THE INFLUENCE OF OVARIAN HORMONES AND AGING ON COGNITION AND NEURONAL MORPHOLOGY

by

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A dissertation submitted to the Graduate Faculty in Psychology (biopsychology subprogram) in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

THE INFLUENCE OF OVARIAN HORMONES AND AGING ON COGNITION AND NEURONAL MORPHOLOGY

by

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Current research indicates gonadal hormones influence cognition. Understanding the effects of ovarian hormones on cognitive function is especially important because, as life expectancy is continually increasing, and hormone production declines with age, individuals, especially females, will spend a larger portion of their lives in a state of hormone deficiency. Therefore, to assess the effects of ovarian hormones on cognitive function, young, gonadally intact and ovariectomized (OVX) female rats were compared on tasks of non-spatial (Object Recognition) and spatial (Object Placement) memory. OVX rats were impaired on both tasks relative to intact rats. To determine possible mechanisms for memory loss, golgi impregnation was used to compare dendritic spine density of pyramidal cell neurons in two brain areas known to influence memory, the prefrontal cortex and hippocampus. OVX rats had significantly lower dendritic spine density for apical and basal dendritic branches in both areas compared to intact. As aging is accompanied by hormone loss, intact young and aged female rats were compared on the same tasks as the intact and ovariectomized rats in order to assess the effects of the aging process on cognitive function and morphology. Young rats demonstrated better performance on both tasks compared to aged rats. Golgi impregnation was then

completed and showed significantly lower dendritic spine density for aged rats compared to young rats on tertiary, apical dendritic branches of neurons in both the prefrontal cortex and hippocampus. To examine whether hormone replacement would be effective at reversing the observed effects of aging, aged female rats were compared on the Object Recognition and Object Placement tasks while treated subchronically with estradiol benzoate (EB) and while treated with vehicle. Results indicated that performance on both tasks was best while receiving the highest dose of EB. Prior to sacrifice, rats received either vehicle or EB treatment. Golgi impregnation assessed morphological changes associated with hormone replacement. Results demonstrated a small, though insignificant, increase in dendritic spine density for secondary, basal dendritic branches of neurons in the prefrontal cortex with EB treatment. Thus, these findings have important implications for designing treatment paradigms for women during the perimenopausal and post menopausal period.

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General Introduction

Recent research has examined the role of gonadal hormones in cognition. Ovarian hormones in particular have been evaluated for their role in memory. Describing the effects of estrogen is especially important because as life expectancy continues to increase, a greater portion of individuals lives, particularly females, will be spent in chronic hormone deficiency, such as the post-menopausal decrease in gonadal hormone levels. It has been noted that both estrogen and progesterone production are reduced by as much as 66% after menopause (Cranton, 1997). Therefore, in addition to the known physical consequences, such as decreased bone density and increased risk of cardiovascular disease (McEwen, 1999), the result that this deprivation has on cognitive ability and brain morphology needs to be examined in greater detail (McEwen, 1999).

It has been shown, for example, that ovarian hormones, estrogen and progesterone, affect memory retention, usually with memory being enhanced by administering these hormones (Dohanich, 2002, See section Gonadal Hormones and Memory for more detail). For example, Rapp et al (2003) demonstrated that ovariectomized rhesus monkeys receiving low dose, cyclic estradiol replacement performed better on tests of spatial working memory compared to vehicle treated animals (Rapp et al, 2003). The morphology of brain structures, including those important for memory, also appears to be influenced by gonadal hormones (See section Gonadal Hormones and Brain Morphology). Specifically, in laboratory animals, a higher level of estrogen in young female rats is associated with higher dendritic spine density in the hippocampus (Gould et al, 1990), an area important for

spatial memory and recognition memory (Broadbent, 2004). In addition, female rats have greater dendritic spine density during proestrus, when estrogen levels are highest, than during other stages of the cycle (Woolley, 1990). Gonadal hormones also influence the structure of the pre-frontal cortex (Hao et al, 2006), an area shown to be important for memory retention (Runyan et al, 2004). For example, Tang et al (2004), found that providing ovariectomized female rhesus monkeys with estrogen increased the number of spinophilin-immunoreactive spines in the prefrontal cortex compared to animals provided with only vehicle (Tang et al, 2004). Interestingly, despite some evidence that gonadal hormones influence the structure, and therefore function, of the prefrontal cortex, very little research has focused on this area, making further study important.

Evidence shows that aging also influences cognition (McEwen, 1999, McEwen, 2002). Experiments have demonstrated that aged animals do not perform as well on memory tasks as young animals (Norris et al, 1996). Specifically, it has been found that working and reference memory are impaired in older rodents compared to younger animals (Schwartz et al, 1998). The morphology of brain structures changes with aging as well. According to Rosenzweig and Barnes (2003), "anatomical and electrophysiological studies indicate that the hippocampus of the aged rat sustains a loss of synapses in the dentate gyrus, a loss of functional synapses in area CA1, a decrease in the NMDA-receptor-mediated response at perforant path synapses onto dentate gyrus granule cells and an alteration of Ca^{2+} regulation in area CA1." (Rosenzweig and Barnes, 2003)

So, while there is evidence that both ovarian steroids and aging can exert an influence on cognition and also morphology, little has been done to examine the association between these factors. Therefore, the aims of this thesis are to provide further information concerning the relationship between circulating gonadal hormones, specifically estrogen, cognitive function and brain morphology in both young and aged female rats.

