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Motherhood, memory and aging : object recognition performance

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Motherhood, Memory and Aging: Object Recognition Performance

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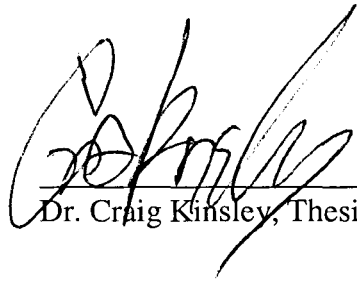
Reproductively experienced female rats have been shown to have attenuated stress responses, improved visual systems, and better memory and learning. This study sought to extend those findings by comparing aged reproductively experienced and aged virgin female rats on an object recognition task, as well as comparing levels of corticosterone and 17β-estradiol and neural activation. Multiparous (MP, 2 reproductive experiences) females performed better on the task and demonstrated quicker habituation to the task than nulliparous (NP, no reproductive experiences) females. No hormonal or neural activation differences were found. The present study contributes to the growing research areas of reproductive experience and cognitive aging.

I certify that I have read this thesis and find that, in scope and quality, it satisfies the requirements for the degree of Master of Arts.



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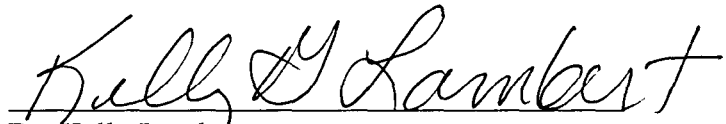

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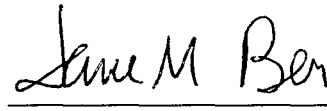

Dr. Jane Berry

MOTHERHOOD, MEMORY AND AGING: OBJECT RECOGNITION PERFORMANCE

By

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From an evolutionary perspective, the purpose of life is not only survival but also reproduction and the successful propagation of one's genes. The importance of reproduction has been illustrated by several leading researchers and the area of study on mating behaviors has grown considerably in the last thirty years. Indeed, reproduction is so important to us that "few domains of human activity generate as much discussion, as many laws, or such elaborate rituals in all cultures" (Buss, 2003, p.1). We as humans are not alone in this. Reproduction is a highly motivated behavior in all species, particularly mammalian. For example, sexually experienced male rats spend more time near receptive females than other males in an open field, even when the other rat is behind a barrier which would prevent copulatory behavior; this effect persists even after repeated trials suggesting that the motivation does not expire (Hetta & Meyerson, 1978). Further, sexually experienced males will run faster toward an ovariectomized (OVX) female treated with estradiol and progesterone (simulating sexual reception) than towards an untreated female (Lopez, Olster & Ettenberg, 1999).

Although the many effects of reproduction on organisms are yet to be fully understood, it is clear that reproduction causes a multitude of both behavioral and neurological changes in reproducing animals. Although reproduction affects males to a degree in species that exhibit paternal behavior, the maternal animals undergo a host of biological changes during and following pregnancy (Kinsley, 1994). Any behavioral changes exhibited, of course, represent changes in the brain and physiology of the animal.

Maternal Behavior

In most mammals, successful propagation of ones genes involves not only mating but also caring for the offspring until they are old enough to be self-sufficient. Maternal behavior is "a highly conserved set of behavioral capacities that are crucial for reproductive success" (Leckman & Herman, 2002, p. 27). In rats, these behaviors are especially crucial because newborn rodents are nearly immobile and cannot thermoregulate, thus they are completely dependent on maternal behaviors for survival. As detailed by Leckman and Herman, these behaviors include nest building, sniffing and licking of pups, pup retrieval, grouping, and crouching (the maternal rat crouches over the grouped pups to enable them to nurse). These behaviors are natural and do not need to be experimentally induced in female rats. Further, the behavioral profile is caused by several factors, including both environmental and hormonal. It is clear that presence of offspring is necessary for performance of maternal behavior. The hormone profile is less clear, but it is known that it is multiply determined: no one hormone can induce or maintain maternal behavior on its own (Lamb, 1975).

Behavioral Changes Due to Reproductive Experience

Not only must new rat mothers be able to nurse and physically care for pups, but a variety of other behaviors are necessary: For example, they must be able to venture out, find and remember the location of food, water, and shelter in the environment for themselves and for their pups. They must also protect themselves and their pups from predators or aggressive males. Thus, the behavioral changes found in maternal rats include not only those which are directly related to the care of the pups, but also those *not*

directly related to caring for the offspring. Improvements in a number of behaviors and abilities have been reported.

Lambert et al. (2005) reported that female rats exposed to pups for 21 days performed better in a dry land maze. The dry land maze task requires rats to find a piece of food in one of several wells in an open field. Primiparous (PP) females, one reproductive experience, performed even better than nulliparous (NP) females although both were exposed to pups; further, NP pup-exposed females outperformed NPs without pup exposure on one version of the task. These results suggest that parity as well as pup-exposure improves foraging ability in the female rat. Similarly, Kinsley and Bardi, et al. (2006) report a very large, significant difference in predatory behavior between NP rats and lactating rats. The task requires the food-deprived female to find and catch a cricket in an open field. The latencies to catch the cricket for NPs were significantly larger than for the lactating females.

The precise mechanisms through which maternal experience causes improvements in foraging ability and predatory behavior is unclear but could be related to any combination of changes in motivation, sensory ability, stress response or spatial learning and memory. In an effort to elucidate these mechanisms, Kinsley and Bardi, et al. (2006) hypothesized that if by changing certain variables, the performance of the lactating females was decreased, it would provide insight into which mechanisms were responsible for improvements. First, motivation levels were examined: perhaps the lactating females were simply more hungry. In order to test the hypothesis, NP females were deprived of food for twice as long. Even being twice as hungry, lactating females

still had significantly smaller latencies. Next, Kinsley and Bardi, et al. attempted to "knock out" certain sensory systems. Zinc sulfate solution temporarily blocks the ability to smell. Saline solution was applied to the nostrils of NPs and zinc sulfate to the lactating females. When tested, the latency to catch a cricket was significantly decreased in lactating females, but not to a degree large enough to account for all of the variance in the original experiment. Next, with the help of a white noise generator, audition was removed; in this trial, lactating females performed as well with the white noise generator (lack of audition) as without in the original experimental condition. Third, Kinsley and Bardi, et al. trimmed the whiskers of lactating females and ran the experiment. When somatosensation was removed, lactating females performed just as well as those lactating females who had a sham whisker trim. The last sensory system tested by Kinsley and Bardi, et al. was the visual system. In a zero lux room, with the help of night vision goggles, lactating females performed significantly worse, now nearly at the level of the NP animals in the original experiment. Interestingly, NPs appeared to perform slightly better, with a smaller latency, in the zero-lux condition. The as yet unpublished results of Kinsley and Bardi, et al. suggest that there are significant changes both to the visual system and the olfactory system in lactating females, most radically to the visual system. A change to the visual system could certainly help account for the increased foraging ability found by Lambert et al (2005).

The discovery that NP animals' performance increased in the zero lux condition is congruent with other findings: First, it is well-known that rodents are nocturnal and are much less fearful in dark environments than in light environments where they are more

exposed. Second, there is much evidence to suggest that reproductively experienced and lactating females are less fearful. For example, in two experiments, Wartella et al. (2003) found that parous rats show less stress reactivity, both behaviorally and neurologically. Multiparous (MP, two litters) and PP females showed less *c*-fos immunoreactivity (IR) in both the CA3 region of the hippocampus and the basolateral amygdala (BLA) following 60 minutes enclosed in a clear restraint tube (the restraint stress paradigm) than NP animals. *C*-fos IR signifies neuronal activation; thus more IR suggests more neuronal activation in those areas. In the second experiment, five groups of animals [NP, PP, MP, primigravid (PG, first pregnancy), multigravid (MG, second pregnancy)], were observed in an open field, a natural stressful environment, and *c*-fos IR was measured postmortem. NP animals were the most reactive to the stress of the open field, as measured both by display of stress and fear behaviors (e.g. freezing) and increased *c*-fos IR. The gravid animals showed less *c*-fos IR than parous animals although the degree of reproductive experience did not seem to make a difference; there was not a significant difference between PPs and MPs or between PGs and MGs.

