measured 1 x 1 m, and the dimensions of the apparatus used in the present study

totaled only a fraction of the dimensions of the arena in question, so the procedure and apparatus used in the present study should have eliminated the problems with spatial cues outside the arena. Thus, the NOR task will be described assuming a rectangular apparatus.

Variations of the NOR task are widespread, an issue of concern that will be later discussed; despite the procedural differences prevalent among studies that use the NOR task, only the procedure used in the current study will be presented. This procedure involves three discrete phases: habituation, familiarization, and test. Habituation occurs in 20 min sessions on day 1 and day 2. The rat is placed in the apparatus and allowed to acclimate to the box. No objects are present during habituation. The familiarization phase starts on day 3, during which the rat is placed in the apparatus with two identical copies of the sample object (which will become the “familiar” object) and allowed to explore for 5 mins. The test phase follows a 2h delay; the rat is placed in the apparatus again, this time with a novel object and a third copy of the familiar object. Using a new copy of the familiar object for each phase reduces olfactory cues that might have resulted from the animal touching the object in a previous phase. On day 4, 24h following the familiarization phase, the rat is placed in the apparatus again with a fourth copy of the familiar object and a second copy of the relatively novel object. Multiple copies of the novel object were also employed for olfactory cue elimination.

Strengths and Weaknesses of the Novel Object Recognition task

Why is the NOR task so widely used? The NOR task can be used to study brain damage, pharmacological effects, and genetic manipulations (Dere, Huston, & De Souza Silva, 2007; Gaskin et al., 2010). The absence of rule-learning of any kind, in

combination with a relatively short test phase, allows the NOR task to be less labor intensive for both the researcher and the subjects compared to other measures of memory (Dere et al., 2007; Winters et al., 2008). Another advantage of a memory test that does not necessitate a food reward is that it does not depend on brain circuitry of reinforcement systems that are not implicated in object recognition memory (Akirav & Maroun, 2006; Dix & Aggleton, 1999; Reger, Hovda, & Giza, 2009; Silvers, Harrod, Mactutus, & Booze, 2007). By excluding such confounding factors, the NOR task can be extended for a variety of experiments. Furthermore, the one-trial nature of the memory task is a design much closer to tasks of episodic memory used in primates and humans than is the DNMS task (Dix & Aggleton, 1999; Clark & Martin, 2005; Reger et al., 2009). Thus it would seem the NOR task produces results that can be extended to recognition memory with greater generalizability and with greater confidence than is true of alternative memory tasks.

Variations of the NOR task have been introduced to assess aspects of recognition memory other than object recognition. In a 1999 study employing the NOR task in a battery of tests for memory of object recognition, Dix and Aggleton were the first to extend the NOR task to both spatial and non-spatial memory in the rodent in a single study. Since then, the NOR has been used to assess memory of object location, (Bisagno, Ferguson, & Luine, 2001; Bowman, Gautreaux, Fernandez, & Luine, 2009; Bussey, Muir, & Aggleton, 1999; de Bruin et al., 2011; Ennaceur, Neave, & Aggleton, 1997; Frye, Duffy, & Walf, 2007; Inagaki, Gautreaux, Luine, 2010; Luine, Jacome, & MacLusky, 2003; Macbeth, Gautreaux, & Luine, 2008; Salas-Ramirez, Frankfurt, Alexander, Luine, & Friedman, 2010; Wallace, Frankfurt, Arellanos, Inagaki, & Luine,

2007; Wallace, Luine, Arellanos, & Frankfurt, 2006), where the subject must recognize that the position of an object has changed relative to its original position; to assess memory of temporal order (Barker, Bird, Alexander, & Warburton, 2007; Barker & Warburton, 2011; Hannesson, Howland, & Phillips, 2004; Hauser, Tolentino, Pirogovsky, Weston, & Gilbert, 2009; Hotte, Naudon, & Jay, 2005; Mitchell & Laiacona, 1998; Nelson, Cooper, Thur, Marsden, & Cassaday, 2011), where the subject must recognize that an object has been presented more recently relative to the time of presentation of another object; and to assess object-in-context memory (Balderas et al., 2008; Barker et al., 2007; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002a; Nelson et al., 2011), where the subject must recognize an object based on the context in which it was originally presented (such as within a red arena). The versatility and ease of use of the NOR task contribute to its popularity among researchers.

The weaknesses of the NOR task lie more in the variety of versions of the task than in any weakness of the task itself. For example, even the calculations used to quantify exploration of rats in the NOR task varies among studies (Gaskin et al., 2010). Researchers have used multiple formulas to denote differences in time spent with objects, varying from a simple difference score used by Albasser and colleagues in a 2009 study (as cited in Gaskin et al., 2010, p. 62), to a ratio of the amount of time spent exploring the novel object relative to the familiar object (t_N/t_F) as used by Gaskin and colleagues in a 2000 study and a 2003 study (as cited in Gaskin et al., 2010, p.61), to a ratio of the difference between time spent exploring the novel object and time spent exploring the familiar object relative to the time spent exploring the familiar object $[(t_N - t_F)/t_F]$ (Barker, Bird, Alexander, & Warburton, 2007). Researchers also differ in the criterion for what

demonstrates intact recognition memory in the rat; some consider scores that significantly differ from the baseline (a ratio of 0.5 in terms of time spent exploring the novel object relative to total exploration time) as demonstrating an intact memory trace of the familiar object, whereas others consider how groups within the study differ from one another (Dere, Huston, & De Souza Silva, 2007). Some investigators even differ in the procedure used for the familiarization phase, with some using a set number of minutes per animal whereas others require a set number of seconds of direct exploration of both objects; for example, Forwood and colleagues stop the familiarization phase once each animal accumulates 25 sec exploration or until 3 min (2005) has passed.

Another issue surrounding the NOR task is the scarcity of literature supporting assumptions made by the NOR task. One assumption of the task is the ability of the rat to visually discriminate between objects and between objects and their surroundings. Indeed, the first study since 1961 to attempt to verify the assumption that rats discriminate objects based on shape was conducted in 2006 (Minini & Jeffery). This assumption is crucial to the NOR task literature, as often experimental objects are chosen based on their shape. In a detailed review of studies involving the NOR in the past decade, Ennaceur names the lack of research in rat perception and object affordances as detrimental to accurate and appropriate interpretation of results from the NOR task (2010). Depending on the setup of the NOR task, the demand on the senses of the rat varies. The fact that the task requires exploration of three-dimensional objects means the rat is using visual, tactile, and olfactory cues for exploration, but the design of an individual test may task one sensory modality over another or even restrict the tool for recognition to a single sense. For example, the task has been modified to test purely

visual recognition memory in the rat (Forwood, Bartko, Saksida, & Bussey, 2007). This study replaced objects with pictures to ensure that the subjects could not use olfactory or tactile clues to discriminate between objects. In terms of the extent to which rats can visually discriminate between similar objects, it has been shown that the rat can visually discriminate between three-dimensional objects that differ by as little as a single feature; the study that demonstrated this capability used a square object and a rectangular object, differing only on edge length, for the subjects to discriminate (Eacott, Machin, & Gaffan, 2001). When using three-dimensional objects, as in the original NOR task design, the demand on the visual system may be increased or decreased by careful selection and treatment of experimental objects. For example, if olfactory cues are removed one can assume the subject used vision to identify and discriminate between objects (Ennaceur, 2010). Removal of olfactory cues may be achieved by choosing experimental objects with surface textures conducive to cleaning with ethanol solution and/or the use of multiple copies of objects throughout test phases.

Besides task parameters influencing which sensory modality is required by the NOR task, they may also influence what type of memory is necessitated. Varying the parameter of the length of delay between familiarization and test phases in turn varies the type of memory assessed by the task, i.e. short-term or long-term memory. Working memory has been falsely considered to be assessed by the NOR task; Ennaceur admits this initial false claim and asserts that working memory is not at all involved (2010). Given that the NOR task is one-trial, the subjects are not attempting to keep any information “on-line”, as working memory is often described. In a one-trial test the subject is not asked to remember anything from a previous trial; working memory requires the recall of

information stored “on-line” from a previous encounter and is thus a more complex process than recognition memory (Clapp & Gazzaley, 2011). Instead, the NOR task involves short- and long-term recognition memory. Delay times vary from studies implementing delays as short as 5 min (Baker & Kim, 2002) and 15 min (Reger et al., 2009) to the 1 h, 2 h, 3 h, 24 h, and 48 h delays more common in the literature. As will be shown in the following sections, delay times can have an effect on recognition memory, and studying this effect can reveal which brain areas are more strongly associated with short- and long-term recognition memory. For example, lesion studies of Prh consistently show poor memory performance in lesioned subjects regardless of the length of delay used (Albasser et al., 2009; Barker, Bird, Victoria, & Warburton, 2007; Barker and Warburton, 2011; Mumby & Pinel, 1994; Ennaceur et al., 1996; Mumby et al., 2007), suggesting that Prh may be critical for both short- and long-term memory. Lesion studies of Hpc are not as consistent in their findings as the literature on Prh, with some studies showing no effect of lesion on memory after both short and long delays (Winters et al., 2004; Forwood et al., 2005; Gaskin et al., 2003), whereas other studies have found impairment at both short and long delays (Broadbent, Squire, & Clark, 2004; Gould, et al., 2001). In terms of Pfc, researchers have found most support for the role of this area in long-term (24 h or longer) recognition memory, as will be discussed in addressing the studies of Akirav and Maroun (2006, 2009) and Barker and Warburton (2011).

Although the NOR task has a simple design, the risk for misinterpretation of the results is high because even slight variations can impact what aspect of recognition memory is being assessed in the rat. Perhaps this risk of misinterpretation is a reason

why consensus on the anatomical brain structures involved in object recognition memory is still evolving.

The Anatomical Bases of Object Recognition Memory

Mishkin's previously described study from 1978, the first animal model for human amnesia, was a catalyst for memory research to narrowly focus on the medial temporal lobe for decades to come (Milner, 2005). The medial temporal lobe is a memory system consisting of the frontal, temporal and parietal lobes, perirhinal cortex, parahippocampal cortex, entorhinal cortex, hippocampal region, and these regions' direct projections (Bayley & Squire, 2003). It is important to note that recognition memory itself is not a single memory process; instead, recognition memory has been proposed to consist of two components: recollection and familiarity (Brown & Aggleton, 2001). More specifically, Brown and Aggleton asserted that one aspect of recognition memory is the ability to distinguish between novel and familiar objects whereas a separate aspect of recognition memory is the ability to recall spatial arrangements of novel and familiar objects (2001). Recalling spatial arrangements is a form of recollection in that the subject has learned one arrangement (whether it be where an object is located in relation to the surrounding environment or whether it be where an object is located in relation to another/other object(s)) and must compare this original arrangement to any new arrangement. The memory trace of the spatial arrangement is separate from the memory trace of the familiar object itself, in isolation from any spatial cues. It is only logical that the brain areas involved in one aspect of recognition memory may not be the same brain areas involved in another aspect of recognition memory. This line of thinking has led to the current dispute and evolving disentanglement of the neural bases of recognition

memory in recent years, and the topic remains controversial. From the time of the introduction of this distinction a number of studies have supported Brown and Aggleton's proposal, and several researchers have gone further to implicate areas outside the conventional medial temporal lobe in recognition memory. For example, speculation as to why research into prefrontocortical involvement in recognition memory in the rat has been rather recent in comparison to research into the medial temporal lobe was highlighted in a 2003 review; this investigation of the literature concluded that there is considerable evidence of interactions between the prefrontal cortex and medial temporal lobes in long-term memory in humans (Simons & Spiers, 2003). Subsequent cross-species studies using anatomical tracing have shown robust interconnections between and among prefrontal and medial temporal areas in the monkey and the rat (Warburton & Brown, 2009). The following studies showing connectivity of several brain areas in the rat brain, not exclusive to the medial temporal lobe, support extending the research effort into the prefrontal cortex in the rat for a more comprehensive understanding of object recognition memory. Namely, the three primary brain sites involved in recognition memory are the perirhinal cortex, the hippocampus, and the prefrontal cortex.

Function of the perirhinal, hippocampus, and prefrontal cortex in object recognition memory.

Lesion studies have helped to model the currently held view of an "integrated neural network" for recognition memory within which the perirhinal cortex, the hippocampus, and the prefrontal cortex incorporate information for identification, location, and recency of objects (Barker, Bird, Alexander, & Warburton, 2007). The role

each region plays in the memory process depends on the particular aspect of recognition memory being tested (such as object location, recency, object recognition).

In a recent study Barker and Warburton showed clear effects of lesions of the perirhinal cortex (Prh), hippocampus (Hpc), and medial prefrontal cortex (mPfc) on novel object recognition memory in rats (2011), and although there is still controversy over the hippocampal and prefrontal contributions to object recognition memory, these recent results reflect the findings from the bulk of the current literature. Importantly, Barker and Warburton tested both short- and long-term object recognition memory in this 2011 study. Inclusion of a long retention interval may be key for demonstrating the role of the prefrontal cortex in recognition memory. The investigators showed that at delays of 5 min and 3 h, Prh-lesioned animals failed to demonstrate intact memory of the familiar object, whereas animals with hippocampal lesions and animals with prefrontal lesions demonstrated intact memory. At the 24 h delay, Prh-lesioned animals still failed to recognize the familiar object, and Hpc still demonstrated intact recognition of the familiar object, but mPfc-lesioned animals demonstrated a lower discrimination ratio at this long delay. This result at the 24 h delay warrants further research into the role of the mPfc in long-term recognition memory.

The following discussion of lesion studies will show that the Prh is the brain area with the most empirical support for having an obligatory role in novel object recognition memory and that the roles of the Hpc and Pfc remain controversial. It may be argued that at this point in time there is solid empirical support against the Hpc in novel object recognition. The Pfc remains perhaps the most controversial brain area of the three in terms of object recognition memory.

Perirhinal cortex: performance of perirhinal-lesioned rats on the NOR task.

The first study to implicate the Prh in object recognition memory involved a DNMS task to assess lesions of the entorhinal (Erh) and Prh cortices (Mumby & Pinel, 1994); animals with bilateral Erh + Prh lesions were impaired in their ability to discriminate novel from familiar objects at delays of 15 sec, 1 min, 2 min, and 10 min. Since the publication of this study, interest in the role of Prh in recognition memory has increased, and a large body of literature exists focusing on the role of the Prh assessed by the NOR task.

Lesion studies of the effect of Prh damage on object recognition memory investigate performance after a variety of delays yet seemingly find the same results. In one study comparing object recognition in rats with Prh lesions to rats with mPfc lesions, only Prh-lesioned subjects were impaired on the NOR task (Barker, Bird, Victoria, & Warburton, 2007). Subjects were tested at delays of 5 min and 2 h, and performance by brain area lesion was similar at both delays. In another study, after brief delays of 1 min and 15 min, Ennaceur and colleagues compared performance of Prh-lesioned animals with that of fornix-lesioned animals (1996). Lesions of the fornix did not hinder object recognition at either delay whereas animals with lesions of the Prh failed to discriminate between novel and familiar object at the 15 min delay. Yet another study of the effect of Prh lesions on short-term object recognition memory showed impaired object recognition after an approximately 1 h delay (Mumby, Glenn, Nesbitt, & Kyriazis, 2002b). Indeed, the Prh is so well supported that investigators are now using the object recognition task to confirm effectiveness of Prh lesions (Aggleton, Albasser, Aggleton, Poirier, & Pearce, 2010).

Because of the empirical support for the Prh in object recognition memory, other more complex aspects of this brain area's involvement in the memory process can be explored. A handful of studies have investigated the influence of the number and length of familiarization trials on object recognition performance, hypothesizing that frequency and duration of exposure to the familiar object may enhance encoding of the stimulus and thus improve object recognition memory. For example, to manipulate the number of familiarization trials, Mumby and colleagues exposed subjects to five familiarization trials on successive days before memory testing and compared performance to subjects that had been exposed to only a single familiarization trial (2007). Experimental animals with Prh lesions could not discriminate between familiar and novel objects after a 24 h delay following the single familiarization trial, whereas when exposed to the multiple familiarization trials, Prh-lesioned animals could make the discrimination after a 24 h delay but not after a 3 week delay (control animals demonstrated intact object recognition at this long retention interval). The duration of the familiarization trial has also been shown to influence object recognition memory in testing. Albasser and colleagues demonstrated the relationship between the amount of exploration during the familiarization phase and the extent of discrimination between novel and familiar object in the test phase by varying familiarization phase duration from 4, 6, and 8 min (2009). The degree to which subjects discriminated between the familiar and novel objects was significantly correlated ($p < .001$) with the length of time of the familiarization trial; most importantly, subjects with lesions of the Prh showed no such relationship ($p = .50$), and upon histological verification of lesion locations, subjects with caudal Prh lesioning showed significant impairment in object recognition (Albasser et al., 2009, p. 118). Both

studies show that despite attempts to strengthen the memory trace of the familiar object, subjects with lesions of the Prh cannot sustain object recognition memory as long as SHAM subjects, supporting the role of the Prh cortex in this memory process.