Mechanisms of estrogenic influences on cognition

The influence of estrogen on memory can occur through both genomic and non-genomic mechanisms. Genomic mechanisms are those which involve transcription. Typically, genomic effects take place over the course of several hours, even days (Deroo and Korach, 2006). The process begins when estrogen diffuses into the cell and binds with nuclear estrogen receptors (ERα or ERβ). This complex can then bind directly with the estrogen response element on the DNA or by interacting with activator protein 1 (AP1) regions of genes. Activation of AP1 sites, in turn, influences transcription, including the transcription of neurotrophics such as neural growth factor (NGF) and brain derived neurotrophic factor (BDNF) which may influence the morphology of neurons in areas of the brain important for memory. For example, Gibbs (1999) found a significant increase in BDNF mRNA in the hippocampus of adult ovariectomized rats after receiving estrogen compared to ovariectomized animals that received vehicle (Gibbs, 1999). The resulting changes in neural morphology may, in turn, influence functions mediated by estrogen, such as ovulation and, in regard to this thesis, cognitive function.

Non-genomic effects of estrogen are effects that do not involve protein synthesis, and are more rapid than genomic effects, often taking place in just seconds or minutes (Deroo and Korach, 2006). In this case, instead of binding with nuclear receptors, as with genomic mechanisms of action, estrogen binds with receptors in or near the cell membrane, thereby influencing cellular activity. For example, Luine et al (2003) found that there was enhancement of both visual and place memory in rats treated with estrogen compared to vehicle treated animals (Luine et al, 2003). Further, this enhancement occurred when treatment was administered only 30 minutes prior to the task, making it unlikely that enough time had passed for genomic changes to occur. It has also been observed that firing rates of neurons have been altered only seconds after applying estrogen (Falkenstein et al, 2000). These rapid changes in neural activity, when occurring in brain structures relevant to learning and memory, may then exert an influence on cognitive function. Additionally, the estrogen, 17α–estradiol, has been found to be effective in activating the extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAP) pathway, a mechanism responsible for the regulation of neuronal plasticity, including brain structures important for cognitive ability. Further, activation of this cell membrane pathway influences the excitability of neurons in brain areas important for memory (Bi et al, 2001).

Gonadal Hormones and Memory

Previous studies have shown a relationship between estrogen and cognitive ability. For example, Gibbs et al (2004), found that chronic estradiol replacement to

young, ovariectomized rats resulted in faster acquisition of a delayed matching to position spatial memory task when compared to those groups not receiving estrogen. A study conducted by Luine et al (1998) examined the effects of estrogen on the spatial memory performance of ovariectomized rats using an 8 arm radial maze. Rats that began radial arm maze trials 12 days after estrogen treatment began performed better than ovariectomized rats (Luine et al, 1998). More recently, Luine et al (2003) demonstrated estrogens lead to rapid enhancement of visual and place memory in rats by measuring ability to discern between old and new objects (visual) and locations (place). Ovariectomized animals that received 17α-estradiol, 17β-estradiol or diethylstilbestrol 30 minutes prior to the tasks were better able to discriminate between the old and new on both the visual and place memory tasks when compared to animals that received only vehicle (Luine et al, 2003).

Animal studies examining memory performance at different stages of the rat estrus cycle have demonstrated better performance on memory tasks during phases of the cycle associated with higher estrogen levels. For example, females in proestrus, when estrogen levels are high, have demonstrated better performance on the Morris water maze compared to those in estrus, when estrogen is lower (Warren and Juraska, 1997). Research using an inhibitory avoidance task demonstrated that female rats in proestrus performed better compared to males and to females in diestrus, when hormone levels are lower (Rhodes and Frye, 2004). Additionally, animals that had been ovariectomized but treated with estrogen performed better on this task than ovariectomized animals treated with only vehicle. Further, administering estrogen to the ovariectomized females immediately upon completion of training was also found

to enhance performance on this task compared to animals that received only vehicle (Rhodes and Frye 2004).

Beneficial effects of estrogen on cognitive performance have also been found in humans. A study using elderly, healthy women who had not previously taken estrogen replacement examined the effects of 3 weeks of estradiol treatment on cognitive ability. Compared to subjects given placebo, subjects treated with estradiol demonstrated significant post-treatment improvement in visuo-spatial ability as measured by a mental rotation task (Duka, 2000). Another study comparing women on estrogen replacement therapy to untreated women found superior verbal learning and memory in treated subjects (Maki, 2001).

It is important to note that not all studies have found that ovarian hormones are beneficial for cognitive performance (Varga et al, 2002). However, many of these animal studies used paradigms that are extremely stressful, such as water maze tasks (Markowska et al, 2002) or tail shocks (Wood et al, 1998). Further, Wood et al (1998) found greater learning impairment in stressed female rats that were intact compared to those that were ovariectomized and better performance in unstressed females that were intact compared to those that were ovariectomized, concluding that the impaired response due to stress is a result of the presence of circulating ovarian hormones and estrogen receptors (Wood et al, 1998). These findings and previously discussed results, therefore, imply that ovarian hormones do enhance cognitive performance when conditions for testing are non-stressful. Recently Daniels (2006) reviewed the literature on estrogenic effects on memory and has offered other explanations for the

lack of consistency in its effects on memory and suggests that the type of task and strategies for solving tasks may also be important determinants in estrogen's actions .