Similar findings were reported by Byrnes and Bridges (2006): Using an elevated plus maze (EPM) task and the open field test, they found that anxiety-like behavior was affected by reproductive experience and hormonal levels which vary with the stages of the estrous cycle. Reproductive experience does not only produce changes to estradiol and prolactin levels during pregnancy and lactation but also causes changes in the future during subsequent estrous cycles. In addition to examining the relationship between stress and reproductive experience and hormonal changes, Byrnes and Bridges also

looked at developmental changes. The EPM consists of two intersecting arms in the shape of a plus creating four 'arms' which extend out from the point of intersection, two of which covered and two of which are open; the entire structure is elevated off of the ground. More time spent in open arms is indicative of less anxiety. Results showed that young PP females (6-8 weeks post-weaning) showed fewer anxiety-like behaviors than NP controls both in the open field and in the EPM. The result was reversed in middle-aged PP and NP females (32-36 weeks post-weaning). In a third experiment, ovariectomized NP and PP females were tested in the EPM; no significant differences were found suggesting that the absence of ovarian hormones eliminated the effects of reproductive experience on anxiety.

In addition to sensory and stress-response changes, other behaviors have been found to be affected by reproductive experience. A new rat mother's performance in many ways is partly contingent on her cognitive abilities and the honing of those cognitive abilities once she has pups to care for. Recent research has indicated that reproductive and pup experience and stimulation is beneficial to learning and memory in female rats (Kinsley et al, 1999). More recently, Pawluski, Walker and Galea (2006) reported that reproductive experience "differentially affects spatial reference and working memory performance." PP females committed significantly fewer errors in a radial arm maze than NP. Stage of estrous cycle and duration of maternal behavior (operationally defined as amount of time spent licking and nursing pups) were also measured. The stage of estrous cycle could not account for the variance between NP and PPs; however, there

was a direct correlation between spatial reference memory performance and total time spent licking and nursing.

Macbeth (2006) compared middle-aged (11 months) MP retired breeders and NP female rats' performance on object recognition and object location tasks as well as examined anxiety behaviors during the EPM. The object recognition and object location tasks, briefly, use the novel-object preference paradigm to determine whether the subject recalls an object from a previous encounter. In both tasks, the subject is allowed to explore two identical objects in a sample trial. In a later recognition trial, one object is either replaced with a novel object (object recognition task) or is moved to a different location (object location task) and the time spent exploring each object is recorded. If the subject recalls the objects or locations from the sample trial, it will spend more time exploring the new object or the object in the new location during the recognition trial. Often, the delays between the sample and test trials are varied to adjust difficulty of the task. On both tasks, Macbeth found that at a two hour inter-trial delay, MP rats significantly differentiated between the old and novel objects/locations whereas the NP rats did not; however, there were no group differences at the four hour inter-trial delay. There were no significant differences between MP and NP on the EPM task.

Brain Plasticity

The next logical step in understanding these changes due to reproductive experience is understanding how they occur. Any change in behavior must be represented by a change, however transient, in the "organization or properties of the neural circuitry that produces the behavior": the brain is not static as was once thought

but rather has the incredible ability to change and to adjust given various environmental and developmental variables (Kolb, Gibb, & Robinson, 2003, p.1). This phenomenon is known as brain plasticity. Kolb, Gibb and Robinson list a limited number of factors which can affect brain plasticity: experience (both pre- and post-natal), psychoactive drugs, gonadal hormones, anti-inflammatory agents, growth factors, dietary factors, genetic factors, disease, stress, and brain injury. Reproductive experience encompasses several of the aforementioned factors, most notably experience and gonadal hormones and stress.

There is much evidence showing a relationship between estrogen and brain plasticity, learning and memory. For example, Foy (2001) reported that estrogen, specifically 17β-estradiol, increased neuronal transmission in the hippocampus. Wise (2006) and Wise et al (2001) also reported that, in both young and aging rats, treatment with 17β-estradiol decreases ischemic injury by 50% and provided evidence that estradiol acts by altering genes that suppress apoptosis and enhance survival. Further, hormone-induced morphological modifications to the hippocampus of rats have also been found, including increases in both long-term potentiaton and dendritic spine density (Kinsley & Trianer, et al, 2006).

In addition to the hormones of pregnancy, PP and MP rats are also exposed to a variety of new sensory stimuli when the pups are born. Several researchers have noted the role of the pups as environmental stimuli as a mechanism by which maternal behavior and its associated changes occur; the pups serve as an enriched environment for the maternal rats which might account for some of the morphological and behavioral changes

seen (Kinsley et al., 1999). It is well documented that enriched environments can provide a buffer against deficits associated with aging. For example, Kolb, Forgie, Gibb, Gorny and Rowntree detail the value of an enriched environment on synaptic plasticity and protection against cognitive aging. Lores-Arnaize et al. (2006) recently replicated those findings, reporting that aged rats raised in enriched environments showed significantly better working memory performance than those raised in standard environments. Thus, the sensory stimulation from the pups may have plasticity effects on the mothers' brains in much the same way that older rats exposed to enriched environments have shown formation of new synapses and new postsynaptic CA1 dendritic spines (Engert & Bonhoeffer, 1999; Maletic-Savatic, Malinow, & Svoboda, 1999). Furthermore, similar benefits are seen when the enriched experience occurs at weaning, as young adults or as adults (Rosenzweig & Bennett, 1996).

Further, Leuner and Shors (2006) report that the presence of pups during the postpartum period reduced the detrimental effects of a stressful event (placement in a restraint tube) to a classical conditioning paradigm.

Duration of Behavioral and Neural Modifications

A logical question at this point would be, how long-lasting are these changes to the parous rat? Do the modifications expire when pups are weaned or do they persist? Interestingly, many of the behavioral changes appear to persist but results are somewhat conflicting. Love et al. (2005) report that reproductive and maternal experience leaves female rats with long-lasting behavioral modifications. Using both a dry land maze (DLM) and an elevated-plus maze (EPM), the cognitive and emotional responses of NP,

PP, and MP rats were assessed every four months from age 5 months to 22 months of age. Averaged across ages, NP animals took longer to reach a baited well in the DLM. At 5 months of age, both PP and MP rats performed better than NPs whereas at 13 months of age only the MPs outperformed the NPs; at all other ages, there were no statistically significant differences. Beginning at 10 months of age, parous animals spent a significantly larger percentage of time in the open arms of the EPM: At 10 and 14 months of age, both PP and MP groups were statistically different from the NP group whereas at 18 and 22 months of age, only the PP group was statistically different from the NP group. Additionally at 20 months of age, all subjects were tested in a novel-stimulus task: MP animals spent significantly more time with the novel stimuli than the NP animals. However, at 23 months of age, animals were subjected to a two-minute swim as a stressful experience and blood samples were taken; plasma was analyzed and no significant differences in corticosterone were detected. Animals were then sacrificed and brains were stained with a Golgi stain which allows quantification and imaging of the neuronal morphology. Reproductive experience did not have a significant effect on any of the neuronal measures.

Gatewood et al. (2005) provided even more compelling data that changes to memory and learning systems were long-lasting: She and colleagues demonstrated that parous rats outperformed nulliparous females consistently on a dry land maze (DLM) when tested at 6, 12, 18 and 24 months. At each time, MP females out performed both PP and NP, finding the baited well significantly faster. At 12 and 18 months, the PP females found the baited well significantly faster than the NPs. On a reversal version of

the DLM (the location of the baited well was switched) performed at 12, 18 and 24 months of age, MPs still consistently performed better than NPs and PPs; additionally, PPs performed significantly better than NPs at both 12 and 24 months of age. In addition to behavioral data, animals' brains were also examined via immunohistochemical staining for signs of neurodegeneration. Brains were stained for amyloid precursor protein (APP), a marker of neurodegeneration and cognitive decline. MPs had significantly less APP in the hippocampus and dentate gyrus than the NPs and the PPs showed a similar trend. This research suggests that reproduction might improve learning and memory long-term in addition to immediately after pregnancy.

There is a great deal of evidence, then, suggesting a strong link between reproduction and maternal memory/learning systems. However, the results of Love et al. (2005) and Gatewood et al. (2005) still leave questions unanswered. In the case of Love et al., why would there have been no hormonal or neural differences at 23 months of age when there were behavioral differences just 3 months prior? In Gatewood et al. (2005) there was no hormonal data; would a significant effect have been found? Furthermore, in both papers, the effects of age and reproductive experience were not linear: at certain times/ages, one group would out perform another but not consistently. Their research is extremely compelling and exciting, but more information is needed to further clarify and document the persistence of differences between reproductively experienced and inexperienced females in old age and to elucidate the mechanisms underlying those differences.

Cognitive Aging

As in humans and many other species, rats exhibit cognitive declines in old age. This characteristic, coupled with their quick reproductive cycle and short life-span, makes them an excellent study-subject in which to assess factors related to increase or decrease the severity or likelihood of these cognitive declines. Further, the evidence, cited above, that increases in learning and memory abilities due to reproductive experience actually persist into old age makes maternal rats an even better subject for research into cognitive aging. The exact mechanisms through which reproductive experience protects the aging brain against cognitive decline are not clear. It is likely, however, that reproductive experience modifies the cognitive apparatus and function through significant alterations of the underlying neural structures; several possible mechanisms are detailed below.