What then, accounted for the sparing of object recognition memory at the 24 h delay in Mumby and colleague's 2007 lesion study? Another interpretation of this result, that other brain areas may be engaged and merely need more exposure to the stimulus to form the memory trace, has been suggested (Albasser et al., 2011; Ennaceur & Aggleton, 1997;). Albasser and colleagues suggested a single familiarization trial might be sufficient for the Prh to be effective whereas multiple familiarization trials might be necessary for other brain regions to come into play in object recognition. In their 2011 study, the investigators showed that Prh-lesioned animals performed comparably to control animals at delays up to 90 min when the design included multiple familiarization trials. Importantly, total exploration time of experimental animals was not reduced by Prh lesion; therefore the exploration of the novel object during the test phase could be compared with confidence to the performance of SHAM animals. Taking into account these studies showing reversal of object recognition memory deficit in Prh-lesioned animals strengthens the conceptualization of the role of Prh and necessitates further research to investigate whether other brain areas are indeed having effect at longer delays.

Hippocampus: performance of hippocampal-lesioned rats on the NOR task.

Lesion studies of hippocampal involvement in the rat NOR task are not all in agreement; however, a 2008 review of object recognition memory concluded that the findings generally support hippocampal involvement in more spatial object recognition

tasks and do not support hippocampal involvement in novel object recognition (Winters, Saksida, & Bussey, 2008). Empirical evidence against Hpc in recognition memory is dominant in the literature (Barker & Warburton, 2011; Bussey, Duck, Muir, & Aggleton, 2000; Forwood, Winters, & Bussey, 2005; Gaskin, Tremblay, & Mumby, 2003; Good, Barnes, Staal, McGregor, & Honey, 2007; Langston and Wood, 2010; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002a; Mumby, Tremblay, Lecluse, & Lehmann, 2005; Winters, Forwood, Cowell, Saksida, & Bussey, 2004;). One possible explanation for results contrary to these is that of procedural differences across studies using the NOR task, as proposed by Winters and colleagues (2004). Several of the studies showing no role of Hpc in the NOR task will be presented here, followed by a discussion of several studies supporting Hpc mediation of novel object recognition. These studies will also be discussed in terms of methodological issues that may have contributed to their results.

Rats with hippocampal damage have maintained their capacity to recognize objects at both short and long delays (Winters et al., 2004; Forwood et al., 2005). In accordance with the majority of Prh-lesion studies on object recognition, Winters and colleagues found significant impairment in animals with combined perirhinal and postrhinal (PPrh) lesions at delays of 30 sec, 13 min, 1 h, and 24 h (2004). These subjects significantly differed from both Hpc and control animals that demonstrated similar performance on the NOR task. In another study, Hpc-lesioned animals were not impaired in anterograde object recognition memory (lesion before familiarization phase) after either a 15 min or a 24 h delay (Gaskin et al., 2003). Conversely, animals lesioned after the familiarization phase were impaired on the test phase. The investigators hypothesized that this retrograde impairment may have been caused by damage to tissue containing contextual

cues animals may have encoded from the familiarization phase. This explanation is plausible considering the extent to which the Hpc is implicated in spatial memory (Winters, Saksida, & Bussey, 2008), but this explanation is speculative.

Further support for the Hpc in spatial memory but not recognition memory tasks was found in Hpc-lesioned animals at delays of 15 min, 1 h, 24 h, and 48 h. This comprehensive study showed lesioned animals retained intact recognition memory at every delay whereas the same lesioned animals were significantly impaired in the spatial memory task (Forwood et al., 2005). Even longer delays were tested in rats with Hpc lesions in object recognition memory performance; hippocampal damage did not impair the lesioned subjects from discriminating novel object from familiar object 24 h, 1 week, and even 3 weeks after familiarization (Mumby et al., 2005). In order for animals to perform over such long delays, animals were exposed to multiple familiarization trials - once daily for 5 days. This result of intact recognition memory in Hpc-lesioned rats after multiple familiarization trials directly contradicts the findings of Broadbent and colleagues (2010), who showed impaired recognition memory in Hpc-lesioned rats despite multiple familiarization trials. Broadbent and colleagues used extensive familiarization in their studies, similar to the extensive familiarization shown to help Prh-lesioned animals overcome impairment due to tissue damage (Albasser et al., 2009, 2011; Mumby et al., 2007). In the 2010 study, rats were exposed to the experimental objects three times a day for four days before testing, suggesting that not even increased familiarization could compensate for the memory deficit caused by Hpc lesion (Broadbent et al.). Studies supporting the role of Hpc in studies involving the NOR task will now be discussed.

In a study employing a battery of learning tests including the NOR task, animals with Hpc lesions showed significant impairment after a 1 h delay (Gould, et al., 2001). One small but critical design flaw was made: novel and familiar objects were not counter-balanced. That is, the same object served as the novel object for each animal; this is a confounding factor emphasized in Ennaceur's previously discussed review of the NOR task, as one object may be preferred by rats over another, thereby confusing object preference for object recognition (2010).

One study demonstrated different effects of Hpc lesion on performance on both the NOR task and a spatial water maze test; lesion size was manipulated to examine the effect of the extent of lesion on performance (Broadbent, Squire, & Clark, 2004). Lesion sizes included 5-30%, 30-50%, 50-75%, and 75-100% of the hippocampal field, and all animals were tested after a 3 h delay. The investigators found that object recognition was impaired only in lesions of at least 75% of the hippocampus, whereas spatial memory was impaired in lesions of 30% and greater. These results suggest that Hpc is more important for spatial memory than for object recognition memory, but they also suggest a role for Hpc in recognition memory with damage of at least 75% of the hippocampal region. The investigators stressed the importance of verifying extent of lesion especially in studies involving the NOR task.

In a more recent study by Broadbent and colleagues (2010), which also supported the role of Hpc in object recognition, another explanation for inconsistent findings of Hpc in object recognition was presented. The investigators again used a 3 h delay to assess memory, this time varying the length of time post-lesion surgery to examine the effect of recovery time on performance. Experimental animals were tested 1 day, 4 weeks, or 8

weeks after surgery. Subjects that had been lesioned 8 weeks prior to assessment performed similarly to controls, and subjects assessed 1 day and 4 weeks after lesioning were “moderately” impaired on the NOR task (Broadbent et al., 2010, p. 8). However, this moderate impairment was not evident in independent NOR tasks, the impairment was only evident after averaging the results of four NOR tasks over four consecutive days. Therefore the investigators concluded that the short delay of 3 h used in the NOR task may not be sufficient to show hippocampal impairment but a longer delay might; one proposal as to why there is debate over Hpc involvement in the NOR task. Similarly, another study employing multiple tests of NOR found impairment in Hpc-lesioned rats; subjects distinguished between 20 different experimental objects over multiple sessions and showed impairment compared to SHAM animals at delays of 10 min, 1 h, and 24 h (Clark, Zola, & Squire, 2000). To summarize the main findings in this 2010 study and the previously described 2004 study, the investigators posit that in order to implicate the Hpc in object recognition memory, lesion size must encompass at least 75% of the Hpc and animals must be subjected to multiple tests of the NOR task.

One glaring methodological inconsistency between the Broadbent and colleagues (2010) study and studies that do not support a role of Hpc in object recognition memory must be addressed: discrimination of the novel object was quantified by performance in 15- and 30-second bins of the test phase, and all animals that showed “perirhinal thinning” were also included in analysis (Broadbent et al., 2010, p. 7). The term perirhinal thinning was mentioned only once in the article and was not defined, putting into doubt the brain areas damaged and thus attribution to strictly the Hpc in discussion

of results. It is unclear in the other publications whether or not the same inclusion rules for analysis were held.

Although methodological inconsistencies exist in both the literature supporting or not supporting the role of Hpc in object recognition memory, the body of literature against a role of Hpc in object recognition memory assessed via the NOR task appears larger and more methodologically sound than the literature supporting a role of Hpc in object recognition memory assessed via the NOR task. It is expected that these findings will be reflected in the results from immunohistochemical analysis in the present study; of the three brain areas under investigation it is anticipated that fos- and zif268-activated cell counts will not be significant in hippocampal tissue.

Prefrontal cortex: performance of prefrontal cortically-lesioned rats on the NOR task.

As controversial as the Hpc contribution to object recognition memory is, there appears to be even less consensus on the involvement of the Pfc in object recognition memory. One aforementioned lesion study did not support a role for the Pfc in this memory process; rats with lesions of the mPfc were able to discriminate between novel and familiar objects and their performance was significantly better than that of rats with Prh lesions (Barker et al., 2007). The delays before testing in the NOR task were rather short: 5 min and 2 h. Similarly, a study employing catecholaminergic depletion to disrupt prefrontal cortical functioning found no impairment on object recognition (Nelson, Cooper, Thur, Marsden, & Cassaday, 2011). Animals with mPfc damage were, however, impaired on both a test of object recency and of object location in this study. It is important to note that on the NOR task, animals were only tested after a 10 min delay

following familiarization, providing no information on the effect of lesion on long-term object recognition memory. The findings of Barker and Warburton (2011), another aforementioned study, suggest long-term object recognition memory should be examined in studies investigating the role of Pfc; the investigators reported a lower discrimination ratio at the 24 h delay compared to 5 min and 3 h delays in mPfc-lesioned rats whereas Hpc-lesioned rats maintained their ability to discriminate between objects at the 24 h delay.

A few studies have supported Pfc in object recognition memory by showing impairment in performance after manipulation of both receptors and protein synthesis in the Pfc (Akirav & Maroun, 2006, 2009; Nagai et al., 2007). For example, Akirav and Maroun (2006) reported that the ventromedial Pfc is essential for long-term object recognition memory. This study will be emphasized largely due to its use of delays (3 h and 24 h) with the Novel Object Recognition task similar to those used in the present study since it is clear after examining the bulk of the literature that even a slight variation on the delays used in the memory task can change the aspect of memory being assessed. For this reason the role of the Pfc in object recognition memory is explored despite evidence to the contrary (Barker et al., 2007, Barker & Warburton, 2011; Nelson et al., 2011).

Akirav and Maroun sought to clarify the role of the ventromedial Pfc (vmPfc), in consolidation and reconsolidation of object recognition memory through the microfusion of anisomycin, a protein synthesis inhibitor, or of D,L-2-amino-5-phosphonovaleric (APV), an NMDA receptor antagonist. Protein synthesis at the time of or shortly following an event is necessary for long-term memory formation (Davis, 1984), thus

anisomycin should interfere with this process. Likewise, APV has been shown to block acquisition of contextual fear (Fanselow, 1994), implicating NMDA receptors in the consolidation of memory. Akirav and Maroun manipulated consolidation and reconsolidation by infusing drug into the vmPfc. Whereas neither drug affected short-term (3 h delay) recognition memory, both drugs impaired long-term (24 h delay) recognition memory, $F = 10.50$, $p < 0.001$, thus supporting the role of the vmPfc in long-term object recognition memory formation. The finding of a role for Pfc in performance of the NOR task was supported by Akirav and Maroun again in 2009; the investigators showed impaired memory performance on the same NOR task following disruption of dopamine D₁ receptors. A recent study showed similar effects on NOR performance after D₂ receptor antagonism and D₃ receptor blockade, again supporting the role of Pfc in object recognition memory through dopamine disruption (Watson, et al., 2011). Given the connectivity via at least three direct and indirect pathways between the Pfc and medial temporal lobe structures, this support for prefrontal involvement in object recognition memory deserves future research (Witter, 2003). Given the aforementioned support for Pfc involvement in long-term recognition memory, it is expected that zif- and fos- activated nuclei will be higher in Pfc sections relative to Hpc sections (due to the inconsistent results from Hpc-lesioned subjects in long-term recognition memory), but no significant difference in either direction is expected between Pfc and Prh given the strong support for Prh at both short and long term delays. It is important to note that immunohistochemistry will only reveal the neural activity that was occurring at the time of NOR testing at the long (24 h) delay.

Factors Influencing Object Recognition Memory

The present study examines two specific variables that have been shown to influence object recognition memory: the age at which subjects are tested and the environment in which they are housed.

Enriched environment and object recognition memory.

The influence of an enriched environment.

One factor that has been shown to affect performance of rats on memory tests is exposure to an enriched environment (Bruehl-Jungeman, Laroche, & Rampon, 2005; Leggio et al., 2005; van Praag, Kempermann, & Gage, 2000). The influence of an enriched environment on neurochemical and neuroanatomical changes in the rat has long been studied (Rosenzweig, 1966). Often described as “a combination of complex inanimate and social stimulation”, an enriched environment for laboratory animals typically consists of group housing or the opportunity for temporary social stimulation with other animals, objects placed in the cage that are repeatedly rearranged and replaced with new objects, and a running wheel for voluntary exercise (Rosenzweig, 1978; as cited in van Praag et al., 2000).

Environmental enrichment (EE) has a number of cognitive benefits beyond enhanced memory; some of the neural influences of EE enhance memory but also have effects on other cognitive processes whereas other neural influences of EE are completely distinct from memory (Dhanushkodi & Shetty, 2008). A large body of research supports the restorative effect of EE on various types of recovery after brain injury (Johansson & Ohlsson, 1996; Kline et al., 2010; Foti et al., 2011; Kovesdi et al., 2011) and is related to the body of literature showing the effect of EE on neurogenesis. Neurogenesis, the production of new neurons in the brain, has been shown to continue beyond the prenatal

period into adulthood in rats as old as 11 months (Kuhn, Dickinson-Anson, & Gage, 1996). Studies of EE have clearly shown its ability to promote neurogenesis in rodents (Kempermann, Kuhn, & Gage, 1997; 1998; Kempermann, Gast, & Gage, 2002; Komitova, Mattsson, Johansson, & Eriksson, 2005).

The exercise aspect of EE due to the placement of a running wheel in the cage has been examined both in isolation from and in conjunction with the other aspects of the EE setup but has been shown to independently enhance neurogenesis (van Praag, Kempermann, & Gage, 1999; Ehninger & Kempermann, 2003; Olson, Eadie, Ernst, & Christie, 2006; Hopkins, Nitecki, & Bucci, 2011). An interesting finding of the investigation by Olson and colleagues was the superior long-term object recognition memory in rats that exercised as adolescents compared to rats that only exercised as adults (2006). This study suggested that EE might be more beneficial earlier in the lifespan. Similar studies have supported this view (Lillard & Erisir, 2011; Williams et al., 2001); however, there may be a U-shaped effect of EE on cognitive ability, as at least one study has shown EE to benefit aged rats but not adult rats on behavioral measures of cognitive ability, suggesting the benefits of EE may be limited to rats with low cognitive ability due to very young or very old age (Paban, Jaffard, Chambon, MalaFosse, & Alescio-Lautier, 2005).

In terms of memory-specific enhancements of EE, many studies support the effect of an enriched environment on spatially related recognition memory in the rat, whereas the effect of EE on NOR is less frequently studied. Animals housed in EE have been shown to have enhanced long-term potentiation (Duffy, Craddock, Abel, & Nguyen, 2001). Long-term potentiation (LTP) is a form of enhanced synaptic transmission

influenced by activity; the prolonged duration of this type of transmission lends itself to efficient storage of information (Bliss & Lømo, 1973; Mainardi et al., 2010; Zhu et al., 2011). The ability of EE to improve spatial memory is clear in studies using the Morris Water Maze (Cao, Huang, & Ruan, 2008; Leggio et al., 2005; Lui et al., 2011; Sozda et al., 2010; Williams et al., 2001). The Morris Water Maze requires the animal to use spatial cues in the environment to find a submerged platform in the center of a circular tub of liquid. These studies show superior ability of enriched animals compared to controls in time taken to find the platform and in more direct routes overall, suggesting that EE is beneficial to strategy development.

Effect of temporary exposure to an enriched environment.

What is the minimum length of EE exposure necessary for rats to reap cognitive benefits? From early on in the history of EE as an experimental manipulation the effectiveness of relatively short lengths of time spent in the environment has been supported, as in a 1977 study that showed equal benefit of rats given just 2 h daily exposure to EE compared to rats given 24 h exposure, both groups for 60 days (Will, Rosenzweig, Bennett, Hebert, & Morimoto). Time spent in EE varies greatly from study to study from short, intermittent exposures to constant exposure of two months, three months, or longer (Escorihuela et al., 1995; Piazz et al., 2011). However, a recent review article reports 41% of enrichment studies use from 1-4 weeks of exposure to the EE condition (Simpson & Kelly, 2011). Despite the common practice of rather short EE exposure durations, there is at least one instance of controversy in the literature. For example, Pereira and colleagues discuss the possibility of an insufficiently long EE

exposure of 3 weeks to explain the failure of EE to elicit cognitive benefits in their study, although this discussion was speculative (2008).