Gonadal Hormones and Brain Morphology

The morphology of brain structures important for memory is also influenced by the level of circulating gonadal hormones. For example, Kadish and van Groen (2002) reported a decreased axonal sprouting response in the entorhinal cortex after lesioning with ibotenic acid in untreated ovariectomized mice compared to control mice and ovariectomized mice receiving estrogen (Kadish and van Groen, 2002). Research using the golgi impregnation procedure has shown that gonadal steroids, such as estrogen, are involved in regulating dendritic spine density in hippocampal pyramidal cells (Gould et al, 1990). Gould et al (1990) reported notable decreases in dendritic spine density in area CA1 of the hippocampus in female rats that were ovariectomized (Gould et al, 1990). However, this decrease was prevented in those animals that received estradiol or combined estradiol and progesterone replacement. It has also been shown that fluctuating levels of gonadal hormones throughout the estrous cycle of the rat result in changes in neuronal morphology within the brain. Specifically, compared to rats in estrus, rats in proestrus, when circulating levels of gonadal hormones are highest, were observed to have a significantly higher dendritic spine density in the CA1 subfield of the hippocampus (Woolley et al, 1990).

Further evidence that ovarian hormones influence the morphology of brain structures has been found in studies using non-human primates. Tang et al (2004) reported an increase in spinophilin immunoreactive spines in the prefrontal cortex of

female rhesus monkeys in association with estrogen treatment (Tang et al, 2004). Further, Leranth et al (2000) found hormone dependent morphological changes in research using African green monkeys. The study demonstrated that, compared to animals that received short term ovariectomy (10 days), longer term ovariectomy (30 days) resulted in an apparently permanent loss of more than 30% of substantia nigra dopamine cells (Leranth et al, 2000). More recently, Leranth et al (2004) compared spine synapse density of intact and gonadectomized St. Kitts vervet monkeys in area CA1 of the hippocampus. The results indicated a significantly lower spine synapse density in this area for the gonadectomized group compared to the controls (Leranth et al, 2004). These findings demonstrate the importance of gonadal hormones in the maintenance of normal cell structure within the brain, including those that are important for cognitive ability.

Aging and cognition

Research has also shown that aging is associated with changes in cognitive ability, possibly due to the decreased levels of hormones being produced (McEwen, 1999). For example, working memory deficits have been observed in aged compared to young rats. Specifically, aged male rats (24 months) were found to be impaired compared to young rats (4 months) on a repeated acquisition task using the Morris water maze in which the position of the escape platform changed between sessions (Frick et al, 1995). Ward et al (1999) compared the ability of middle aged and aged male rats on a contextual fear conditioning task. After being trained on the task, animals either received a dorsal hippocampal lesion or sham

surgery at intervals of 1, 7, 14 or 28 days after training. When given retention trials, the aged animals were observed to have deficits in contextual memory for all intervals while middle-aged animals demonstrated only a temporally based deficit. Similarly, in a study comparing young (3 months) and aged (24^{+} months) female gerbils, aged animals were found to have working memory deficits compared to the young on performance on the radial arm maze (Schwartz, 1998). Other findings have shown that long term potentiation in the hippocampus of aged animals decays more rapidly compared to younger animals and that this decay is associated with greater spatial memory impairment (Norris et al, 1996). These findings suggest, therefore, that increased age in animals, both male and female, is associated with impaired cognitive function relative to younger animals.

Human studies comparing the young and aged have also demonstrated age related deficits in cognitive ability. Using a working memory task, the Operation Span, participants were required to perform a memory task and verify math equations simultaneously (Smith et al, 2001). While both young and aged participants showed an increased latency and decreased accuracy when performing the 2 tasks concurrently compared to separately, performance was significantly affected by age with older subjects performing less well than younger subjects. Other experiments have shown decreases in scores on memory tests as a function of age as well, regardless of the sex of the subject (Choudhury et al, 2003). These findings indicate that aging in humans results in changes in cognitive ability similar to the changes observed in animal studies.

Aging and Hormone levels

It has been documented that gonadal hormone production decreases with age (McEwen, 1999, Morrison et al, 2006). The age associated decrease in circulating gonadal hormones also tends to be greater in females than in males. For example, Bowman et al (2006) found, in comparing 20-month-old Fischer 344 rats, that serum estradiol levels were approximately 50% lower in the females than the males. The dramatic drop in estrogen levels in females is likely the result of a rapid reduction in the number of ovarian follicles (Faddy et al, 1992) as well as the decrease in volume of the ovaries (Andolf et al, 1987) that occurs with age.

Aging, Hormone Replacement and Cognitive Function

As gonadal hormone production decreases with aging, some research has examined the effects of hormone replacement, usually estrogen, on aged females. For example, in a study on cognitive functioning in aging female rats, better performance on tasks of working memory was seen in rats receiving hormone replacement compared to untreated rats (Markowska et al, 2002). Notably, on the delayed non-matching to position task, using intertrial delays of 1 – 30 minutes, performance deficits progressed from long delays to short delays in untreated rats. This decline in performance was lessened in rats receiving estrogen treatment (Markowska et al, 2002). Further, in a study using the Morris water maze, beneficial effects were observed in aged, ovariectomized female rats that were receiving ovarian hormone replacement relative to control animals not receiving hormone

replacement (Markham et al, 2002). Specifically, compared to rats receiving estrogen or estrogen plus progesterone, control animals showed significant overnight forgetting during task acquisition. This forgetting was prevented in rats that received hormone replacement (Markham et al, 2002). However, it should be noted that subjects receiving chronic estrogen treatment in this study only demonstrated enhanced performance when primed with additional estrogen, creating a cyclical pattern of hormone fluctuation. Therefore, it is possible that over time, sensitivity to estrogen decreases, making the priming with additional estrogen necessary to achieve the same response as the rats aged. Vaucher et al (2002) also showed that, while aging was associated with poorer memory, estrogen treatment enhanced memory function. Specifically, ovariectomized, aged female mice (24 months) treated with estrogen (5mm silastic capsules resulting in circulating estrogen levels of 12 – 18 ng/ml) had better recall for a previously encountered object compared to untreated ovariectomized mice (undetectable circulating estrogen levels). Therfore, rodent models of aging are supportive of estrogen replacement improving memory function in age-related cognitive impairment.