As previously mentioned, research has shown that exposure to an enriched environment helps to counteract some of the detrimental effects of aging in rats via a greater neuronal plasticity and fewer spatial and working memory deficits (Lores-Arnaiz et al., 2006) and pups provide a highly enriched environment for the mother. Another potential mechanism is the stress system: Sandi and Touyarot (2006) found that mid-life stress increases the likelihood of cognitive decline in early old age. On a cellular level, Magarinos and McEween (1995) showed that stress can induce atrophy of hippocampal neurons. Reproductive experience attenuates the stress response (Byrnes & Bridges, 2006) and that relationship could then affect the degree of cognitive decline.

A third possibility is linked to the activity of the estrogen hormones. As mentioned above, there is a definite relationship between the hormones of pregnancy and memory performance (Foy, 2001; Kinsley & Trainer, et al., 2006). Previous research also suggested that the natural fluctuations of the hormones of pregnancy and motherhood affect dendritic spine density (Rasia-Filho, Fabian, Rigoti & Achaval, 2004). Bodo and Rissman (2006) report multiple findings which suggest a role for the estrogen receptor β (ER-β) in learning and memory: the details are unclear but it is known that ER-β is involved in visuospatial learning and in its absence, learning is inhibited. Furthermore, "several neurotransmitter-containing neurons in the rat paraventricular nucleus coexpress ER-β including vasopressin, oxytocin, prolactin..." (Bodo & Rissman, 2006, p. 217). These findings suggest particular relevance to maternal behavioral changes because of the common hormones involved. Furthermore, even if estrogen levels during old age do not differ as a factor of parity, there could be residual effects from the prior differences due to reproductive experience (De Kleijn, Van der Schouw, & Van der Graaf, 1999).

Research in this area is not limited to studies of reproductive experience but extends to studies of cognition, learning and memory and focuses more precisely on estrogen. Ovariectomized (OVX) female rats performed significantly worse than intact females on both an object recognition task and an object location task; additionally, OVX females had significantly lower dendritic spine densities in the medial prefrontal cortex and the CA1 region of the hippocampus (Wallace, Luine, Arellanos, & Frankfurt, 2006). Furthermore, when OVX rats received acute 17α- or 17β-estradiol injection, there was a

rapid enhancement of object recognition and object location performance (Luine, Jacome & MacLusky, 2003). Holmes, Wide and Galea (2002) reported that estradiol levels affect working memory performance on a hippocampal-dependent spatial task, a version of the radial arm maze. Interestingly, there is not a direct relationship: low levels of estradiol injected daily improved spatial working memory whereas higher levels impaired performance (Holmes, Wide & Galea, 2002).

Sex hormones are not alone in their involvement in memory and learning systems: corticosterone is also implicated. In humans, increased levels of glucocorticoids frequently occur with cognitive impairment and dementia and could be used as a predictive tool (Karlamangla, Singer, Chodosh, McEwen & Seeman, 2005). Karlamangla et al. (2005), studied high-functioning older adults (N=538) in a longitudinal study and found that participants whose cortisol levels put them in the top three quartiles had a higher risk of cognitive impairment at the 7-year follow up. Cognitive impairment was operationally defined by a set decrease in performance on the short portable mental status questionnaire.

Sandi and Touyarot also explored the relationship between stress and cognitive deficits during early aging (2006). As predicted, mid-life stress was negatively correlated with cognitive performance: learning abilities were tested in male Wistar rats who were assigned to a chronic stress (which induced an increased corticosterone response) or control condition. Subjects in the chronic stress condition performed worse than controls in the Morris water maze. The relationship between glucocorticoids and cognitive

impairment is particularly relevant to the present study due to the aforementioned relationship between reproductive experience and stress response.

Neural Changes

In addition to hormonal correlates with memory and learning systems, it is necessary to look at the neuronal morphology of certain brain regions to identify any relationships with reproductive experience, learning and memory, hormonal exposure and/or stress systems. Keyser-Marcus et al. (2001) found a significant relationship between the neuronal morphology in the medial preoptic area (mPOA) and pregnancy/pregnancy-like steroidal administration: Compared with ovariectomized (OVX) and diestrus females, there was an increase in cell-body size in the mPOA of late-pregnant females. Similar effects were found in OVX females treated with estradiol and progesterone. Upon closer analysis, increases were also found in dendritic number and length. Interestingly, changes were also found in lactating females whose hormone levels were significantly lower; this finding supports the idea that hormones alone are not responsible for these changes. Rather, pups are providing an enriched environment for the maternal rat.

Changes have also been found in the hippocampi of late pregnant and lactating females in that there was a greater density of dendritic spines than in virgin females regardless of stage of estrous (Kinsley & Trainer, et al., 2006). Kinsley and Trainer, et al. also found that OVX females treated with estrogen and progesterone had similar increases in dendritic spine density. Gould, Woolley, Frankfurt and McEwen (1990) reported a similar relationship: OVX females showed a decrease in the dendritic spine

density in the CA1 region of the hippocampus and administration of estradiol and progesterone prevented the decrease. However, no changes were found in the CA3 region of the hippocampus or the dentate gyrus, two areas where differences have previously been found (Gatewood et al., 2005). However, Magarinos and McEwen (1995) reported that repeated, daily stress of male rats caused atrophy of dendrites in the CA3 region of the hippocampus. The stress had to occur for 21 days in order to produce these neural changes. The type of stress (restraint versus multiple) had no effect on the dendritic atrophy but did have a differential effect on hormonal response and weight loss.

Rasia-Filho, Fabian, Rigoti and Achaval (2004) found a complex relationship between changes in the dendritic spine density in the medial amygdala and on parity and stage of estrous; no overall directional effect was found. The amygdala has also been implicated in studies on memory: Huff and Rudy (2004) describe how the basolateral region of the amygdala modulates context memory formation. McGauh (2004) supports this finding with a review of studies demonstrating the role of the amygdala in emotionally-arousing or stressful memories.

Another valuable tool is using immunohistochemical (IHC) techniques to examine specific areas of the brain which may be related to any behaviors being studied. IHC methods have been used to visualize *c*-fos as a means of identifying neurons which are activated in response to various experiences or stimuli. For example, as described previously, MP and PP females showed less *c*-fos immunoreactivity in both the CA3 region of the hippocampus and the basolateral amygdala (BLA) following 60 minutes

enclosed in a clear restraint tube (the restraint stress paradigm) than NP animals (Wartella et al, 2003).

Similarly, Zhu, Brown, McCabe and Aggleton (1995) examined eight brain areas using IHC techniques to detect *c*-fos following a novel object test. There were significantly more *c*-fos stained cells in the perirhinal cortex, temporal cortex, occipital cortex and anterior cingulate cortex in subjects shown novel objects as opposed to familiar objects.

Current Study

Due to the complexity of memory and learning systems, it is difficult to pinpoint precisely one or even a few causes of change. Several of the studies cited above attempted to do this but some are conflicting and none provide a truly multidimensional perspective. In the present study, I sought to further elucidate those mechanisms involved in age- and reproductive experience-related changes in cognitive abilities. Using a non-spatial test of memory, the object recognition task, behavioral differences were compared between MP and NP groups of 14-month-old Sprague Dawley rats.

There are many different accepted methods of running the object recognition task. As discussed previously, the vast majority of them have the same procedure which is based on the novel object paradigm. The novel object paradigm holds that an animal will spend more time investigating/exploring a novel object than a previously encountered object. The object recognition task uses the paradigm to measure object recognition memory: if the animal does not remember the object it will be treated as "novel." The task is widely accepted and is appealing in that it does not require any invasive

procedures, food deprivation or extensive training. Furthermore, as the test is conducted in an open field, I was also able to evaluate the possible role of stress. It was hypothesized that MPs would perform better on the task, spending more time exploring the novel object than the old object, would be more exploratory and would exhibit more behaviors indicative of a lack of fear (more rearing, more ventures into the open field). As the test was conducted over three, progressively more difficult days, it was hypothesized that there would also be an effect of the inter-trial delay with performance on the object recognition task declining as difficulty and inter-trial delay increased.

In addition to the behavioral task, hormone levels (progesterone, 17β-estradiol and corticosterone) were also analyzed in order to obtain a more comprehensive understanding of these changes. It was hypothesized that there would be differences between groups on all three hormone levels, with corticosterone levels higher in the NPs during testing and no direction predicted for estradiol and progesterone.