An insufficient number of studies have focused on the direct impact of EE on NOR task performance, instead, the literature tends to include EE as a mediating factor, as in Tang and colleagues 2001 study of NMDA receptor function in NR2B transgenic mice. Tang and colleagues found that in mice, exposure to EE significantly increased exploration of objects in the NOR task when compared to controls, even at a minimal exposure of 3 h daily for two weeks (Tang, Wang, Feng, Kyin, & Tsien, 2001). Also representative of a study that examines the effect of EE on NOR task performance is that of Gobbo and O'Mara (2004). This 2004 study found that subjects in the enriched condition had significantly higher means for object exploration compared to standard housed subjects, though the main purpose of the study was to examine the mediating effect of EE on cognitive function following global ischemia. The ability of enriched housing conditions to enhance general memory function and, specifically, performance on the NOR task as supported by the aforementioned literature supports a need for the present study.

Age and Object Recognition Memory

The influence of age on novel object recognition task performance.

It is difficult to assess the literature on the factor of age on performance on the NOR task when age of laboratory animals is rarely taken into serious consideration (McCutcheon & Marinelli, 2009). A review of this issue, titled "Age Matters", emphasizes the influence of age on behavior and cognitive functions as a factor that should not be ignored. The review revealed inconsistency in the literature as to what age

constitutes “young,” “adolescent,” and “adult” in the rat. Memory may be significantly influenced by age given that some brain areas (already discussed in this paper in relation to memory) continue to undergo neuronal change, particularly at receptors and the synapse in the Pfc, as late as postnatal day 60 (Counotte et al., 2011; McCutcheon & Marinelli, 2009). Evidence for the functional effects of age on memory task performance is clear: specifically in the Sprague-Dawley strain of rats, adolescents (35-70 days old) have shown significantly more reactivity to novel objects when compared to adult Sprague-Dawleys (over 70 days old) (Silvers, Harrod, Mactutus, & Booze, 2007). Periadolescent (the developmental age surrounding the onset of puberty, approximately 40 days postnatal) rats in general exhibit hyperactivity basally and under learning tests, leading to better performance on simple learning tests compared to both younger and older rats but poorer performance on complex learning tests compared to younger and older rats (Spear, 1983). This evidence suggests age differences should be expected when animal cohorts of different age groups are being tested. The results of the following studies of the effect of EE on NOR task performance are examined with consideration of the age of subjects at the time of testing.

The effect of age and enriched environment on NOR task performance.

The effect of EE on performance on the NOR task is not as clear as the effect of EE on spatial memory tasks. In fact, investigators have found EE rats to have reduced total exploration times during the familiarization phase for objects compared to control rats (Bruehl-Jungerman, Laroche, & Rampon, 2005; Viola et al., 2010). As Viola and colleagues explained:

Here, the EE group expended less time exploring the objects, which could indicate reduction of motivation, curiosity and/or interest for objects, probably because these animals previously experienced more stimulating environmental conditions (learning, social and physical), which make the novelties not so appealing. (2010, p. 19)

This evidence warrants careful examination of studies of EE on object recognition memory with the NOR task that is seemingly not necessary in studies of EE on spatial recognition memory with the Morris Water Maze. The EE design may reduce the general appeal of novel objects and thus complicate the NOR task as a measure of object recognition memory. However, the paper from Viola and colleagues continues on to report no counter-intuitive effect of EE on the investigation ratio in the test phase after familiarization: EE animals explored the novel object significantly more than they did the familiar object, demonstrating that EE animals were possibly even more efficient than were control animals in their ability to encode the memory despite lower investigation times (Viola et al., 2010). These animals were tested at the relatively young age of under 3 months. Bruel-Jungerman and colleagues also found that EE decreased exploration time during the familiarization phase but increased exploration the novel object significantly more than the familiar object up to a 48 h delay (2005). Exposure to EE was 3 h daily for 14 d. Subjects in this study were only described as “adults” but the age of the subjects was not given.

Like the young rats presented in the previous studies, aged EE rats showed reduced total exploration during the familiarization phase compared to aged standard-housed rats but showed significantly higher investigation ratios during the test phase

compared to the controls; these rats were aged 18 months (Escorihuela et al., 1995). Similar findings were reported in a more recent study of the effect of EE on performance in the NOR task in rats aged 18 months (Leal-Galicia, Castaneda-Bueno, Quiroz-Baez, & Arias, 2008), supporting the mediatory effect of EE on NOR for aged rats.

Other studies have found EE to reduce investigation ratios in the NOR task. A 2009 study found that EE decreased time spent exploring the novel object for adult Wistar Rats aged 3 months (Pamplona, Pandolfo, Savoldi, Prediger, & Takahashi). Subjects were raised in the EE condition from postnatal day 21 to adulthood and then given a battery of cognitive tests. Similar to the two previously discussed studies, EE rats explored objects significantly less during the familiarization phase of the NOR task compared to control rats. Contrary to the two previously discussed studies, the EE rats also had lower investigation ratios during the test phase compared to controls, suggesting that EE was detrimental to performance on the NOR task.

An Alternative to Lesion Studies: The Immediate-Early Genes

The immediate-early genes.

While lesion studies provide information about the necessity of specific structures within the brain's memory system, the information is limited to these specific isolated structures and does not allow for a more comprehensive view of the memory system. Indeed, it has been suggested that since there are multiple pathways projected from areas involved in spatial or recognition memory, other brain areas could provide sufficient information for a memory process to occur, compensating for lesion to the specific area (Aggleton & Brown, 2005). One way to monitor this phenomenon is to simultaneously investigate the neural activity of multiple brain structures during a specific memory

process; this can be achieved through study of the immediate-early genes. The immediate-early genes were first conceptualized as a group of 10-15 genes essential for establishing long-term potentiation, and thus as essential for cognitive functions such as memory formation (Lanahan & Worley, 1998). These genes are induced by synaptic activity in response to some sort of stimulus, allowing investigators to map the expression of these genes via both immunohistochemistry and in-situ hybridization after controlled stimulation (Okuno, 2011).

Careful mapping of the upregulation and downregulation patterns over time of two common IEG's, zif268 and c-Fos, has suggested that zif268 serves as a better marker for "ongoing" activity whereas c-Fos serves as a better marker for "transient" activity (Zangenehpour & Chaudhuri, 2002, p. 225). Therefore, a combination of IEG expression analysis may allow for a more comprehensive understanding of memory processes if both long- and short-term manipulations are involved in a single study. Zif268 and c-Fos are the IEG's relevant to the present study.

c-fos.

The focus of c-Fos imaging studies of rodent object recognition tends to be to dissociate the neural activity that occurs with exposure to novel objects from the neural activity that occurs with exposure to familiar objects. For this reason, most of the literature examines Fos expression during exposure to either novel or to familiar stimuli rather than during exposure to both a novel and a familiar stimulus; this then means that there is a gap in the literature on Fos expression while an animal is actively discriminating between the novel and the familiar.

It is well established that the rat brain shows increased Fos expression in the perirhinal cortex in response to novel stimuli. Perirhinal activation to novel stimuli was established with the first study to use Fos expression as indicative of recognition memory processing in specific brain areas (Zhu, Brown, McCabe, & Aggleton, 1995). In this study, Fos expression was significantly higher in perirhinal cortex, area TE of the temporal cortex (the inferior temporal cortex, adjacent to the perirhinal cortex), the occipital association cortex, and the anterior cingulate gyrus. Conversely, Fos expression levels were low in the hippocampus and mediodorsal nucleus of the thalamus, although not significantly so. Zhu and colleagues conducted a follow-up study to control for eye movements and related motor movements during testing that possibly contributed to the Fos expression in the occipital association cortex in the previous study (1996). With a new design restricting the vision of a novel object to one eye and the vision of a familiar object to the other eye, simultaneously, the investigators were able to quantify Fos expression in the hemisphere exposed to the novel object and compare it to Fos expression in the contralateral hemisphere exposed to the familiar object. The researchers found significantly higher Fos expression in the perirhinal cortex, area TE, and the ventrolateral geniculate nucleus. No increase in Fos activation was found in hippocampal areas. Other studies support this increase in Fos expression in Prh with exposure to novel stimuli compared to exposure to familiar stimuli (Aggleton & Brown, 2005; Albasser, Poirier, & Aggleton, 2010; Wan, Aggleton, & Brown, 1999). Although the present study does not isolate exposure to the familiar object from exposure to the novel object as the formerly presented papers have, it is expected that Fos expression will

be higher in subjects who exhibited intact novel object recognition behaviorally compared to subjects who failed to discriminate.

In all but one of these studies, hippocampal areas were included for analysis and failed to show increase in Fos expression during exposure to a novel stimulus (Zhu et al., 1995; 1996; Aggleton & Brown, 2005; Albasser et al., 2010). The single study found increased and decreased activity levels in the hippocampus upon exposure to a novel stimulus; specifically, Fos expression was significantly higher in areas CA1 and CA3 and significantly lower in the dentate gyrus (Albasser *et al*, 2010). These unexpected findings are explained by the investigators in terms of procedural differences in their study that may have allowed for spatial cues in test performance. As previously discussed in the section describing performance of Hpc-lesioned rats on the NOR task, hippocampal involvement is supported in tasks of spatial memory (Winters, Saksida, & Bussey, 2008). Without more empirical support, one cannot implicate hippocampal increased neural activity as a response to novel stimuli, and the investigators acknowledged this.

zif268.

The IEG *zif268* is also called *Egr1*, *Krox24*, *ZENK* and *NGFI-A* (Bozon, Davis, & Laroche, 2002; Soulé et al., 2008). The role of *zif268* in object recognition memory processes has been the focus of several studies. The expression of *zif268* in response to the NOR task is much more clearly understood compared to c-Fos because investigators have studied the expression under normal procedures for the NOR task, that is, without trying to disentangle expression to novel objects versus expression to familiar objects.

Jones and colleagues demonstrated that *zif268* is necessary for long-term memory as assessed by the NOR task (2001). Both rats with *zif268* mutations and wild-type rats

were able to discriminate between the novel and familiar objects at a short delay (10 min), but animals with mutated *zif268* were unable to maintain the memory trace of a novel object at a long delay (24h), whereas wild-type rats still exhibited novel object recognition at the long delay, suggesting that *zif268* is critical for retaining a long-term memory (Jones et al., 2001). Bozon and colleagues conducted a study preceding that of Jones and colleagues and the inability to perform on the NOR task in *zif268* mutant animals also suggested that the gene is necessary for establishment of a long-term memory (2002). These studies were all focused on object recognition memory, but investigations into the role of *zif268* in other types of recognition memory have been conducted as well. During spatial-type object recognition memory, *zif268* has been shown to be upregulated in the hippocampal formation, such as in the dentate gyrus (Soulé et al., 2008). Thus, *zif268* seems to respond similarly to Fos in response to tasks of spatial memory.

In terms of non-spatial object recognition memory, *zif268* seems to have a particular role in the recall of a long-term object recognition memory (Akirav & Maroun, 2006, 2009; Bozon, Davis, & Laroche, 2003; Davis et al., 2010; Kelly, Laroche, & Davis, 2003; Lima et al., 2009; Winters, Tucci, & DaCosta-Furtado, 2009); the typical “long-term” delay is that of 24h post the initial exposure to a novel object.

To gain a more comprehensive understanding of what is happening to the memory trace outside of the commonly studied delays (like the 24 h delay), researchers have examined the time-dependent nature of the object recognition memory trace. For example, in a four-phase investigation, Romero-Granados and colleagues studied the role of temporal lobe structures in the consolidation, reconsolidation, storage, and expression

of object recognition memory in rodents (2010). One of the four experiments in this comprehensive study examined the role of hippocampus, somatosensory cortex, perirhinal cortex, entorhinal cortex, and prefrontal cortex in object recognition memory by mapping zif268 expression; investigators modeled a timeline of the object recognition memory trace following initial acquisition of a memory for a novel object in the NOR task. Based on zif268 activation levels, the hippocampus, somatosensory cortex, and perirhinal cortex were shown to be most involved in consolidation of the memory trace within hours of the test whereas the prefrontal cortex and entorhinal cortex were shown to be most involved in consolidation of the memory trace during a second exposure (“reactivation”) to the novel object 24h following the test. Both prefrontal and entorhinal cortex showed significantly higher zif268 expression when compared to hippocampus, somatosensory cortex, and perirhinal cortex and when compared to control animals (“nonreactivated” subjects who had been exposed to the initial NOR task 24h earlier but not again at the 24h delay). This study suggests that higher activity levels of zif268 in the rhinal and prefrontal cortices at 24h recall supports the role of these brain areas in reactivation of object recognition memory.

How does enriched environment influence immediate-early gene activity?

How might IEG activation levels be affected by exposure to an enriched environment? In an investigation of the ability of EE to reverse the effects of seizure following kainite acid treatment, non-kainate acid controls exposed to EE showed significantly higher Zif268 expression after just 7-10 days of exposure than did kainite acide-treated subjects (Koh, Chung, Xia, Mahadevia, & Song, 2005). Paired with the finding of increased Fos expression in the dentate gyrus after performing a water maze

task following only 11 d in an enriched environment (Puurunen, Koistinaho, Jolkkonen, & Sivenius, 2001), it could be expected that EE will lead to increased expression of both Zif268 and Fos in the rat brain, acting as a positive mediator of IEG expression in memory-related brain areas following performance on the NOR task. The upregulation of IEG expression following EE has been supported in the literature for over a decade (Wallace et al., 1995).

Hypotheses

Generally, the presented research has shown that the Prh, Hpc, and Pfc are involved in object recognition memory, and the roles of Prh and Pfc seem particularly important to long-term memory. The influence of age on object recognition memory is not deducible from studies of rats older than “adolescent” but younger than “aged,” but evidence has shown that adolescent rats tend to exhibit more exploratory behavior compared to adults. Contrastingly, animals of “old” age tend to exhibit poorer recognition memory when compared to adults, but EE may mediate the negative influence of age on memory performance. Enrichment also generally increases both Fos and Zif268 expression in memory-related brain areas during memory tests.

The present study examined the effect of EE on NOR task performance on periadolescent and adult male Sprague-Dawley rats after a 2h and a 24h delay. Immunohistochemistry revealed neural activity after reactivation of the object recognition memory at the 24h delay through Fos and Zif268 in Prh, Hpc, and Pfc. Since it has been shown that EE generally increases IEG expression, it was hypothesized that EE rats compared to standard rats showed higher neural activity in all three brain regions but particularly in Prh and Pfc due to the long delay. Moreover, cognitive maturity

should significantly increase Fos activated-cell counts, and environment should significantly increase zif268 activated-cell counts. Behaviorally, all rats were hypothesized to exhibit stronger memory at the 2h delay compared to the 24h delay. Despite the evidence for younger rats to show more reactivity to novel stimuli, adults were hypothesized to show stronger recognition memory compared to periadolescents; due to the elongated period of development of the PFC (Van Eden & Uylings, 1985) and the literature supporting the role of the mPFC in long-term memory, animals were classified as “cognitively mature” or “cognitively immature” to reflect this hypothesis. Lastly, EE was hypothesized to have a less significant effect in adults compared to periadolescents due to evidence supporting the limited effectiveness of EE to mediate memory performance in healthy, normal, adults.

Methods

Subjects

Forty male Sprague-Dawley rats were used in this study. All rats (76-125g) were received from Charles River Laboratories (Wilmington, Massachusetts) at the beginning of the study. Subjects were immediately housed in pairs upon arrival at the facility and maintained undisturbed for 5 days prior to the start of the experiment. Rats were then housed in environmentally enriched or in standard conditions (see following). All rats were maintained in a temperature and humidity controlled environment under a 12:12 hour light and dark cycle. Food and water were provided *ad libitum*. Rats were tested as periadolescents (aged 59-63 days) or as mature adults (aged 129-133 days), depending on experimental group assignment (see following). Animals were habituated to handling before behavioral testing began. All procedures were approved by the Connecticut

College Institutional Animal Care and Use of Laboratory Animals before commencement of the experiment.