Similar results to those seen with rats have been obtained in studies using primates. For example, Rapp et al (2003) found that estrogen treatment improved cognitive function in aged rhesus monkeys that were ovariectomized in comparison to vehicle treated subjects. Specifically, the estrogen treated monkeys demonstrated enhanced performance on a visuospatial task of working memory involving locating a food reward after progressively longer inter-trial delays compared to vehicle treated monkeys (Rapp et al, 2003). Results from studies using humans, however,

are often less definitive. There are studies showing potential cognitive benefits of hormone replacement therapy for aged human females, such as an association between higher estradiol levels and better delayed verbal memory and retrieval efficiency (Drake et al, 2000). Other studies, however, have shown no effect on cognitive performance in subjects receiving estrogen replacement (Wang et al, 2000). However, problems with the generalizability of the results of such studies arise because, often, women receiving hormone replacement have a higher level of education, higher socioeconomic status and are in better overall health than women who do not receive treatment (Taber at al, 2001). Therefore, it is possible that estrogen may be beneficial when the above-mentioned parameters are controlled, making further study warranted.

Aging and brain morphology

Other research has found aging to be associated with morphological changes in brain structures, including those important for memory. For example, anatomical studies have shown that the aged rat experiences a loss of synapses in the dentate gyrus as well as a loss of functional synapses in the CA1 subfield of the hippocampus (Rosenzweig and Barnes, 2003). It has also been found that pyramidal cells in area CA3 of the hippocampus in aged rats (24 months) have a diminished ability to form new synapses in response to deafferentiation lesions compared to young rats (3 months) (Schauweker et al, 1995). Additionally, decreases in dendritic spine density have been observed with aging in rats.

Specifically, in the frontal cortex, pyramidal neurons showed lower dendritic spine density on terminal dendritic branches in aged (24 months) compared to young (3 months) rats (Mervis et al, 1991). The dendritic spine density was restored, however, in rats that were treated with neural growth factor for a period of 4 weeks. Such studies provide evidence that aging is associated with morphological changes in the brain of rodent and that, in some instances, appropriate treatment can reverse these changes.

Similar results have been found in humans. Specifically, Pantel et al (2003) conducted a comparison of subjects without aging associated cognitive impairment, with aging associated cognitive impairment and with Alzheimer's disease (Pantel et al, 2003) The volume of the parahippocampal gyrus was assessed using an MRI and it was found that subjects with aging associated cognitive impairment had a significantly smaller volume (-12%) of the right parahipocampal gyrus in comparison to those who were cognitively unimpaired. Those who had Alzheimer's disease, however, were found to have a significantly smaller volume of the parahippocampal gyrus on both the right (-24%) and left (-29.6%) in comparison to subjects without aging associated cognitive impairment, suggesting a relationship between brain morphology and cognitive function. Driscoll et al (2003) demonstrated a decreased bilateral, right, left, anterior and posterior hippocampal volume in aged subjects (aged 60-85 years) compared to young subjects (aged 20-39 years) using magnetic resonance imaging and proton magnetic resonance spectroscopy, which suggests decreased neuronal density in this structure (Driscoll et al, 2003). There was also an impaired ability to perform

hippocampal dependent tasks, the virtual water maze and transverse patterning discrimination task, among the aged subjects, indicating that the hippocampus may be undergoing structural changes due to aging that impair cognition (Driscoll et al, 2003). These findings are supportive of humans experiencing age related morphological changes in the brain similar to that seen in animals. Additionally, as some age-related morphological changes have been reversed with treatment in animals, further research in this area may provide potential therapies for aging humans as well.

Specific Aims

As reviewed above, research has shown that both ovarian hormones and aging influence memory (see sections Gonadal Hormones and Memory, Aging and Cognitive Function) and have an effect on the morphology of brain structures associated with cognitive function (see sections Gonadal Hormones and Brain Morphology, Aging and Brain Morphology). However, most of the recent research has focused on the effects of hormone replacement, rather than loss of hormones. Life expectancy of the human population is constantly increasing, though, and gonadal hormone production decreases with age, so more time will be spent by individuals, especially females, in a state of hormone deprivation. Also worth noting is the fact that the majority of animal research examining the influence of hormones on cognitive function uses young animals. However, as discussed previously, memory loss often occurs in conjunction with aging, as does a decline in circulating gonadal hormone levels and both circulating gonadal hormones and

aging appear to influence brain morphology. Therefore, the effect of hormone deprivation, rather than replacement, as well as the role of age associated changes on areas of the brain related to memory, need to be examined further.

The goals of the current thesis are to examine the extent to which gonadal hormones contribute to cognitive function, specifically memory, in young and aging rats. Further, the relationship between changes in cognitive function and neuronal morphology will also be examined. To address these questions, the current research seeks to determine whether ovarian hormone deprivation in young female rats affects memory and neuronal morphology. Aging also results in a decrease in gonadal hormone production, analogous to that seen with ovariectomized young animals. Therefore, intact young and aged female rats will be compared as well, to examine whether aging results in memory and morphological changes similar to ovariectomy. As any changes observed in association with aging may be due, at least partially, from decreased production of gonadal hormones, the current thesis will also examine the potential benefits of hormone replacement therapy for enhancing cognitive performance and maintaining normal neuronal morphology by comparing aged female rats, both treated and untreated with estradiol.