Finally, immunohistochemistry was used to visualize *c*-fos immunoreactivity in four areas of the brain. The object recognition task has been shown to be dependent on three main areas: the perirhinal cortex (PRh), the temporal cortex (TE), and the occipital or visual cortex (VC). These areas have been implicated and shown to be involved in visual object recognition by several researchers (Ennaceur, Neave & Aggleton, 1996; Aggleton, Keen, Warburton & Bussey, 1997; Bussey, Duck, Muir, & Aggleton, 2000; Zhu, Brown, McCabe & Aggleton, 1995). Additionally, immunoreactivity in the basolateral amygdala (BLA) was examined due to its relationship to fear and anxiety. It

was predicted that there would be a main effect of reproductive experience in a complementary way to behavioral performance on the final day of the task.

Method

Animals

Sixteen female Sprague Dawley rats were purchased from Zivic Miller for this experiment; all subjects were age-matched and arrived at approximately 110 days of age. Prior to arrival, eight females had been mated and delivered one litter (primiparous, PP) and the remaining eight were virgins (nulliparous, NP). Upon arrival, all subjects were double-housed with another female of the same reproductive status. Subjects were housed in a 20 × 45 × 25 cm clear polypropylene cage. The bottoms of the cages were covered with pine chip bedding. The tops of the cages are wire lids which provided food (Purina Rat Chow) and water *ad libitum*. The animal housing room was kept on a 14:10 reverse light/dark cycle with the light cycle beginning at 1630h. Human contact was limited to feeding, cleaning of cages and re-housing during any subsequent mating and weaning. Procedures pertaining to all animals in this study were approved by the Institutional Animal Care and Use Committee (IACUC; 06-05-6) of the University of Richmond.

Experimental groups

At six months of age, the eight PP subjects were mated for a second time. Cage-mates were placed in a cage with a stud male for seven days and were then separated and housed singly throughout pregnancy and lactation. All subjects mated and delivered

successfully approximately three weeks later. Pups were then weaned 21 +/- 1 day after delivery. The now multiparous (MP) females were housed singly until two months before testing in order to minimize the stress of being repeatedly marked as is customary in a double-housing situation when the subjects need to be distinguished.

Testing began at approximately 14 months of age and took place over a three-week span. Two subjects, one from each experimental group, were removed from analyses due to absence of behavioral data during testing; thus for all statistical analyses $N = 14$ unless otherwise noted.

Materials

Behavioral Assessment. An object recognition task (described below) was used to evaluate memory capabilities. The task was performed in the laboratory's "open field" maze, a 1 × 1 × 1 m. The objects used are all common, household items of a cylindrical shape and consisted of two plastic water bottles, two diet coke cans, two cans of pears, two hydrogen peroxide bottles (brown plastic), two hard plastic cups (red), and two small wine bottles (187 ml).

Hormonal Assessments. Hormones were extracted from fecal samples. Samples were taken twice: first, prior to the start of the behavioral task for baseline assessment and second, on the final day of the task, to measure changes related to the experimental settings. Commercial Enzyme Immunoassay (EIA) kits (AssayDesign) were used to determine levels of metabolized corticosterone, estradiol and progesterone. The progesterone kit was faulty and therefore no progesterone data was recovered.

Neural Assessments. Following transcardial perfusion, a cryostat was used to cut 40 μm sections from the brain. Analysis of neuronal activation was performed using immunohistochemistry. The above techniques require the use of the following substances and kits: sodium pentobarbital, 4% paraformaldehyde (PF), phosphate-buffered saline (PBS), 20% sucrose/PBS solution, 5% dimtheylsulfoxide (DMSO), hydrogen peroxide (H_2O_2), normal goat serum (NGS), .25% Triton-X-100, *c*-fos primary antibody (ImmumnoStar, Inc, Hudson, WI), biotintylated anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA), ABC kit (Vector Laboratories, Burlingame, CA), nickel sulfate, diaminobenzidine, ethyl alcohol and xylene. Microplate wells, slides, cover slips, mounting medium, and microscopes will also be used.

Procedures

Behavioral Procedures. Testing began approximately eight months following the final weaning of the MPs litters. All subjects were approximately 14 months of age. The behavioral test is an object recognition task which was performed using the general methods of Ennaceur and Aggleton (1994) and as described below.

The object recognition task is a four day test. The first day consisted only of habituation: subjects were exposed to the open field for two trials of 15 minutes each, separated by 90 minutes and no data was taken. Many studies in the literature (e.g. Aggleton, Keen, Warburton & Bussey, 1997; Beck & Luine, 1999; Bussey, Ducke, Muir & Aggleton, 2000; Ennaceur, Neave & Aggleton, 1996; Ennaceur, Michalikova, Bradford, & Ahmed, 2005) have used repeated habituation periods as opposed to a single, longer habituation period in order to better simulate the two-trial design of the testing

days. A longer delay between the habituation trials was chosen in order to further acclimate subjects to the experimental design. The total habituation time is 30 minutes which is in accordance with the methods of Luine, Jacome & MacLusky, 2003; Ennaceur & Aggleton, 1997 and Macbeth, 2006. Only one day of habituation without object exposure was chosen based on the literature and to minimize stress and habituation of the subjects.

Testing days (referred to hence forth as Day 1, Day 2 and Day 3) begin 48 hours after the habituation day (in accordance with the methods of Ennaceur & Aggleton, 1994; Ennaceur & Aggleton, 1997; Bussey, Ducke, Muir & Aggleton, 2000). Days 2 – 4 were comprised of a sample trial and a recognition trial of three minutes each for three days. During the sample trial, the rat was placed into the open field with two identical common objects (e.g. water bottles) and her behavior was recorded. Objects were placed 60 centimeters from the two back corners of the open field, with one object in the left back corner and the other object in the right back corner. During the recognition trial, the rat was placed back into the open field with one of the initial objects (e.g. a water bottle) and a novel object (e.g. a diet coke can) in the same locations as during the sample trial. The delay between the sample trial and the recognition trial increased each day of the task as follows: one hour (Day 1), two hours (Day 2), and four hours (Day 3). All subjects were sacrificed and transcardially perfused 60 to 90 minutes following the test trial on Day 3.

Three days of testing were chosen to increase the likelihood of seeing group differences and to minimize the chance of ceiling or floor effects. The specific delay

times were chosen because they are well within the range of delays used in object recognition tasks.

Each day the sets of objects were changed as to eliminate any carryover effects from day to day; thus three pairs of objects were used over the course of testing. All object pairs and location (left or right side of field) were counterbalanced across days and experimental groups.

Behavior in the open field from Day 1, Day 2 and Day 3 was recorded on videotape. Video was analyzed by the researcher who was blind to the subjects' experimental condition and to the identity of the objects as new or old. The following behaviors were recorded and operationally defined as follows: the time spent investigating each object in seconds (Exploration was defined as smelling or directing the nose toward the object at a distance of less than 2 cm, "whisking" the object, or touching the object with front paws; walking around the object was not defined as exploration.), the number of approaches to each object (frequency of explorations; Subject must have turned away and then turned back towards the object to count as distinct approaches.), the number of ventures into the middle of the open field (subject must have moved more than 10 cm away from wall), and the number of times subject reared up on hind legs (this behavior was not counted if the subject used one of the objects for support or if done during investigation of object). This design resulted in data from six trials over three days.

Hormonal procedures. In order to measure corticosterone, 17-β estradiol and progesterone levels, 0.1g of fresh fecal samples were collected for each subject in

duplicate on two occasions: first, before the start of behavioral testing and second on the final day of behavioral testing. Subjects were placed in a clean cage until they defecated, which in all cases occurred immediately or within less than five minutes. During this time, a sample of vaginal cells was taken. A small pipet filled with .1 ml of saline was inserted vaginally, saline was released and then recollected. The subject was returned to her home cage and the sample was weighed and frozen until assay. Vaginal cytology was examined under the microscope. No group patterns were found.

Hormones were then measured using three kits: a corticosterone enzyme immunoassay kit, a 17-β estradiol enzyme immunoassay kit and a progesterone enzyme immunoassay kit (Assay Designs, Ann Arbor, MI). For each kit, one sample of 0.1g from each subject was dissolved in 1ml of 100% methanol. Samples were homogenized and vortexed for hormone extraction. Extracted corticosterone, 17-β estradiol and progesterone were centrifuged for ten minutes and dissolved in an assay buffer (tris buffered saline) prior to incubation in donkey anti-sheep IgG antibody, goat anti-rabbit IgG antibody and goat anti-mouse IgG antibody, respectively. The assay procedure followed the Assay Designs protocols for Catalog numbers 900-097 (corticosterone), 900-008 (estradiol), and 900-011 (progesterone). Each sample was analyzed in quadruplicates, and a single mean value in pg/mL was used for statistical analysis. Due to an error with the progesterone controls, the progesterone concentrations were unable to be determined and thus were excluded from any statistical analysis.