Housing Conditions

Subjects were randomly assigned either to standard housing or to enriched housing. Rats assigned to enriched environment (EE) were to be exposed to 2 weeks of the enriched environment as either (prefrontal-) cognitively immature rats or as (prefrontal-) cognitively mature rats. Cognitive maturity of the prefrontal cortex in this study was defined in accordance with the findings of Van Eden and Uylings (1985) who distinguished three phases of prefrontal cortical development, asserting that neuronal development in the rat Pfc continues between postnatal day 30 and postnatal day 90. Thus, periadolescent rats aged 41-45 days at the beginning of exposure to the enriched environment were considered “cognitively immature”, and adult rats aged 111-115 days at the beginning of exposure to the enriched environment were considered “cognitively mature”. Regardless of age, exposure to the EE condition occurred for 2 consecutive weeks prior to memory testing for EE rats; standard housing was maintained otherwise from arrival until sacrifice through memory testing for SE rats.

Both enriched and standard housing cages were lined with wood shavings and underwent the same weekly cleaning procedures. Both EE and SE groups were housed in pairs. Enrichment cages were equipped with plastic shelters, small toys, and a running wheel (Figure 1). These enrichment objects were rearranged twice a week as well as were rotated with different enrichment objects for novelty stimulation. Twice weekly EE rats also experienced 30 minutes of social stimulation, a period of time during which 4-6 rats were placed in the same large circular arena for social interaction. The social arena

was lined with wood shavings and equipped with toys, shelters, and cylindrical tubes for added stimulation. The impact of such an enriched environment on rodent cerebral chemistry is long established in the literature (Krech, Rosenzweig & Bennett, 1960), and more current studies have specified improved cognitive abilities, anatomical changes, neurogenesis, and catecholaminergic enhancement among a number of changes in response to exposure to an enriched environment (see van Praag, Kempermann & Gage, 2000 for a review).

Novel Object Recognition Task

Apparatus.

The NOR observation chamber consisted of a red rectangular plexiglass box (30 x 56 cm) with walls 30 cm high. The floor of the chamber was covered with wood shavings and was removable for cleaning purposes. A video camera was positioned over the chamber for later off-line analysis of the familiarization and test phases. Two glass jar lids were secured to the floor of the apparatus at a distance of 18 cm diagonally from each other. Experimental objects were secured with Gorilla Glue adhesive onto the bottom of glass jars (6.5 x 7.0 cm) that could be inverted and screwed into the lids. This design afforded changing of object type and object location while also preventing displacement of the objects by the rats. The object-atop-jar design also prohibits the rodents from sitting on the objects and complicating exploration quantification.

Procedure.

Behavioral testing began at age 55-59 days for cognitively immature rats and at age 125-129 days for cognitively mature rats. The Novel Object Recognition task is

composed of three phases: habituation phase, familiarization phase, and test phase and was carried out according to Gaskin et al. (2010).

The habituation phase occurred over 2 consecutive days during which the rat was placed in the empty apparatus and allowed to explore for single 20-min sessions both days. Regarding the habituation phase of the NOR task, Ennaceur and colleagues (2010) suggested the possibility of impaired ability of the rat to detect differences between a novel and a familiar object when objects are mounted atop a jar. In order to address this concern, two jars without mounted objects were screwed into place for the habituation phase in order to make the objects themselves more prominent relative to the jars come testing. The familiarization phase was implemented the day immediately following the second habituation phase. During familiarization the rat was placed in the apparatus with two identical objects and was allowed to explore for 5 min.

Lastly, recognition memory was tested 2h following the familiarization phase and reactivated after 24h. During the 2h test phase, the rat was placed in the apparatus with a third copy of the previously presented sample object and one novel object and was allowed to explore for 5 min. During the 24h test phase, a fourth copy of the sample object was used and a second copy of the novel object was used.

Across all three phases each rat was placed in the chamber facing the same wall so as not to influence the subject with regard to either object. After each trial, the objects and apparatus were thoroughly cleaned with 70% Ethanol solution to remove olfactory clues. Combinations and locations of objects were balanced between groups to reduce risk of preference for specific type or location of objects.

Objects.

The experimental objects were constructed of wood and measured between 2 and 5 cm in width and between 5 and 10 cm in height (Figure 4). Each sample object existed in 4 copies and each novel object existed in 2 copies so that no object was used more than once with the same subject. All objects had been chosen according to the guidelines presented in Ennaceur and colleagues (2010) that suggest taking into account both color, texture, hue, and the presence or absence of protrusions when choosing test objects. No red objects were used, as the testing chamber itself was red. Such considerations ensure that the rats can perceive differences between objects and that all objects have the same affordances to the rats to prevent preference.

Analysis of Behavior

Exploration was defined as time spent in a pre-determined circular zone no farther than 2.5 cm from each object. The times spent by rats exploring objects in the familiarization and testing phases were recorded automatically by ANY-maze video tracking software, and video files were saved for later off-line analysis. The investigation ratio was determined by equating time spent investigating the novel object with total time spent investigating ($t_N / t_N + t_F$) during the test. Individual difference scores ($t_N - t_F$) were also calculated for comparison. Animals that failed to explore one or both objects in the familiarization phase or in either the 2 h and 24 h test phases were excluded from all phases of the study. This rule of exclusion was established *post hoc*; similar exclusion criteria have been used in previous studies (Anderson et al., 2004; Cyrenne & Brown, 2011).

Tissue Preparation

Ninety minutes post completion of the 24h test phase of the NOR task rats were individually euthanized in a carbon dioxide chamber. Rats were transcardially perfused

with 0.1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M PB. Brains were then extracted and stored in 4% paraformaldehyde solution (for 24h) then transferred to a 30% sucrose solution until slicing. Coronal sections were cut at 40µm on a -20°C cryostat. Sections were collected and stored in cryoprotectant at 4°C.

Immunohistochemistry

Coronal sections from each rat were chosen to represent the prefrontal cortex (approximately 2.70mm anterior to Bregma), the hippocampus (approximately 3.14mm posterior to Bregma), and the perirhinal cortex (approximately 4.16mm posterior to Bregma (Paxinos & Watson, 1998)).

c-Fos.

The staining for c-Fos was adapted from the avidin-biotin-horseradish peroxidase (ABC) method as described by Grahn et al. (1999). Sections were washed in 0.01M PBS for three-10 minute sessions then underwent a 24h incubation with a 1:8000 dilution of polyclonal rabbit anti-Fos primary antibody (Santa Cruz Lot #c270) in a blocking solution of 1% normal goat serum, 1% bovine serum albumin, and 0.25% Triton- X100 (30%), in 0.01M PBS. Following incubation the tissue was washed in 0.01M PBS for 10 minutes and incubated for 2 h in biotinylated goat anti-rabbit secondary antibody (Jackson Laboratories Lot #90982) diluted in 1:200 in blocking solution.

After incubation the sections were again washed in 0.01M PBS for three-10 minute sessions and then incubated for 1 h with avidin-biotin complexed with horseradish peroxidase (Vectastain Elite ABC kit) in 0.01M PBS. The sections were then washed in 0.1M PB for three-10 minute sessions and placed in a solution containing diaminobenzidine (DAB), cobalt chloride, ammonium chloride, nickel ammonium

sulfate, and glucose oxidase, in 0.1M PB for 10 minutes. The reaction was started by the addition of β -D-glucose solution. The reaction sat for approximately 15 minutes before the tissue was placed in 0.01M PBS to end the reaction.

zif268.

Separate from the Fos-labeled sections, sections from prefrontal cortex, hippocampus, and perirhinal cortex for each animal were selected and stained for zif268. The staining method for zif268 was adapted from the method as described by Filipkowski, Rydz, and Kaczmarek (2001). Sections were washed in 0.01M PBS for three-10 minute sessions then underwent a 24h incubation with a 1:3000 dilution of Egr-1 (zif268) rabbit polyclonal IgG primary antibody (Santa Cruz Biotechnology Lot #K1011) in a blocking solution of 1% normal goat serum, 1% bovine serum albumin, and 0.25% Triton- X100 (30%), in 0.01M PBS. Following incubation the tissue was washed in 0.01M PBS for 10 minutes and incubated for 2 h in biotinylated goat anti-rabbit secondary antibody (Jackson Laboratories Lot #90982) diluted in 1:200 in blocking solution.

After incubation the sections were again washed in 0.01M PBS for three-10 minute sessions and then incubated for 2 h with avidin-biotin complexed with horseradish peroxidase (Vectastain Elite ABC kit) in 0.01M PBS. The sections were then washed in 0.1M PB for three-10 minute sessions and placed in a solution containing diaminobenzidine (DAB), cobalt chloride, ammonium chloride, nickel ammonium sulfate, and glucose oxidase, in 0.1M PB for 10 minutes. The reaction was started by the addition of β -D-glucose solution. The reaction sat for approximately 15 minutes before the tissue was placed in 0.01M PBS to end the reaction.

Immediate-early gene product cell counts.

To compare numbers of activated cells across the two conditions in this study, estimates of Fos-activated cells were made in each region of interest, and estimates of zif268-activated cells were also made in each region of interest for the separate zif268-stained sections. Regions of interest within the prefrontal cortex (93 μm x 133 μm), the hippocampus (594 μm x 442 μm), and within the perirhinal cortex (125 μm x 50 μm) was framed for counting. Labeled nuclei (black) were identifiable against the background due to staining. Cells were counted by hand.

Statistical Analysis

Behavioral and neural measures were statistically analyzed with IBM SPSS 19.0 for Windows Professional 2011. A p value of < 0.05 was considered significant unless otherwise noted.

Results**The NOR Task**

A two-way repeated measures ANOVA was performed to determine the influence of maturity and environment on exploration time of the familiar object in each of the three phases of the NOR task: familiarization, 2 h test, and 24 h test. For within-subjects effects, there was a significant main effect of time, $F(2, 46) = 48.98, p < .001$. Follow-up pairwise comparisons were conducted and confirmed that object exploration time decreased significantly from the familiarization phase ($M = 18.70$) to the 2 h test ($M = 8.77$), $t(26) = 2.12, p < .001$, and object exploration time decreased significantly from the 2 h test to the 24 h test ($M = 6.53$), $t(26) = 6.26, p = .04$. There was no significant interaction effect between time and maturity, $F(2, 46) = 1.16, p = .32$ (see Figure 5) or

between time and environment, $F(2, 46) = .15, p = .82$ (see Figure 6); however, the interaction effect between time, maturity, and environment revealed a trend toward significance, $F(2, 46) = 2.62, p = .08$.

A MANOVA was performed to determine the influence of maturity and environment on investigation ratios at the 2 h and at the 24 h delay. For within-subjects effects, there was no significant main effect for time, $F(1, 23) = 3.04, p = .09$, but there appeared to be a difference between investigation ratios at the 2 h delay ($M = 0.51$) and investigation ratios at the 24 h delay ($M = 0.43$), indicating that investigation ratios tended to be lower at the longer delay. For between-subjects effects, the main effect for maturity was not significant, $F(1, 23) = 1.05, p = .32$, but the main effect for environment was significant, $F(1, 23) = 4.88, p = .04$ (see Table 1), revealing that the investigation ratios of enriched subjects ($M = 0.56$) were significantly higher than investigation ratios of standard subjects ($M = 0.40$).

There was no significant interaction effect between maturity and environment indicated in the MANOVA, $F(1, 23) = 0.50, p = .49$. However, post-hoc comparisons were conducted to investigate the main effects at each delay and revealed significant differences within age groups; it was revealed that adult enriched subjects had significantly higher investigation ratios ($M = 0.66$) than did adult standard subjects ($M = 0.46$), $F(1, 12) = 5.59, p = .04$ at the 2 h delay (see Figure 7). Likewise, it was revealed that the investigation ratios of adult enriched subjects ($M = 0.62$) were significantly higher than were the investigation ratios of adult standard subjects ($M = 0.36$), $F(1, 12) = 5.33, p = .04$ at the 24 h delay (see Figure 8); the effect was larger at the 2 h delay ($p = .038$) than at the 24 h delay ($p = .041$).

Student's *t*-tests were performed to examine the effect of environment on total time spent exploring objects during the familiarization phase. Periadolescent subjects in the enriched condition ($M = 13.95$) appeared to spend less time exploring objects than did periadolescent subjects in the standard condition ($M = 21.13$), but the difference was at, not less than, the .05 significance level, $t(17) = -2.15, p = .05$ (see Figure 9). The effect of environment on total object exploration time during the familiarization phase did not approach significance for adult subjects, $t(17) = -.32, p = .76$.

Immunohistochemistry for Immediate-Early Gene Products

Staining with antibodies recognizing the immediate-early gene products cFos and zif268 was conducted to determine the effect of environment and cognitive maturity on prefrontal cortical, hippocampal, and perirhinal cortical neuronal activity, and to correlate neuronal activity with memory task performance (see Figures 16 & 17). Sections from each subject were taken to represent the prefrontal cortex (IL and PL; collectively, the 'mPFC') (see Figure 2), hippocampus (CA1, CA2, and CA3), and perirhinal cortex (in its entirety) (see Figure 1). Sections were selected and stained for cFos separately from sections stained for zif268.

cFos.

Cell count comparisons were performed in the medial prefrontal cortex between groups after staining for the immediate-early gene product cFos. ANOVA statistical analysis revealed a significant effect of maturity, $F(1, 18) = 4.79, p = .04$, indicating that prefrontal neuronal activity was significantly higher in adults ($M = 34.35$) compared to periadolescents ($M = 22.51$) at the time of the 24 h test (see Figure 10). The ANOVA revealed no significant effect of environment $F(1, 18) = 0.78, p = .39$.

In the encompassed CA1, CA2, and CA3 regions of the hippocampus, ANOVA analysis revealed no significant effect of maturity, $F(1, 18) = 0.39, p = .54$, or of environment, $F(1, 18) = 1.58, p = .23$ (see Figure 11) on cFos cell counts.

In the perhinal cortex, ANOVA analysis revealed no significant effect of maturity, $F(1, 18) = 1.62, p = .23$, or of environment, $F(1, 18) = 0.002, p = .96$ on cFos cell counts. However, perirhinal cell counts appeared to be higher for adult subjects ($M = 4.41$) compared to periadolescent subjects ($M = 2.18$) (see Figure 12).

In general, cell counts appeared to be higher in the prefrontal cortex than in either the hippocampus or perirhinal cortex. In order to make cross-brain area comparisons of the effects of environment and cognitive maturity on immediate-early gene product expression, mean particle counts were divided by the region of interest (encompassing each of the medial prefrontal cortex, hippocampus, and perirhinal cortex) and expressed as “particle per square millimeter”.

To test the hypothesis that the effects of environment and maturity on immediate-early gene product expression would vary across brain areas, a two-way repeated measures MANOVA with brain area (prefrontal cortex, perirhinal cortex, and hippocampus) as a within-subjects factor was run for cFos (see Table 4) mean particle counts.

The analysis showed that there was no statistically significant difference in the effect of environment across brain areas on mean particle expression after staining for cFos, $F(2, 14) = .11, p = .90$. Analysis also revealed that there was no statistically significant difference in the effect of cognitive maturity across brain areas on mean particle expression after staining for cFos, $F(2, 14) = 1.37, p = .29$.

zif268.

Comparisons of cell counts between groups were performed in the medial prefrontal cortex, hippocampus, and perirhinal cortex after staining for the immediate-early gene product zif268. ANOVA statistical analysis revealed no significant effect of maturity or environment on IEG product levels in any of the three brain areas (see Figures 13, 14, 15). The effect of environment approached significance in hippocampal cell counts, $F(1, 18) = 4.38, p = .06$, indicating that hippocampus neuronal activity tended to be higher in enriched subjects ($M = 3.52$) than in standard-housed subjects ($M = 2.20$), but this finding did not reach significance. Similar to the Fos staining, cell counts appeared to be higher in the prefrontal cortex than in either the hippocampus or perirhinal cortex.

To test the hypothesis that the effects of environment and maturity on immediate-early gene product expression would vary across brain areas, a two-way repeated measures MANOVA with brain area (prefrontal cortex, perirhinal cortex, and hippocampus) as a within-subjects factor was run for zif268 (see Table 5) mean particle counts.

Cell counts from Fos and zif268 staining were then correlated with NOR task performance to investigate whether neural activity was related to demonstration of intact memory. Sample size limited statistical analysis to the impact of the main effects of environment and enrichment.

Repeated measures MANOVA showed that there was no statistically significant difference in the effect of environment across brain areas on mean particle expression

after staining for zif268, $F(2, 20) = .34, p = .61$, and there was no statistically significant difference in the effect of cognitive maturity across brain areas on mean particle expression after staining for zif268, $F(2, 20) = .07, p = .83$. The analysis did reveal a significant interaction effect among environment and maturity on mean zif268 particle expression that varied across brain area, $F(2, 20) = 5.54, p = .03$ (see Figure 18).