Specific Aim 1

Effects of chronic hormone deprivation on memory and neuronal morphology

Young, gonadally intact and ovariectomized female rats will be compared on tasks of visual and spatial recognition memory. As prior research has shown a relationship between estrogen and cognitive function, it is hypothesized that ovariectomized rats will show a decrease in performance compared to the intact rats on both the visual and spatial recognition tasks. Additionally, ovarian hormones have been shown to influence brain morphology. Therefore, to determine possible mechanisms for memory loss, golgi impregnation will be used to compare dendritic spine density, "thought to represent the neuroanatomic basis of learning and memory" (Mervis et al, 1991), in two areas of the brain known to have a role in memory, the prefrontal cortex and hippocampus. It is hypothesized that a decrease in spine density will be found in the ovariectomized females.

Specific Aim 2

The effect of age on memory and neuronal morphology

To assess the role of the aging process on memory, intact young and aged female rats will be compared on the same visual and spatial memory tasks as the intact and ovariectomized rats in aim 1, to determine if aging results in cognitive impairment similar to the relationship seen between ovarian hormones and cognition. As aging is accompanied by hormone loss, it is hypothesized that aged rats will show behavioral and morphological changes similar to the chronically hormone deprived, young female rats. Therefore, the same golgi impregnation procedure used in specific aim 1 will be applied to determine whether possible changes in dendritic spine

density with aging are similar to those that occur in response to ovarian hormone deprivation in young animals.

Specific Aim 3

The effect of estrogen treatment on memory and neuronal morphology in aged rats

As both circulating ovarian hormones and aging have been shown to influence cognitive function and dendritic spine density, the effects of estrogen treatment to aged rats will be evaluated using the same visual recognition and spatial recognition task as in the previous specific aims and golgi impregnation will also be applied. It is hypothesized that treatment of aged female rats with estrogen will be associated with an increase in dendritic spine density in both the prefrontal cortex and hippocampus and better ability to discriminate on the memory tasks compared to the untreated aged rats. However, as discussed previously, some evidence suggests older subjects that have experienced long-term estrogen deprivation are less sensitive to the effects of hormone treatment. Thus, this aim will further the understanding of the role of gonadal hormones and the aging process on memory through investigating possible changes induced in the morphology of memory related brain structures.

Specific Aim 1

Effects of chronic hormone deprivation on memory and neuronal morphology

This specific aim has been published (Wallace et al, 2006). Young, gonadally intact and ovariectomized female rats will be compared on tasks of visual (Object Recognition) and spatial recognition (Object Placement) memory in order to assess the influence of gonadal hormones on memory. As prior research has shown a relationship between estrogen and cognitive function (see section Gonadal Hormones and Memory), it is hypothesized that intact rats will demonstrate enhanced performance on both the visual and spatial recognition tasks compared to ovariectomized rats. Further, to determine possible mechanisms for memory loss, golgi impregnation will be used to compare dendritic spine density in two areas of the brain known to have a role in memory, the prefrontal cortex and hippocampus. As there is existing evidence that dendritic spine density is affected by ovarian hormone levels (Gould et al, 1990, Woolley, 1990) it is hypothesized that lower spine density will be found in the ovariectomized females compared to the intact females.

Subjects

Sixteen intact female Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, Indiana), aged 2 months on arrival, were used for this study. Animals were cared for in accordance with the NIH Guide for Care and Use of Animals and the experiment was approved by the Institutional Animal Care and Use Committee.

The rats were double housed and free access to food and water was permitted for the entire duration of the experiment. Animals were maintained in a 12-hour light/dark cycle (lights off 7:00pm). Daily vaginal smears were taken for the first 2 weeks to ensure normal cycling. All animals were found to have normal cycles.

Methods

General Methods

Animals received both non-spatial (Object recognition) and spatial (object placement) memory assessments (Luine et al, 2003). The trials consisted of a sample trial (T1) and a recognition/retention trial (T2). During T1, two identical objects were placed at one end of an open testing field and the amount of time the rat spent exploring the objects was recorded for 3 minutes. Exploration consisted of actions such as touching, whisking and climbing on the objects as long as the objects were not being used as a vantage point to see over the enclosure. Following the sample trial, an intertrial delay of 4 hours for Object recognition and 2 hours for Object Placement was initiated. At the end of the delay, the retention trial was administered, with either one of the objects being replaced (Object recognition, or the location of one of the objects being changed. The animal was placed in the testing field for 3 minutes and the amount of time spent exploring the new object or location and the old object or location was recorded. The recognition and placement tasks are useful measures of memory for several reasons. The tasks utilize spontaneous behavior, so extensive training is not necessary. Also, as there is no contingency rule to be learned, trials can be administered to the same subjects repeatedly. Further, no food or water

deprivation is required, which lessens the amount of stress experienced by the animals.

Procedures

Animals were provided with a one week acclimation period, which included daily handling, after arriving at the laboratory. Following acclimation, an open field paradigm was utilized to allow animals to habituate to the testing field. This involved placing the rat in a field composed of sectors measuring 9"x 9", 3 sectors in length, and 5 sectors in width. Animals were allowed to explore the open field for 6 minutes. During this time, behaviors such as visits to a sector, rearing up on hind legs, placing paws on the walls of the testing field, grooming and defecation were noted. The number of observations of each behavior in individual sectors was recorded for each animal.

Habituation to the memory tasks was next given. The same testing field used earlier was reduced to 3 sectors in length and 3 sectors in width. For Object Recognition, a sample trial (T1) was provided in which two identical objects were placed at one end of the testing field and a 3-minute exploration time was allowed. The total amount of time the animal spent with either object, evidenced by touching, sniffing, whisking or otherwise interacting with the objects (other than using the object as a vantage point to see over the enclosure) was recorded using a stopwatch. The experimenter was positioned at the far end of the testing field while making all observations. To allow the rats to become accustomed to the inter-trial delay, delays of 1 minute, 10 minutes, 1 hour, 2 hours and 4 hours, respectively, were then enforced. At the end of the delay, one of the objects was replaced with a novel

object, and the animal was returned to the testing field. Three minutes exploration time was again provided (T2) and the time the animal spent with each of the 2 objects was recorded separately. Habituation was considered complete when the animals could successfully differentiate between the old and new object, defined as spending significantly greater time exploring the new object compared to the old object, at a 4-hour delay, which young, intact female rats have previously been shown capable of completing (Luine, 2002, Bisagno et al, 2003).