Neural Procedures. All subjects were injected with a lethal dose of sodium pentobarbital (100 mg/kg). They were then transcardially perfused with PBS followed by

PF. The brains were postfixed in the PF for three hours and then placed in 20% Sucrose/PBS solution until staining began.

The fixed brains were then blocked for the areas of interest (basolateral amygdala, visual cortex, perirhinal cortex and temporal cortex) and cut at a thickness of 40 μm. A total of twelve sections were taken for each brain area of interest and alternate sections were saved for staining. Sections were cut using the Paxinos and Watson stereotaxic atlas (1998) as a guide, anterior to posterior, with the following landmarks: Amygdala sections were taken at 1500 μm posterior to the start of the hippocampus. Visual cortex, perirhinal cortex and temporal cortex sections were taken immediately following the sections for the amygdala at approximately 2000 μm posterior to the start of the hippocampus. Sections were taken off the cryostat and placed in a PBS-filled 12-well microplate with two sections per well.

Immediately after all sections were cut from each brain, the tissues were immersed in a 5% DMSO solution for 10 minutes, followed by a 3% H_2O_2/1% NGS solution for 20 minutes. Next sections were washed eight times with PBS and left overnight in PBS. The following day, sections were placed in a blocking solution (3% NGS, 0.025% Triton-X-100) for two hours. Sections were then exposed to the *c*-fos primary antibody (diluted 1:4000 with blocking solution) for 18 to 24 hours. The next day, tissues were washed six times with PBS and then exposed to the secondary antibody (diluted 1:500 with blocking solution) for two hours. Following exposure to the secondary antibody, tissues were again washed with PBS (three times) and then immersed in the ABC kit solutions for 90 minutes. Next, tissues were washed with PBS

(twice) and placed in a nickel sulfate/DAB/H_2O_2 for 6 to 10 minutes. Tissues were washed twice more with PBS and painted onto subbed slides. Tissues were left to dry overnight onto the slides. Once dry, tissues were cleared of background staining and dehydrated using a series of alcohols (50% to 100%) and xylene and then coverslipped.

Image analysis and quantification. Only brain tissues that were adequately stained as compared to negative control samples were quantified and analyzed. All stained tissue was analyzed using the Bioquant Image Analysis System. A portion of each area of interest, basolateral amygdala (BLA), visual cortex (VC), perirhinal cortex (PRh) and temporal cortex (TE) was analyzed for total number of stained objects. As the areas are quite large, only a portion of the area ("a punch") was quantified with the goal of being representative but not exhaustive. For each area, a 600 × 450 μm area was quantified in both the left and right hemisphere. Again based on the Paxinos and Watson (1998) stereotaxic atlas, the BLA was identified as being approximately 900 μm laterally and 1680 μm dorsally of the base of right/left hemisphere. The BC was identified as being approximately 900 μm laterally and 1100 μm dorsally of the midline above the corpus callosum. The PRh was identified as being directly medial to the rhinal fissure. The TE was identified as being 1500 μm dorsal to the rhinal fissure. The total number of stained objects in each area were averaged for each subject and used in statistical analyses.

Statistical Analyses

A series of 3 one-tailed paired-sample *t*-tests for each group (MP and NP) were performed to compare the mean amount of time spent exploring the old versus the novel object on each of the three days of testing

Mixed analyses of variance (ANOVAs) with reproductive experience (2; MP or NP) as a factor and trial (3; Day 1, 2 or 3) as a repeated measure were performed to analyze the following behavioral dependent variables: Percentage of time exploring the novel object, total time (sec) exploring objects in both the sample and recognition trial; total number of times subject reared up on two legs; and, total number of times subject entered the center of the open field.

Independent samples *t*-tests were performed to compare MP and NP subjects on baseline corticosterone levels, during testing levels, and average estradiol levels. Similarly, an independent samples t-test comparing MP and NP subjects was performed for each of the four brain areas quantified: BLA, VC, TE and PRh.

Results

Each variable was tested for normality using a one-sample Kolmogorov-Smirnov test; all variables were normally distributed.

Behavioral Analyses

In order to test subjects' performance on the object recognition task, three paired-sample, one-tailed *t* tests, one for each day of testing, were run for both reproductive experience groups to compare the amount of time spent exploring the old versus the

novel object. As shown in Figure 1, on Day 1 of testing, NP subjects did not significantly differ in the amount of time spent exploring the old object (M = 6.86, SD = 3.82) and the novel object (M = 8.43, SD = 4.16), $t(6)$ = -.921, N = 7, p = .20; however, MP subjects did significantly differ in the amount of time spent exploring the old object (M = 5.71, SD = 4.12) and the novel object (M = 14.00, SD = 9.03), $t(6)$ = -2.185, N = 7, p = .04. As shown in Figure 2, on Day 2 of testing, NP subjects did not significantly differ in the amount of time spent exploring the old object (M = 7.00, SD = 4.00) and the novel object (M = 10.07, SD = 6.21), $t(6)$ = -1.248, N = 7, p = .13; however, MP subjects did significantly differ in the amount of time spent exploring the old object (M = 9.29, SD = 5.28) and the novel object (M = 15.29, SD = 6.40), $t(6)$ = -2.253, N = 7, p = .03. As shown in Figure 3, on Day 3 of testing, NP subjects did not significantly differ in the amount of time spent exploring the old object (M = 6.50, SD = 4.31) and the novel object (M = 8.00, SD = 2.24), $t(6)$ = -.685, N = 7, p = .26; MP subjects nearly significantly differed in the amount of time spent exploring the old object (M = 5.43, SD = 1.72) and the novel object (M = 10.29, SD = 6.52), $t(6)$ = -1.752, N = 7, p = .06.

To further evaluate object recognition performance, a mixed analysis of variance (ANOVA) was performed with percentage of time exploring the new object during the test trial as the dependent variable, day (3) as a repeated measure, and reproductive experience (2) as a factor. There were no statistically significant main effects of reproductive experience, $F(1,12)$ = 1.165, N = 14, p = .30 or of day, $F(2,24)$ = .027, N = 14, p = .97; and there was not a significant interaction of trial and reproductive experience, $F(2,24)$ = .186, N = 14, p = .83.

Additionally, a mixed analysis of variance (ANOVA) was performed with total time spent exploring both objects during the sample trial as the dependent variable, day (3) as a repeated measure, and reproductive experience (2) as a factor. There were no statistically significant main effects of reproductive experience, $F(1,12) = 1.235$, $N = 14$, $p = .29$ or of day, $F(2,24) = 1.695$, $N = 14$, $p = .21$. The interaction between reproductive experience and trial approached significance, $F(2,24) = 3.253$, $N = 14$, $p = .056$ (see Figure 4). A series of two-tailed paired-sample t tests revealed that for MP subjects the amount of time exploring differed only between the Day 1 ($M = 21.64$, $SD = 7.14$) and Day 3 ($M = 14.57$, $SD = 5.16$), $t(6) = 5.182$, $N = 7$, $p < .01$. There were no other significant differences between days for either the NP group or the MP group during the sample trial (see Tables 1 and 2).

A second mixed ANOVA was performed with the total time spent exploring both objects during the recognition trial as the dependent variable, day (3) as a repeated measure, and reproductive experience (2) as a factor. Again, there was not a significant main effect of reproductive experience, $F(1,12) = 1.927$, $N = 14$, $p = .19$; however, there was a significant main effect of day: $F(2,24) = 4.058$, $N = 14$, $p = .03$ (see Figure 5). The interaction between reproductive experience and trial was not significant: $F(2,24) = 1.269$, $N = 14$, $p = .30$. A series of two-tailed paired-sample t tests revealed for each group, MP and NP, there were no significant differences between days (see Tables 1 and 2).

Anxiety behaviors (see Table 3) were also compared using a mixed ANOVA with day (3) as a repeated measure, and reproductive experience (2) as a factor. The first

analysis, with number of rearings as the dependent variable, had a significant main effect of day, $F(2,24) = 4.063$, $N = 14$, $p = .03$, but no significant main effect of reproductive experience, $F(1,12) = 1.702$, $N = 14$, $p = .22$, and no significant interaction, $F(2,24) = .442$, $N = 14$, $p = .65$. The second analysis, with number of ventures into the center of the open field, did not have a significant main effect of day, $F(2,24) = 2.238$, $N = 14$, $p = .13$, or of reproductive experience $F(1, 12) = .167$, $N = 14$, $p = .69$; the interaction was also not significant, $F(2,24) = .959$, $p = .40$.