Follow-up one-way ANOVA analysis of each brain area revealed the interaction effect among environment, maturity, and brain area on mean zif268 particle expression. ANOVA analysis revealed that the two-way interaction effect between maturity and environment on zif268 particle expression was modified by brain area in the prefrontal cortex, $F(2, 16) = 7.54, p = .01$ (see Figure 18a). ANOVA analysis revealed that the two-way interaction between maturity and environment on zif268 particle expression was not modified by brain area in the hippocampus, $F(2, 14) = 2.88, p = .11$ (see Figure 18b), nor in the perirhinal cortex, $F(2, 14) = .07, p = .79$ (see Figure 18c).

For cFos, mean cell counts for all three brain areas were correlated with investigation ratios at the 24 h delay of the NOR task (see Table 2). Given the time dependent nature of cFos, the neuronal nuclei reflected the neural activity for each subject at the time of the 24 h test. No significant correlations were found among Fos levels in the three brain areas and the 24 h investigation ratios when each environment was considered separately. One significant correlation was found among one of the three brain areas and the 24 h investigation ratio when each age group was considered separately: in periadolescents, Fos neuronal nuclei counts in the hippocampus were significantly negatively correlated with 24 h investigation ratios, $r = -.79, p = .02$,

indicating that higher levels of neuronal activity in the hippocampus was associated with lower investigation ratios.

The immediate-early gene product zif268 has been shown to reflect neuronal change over a long time period (Romero-Granados et al., 2010), therefore cell counts after staining for zif268 were correlated with both 2 h and 24 h investigation ratios. Pearson's r analyses revealed no significant correlations for 2 h.

No significant correlations were found among levels of zif268 in the prefrontal cortex, hippocampus, or perirhinal cortex and the 24 h investigation ratios when enriched and standard subjects were examined separately (see Table 3). Two significant correlations at the $p < .05$ significance level were found among two of the three brain areas and the 24 h investigation ratios when age groups were examined: in periadolescents, zif268 neuronal nuclei counts in the hippocampus were significantly positively correlated with 24 h investigation ratios, $r = .91$, $p < .01$, indicating that higher levels of neuronal activity in the hippocampus was associated with higher investigation ratios. Also in periadolescents, zif268 particle counts in the perirhinal cortex were significantly negatively correlated with 24 h investigation ratios, $r = -.72$, $p = .04$, indicating that higher levels of neuronal activity in the perirhinal cortex was associated with lower investigation ratios.

Discussion

The present study examined the effect of environment and cognitive maturity on long-term reactivation of recognition memory in male Sprague-Dawley rats. The study was conducted in order to add to the body of literature on recognition memory, a critical component of episodic memory (Winters, Saksida, & Bussey, 2008). The development

of valid rodent models of recognition memory is necessary for understanding human episodic memory, a group of memory processes affected by trauma and disease. The NOR task is a useful and popular tool among investigators for studying recognition memory due to its adaptability and ease of use (Dere, Huston, & DeSouza Silva, 2007; Gaskin et al., 2010). However, the literature surrounding the NOR task contains discrepancies regarding what brain areas are implicated in performing the task.

Whereas the perirhinal cortex is consistently implicated in the NOR task, results implicating the hippocampus and the prefrontal cortex have been incongruent (Barker and Warburton, 2011). In addition, few studies have focused on the role of the prefrontal cortex. These few studies have revealed a particular role, however, for the mPfc at delays of 24 h (Akirav & Maroun, 2006, 2009; Barker & Warburton, 2011, Watson et al., 2011). Thus the present study sought a comprehensive analysis of the neural activity in all three brain areas in response to long-term reactivation of memory for an object. In contrast with the majority of the aforementioned studies that have utilized lesioning to assess the extent of involvement of specific brain areas, the present study utilized immunohistochemistry for immediate-early gene products for a less invasive and more precise means of analysis.

Behaviorally, all animals were predicted to exhibit stronger memory for the familiar object at the 2 h delay compared to after the 24 h delay. The strength of the memory trace was assessed by an investigation ratio, which calculated the amount of time the animal spent exploring the novel object relative to the amount of time spent exploring the novel and the familiar object. More time spent exploring the novel object indicates recognition of the familiar object as an object already explored. Although there was no

significant main effect of time, there appeared to be a decrease between investigation ratios at the 2 h delay and investigation ratios at the 24h delay, indicating that the memory trace tended to weaken over time.

Effect of Cognitive Maturity on NOR Task Performance

In terms of the effect of cognitive maturity on recognition memory, the hypothesis that adults would exhibit stronger recognition memory compared to periadolescents was not supported at a level of statistical significance, but adults did tend to demonstrate superior memory performance (see Figures 7 and 8). Investigation ratios demonstrated that adults performed better than did periadolescents at both delays and across environmental groups (see Table 1). Among periadolescents, only enriched subjects at the 2 h delay demonstrated recognition of the familiar object (indicated by an investigation ratio greater than .50).

One weakness of the present study was the failure to establish cognitive maturity *post hoc*. The present study did not go beyond postnatal days as a measure of cognitive maturity but attempted to use immunohistochemistry results to describe cognitive development instead. At the beginning of experimenting, animals postnatal day 41-45 constituted the periadolescent group and animals postnatal day 111-115 constituted the adult group. Many rodent studies fail to document age by postnatal day, using body weight as an indicator of age instead (McCutcheon & Marinelli, 2009). In order to accurately determine cognitive maturity, future studies should go beyond postnatal day as an age indicator and analyze cell layer development in each subject. Such an analysis would account for the individual differences that may occur in adherence to postnatal day markers of cognitive maturity.

The determination as either adolescent (35-70 days) or adult (over 70 days) for the subjects of the present study is established in the literature for the Sprague-Dawley strain (Silers, Harrod, Mactutus, & Booze, 2007). According to this literature, periadolescent (the earlier phase of adolescence, approximately 40 days postnatal) Sprague-Dawley rats generally display hyperactivity at the basal level and during learning task performance. The authors describe a U-shaped performance curve across ages for complex learning tasks, in which the performance of periadolescents is superior to the performance of younger and older rats. Silvers and colleagues attributed the superior performance to the general hyperactivity of periadolescence. In reference to the present study, the NOR task may not be considered a complex learning task despite the incorporation of a long delay; therefore, the hyperactivity typical in an adolescent Sprague-Dawley might then explain the poor performance of the group compared to adults.

Effect of Environment on NOR Task Performance

The hypothesis that exposure to an enriched environment would significantly improve NOR task performance was supported in the results. Furthermore, it was predicted that an enriched environment would benefit periadolescent subjects to a greater extent than for adult subjects, but this hypothesis was not supported. Results indicated that the effect of environment on recognition memory was not significant for periadolescents but was significant for adult subjects at the 2 h and 24 h delay. At both delays, enriched adults demonstrated significantly higher investigation ratios than did standard-housed adults. The adult enriched subjects were the only subjects to demonstrate intact recognition memory at the long delay (indicated by an investigation

ratio greater than .50). These results for the adult subjects support the body of literature on the general cognitive benefits of an enriched environment but stand in contradiction to studies that have found an enriched environment to be more beneficial early on in the lifespan (Lillard & Erisir, 2011; Williams et al., 2001).

Conflicting results in studies of the effect of an enriched environment on recognition memory are evident in the literature (Bruehl-Jungerman et al., 2005; Pamplona, Pandolfo, Savoldi, Prediger, & Takahashi, 2009; Viola et al., 2010). The 2010 study of Viola and colleagues and the 2005 study of Bruehl-Jungerman found EE to reduce total exploration time during the familiarization phase. Viola and colleagues proposed that an enriched environment might reduce the appeal of novelty (2010). Given these findings, the present study examined whether environmental enrichment had affected total object exploration time during the familiarization phase. The effect of environment approached significance for periadolescent subjects, indicating that periadolescents in the enriched condition spent less time exploring objects in the familiarization phase than did periadolescents in the standard condition. Statistical analyses revealed that this trend did not occur in the adult groups.

The objects used in the NOR task in the present study may have been less appealing for the periadolescent subjects who were exposed to similar objects in the enriched environment than for the periadolescent subjects who were housed under standard conditions. Whereas this phenomenon may have been the case in the present study with regard to adolescent memory task performance, it fails to explain the significant positive effect of the enriched environment on adult memory task performance. Conflicting results suggest that future studies using the NOR task take into

account its possible interference with novelty appeal when testing animals that have been exposed to an enriched environment.

One confounding factor in the present study was the number of animals excluded based on failure to perform (freezing episodes) in any one of the three phases of the NOR task. Fewer animals might have been excluded if the length of each test phase were based on individual accumulation of object exploration rather than based on a set length of time. For example, a previous study ended test phases once each subject accumulated 25 total seconds of object exploration; if the 25 sec were not accumulated by 3 min, the trial was ended (Forwood et al., 2005). Ensuring subjects spend equal time exploring objects would also reveal more meaningful comparisons, as the amount of time spent with the novel object compared to time spent with the familiar object would be examined relative to one constant denominator.

The differences in memory performance between adults and periadolescents may be attributed to varying stages of cognitive development. Results from immunohistochemistry for immediate-early gene products can explain how memory task performance was related to neural activity (see section to follow).

Overview of Immunohistochemistry for Immediate-Early Gene Products

The quality of brain tissue varied across brain areas. The anterior tissue sections of the prefrontal cortex appeared more damaged than did the hippocampal and perirhinal sections (see Figures 16 and 17). Consequently, more antibody might have penetrated the tissue than if the tissue had been more intact. The quality of the tissue might explain the higher mean prefrontal cell counts for both immediate-early gene products across conditions. Damaged tissue also appeared to have higher background staining, thereby

cell counting was more difficult; as a result, all counting was done by hand rather than by a computer software program.

To examine the impact of environment and cognitive maturity on cFos and zif268 expression comparatively across the prefrontal cortex, hippocampus, and perirhinal cortex, mean particle counts were expressed as particle per square millimeter. Hippocampal particle counts were thus extremely low, under .10 particles/mm², which can be attributed to the hippocampal-independent nature of the Novel Object Recognition task and the large size of the hippocampus relative to the medial prefrontal and the perirhinal cortices.

Immunohistochemistry for cFos

In support of the hypothesis, prefrontal cFos cell counts were significantly higher in adults than in periadolescents. Prefrontal development continues into late adolescence, and as expected, periadolescents had significantly lower levels of cFos compared to adults. This finding further supports the behavioral results of the NOR task, where periadolescents failed to demonstrate object recognition (investigation ratio of .50 or greater) at the 24 h delay. Given that staining for cFos revealed brain activity at the time of the 24 h test, this finding supports the literature for a role of the medial prefrontal cortex in long-term recognition memory.

Hippocampal cFos cell counts did not significantly differ among groups. This finding supports the hypothesis that, based on the literature on the role of the hippocampus in object recognition memory, no significant immediate-early gene product levels would be revealed. The hippocampus is extensively implicated in spatial memory as opposed to recognition memory (Winters, Saksida, & Bussey, 2008). Whereas the

NOR task can be manipulated to assess spatial memory, no such manipulation occurred in the present study and spatial cues were purposefully limited during testing; the lack of significant differences of hippocampal cFos cell counts between groups reflect these efforts to limit recruitment of spatially-related memory in the task.

Contrary to the hypothesis for neural activity in perirhinal cortex, no significant difference between groups was found. This result was unexpected given the support for the role of perirhinal cortex in the literature; in 2010 Albasser and colleagues published the first study showing the association between increases in cFos expression in perirhinal cortex and the active discernment between novel and familiar *after having confirmed* the ability of subjects to discern novel objects from familiar objects. The present study was unable to do so with the limited sample size for usable brain tissue.

The effect of environment and cognitive maturity on cFos activation across brain areas.

The finding that the effect of environment and maturity on cFos activation did not vary among brain regions was surprising. It was expected that the effect of cognitive maturity on Fos expression would be significantly greater in prefrontal and perirhinal cortices relative to the hippocampus. This finding might be partially explained by the number of subjects that failed to demonstrate intact recognition memory at the 24 h delay; if subjects were not accurately and actively discriminating between the novel and familiar object at the time the Fos expression captured, then the prefrontal and perirhinal cortices would not have been engaged to the same extent as when subjects with intact memory traces were recalling the original memory.

Akirav and Maroun (2006, 2009) have shown the obligatory role of medial prefrontal cortex in object recognition memory at long-term delays of 24 h, suggesting that the enriched adult subjects in the present study who produced strong investigation ratios at the 24 h delay should have been supported by an interaction effect in the prefrontal cortex. More recently, a study has been conducted for the purpose of isolating the role of the perirhinal cortex in object recognition memory at a 24 h delay (Seoane, Tinsley, & Brown, 2012); the investigators showed that infusion of antisense Fos oligodeoxynucleotide directly into the perirhinal cortex impaired object recognition memory at both a 3 h and a 24 h delay. As this result suggests a critical role for Fos as a perirhinal mechanism of object recognition memory, an interaction effect between environment and cognitive maturity on Fos expression in the perirhinal cortex was expected to reflect the ability of enriched adult subjects and the inability of enriched periadolescents to discriminate between novel and familiar objects at the long-term delay.

Immunohistochemistry for zif268

Immunohistochemistry for the immediate-early gene product zif268 was predicted to reveal the influence of environment, but no significant effect of environment or maturity on zif268 levels in any of the three brain areas was found. This result is surprising given the number of studies that have found upregulation of zif268 in response to non-spatial object recognition memory in rodents, particularly given findings of significantly higher upregulation in the prefrontal cortex (compared to hippocampus, somatosensory cortex, and perirhinal cortex) in response to reactivation of an object recognition memory at a 24 h delay (Romero-Granados et al., 2001). Similar upregulation was expected in the present study, and the influence of an enriched

environment was predicted to mediate this difference between groups. Indeed, the mean prefrontal cell counts for zif268 in enriched adult subjects was 40.4 particles, compared to 26.5 particles in standard-housed adult subjects, but insufficient sample sizes precluded establishing any significant differences. A replication of the present study with a larger sample would have the opportunity to investigate whether zif268 is upregulated in the prefrontal cortex in enriched subjects who demonstrate intact long-term recognition memory. Given the high investigation ratios and zif268 prefrontal cell counts for the enriched adult subjects, it is reasonable to predict that such a study would find a significant positive correlation between memory task performance and expression of zif268.

Zif268, regarded as a gene for neuronal plasticity in the rat brain, has been shown to be sensitive to even overnight exposure to an enriched environment (Staiger et al., 2000). The focus of studies on zif268 has increasingly considered its role in experience-dependent plasticity over the long-term (Maddox, Monsey, & Schafe, 2011). However, *stressful* experiences, such as fear conditioning, might induce zif268 expression more consistently given the preference in the literature to explore zif268 expression as affected by stressful experience rather than enriching experience. It has also been proposed that increased zif268 expression in response to experience-specific change to the rat brain must occur within a critical period, no later than three weeks postnatal (Tashiro, Makino, & Gage, 2007). These findings from the literature offer possible explanations for the difficulty in drawing conclusions from the zif268 data in the present study.

The effect of environment and cognitive maturity on zif268 activation across brain areas.

The interaction effect between environment and cognitive maturity on zif268 particle expression was significantly different across brain areas. Whereas there was a significant interaction effect between environment and cognitive maturity on zif268 activation in the prefrontal cortex, this interaction effect did not occur in the perirhinal cortex or in the hippocampus. The nature of the interaction effect in the prefrontal cortex occurred as follows: enrichment led to increased zif268 particle expression for adult subjects and led to decreased zif258 particle expression for periadolescents subjects, and the reverse was found for subjects in the standard housing condition: whereas standard housing decreased zif268 particle expression for adult subjects, standard housing increased zif268 particle expression for periadolescents subjects.

Interestingly, this interaction effect of prefrontocortical activity mirrors the behavioral findings for the adult subjects, in the sense that environmental enrichment resulted in significantly higher investigation ratios at both delays for adult subjects but not for periadolescents subjects. Increased zif268 activation in the prefrontal cortex for enriched adult subjects suggests that environmental enrichment contributed to the ability of this group to maintain the initial memory trace through the long-term delay and that the prefrontal cortex played a critical role in this memory process. On the other hand, decreased zif268 activation in the prefrontal cortex for periadolescent subjects suggests that environmental enrichment failed to stimulate zif268 expression in this area, and that the prefrontal cortex was not engaged to the same extent as seen in adult subjects who demonstrated object recognition memory at the long-term delay. [will insert before discussion of reduced object exploration among periadolescents during familiarization phase]

Given the support for the role of the perirhinal cortex in object recognition memory, an interaction effect of environment and cognitive maturity was expected in perirhinal zif268 expression, parallel to the interaction effect on prefrontocortical zif268 expression. One study in particular supports this unexpected finding; Romero-Granados and colleagues found that based on zif268 activation levels, the perirhinal cortex was shown to be most involved in consolidation of the memory trace after a 1 h delay, whereas the prefrontal cortex and entorhinal cortex were shown to be most involved in consolidation of the memory trace during a second exposure (“reactivation”) to the novel object 24 h following the test (2010). Both prefrontal and entorhinal cortex showed significantly higher zif268 expression when compared to perirhinal cortex and when compared to control animals. This study suggests that perirhinal cortex may be engaged in short-term memory but not in reactivation of a memory after a long delay, as was used in the present study. However, this study is an anomaly among the literature that supports an obligatory role for the perirhinal cortex in long-term object recognition memory, a role that continues to be supported in the most recent literature (Balderas, Morin, Rodriguez-Ortiz, & Bermudez-Rattoni, 2012).