Habituation was then given for the Object Placement task. Using the same testing field, a sample trial was provided in which 2 identical objects were placed at one end of the field and a 3-minute exploration time was allowed (T1). The total time the animal spent interacting with the objects, evidenced by touching, sniffing, whisking or otherwise interacting with the objects (other than using the object as a vantage point to see over the enclosure) was recorded using a stop watch. Inter-trial delays of 10 minutes, 1 hour and 2 hours, respectively, were then enforced. At the end of the delay, one of the objects was moved to a new position and the animal was returned to the testing field. Three minutes exploration time was again provided (T2) and the amount of time the animal spent interacting with the object in the new position versus the old position was then recorded. Habituation was considered complete when the rats could successfully differentiate between the objects in the old and new position at a 2 hr delay (Bisagno, 2003).

Surgery

Upon completion of habituation to memory tasks (see above), half of the

animals were ovariectomized and half received a sham surgery under isoflourane anaesthetic using aseptic procedures. Ovariectomized rats had the ovaries removed bilaterally by making an incision through the abdominal wall and exposing the ovary. The oviduct was then ligated and the ovary removed. The abdominal wall was then closed with chromic gut sutures and the skin was closed with wound clips. Topical antibacterial ointment was then applied to prevent infection. Sham surgery subjects received the same incisions as the ovariectomized animals and were sutured in the same way, but the ovaries were palpated instead of removed. Subjects were assigned to groups so that the two groups did not differ in pre-surgery performance. All rats were returned to their home cages following the surgical procedure. A one-week recovery period was provided before testing resumed.

Post-Surgical Testing

After the recovery period, Object Recognition and Object Placement testing resumed. The animals were tested once a week on 2 consecutive days, with intact rats at random cycle stages, Object Recognition followed by Object Placement, at the longest delay completed (4 hours for Object Recognition, 2 hours for Object Placement), for 7 weeks.

Golgi Impregnation

After completion of post-surgical testing, one week later animals were sacrificed by rapid decapitation and the brains were removed for golgi impregnation. The brains were then blocked by slicing through the optic chiasm and removing the

cerebellum and brain stem. The staining procedure was carried out using FD Rapid GolgiStain kit (FD NeuroTechnologies, Inc). Brains were first rinsed in a 0.1 M phosphate buffer to protect the tissue. Tissue was then immersed in the Golgi-Cox solution, a commercial combination of potassium dichromate and mercuric chloride, for 14 days. Brains were then transferred into a sucrose-based solution and stored at 4˚ C for 2-7 days. Next, the frontal cortex and hippocampus of the brain was sliced into 100 μm sections on a cryostat and mounted on gelatin coated slides, yielding approximately 6 sections for the prefrontal cortex and 12 sections for the hippocampus. To protect specimens from ice damage while in the cryostat chamber, specimens were flash frozen before sectioning using Histofreeze-2000 (Fischer Scientific). A drop of the sucrose solution was then placed on each of the sections on the slides and the excess absorbed off with filter paper. Slides were then allowed to air dry at room temperature. Once sufficiently dry, sections were rinsed in distilled water and placed in solution containing silver nitrate for 10 minutes. Slides were then rinsed again in distilled water and dehydrated in 50%, 75%, 95% and 100% ethanol, respectively. Sections were then cleared in Protocol (Fischer Scientific) and coverslipped in Permount®.

Morphological analysis

Dendritic spine density of pyramidal neurons in the medial pre-frontal cortex (layer II – III) (Fig. 1) and hippocampal subfields CA1 and CA3 (stratum lacunosum and stratum oriens) (Fig. 2) was analyzed using the Spot Advanced program (Diagnostic Instruments, Inc., 2001-2002). For each animal entered into the data, the

spines of 6 tertiary apical dendrites and 6 secondary basal dendrites, only one each per neuron in the areas mentioned were counted. Dendrites that were counted had to meet several criteria. First, the cell body had to be located within the area of interest. Second, the length of the branch being counted had to be unbroken. Third, the length of the dendritic branch being counted had to be isolated well enough for the view to be unobstructed. These criteria were applied to ensure the same portions of the dendritic branches were being analyzed both within and between animals. Dendrites meeting these criteria were photographed with the Spot Advanced program under the 100x objective and the length of the branch was measured using the Spot Advanced program as well. The spines were counted by two different investigators and the mean of the two counts was obtained and used to calculate the spine density per 10 micrometers. Comparison of ovariectomized versus intact spinal density was completed using a two way ANOVA (group x brain area) and two sample T-test.