Hormonal Analyses

Baseline and during testing levels in (pg/mL) of 17-β estradiol were averaged and an independent samples *t*-test was conducted to compare NP ($M = 418.67$, $SD = 432.88$) and MP ($M = 127.47$, $SD = 78.11$) groups. The means were not significantly different, $t(12) = 1.75$, $N = 14$, $p = .11$. After removing one outlier, baseline levels (pg/mL) of corticosterone were compared between the NP ($M = 1339.88$, $SD = 622.36$) and the MP ($M = 812.18$, $SD = 409.11$) groups. The means were not significantly different, $t(11) = 1.77$, $N = 13$, $p = .11$. Corticosterone levels during testing were also compared between the NP ($M = 722.61$, $SD = 328.81$) and the MP ($M = 876.93$, $SD = 463.62$) groups. The means were not significantly different, $t(12) = -.718$, $N = 14$, $p = .49$.

Neural Analyses

Number of *c*-fos stained neurons in four brain areas, basolateral amygdala (BLA), visual cortex (VC), perirhinal cortex (PRh), and temporal cortex (TE), were compared between multiparous and nulliparous subjects in a series of four *t* tests. No significant differences were found in any of the four areas: in the BLA, $t(11) = 1.024$, $N = 13$, $p = .33$; in the

VC, $t(11) = -.631$, $N = 13$, $p = .54$; in the PRh, $t(11) = -.071$, $N = 13$, $p = .95$; and in the TE, $t(11) = -.569$, $N = 13$, $p = .58$.

Correlations

In order to check for any potential covariates or relationships, behavioral, hormonal and neurological variables were correlated (see Table 4). There were no significant correlations.

Discussion

This study examined long-term effects of aged female Sprague Dawley rats on an object recognition task. Past research indicates that there are long lasting effects of reproductive experience on memory and learning systems and this research supports and extends those findings to non-spatial memory. Multiparous (MP) females were successfully able to differentiate between the old and novel object on two of the three days of testing. Nulliparous (NP) females did not differentiate between the old and novel object on any day of testing. Despite these findings, there was not a significant difference between the MP and NP groups with regards to the percentage of time spent exploring the novel object on any of the days of the task. There were no differences due to reproductive experience with regards to other behaviors (overall exploration, rearing, ventures into the open) although there were differences across days. There were also no group differences in any of the hormonal analyses or neural analyses.

As hypothesized, reproductive experience played a role in subjects' ability to differentiate between an old and novel object in the object recognition task. MP subjects

spent significantly more time exploring the novel object than the old object on the first two days of testing. On the third day, the difference approached significance. The fact that the MP subjects did not differentiate between objects as well on the third day is supportive of the hypothesis that performance would decline as inter-trial delay increased. In contrast, NP subjects did not significantly differ in their exploration of the old and novel objects on any day of testing. Performance in the NP group did not appear to decline across days. The results of the analysis comparing percentage of time spent exploring the novel object did not support the hypotheses: there was not a significant effect of reproductive experience nor of time.

It was also hypothesized that MP subjects would exhibit more exploratory behavior overall. While there was not a significant main effect of reproductive experience, there was an interaction between reproductive experience and day of testing during the sample trial, which suggests that reproductive experience affects the rate at which an animal habituates to the object recognition task. The MP subjects explored more during the first day of the task than the third day. There were no significant differences in the NP group.

While analyses did not support the hypotheses that MP subjects would engage in more behaviors indicative of a lack of fear, there was a significant effect of time for rearing behaviors. Although there were no differences between groups or days on the number of ventures into the center of the open field, both groups exhibited more rearing behaviors as testing progressed. This suggests that both groups were habituating to the testing environment, if not the object recognition task itself.

Group differences in 17β-estradiol and corticosterone levels were predicted. Corticosterone levels were hypothesized to be higher in NP subjects than in MP subjects, particularly during testing. There were no significant group differences in baseline corticosterone levels nor were there significant differences in levels during testing. Group differences in estradiol were also hypothesized, but groups did not significantly differ.

It was predicted that there would be a main effect of reproductive experience in a complementary way to behavioral performance on the final day of the task. There were no significant differences between MP and NP subjects in *c*-fos immunoreactivity in any of the four areas of the brain: BLA, PRh, TE, and VC. However, this is not surprising in that subjects appeared to have habituated by the final day of testing.

The current study was certainly limited by sample size which had a large effect on the analyses' power. It is likely that many of the non-significant trends would have reached statistical significance had a larger group of animals been used. Although there were benefits to repeated days of testing in that different inter-trial delays could be tested, it also allowed for habituation to the task as well as the testing environment. Because of the habituation, neural and hormonal analyses were limited: Had analyses been conducted after the first day of testing, it is possible that group differences in neural activation and hormone levels would have been evident. Additionally, the open field may not have been stressful enough for the subjects to adequately be able to examine the role of corticosterone in cognitive functioning and to compare levels between groups.

Furthermore, the effects found in the present study are in part more modest or different than others cited (Byrnes & Bridges, 2006; Gatewood et al, 2005; Macbeth, 2006). There are a number of reasons for this: Andrews (1996) reported that there are substantial differences in learning, memory and the effects of aging between strains or even in groups of the same strain but obtained from different suppliers. There is evidence which suggests that early rearing environment and postnatal handling can effect in cognitive and neuroendocrine functions in aging female rats (Meaney, Aitken, Bhatnagar, & Sapolsky, 1991). The subjects in this study were ordered from a supplier company whose policies may differ from those in the animal facility at the University of Richmond and other institutions. Moreover, differences between the results of the present study and a similar study by Macbeth could be attributed to differences in the ages of the subjects as well as the precise number of reproductive experiences: Subjects in Macbeth's study were only 11 months old and were retired breeders who likely had more than two pregnancies. There were no main effects of reproductive experience in any of the other behaviors analyzed; this could be due to the age of the animals. It is likely that the age of the animals has a larger effect than the subjects' reproductive experience. The addition of groups of young reproductively experienced and virgin females might have clarified any baseline effects of reproductive experience.

Despite the limitations, these results have multiple implications for the role that parity plays in old age. Although group differences were modest, those differences suggest that there are long-lasting effects of reproductive experience. Aged reproductively experienced females performed better on the object recognition task and

appeared to habituate better as well. This suggests a long-lasting benefit to visual object recognition and memory systems as well a greater awareness of environment. Evolutionarily, these differences could have helped an animal recognize a food source, and encouraged exploratory behavior to find food.

There were no differences between corticosterone or estradiol levels; although this was not hypothesized, it is congruent with other findings. Stress differences due to reproductive experience have been reported (Wartella et al, 2003; Byrnes & Bridges, 2006), however, the subjects were not aged. There were no differences in estradiol levels between MP and NP groups, but due to previous repeated higher levels in MPs during pregnancy, estradiol could still be affecting some of the cognitive and behavioral differences between groups (De Kleijn, Van der Schouw & Van der Graaf, 1999).

The lack of differences in *c*-fos IR also did not support hypotheses. However, other studies of the effects of reproductive experience in aged rats have failed to find significant brain differences (Love et al, 2005; Macbeth, 2006). Additionally, the results do not mean that there were no differences between the subjects' brains; perhaps brain differences in aged animals are more subtle and require larger sample sizes and more specific analyses. Different analyses examining neurodegenerative markers or neuronal morphology should be used in future studies.

These results demonstrate that in rats the effects of reproductive experience in old age extend beyond spatial learning and memory (Love et al, 2003; Gatewood et al, 2003) and include object recognition memory. This relationship should be further studied. It is possible that an increased object recognition memory serves as a mechanism for

increased spatial memory and location recognition. Furthermore, the results of this study and similar studies go beyond the laboratory and academic setting and are valuable to society at large.

As the 'Baby Boomer' generation reaches old age and as humans continue to live to older ages, the proportion of aged people is continually increasing, and as such cognitive functioning in older adults is increasing in importance in society, in the health care system and in the private sector (Josef van der Staay, 2002). As there is currently no known cause or prevention for Alzheimer's disease or dementia, the identification of related factors is crucial. Predictive variables (e.g. mid-life stress levels) and protective factors (e.g. enriched environment) are invaluable and as more correlative factors are found, the better the health care system can aid people suffering from these diseases and also those who are undergoing typical aging-related cognitive declines.