Correlation Between Memory Task Performance and Neural Activity

The number of animals in each experimental group after exclusion based on unusable tissue limited the results of the study; meaningful differences could only be analyzed in terms of the main effects of cognitive maturity and environment. These correlations revealed no significant relationship between memory task performance and neural activity among adult subjects, but one significant finding as revealed among

periadolescents subjects: cell counts for zif268 in the hippocampus were positively correlated with investigation ratios.

Before Bonferroni corrections, two significant correlations at the $p < .05$ significance level were found among two of the three brain areas and the 24 h investigation ratios when age groups were examined. However, after Bonferroni corrections, only the correlation between periadolescent investigation ratios and zif268 cell counts were significant.

Inconsistency in levels of both immediate-early gene products relative to memory task performance might reflect transition of some of the periadolescents subjects to greater cognitive maturity. Indeed, incongruent expression of cFos and zif268 has been shown to occur in individual adolescent rats in at least one previous study. In a 2009 study examining the effect of cocaine on cFos- and zif268-induced particle expression, expression of cFos and zif268 was significantly correlated in adults whereas expression of the two immediate early gene products was not related (Caster & Kuhn, 2009); researchers concluded that cognitive developmental changes in transcription factor induction could account for the difference between adolescent and adult rats. In simplified terms, regulated doses of cocaine did not elicit congruent expression of immediate early gene products in adolescents as a result of their cognitive immaturity. The finding in the current study (where expression of zif268 particles was significantly positively correlated with memory task performance and expression of cFos was not) could only be examined among the periadolescent group as a whole due to sample size, but a replication of the present study could examine whether exposure to an enriched

environment elicits incongruent expression of cFos and zif268 in cognitively immature rats.

Conclusions

Despite the limited body of literature on prefrontal cortical involvement in NOR task performance, the existing publications have implicated this brain area, particularly the medial prefrontal cortex, in long-term recognition memory. Given the delayed development of the prefrontal cortex, future studies must meticulously account for the age of the subjects. There is still much work to be done to determine whether the use of the NOR task is viable in studies involving environmental enrichment; experimental objects similar to those in an enriched housing setup may reduce novelty appeal and general appeal to explore at the time of memory testing. The current study showed that exposure to an enriched environment significantly improved object recognition memory performance in adult rats compared to standard housed adult rats. When examined in relation to memory task performance, significantly high levels of expression of cFos particles in the adult prefrontal cortex likewise support the need for prefrontal cortical maturation to discriminate between the novel and familiar after a long delay. This study provides encouragement for continued research into prefrontal cortical function and development in the formation of memory.

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Table 1

Mean investigation ratios from test phases for the NOR task (2 h and 24 h delays),

N = 27

		<i>2 h delay</i>	<i>24 h delay</i>
		<i>Investigation Ratio</i>	<i>Investigation Ratio</i>
<i>Environment</i>	<i>Maturity</i>		
Enriched		0.59	0.54
	periadolescent	0.53	0.48
	adult	0.66	0.62
Standard		0.45	0.35
	periadolescent	0.44	0.33
	adult	0.46	0.36

Table 2

Mean cFos cell counts and 24 h investigation ratios

<i>Variable</i>	<i>Brain Area</i>	<i>Correlation</i>
Enriched	Pfc	-.04
	Hpc	.62
	Prh	.22
Standard	Pfc	.46
	Hpc	.24
	Prh	.26
Periadolescent	Pfc	.19
	Hpc	-.79*
	Prh	.67
Adult	Pfc	.21
	Hpc	.66
	Prh	.02

* $p < .05$; ** $p < .01$

Table 3

Mean zif268 cell counts and 24 h investigation ratios

<i>Variable</i>	<i>Brain Area</i>	<i>Correlation</i>
Enriched	Pfc	-.40
	Hpc	.31
	Prh	-.18
Standard	Pfc	.16
	Hpc	-.10
	Prh	-.42
Periadolescent	Pfc	-.03
	Hpc	.91**
	Prh	-.72*
Adult	Pfc	-.08
	Hpc	-.11
	Prh	-.16

* $p < .05$; ** $p < .01$

Table 4

Mean cFos particle counts across prefrontal cortex, hippocampus, and perirhinal cortex**Brain Area*

prefrontal cortex		adult	periadolescent
	enriched	2.59	1.94
	standard	2.61	1.52
hippocampus		adult	periadolescent
	enriched	0.01	0.00
	standard	0.02	0.01
perirhinal cortex		adult	periadolescent
	enriched	0.56	0.48
	standard	0.98	0.11

*particles/area (mm²)

Table 5

Mean zif268 particle counts across prefrontal cortex, hippocampus, and perirhinal cortex*

Brain Area

prefrontal cortex		adult	periadolescent
	enriched	3.28	1.52
	standard	2.14	3.53
hippocampus		adult	periadolescent
	enriched	0.01	0.02
	standard	0.01	0.01
perirhinal cortex		adult	periadolescent
	enriched	0.30	0.34
	standard	0.46	0.43

*particles/area (mm²)

Figure captions

Figure 1. Coronal section showing perirhinal cortex and areas CA1, CA2, and CA3 of hippocampus.

Figure 2. Coronal section showing areas infralimbic and prelimbic (collectively known as the medial prefrontal cortex) of prefrontal cortex.

Figure 3. The shelters, running wheel, and toys used in enriched environment subject cages.

Figure 4. The four experimental objects that served as the novel or the familiar object used in the NOR task.

Figure 5. Total time spent exploring the novel and the familiar object during the familiarization phase, 2 h delay test phase, and 24 h delay test phase. Object exploration time decreased significantly from the familiarization phase ($M = 18.70$) to the 2 h test ($M = 8.77$), and from the 2 h test to the 24 h test ($M = 6.53$). There were no significant effects between groups by maturity.

Figure 6. Total time spent exploring the novel and the familiar object during the familiarization phase, 2 h delay test phase, and 24 h delay test phase. There were no significant effects between groups by environment.

Figure 7. Investigation ratios at the 2 h delay test phase. Adult enriched animals demonstrated significantly higher investigation ratios than did adult standard-housed animals at the 2 h delay.

Figure 8. Investigation ratios at the 24 h delay test phase. Adult enriched animals demonstrated significantly higher investigation ratios than did adult standard-housed animals at the 24 h delay.

Figure 9. Effect of maturity on total time spent exploring the novel and the familiar object during the familiarization phase for enriched versus standard subjects. The difference between enriched and standard-housed periadolescents approached significance ($p = .05$). There was no significant effect between enriched and standard-housed adults.

Figure 10. The effect of maturity on mean cell counts in the Pfc after staining for cFos. Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task. Adult subjects demonstrated significantly higher Pfc neural activity compared to periadolescents.

Figure 11. The effect of maturity on mean cell counts in the Hpc after staining for cFos. Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task. No significant differences were found between groups.

Figure 12. The effect of maturity on mean cell counts in the Prh after staining for cFos. Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task. No significant differences were found between groups.

Figure 13. The effect of environment on mean cell counts in the Pfc staining for zif268. Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task. No significant differences were found between groups.

Figure 14. The effect of environment on mean cell counts in the Hpc staining for zif268. Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task. No significant differences were found between groups.

Figure 15. The effect of environment on mean cell counts in the Prh staining for zif268. Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task. No significant differences were found between groups.

Figure 16. Examples of Pfc, Hpc, and Prh sections stained with cFos antibody under 100X magnification. Arrows indicate an example of a cFos cell, which appears as a solid black circle. Background staining occurred but as lighter, irregularly shaded circles, allowing the investigator to distinguish between cell bodies and cFos cells. Background staining from cell bodies was most frequent in prefrontal sections. Adult rats in the enriched condition showed significantly higher Fos activated cell counts in the Pfc; in periadolescents, Fos neuronal nuclei counts in the Hpc were significantly negatively

correlated with 24 h investigation ratios.

Figure 17. Examples of Pfc, Hpc, and Prh sections stained with zif268 antibody under 100X magnification. Arrows indicate an example of a zif268 cell. Background staining from cell bodies was most frequent in prefrontal sections. ANOVA statistical analysis revealed no significant differences between groups at the 2h delay, but at the 24h delay, zif268 mean cell counts in the Hpc were significantly positively correlated with investigation ratios, and mean cell counts in the Prh were significantly negatively correlated with 24 h investigation ratios.

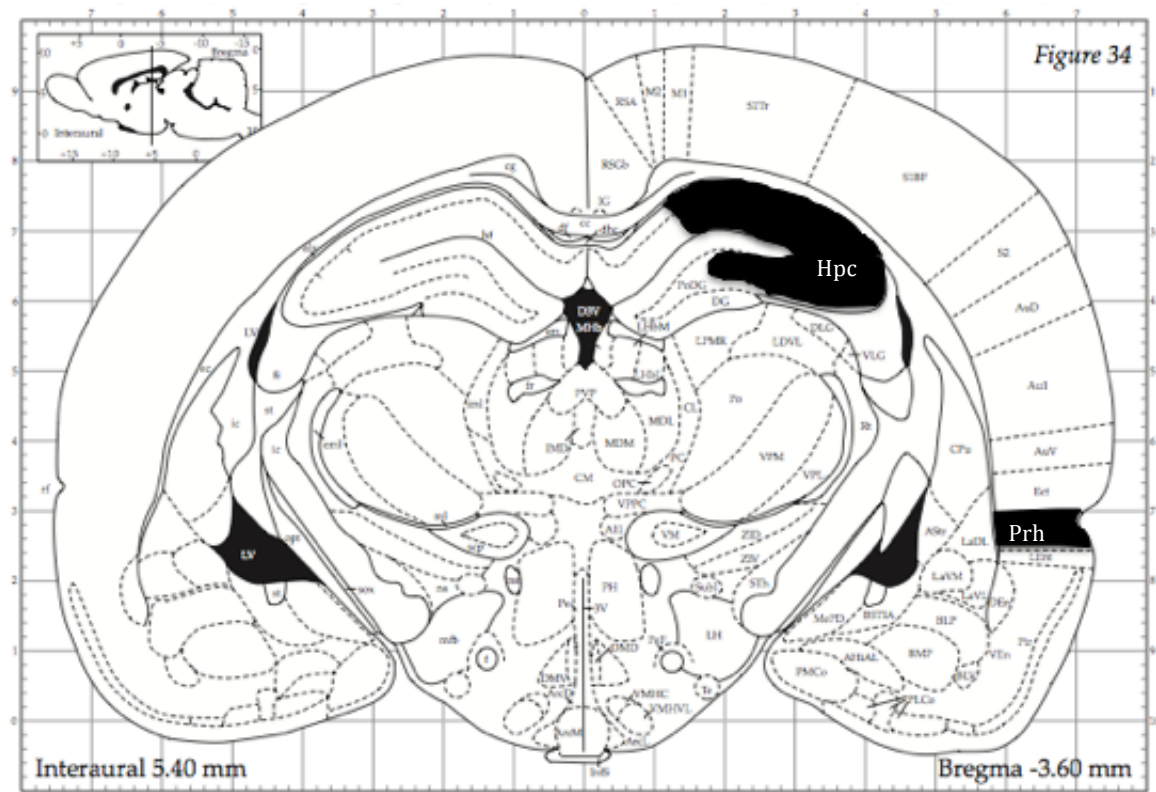


Figure 1. Coronal section showing perirhinal cortex and areas CA1, CA2, and CA3 of hippocampus.

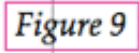


Figure 2. Coronal section showing areas infralimbic and prelimbic (collectively known as the medial prefrontal cortex) of prefrontal cortex.



Figure 3. The shelters, running wheel, and toys used in enriched environment subject cages.

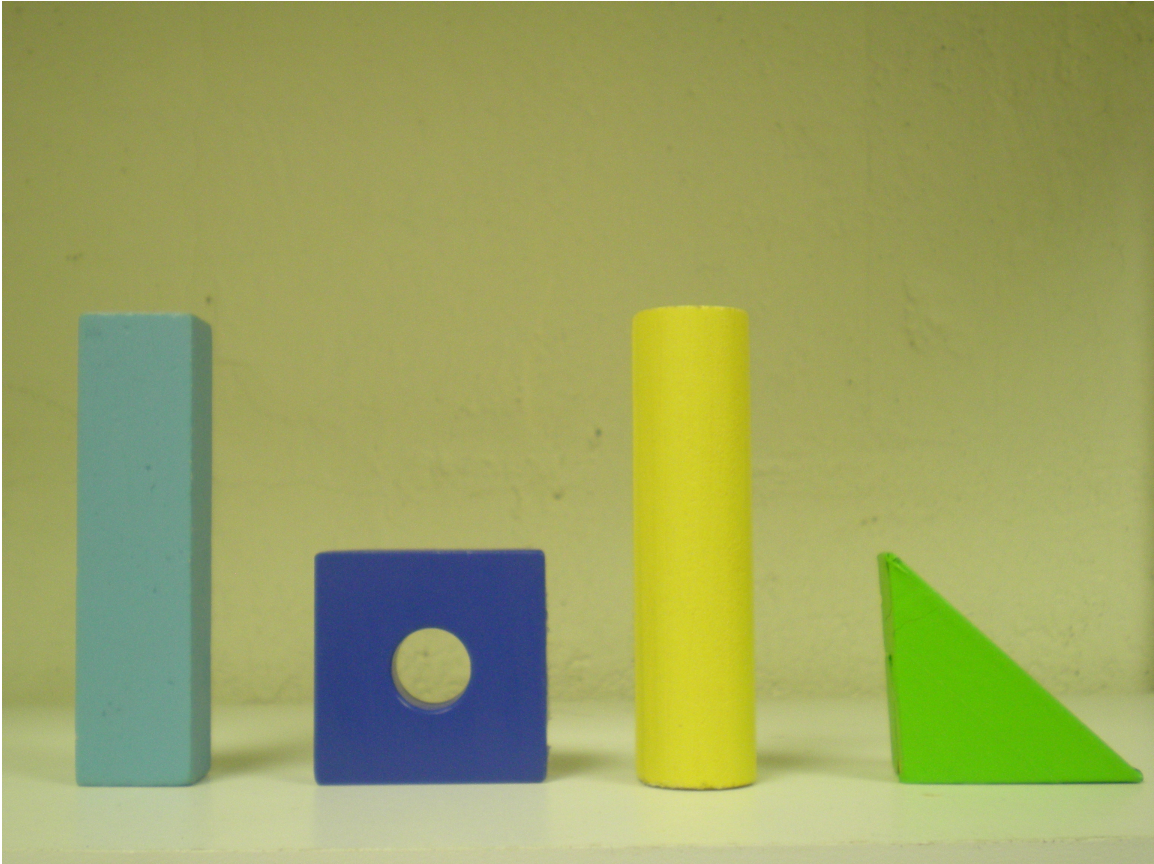


Figure 4. The four experimental objects that served as the novel or the familiar object used in the NOR task.

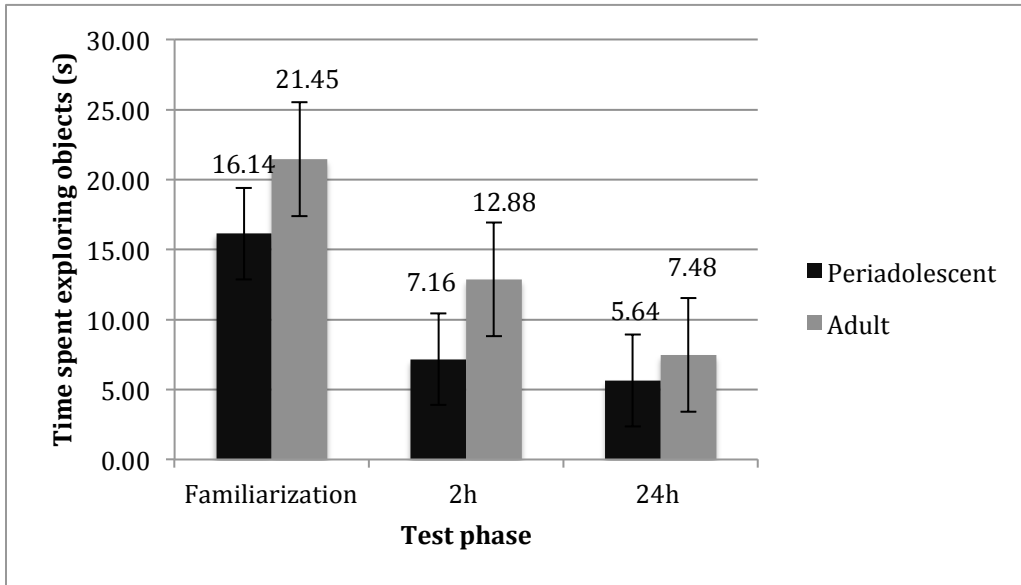


Figure 5. Total time spent exploring the novel and the familiar object during the familiarization phase, 2 h delay test phase, and 24 h delay test phase. Object exploration time decreased significantly from the familiarization phase ($M = 18.70$) to the 2 h test ($M = 8.77$), and from the 2 h test to the 24 h test ($M = 6.53$). There were no significant effects between groups by maturity.