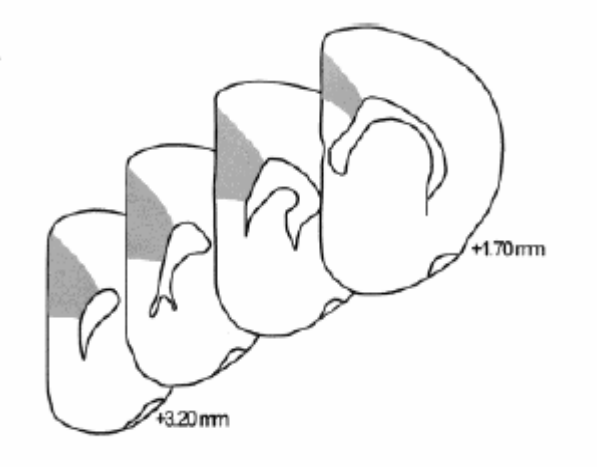

Fig. 1. Diagram of coronal sections of rat medial prefrontal cortex. Shaded areas indicate layers II/III of the prefrontal cortex. Coordinates indicate position relative to Bregma. (Wellman, C., 2001)

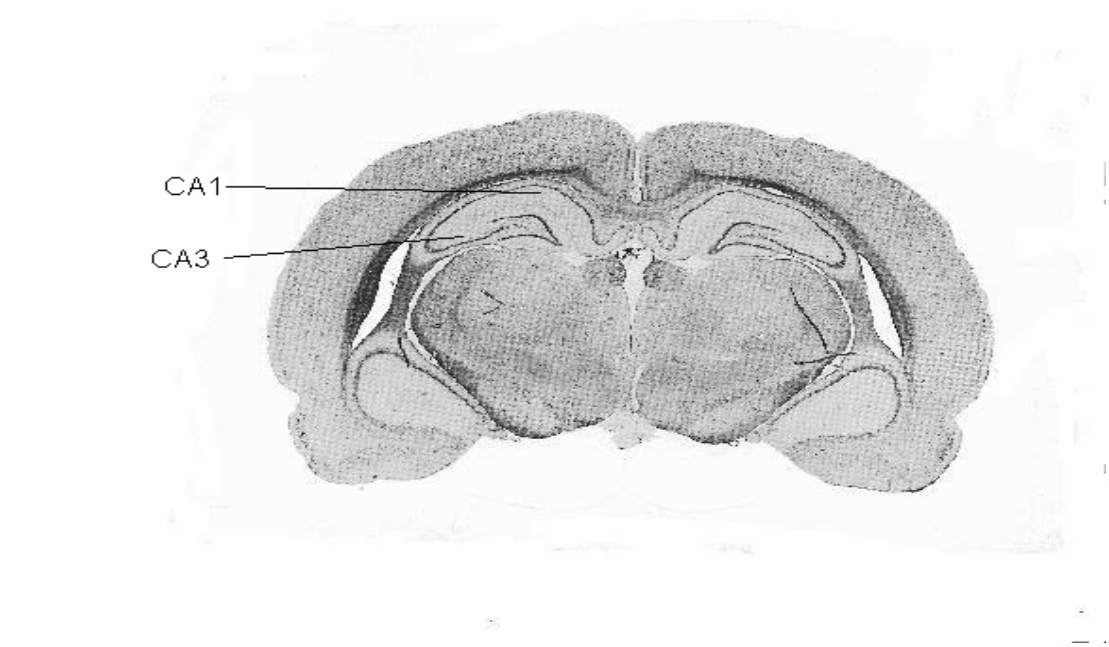

Fig. 2. Photograph of coronal section of hippocampus.
Structure is outlined and areas CA1 and CA3 are labeled, indicating areas used in dendritic spine counting. (Pelligrino, L., Pellegrino, A., Cushman, A. (1979). A Stereotaxic Atlas of the Rat Brain. © 1967, 1979 Plenum Press, NY)

Statistics

Behavior data for the Object Recognition and Object Placement tasks prior to surgery was analyzed using a two–way ANOVA (group x object or group x location, respectively). Post-surgical Object Recognition data was analyzed using two-way ANOVA (group x week) and Repeated Measures ANOVA (group x object x week). Paired t- tests were used for post hoc analysis. Post-surgical Object Placement data was analyzed using two-way ANOVA (group x week) and Repeated Measures ANOVA (group x location x week). Paired t –tests were used for post hoc testing. Analysis of dendritic spine density was completed using two –way ANOVA (group x brain area) and two-sample t-tests. All statistics were completed using Number Cruncher Statistical Systems (NCSS)2000 software (©1999 Jerry Hintze).

Results

Prior to surgery, subjects in both the OVX and sham OVX groups were able to discriminate between old and new objects on the OR task with a 4 hour inter-trial delay between the sample trial and the recognition trial (Two-way ANOVA, group x object, indicated a significant object effect, $F(1,28)= 20.61$, $p<0.001$). Thus, both groups spent significantly greater time with the new object as compared to the old object prior to surgery. Fig. 3 shows the results of pre- and post-surgical OR testing as a ratio, time spent with the new object/time with the old + new object. Analysis of the post-surgical ratios by two-way ANOVA (group x week) showed a significant group effect ($F(1,6) = 19$, $p<0.00003$) and no significant week or group x week interactions, indicating that OVX was associated with a decline in performance of OR

following surgery. Gonadally intact rats had ratios of 0.6 and above while OVX rats performed at chance level, approximately 0.5, an indication of spending the same amount of time exploring the old and the new object. Additional analyses of the exploration times were conducted by a repeated measures ANOVA (group x object x week.). Results showed a significant group x object interaction ($F(1,14)=12.75$, $p<0.0004$). Post hoc testing by a paired t-test determined whether subjects significantly discriminated between the old and new objects at each week of behavioral testing. Neither group was able to discriminate between objects 1 week after surgery ($p > 0.05$). At weeks 2,3,5,6,7 after surgery, the OVX subjects could not significantly discriminate between the old and new objects while the intact, sham OVX subjects significantly discriminated at each week from weeks 2-7 ($p<0.05$).