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Table 1

Total Object Exploration Time

	Time Spent Exploring Objects (sec)			
	Nulliparous (NP)		Multiparous (MP)	
	M	*SD*	*M*	*SD*
Sample Trial				
Day 1	16.43	5.50	21.64	7.15
Day 2	11.00	5.95	19.71	12.74
Day 3	16.83	9.30	14.57	5.16
Recognition Trial				
Day 1	15.29	6.58	21.14	11.19
Day 2	17.07	8.17	24.80	9.37
Day 3	14.50	3.69	15.71	6.10

Table 2

Total Exploration Time Compared Across Days

	Nulliparous (NP)	Multiparous (MP)
Sample Trial		
Day 1 – Day 2	$t(6) = 1.698$	$t(6) = .659$
Day 2 – Day 3	$t(6) = -1.984$	$t(6) = 1.523$
Day 1 – Day 3	$t(6) = -.095$	$t(6) = 5.820$ ***
Recognition Trial		
Day 1 – Day 2	$t(6) = -.504$	$t(6) = -1.097$
Day 2 – Day 3	$t(6) = 1.096$	$t(6) = 4.015$ ***
Day 1 – Day 3	$t(6) = 0.250$	$t(6) = 2.168$

Note. *** $p < .01$

Table 3

Anxiety Behavior Descriptive Statistics

Variable	Nulliparous (NP)		Multiparous (MP)	
	M	*SD*	*M*	*SD*
Rearing on Two Legs				
Day 1	5.00	2.45	9.14	5.76
Day 2	8.14	4.74	12.14	8.02
Day 3	10.14	7.63	11.86	5.59
Ventures into Open Field				
Day 1	2.14	0.90	3.43	2.07
Day 2	3.86	2.91	4.43	3.15
Day 3	4.14	2.54	3.57	2.44

Table 4

Correlation Matrix

	Explore	Open	Rear	Estrad.	Cort.	BLA	VC	PRh	TE
Average Object Exploration (Explore)	1	.256	.240	-.292	.130	.298	.240	-.028	-.033
Ventures into Open Field (Open)		1	.207	.228	-.248	.002	-.069	.192	.078
Rearing (Rear)			1	-.018	-.458	.085	-.006	-.354	-.069
Average Estradiol (Estrad.)				1	.228	.200	-.362	.081	.249
Average Corticos-terone (Cort.)					1	.186	-.433	.333	-.029
Basolateral Amygdala (BLA)						1	.519	.412	-.094
Visual Cortex (VC)							1	.238	-.026
Perirhinal Cortex (PRh)								1	.415
Temporal Cortex (TE)									1

Figure 1

Object Recognition Performance: Day 1

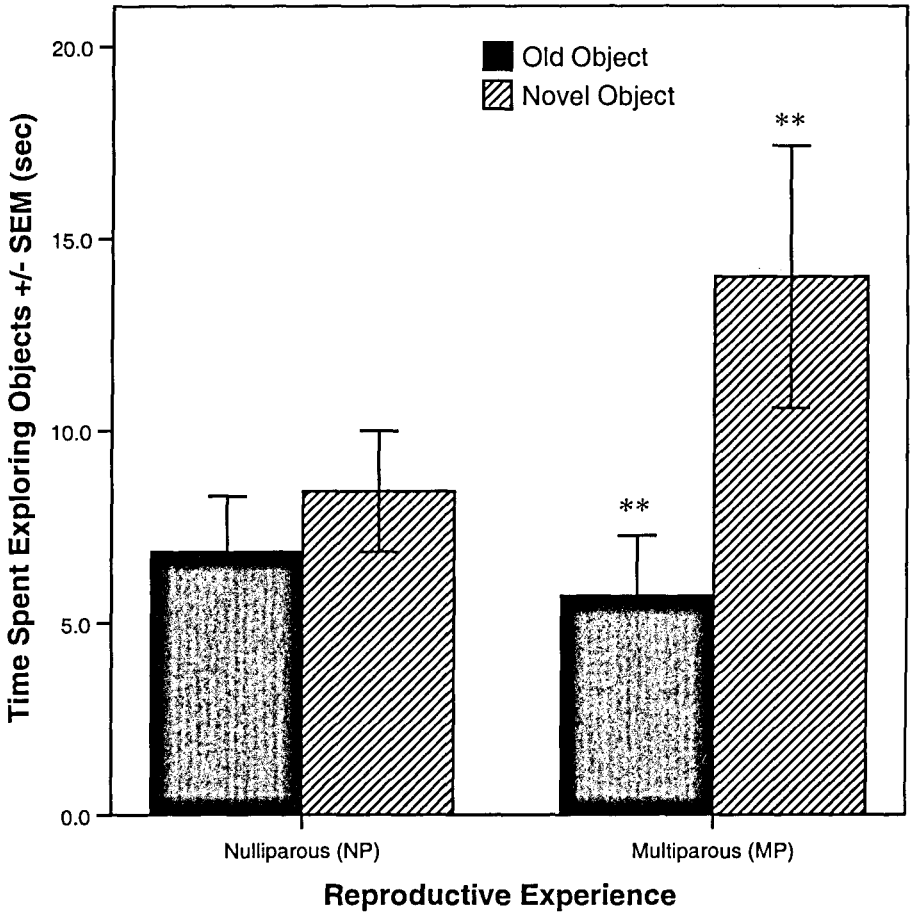

Figure 1. Object recognition performance on Day 1 of testing as measured by mean seconds exploring the old versus novel object for NP subjects ($n = 7$) and MP subjects ($n = 7$).
Note. * $p < .1$; ** $p < .05$

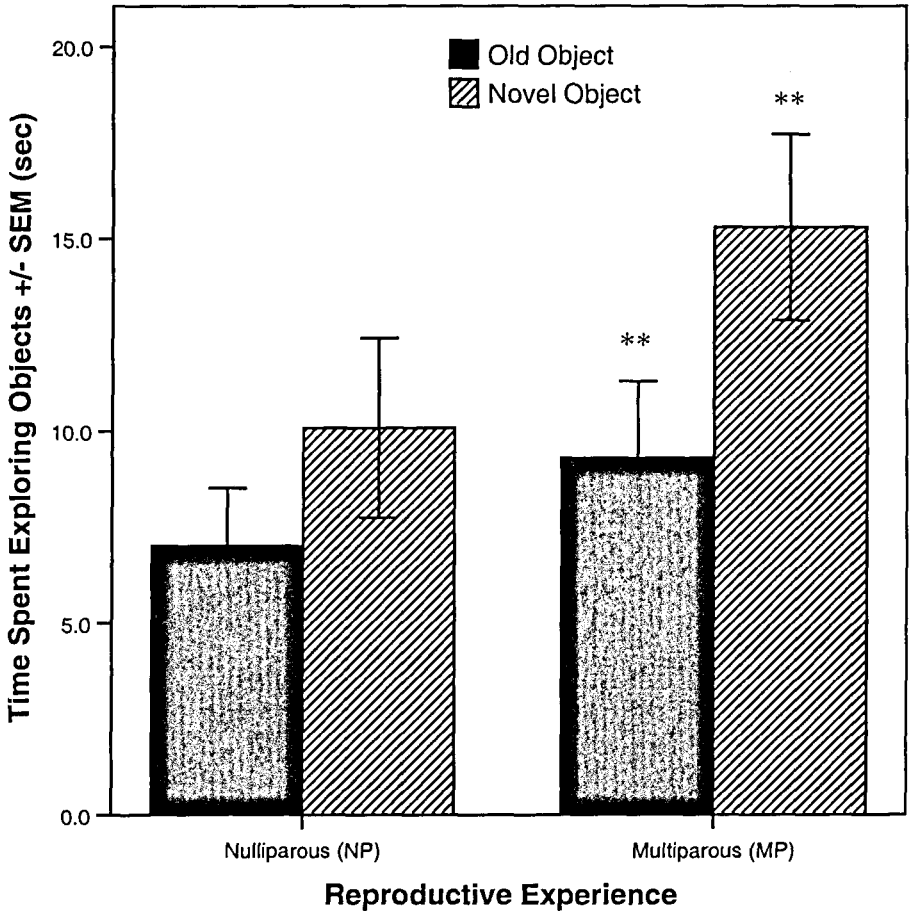

Figure 2. Object recognition performance on Day 2 of testing as measured by mean seconds exploring the old versus novel object for NP subjects ($n = 7$) and MP subjects ($n = 7$).
Note. * $p < .1$; ** $p < .05$