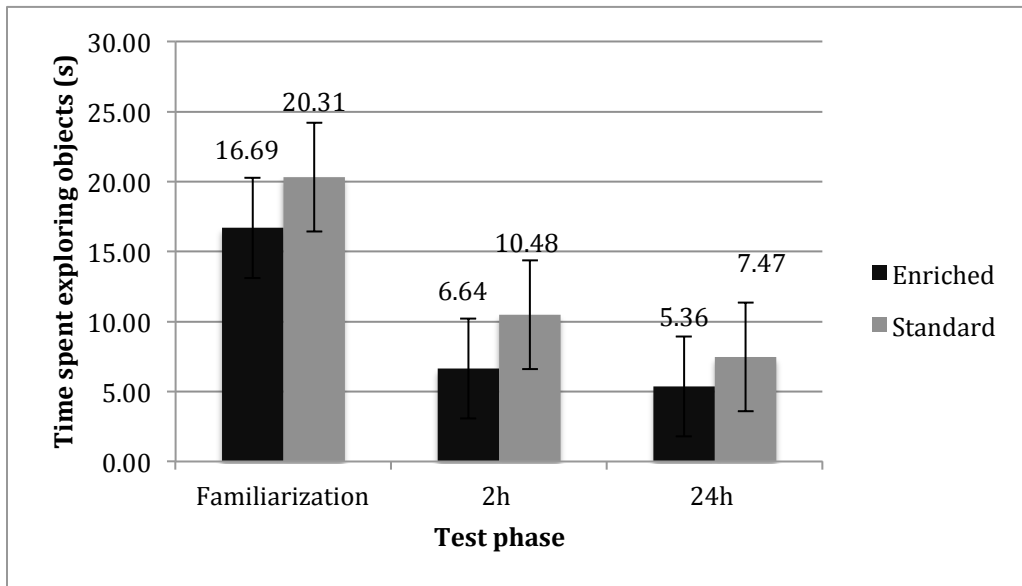


Figure 6. Total time spent exploring the novel and the familiar object during the familiarization phase, 2 h delay test phase, and 24 h delay test phase. There were no significant effects between groups by environment.

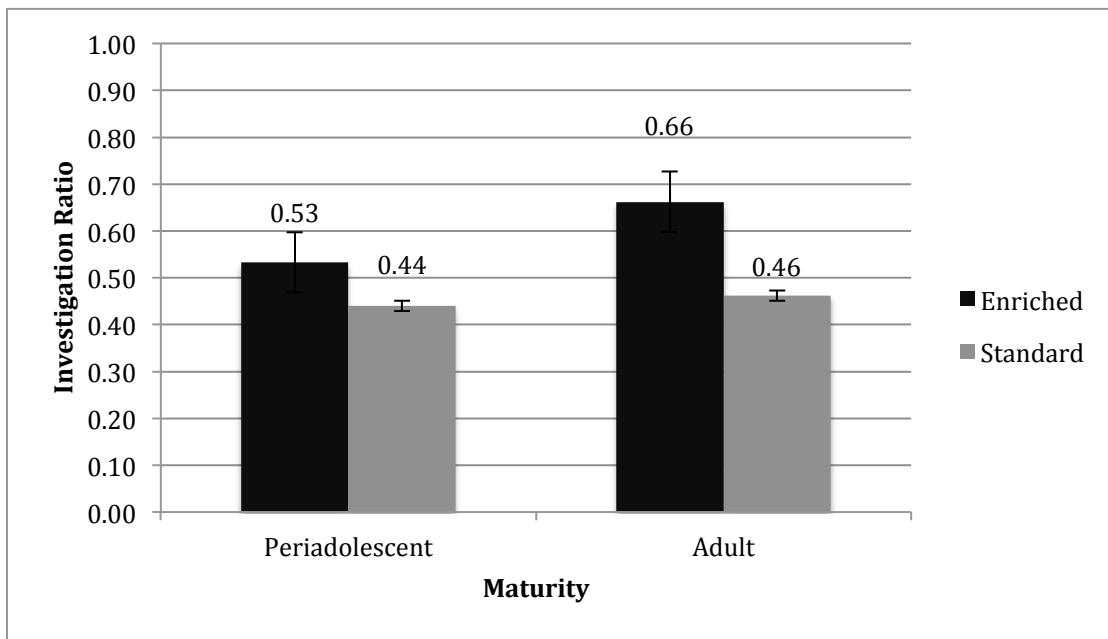


Figure 7. Investigation ratios at the 2 h delay test phase. Adult enriched animals demonstrated significantly higher investigation ratios than did adult standard-housed animals at the 2 h delay.

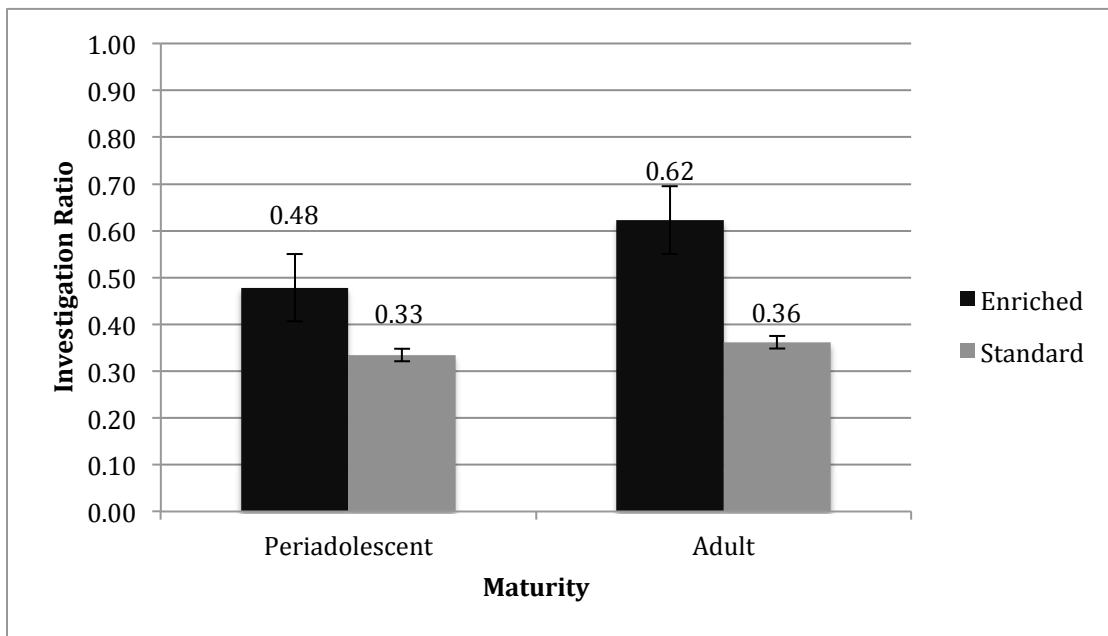


Figure 8. Investigation ratios at the 24 h delay test phase. Adult enriched animals demonstrated significantly higher investigation ratios than did adult standard-housed animals at the 24 h delay.

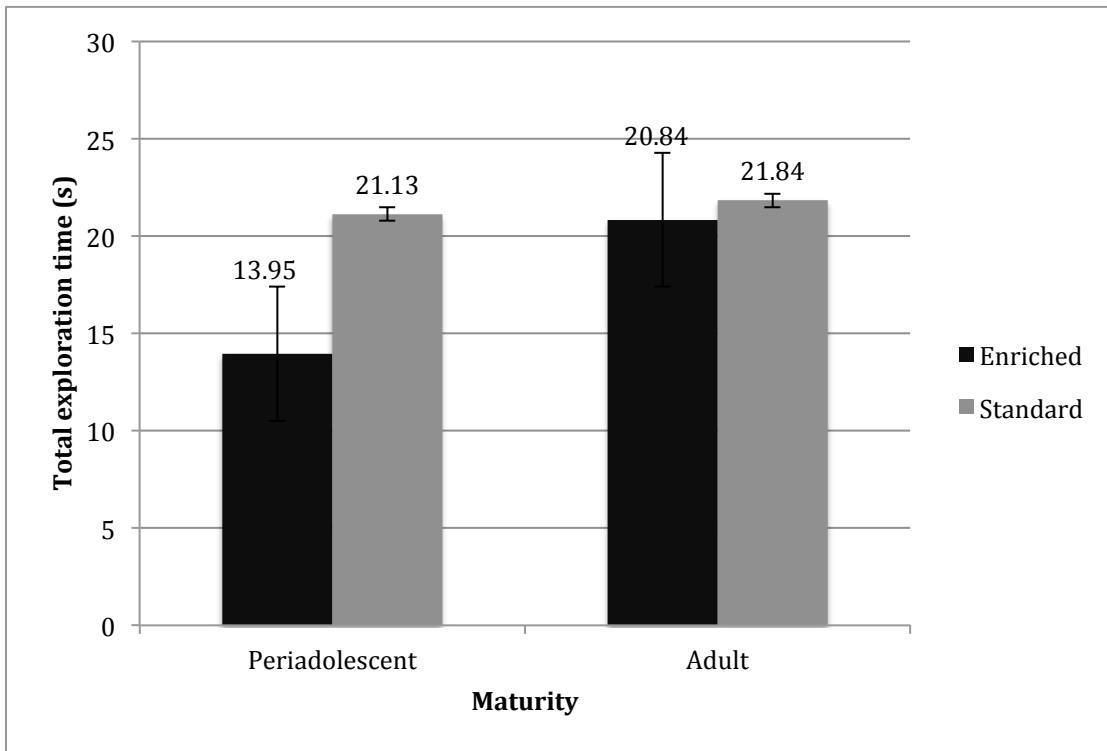


Figure 9. Effect of maturity on total time spent exploring the novel and the familiar object during the familiarization phase for enriched versus standard subjects. The difference between enriched and standard-housed periadolescents approached significance ($p = .05$). There was no significant effect between enriched and standard-housed adults.

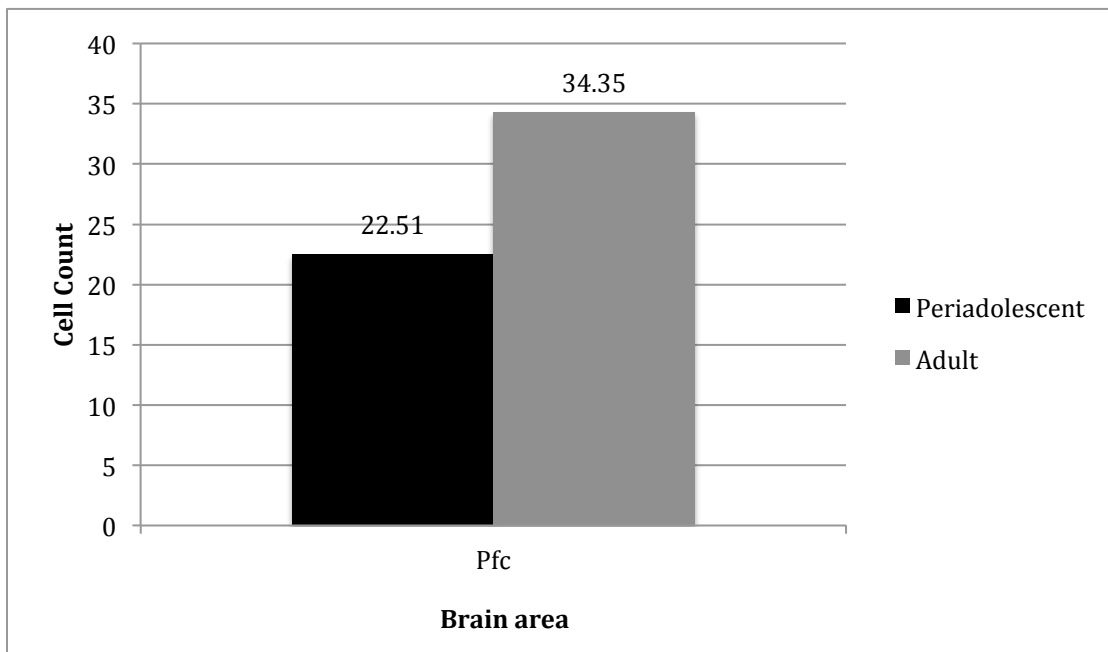


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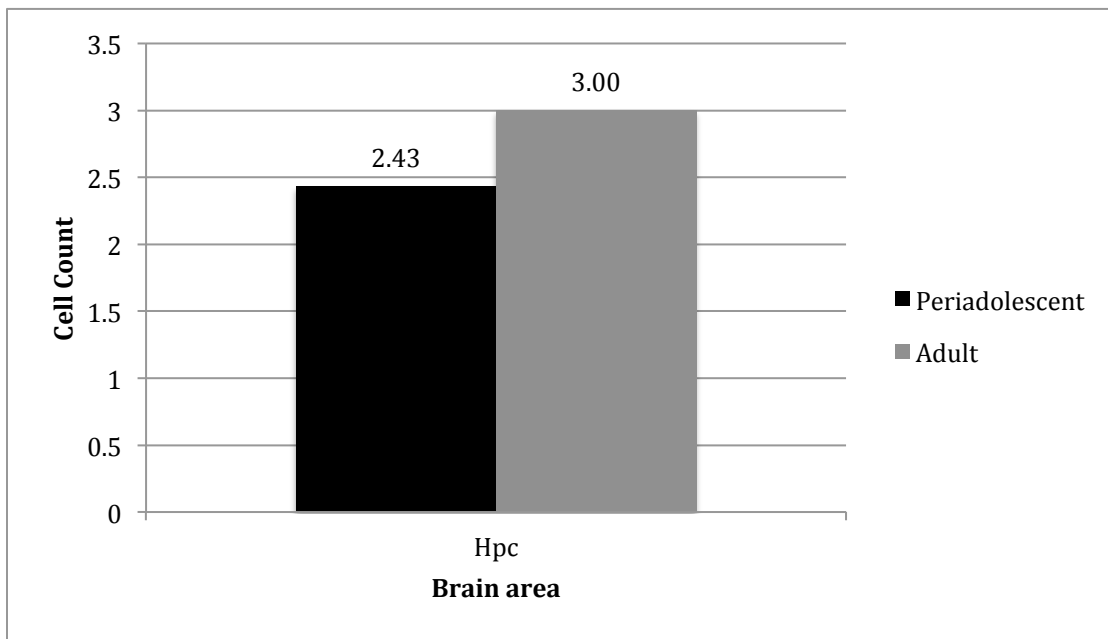


Figure 11. The effect of maturity on mean cell counts in the Hpc after staining for cFos. Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task. No significant differences were found between groups.

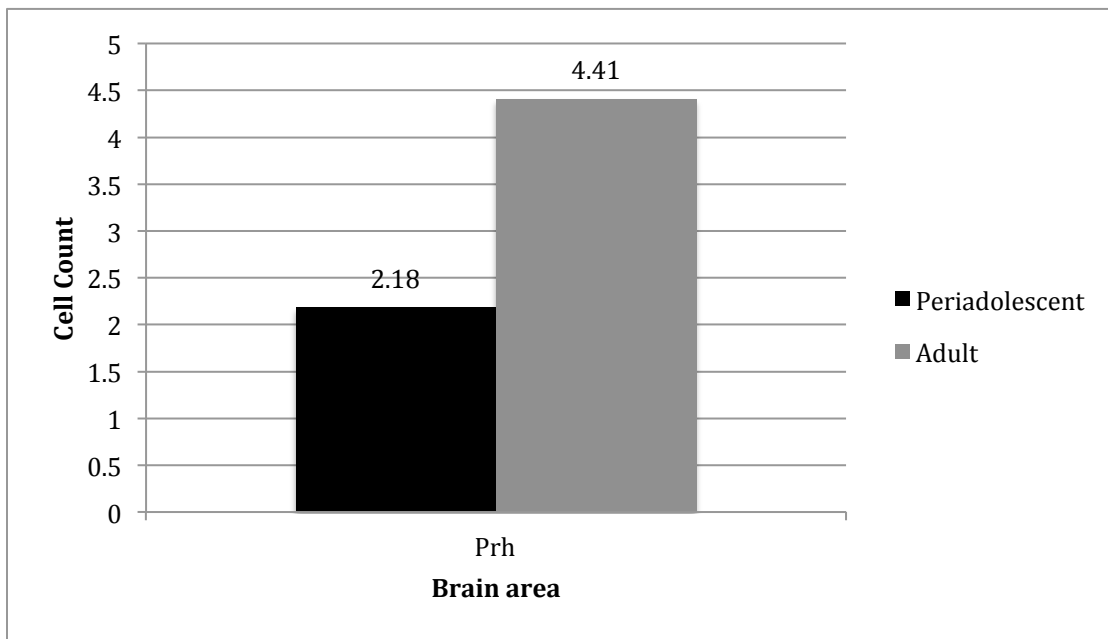


Figure 12. The effect of maturity on mean cell counts in the Prh after staining for cFos. Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task. No significant differences were found between groups.

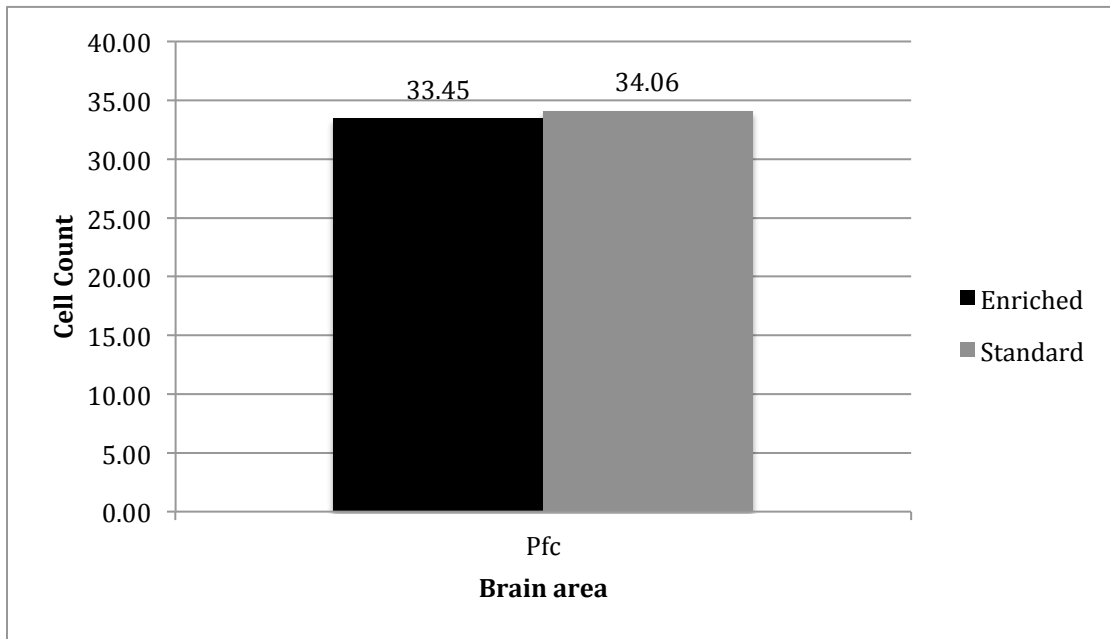


Figure 13. The effect of environment on mean cell counts in the Pfc staining for zif268.

Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task.

No significant differences were found between groups.

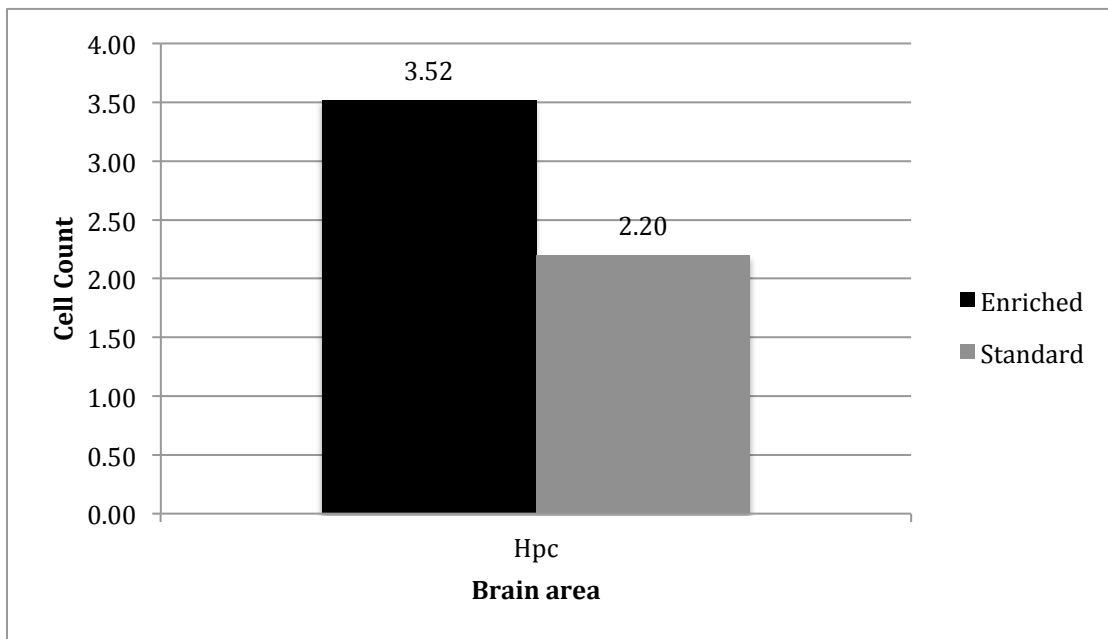


Figure 14. The effect of environment on mean cell counts in the Hpc staining for zif268.

Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task.

No significant differences were found between groups.

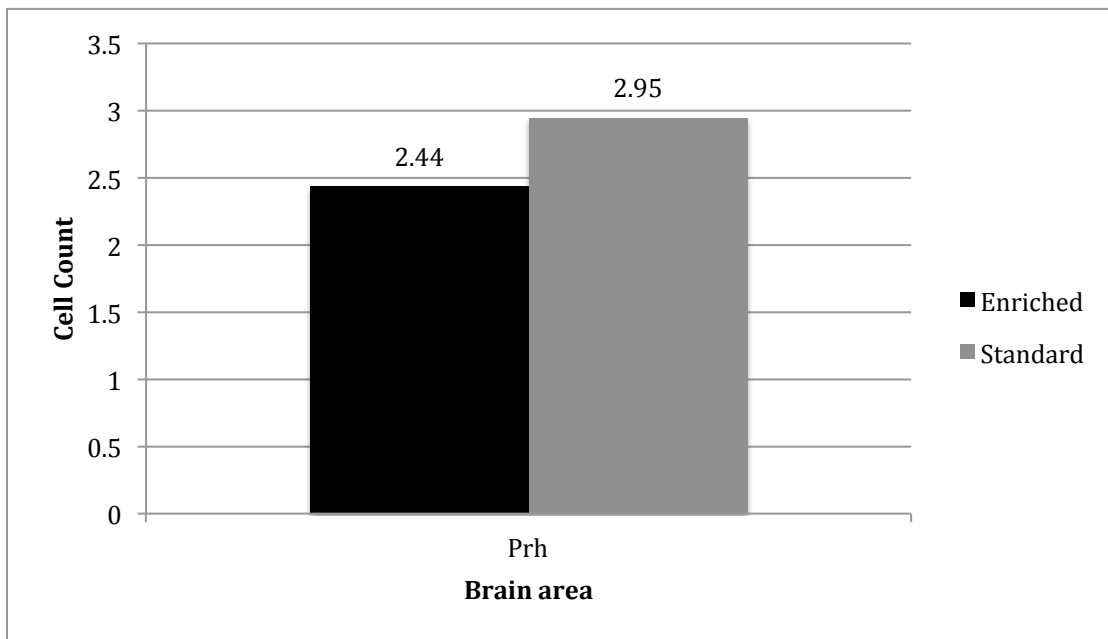


Figure 15. The effect of environment on mean cell counts in the Prh staining for zif268.

Cell counts reflect neural activity at the time of the 24 h delay test phase of the NOR task.

No significant differences were found between groups.

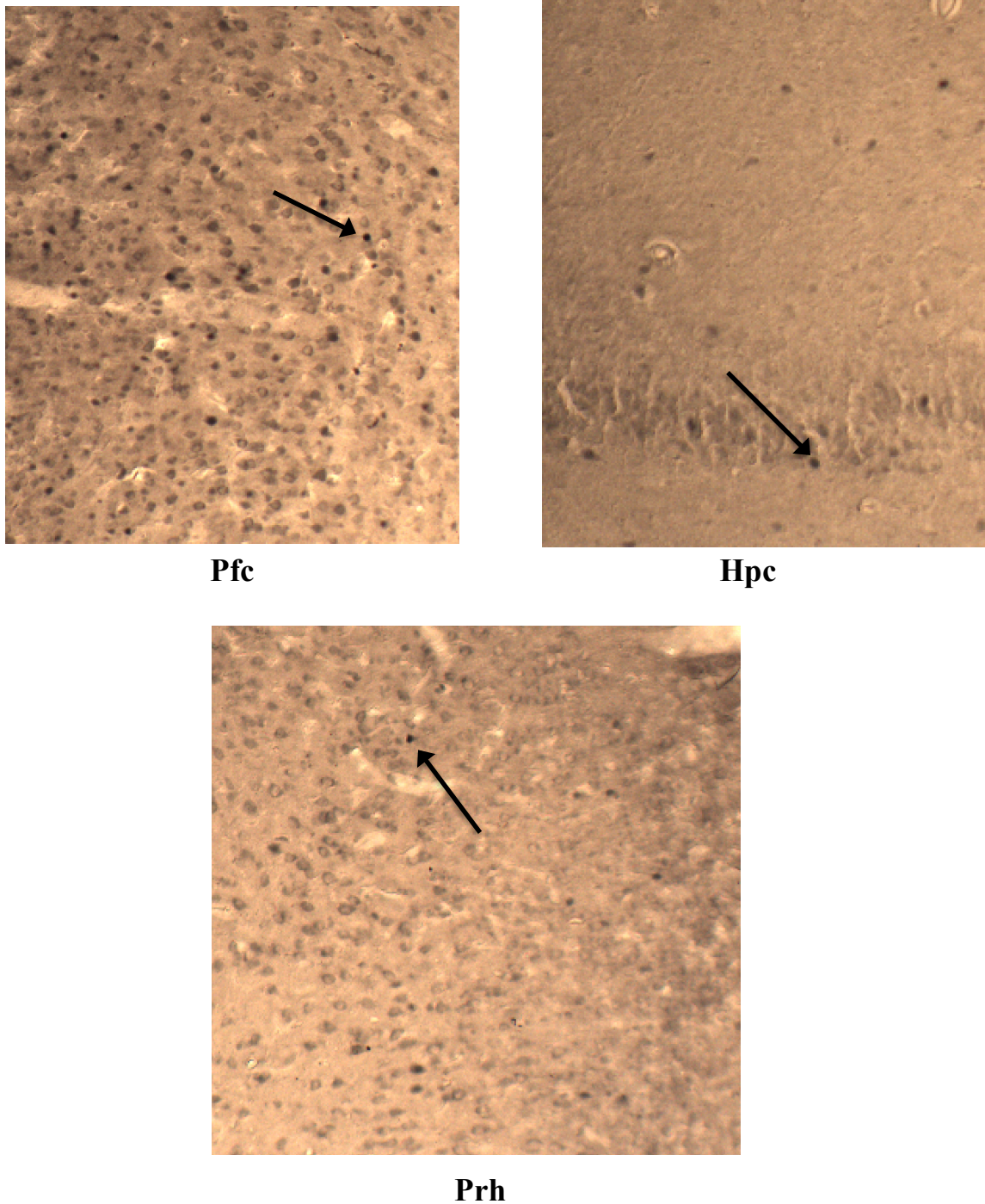


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investigator to distinguish between cell bodies and cFos cells. Background staining from cell bodies was most frequent in prefrontal sections. Adult rats in the enriched condition showed significantly higher Fos activated cell counts in the Pfc; in periadolescents, Fos neuronal nuclei counts in the Hpc were significantly negatively correlated with 24 h investigation ratios.

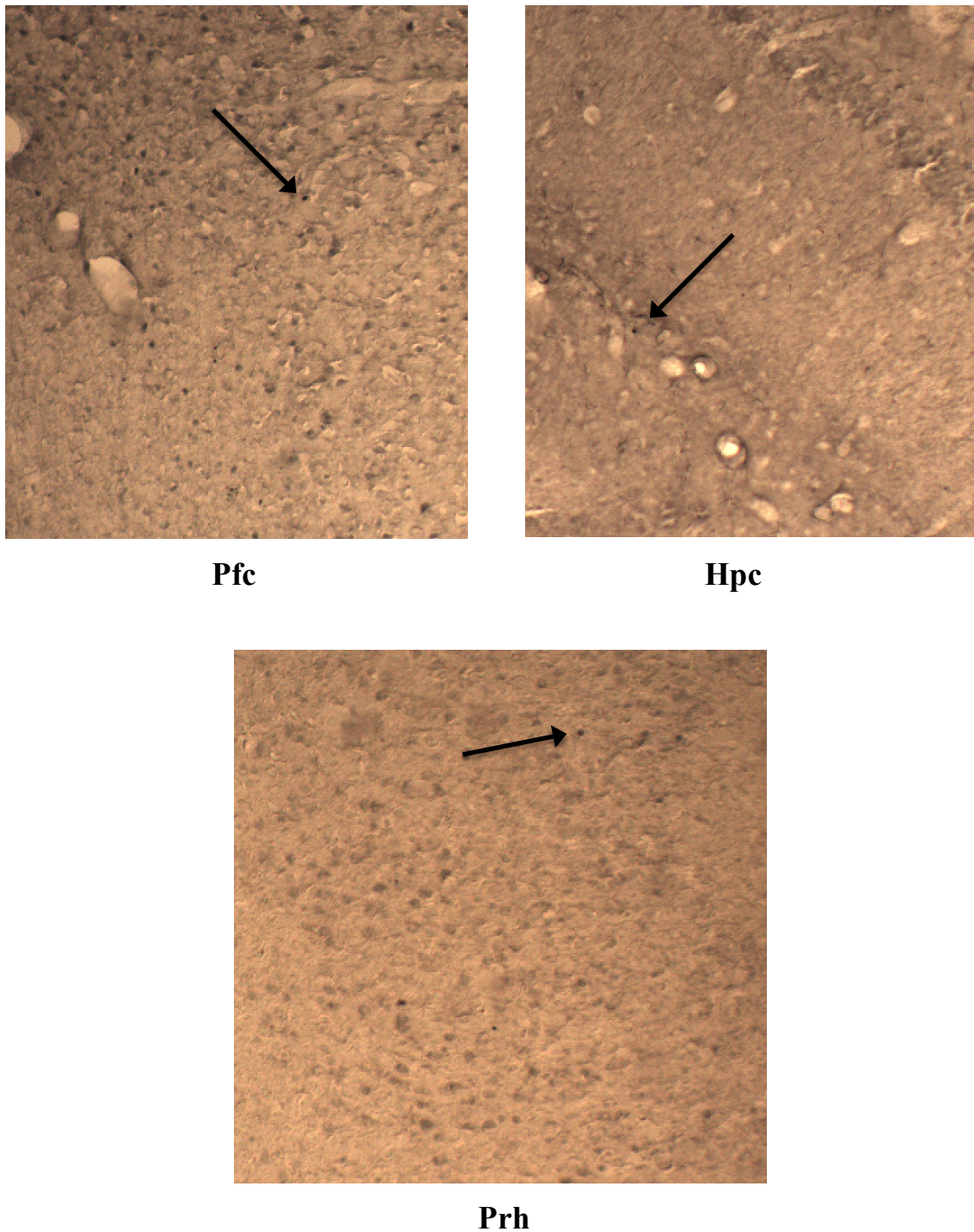


Figure 17. Examples of Pfc, Hpc, and Prh sections stained with zif268 antibody under 10X magnification. Arrows indicate an example of a zif268 cell. Background staining from cell bodies was most frequent in prefrontal sections. ANOVA statistical analysis

revealed no significant differences between groups at the 2h delay, but at the 24h delay, zif268 mean cell counts in the Hpc were significantly positively correlated with investigation ratios, and mean cell counts in the Prh were significantly negatively correlated with 24 h investigation ratios.

Figure 18.