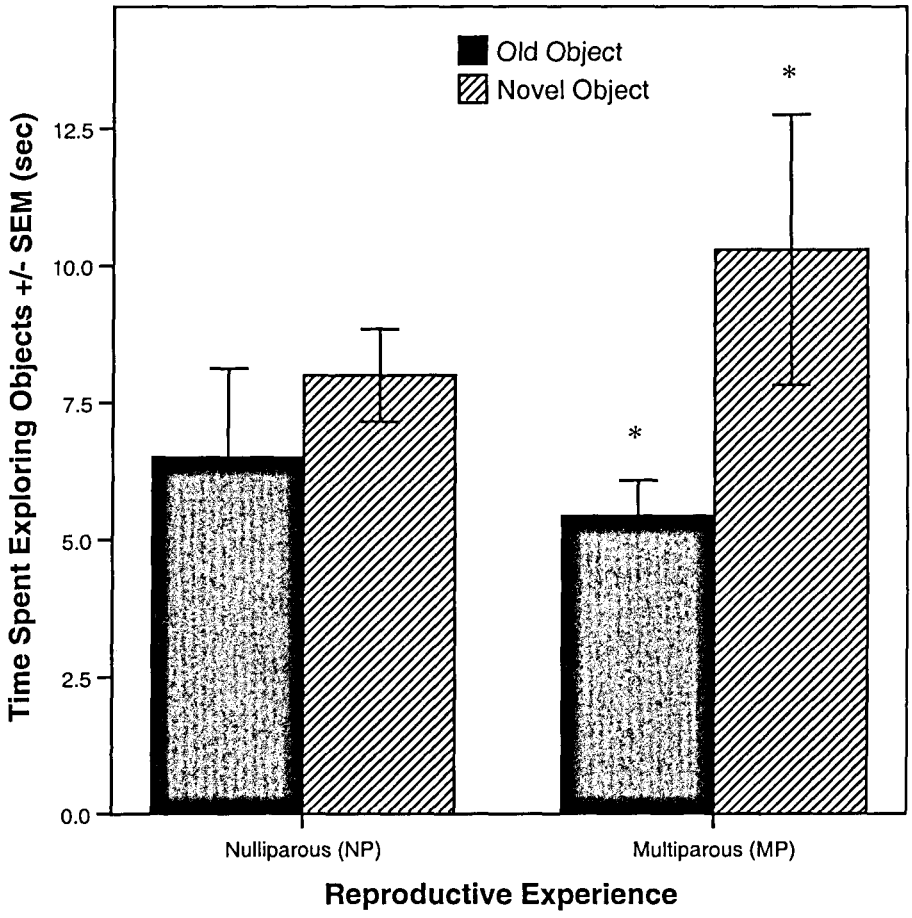

Figure 3. Object recognition performance on Day 3 of testing as measured by mean seconds exploring the old versus novel object for NP subjects ($n = 7$) and MP subjects ($n = 7$).
Note. * $p < .1$; ** $p < .05$

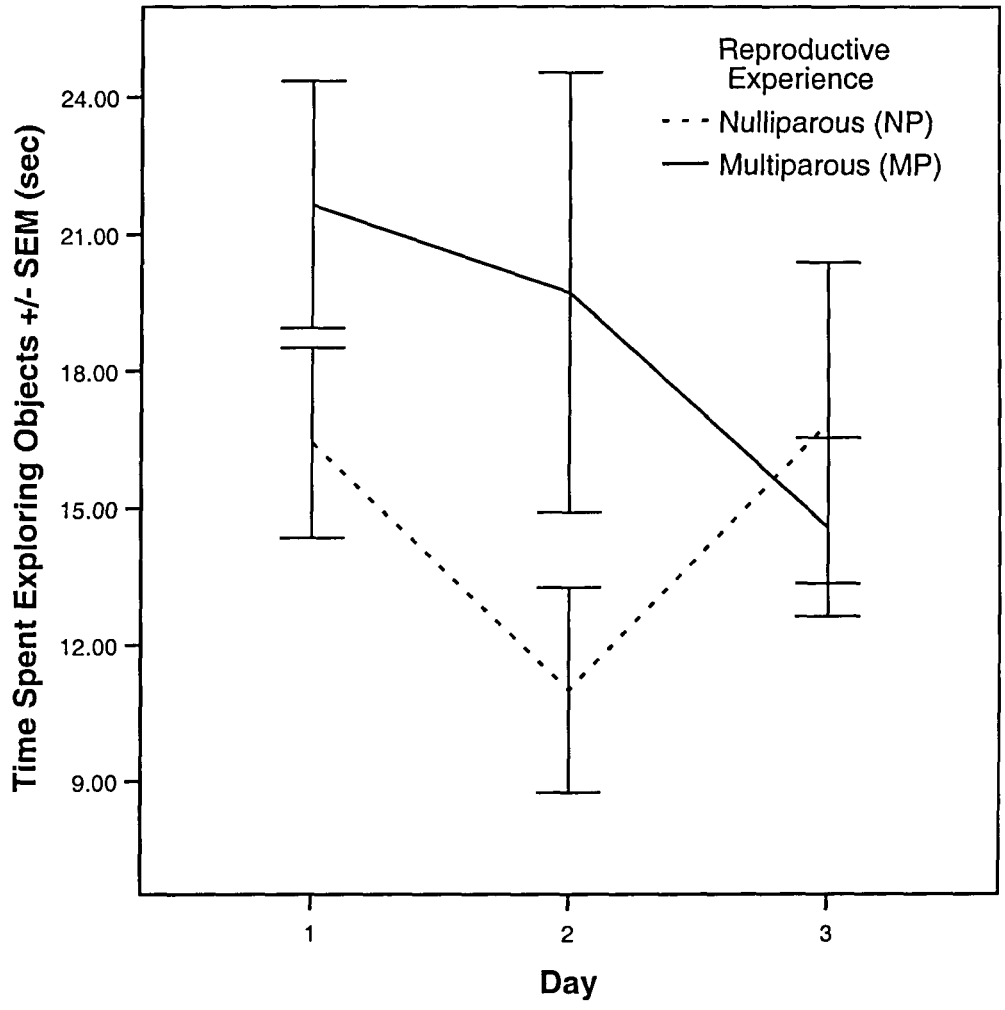

Figure 4. Total time spent exploring objects during the sample trial for NP subjects ($n = 7$) and MP subjects ($n = 7$).

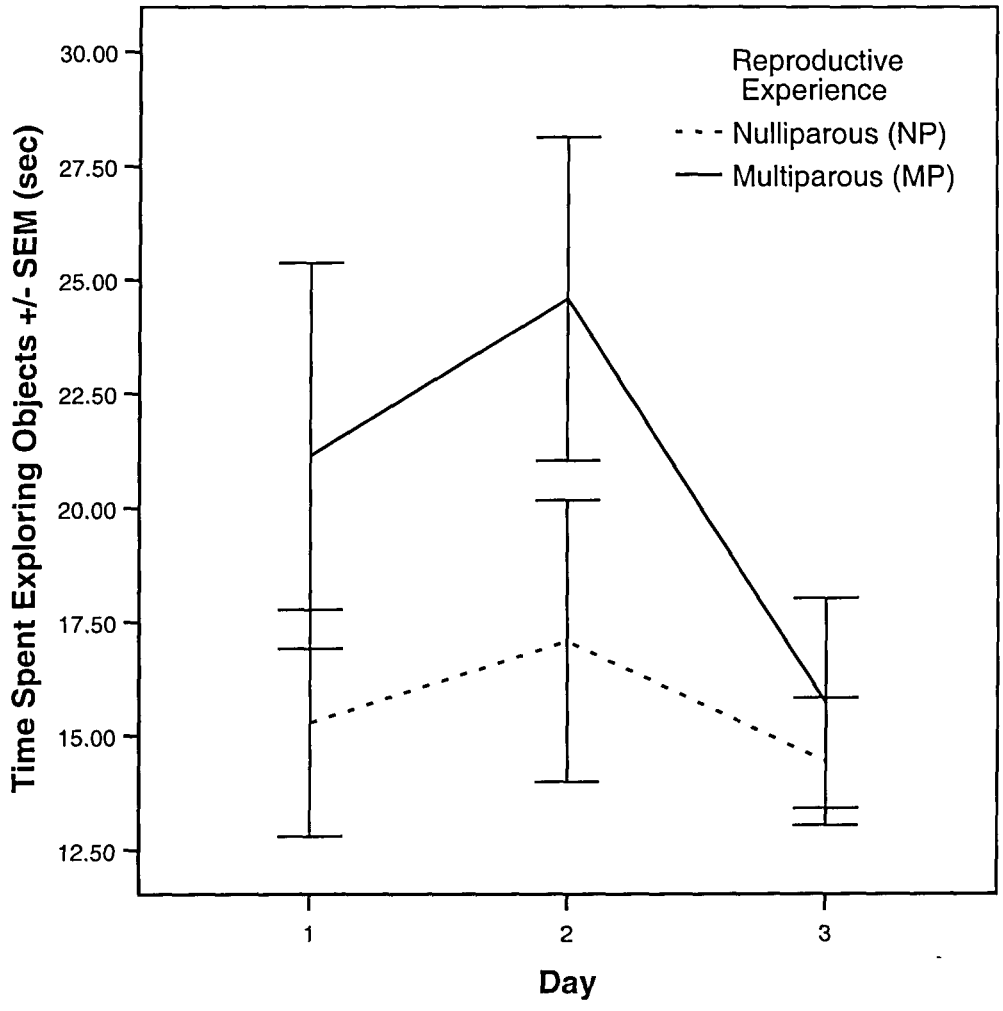

Figure 5. Total time spent exploring objects during the recognition trial for NP subjects ($n = 7$) and MP subjects ($n = 7$).