Similar to OR test results, all subjects discriminated between objects at old and new locations with a 2 hour inter-trial delay on the OP task prior to surgery. Two-way ANOVA, group x location, demonstrated a significant location effect ($F(1,28)= 5.60$, $p<0.02$), indicating both groups spent significantly greater time exploring the object at the new location relative to the old location. Fig. 4 shows the results of OP testing as a ratio, time spent at the new location/time at old + new location. Post-surgical, two-way ANOVA (group x week) of the ratios showed no significant group or week effect, but a significant group x week interaction ($F(1,6) = 2.22$, $p<0.047$). Post-hoc analysis by two sample T-tests showed that OVX rats performed significantly worse than the intact group beginning at 4 weeks after surgery and continuing through week 7 post-surgery. Intact subjects maintained ratios above 0.6 while ratios in OVX groups slowly declined from 0.6 to 0.45 at week 7 post OVX. In

addition, the data were analyzed by a repeated measures ANOVA (group x location x week) in order to determine whether animals could significantly discriminate between old and new locations. A group x location interaction ($F(1,14)=20.11$, $p<0.0005$) was observed, as well as a group x location x week interaction ($F(6,14)=2.30$, $p<0.04$). Ability to discriminate between old and new locations was tested by paired T-test and showed that both groups could significantly discriminate between old and new locations at weeks 1 and 2 after surgery ($p<0.05$). From weeks 3-7 post-surgery, however, OVX animals could no longer significantly discriminate between locations ($p>0.05$), while intact, sham OVX subjects retained the ability to significantly discriminate between objects at the old and new locations ($p<0.05$).

Following behavioral testing, spine density was measured in the prefrontal cortex (layers II and III) and subfields CA1 and CA3 of the hippocampus. Spines of tertiary apical dendritic branches and secondary basal dendritic branches of pyramidal cells were counted. The average spine density per 10 μm is shown in Table 1, and photomicrographs of high and low spine densities are shown in Fig. 5, respectively. Analysis by two way ANOVA (group x brain area) indicated a significant effect by area, $F(5, 60)=14.93$, $p<0.0001$, and a group x area interaction, $F(5, 60)=8.97$, $p<0.000002$. Two sample T-tests indicate a significant difference in spine density between intact and OVX animals in both apical and basal dendrites in the PFC and CA1 region of the hippocampus ($p<0.05$). Specifically, in the prefrontal cortex, tertiary apical dendritic spine density was 30% lower for OVX than intact subjects and basal dendritic spine density was 53% lower. Tertiary apical spine density in area CA1 was 17% lower for OVX than intact subjects and basal dendritic spine density

was 48% lower for OVX than intact rats. In CA3, no significant differences in spine density were found between groups.

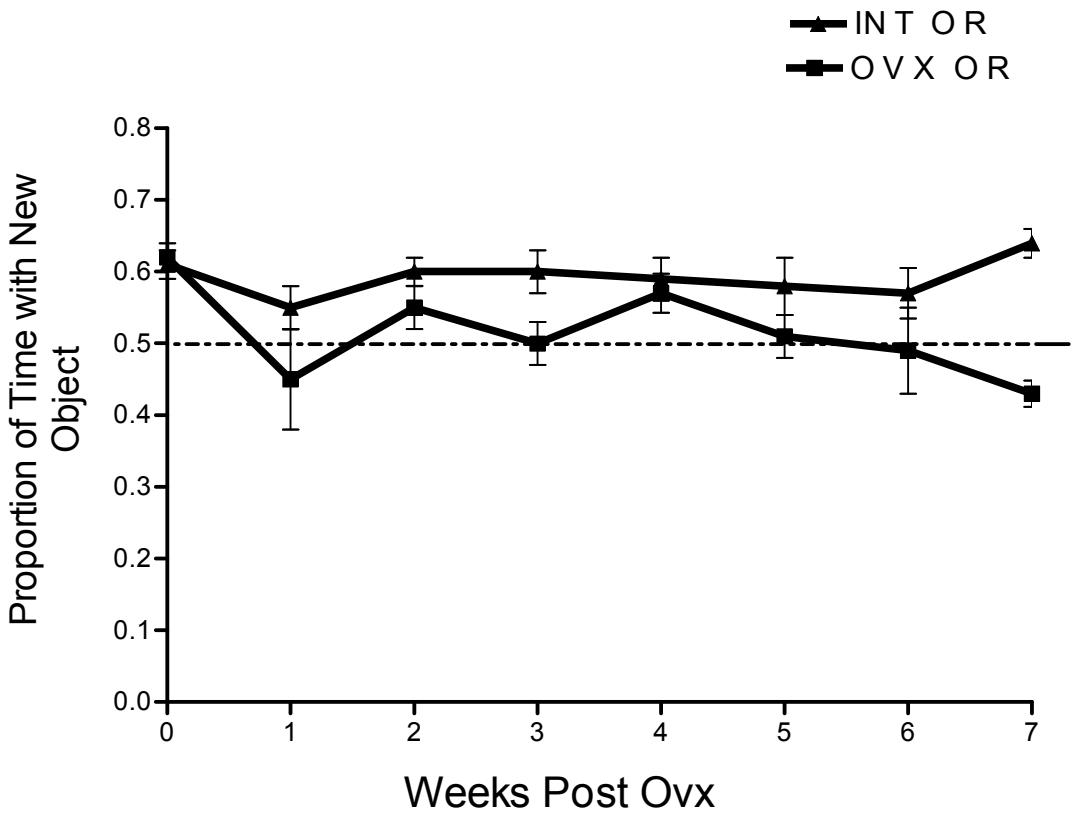

Fig. 3. The Effect of OVX on Object Recognition
The exploration ratio ± SEM (time with new object/time with old + time with new Object) is shown prior to OVX (0 week) and for 7 weeks post OVX for Intact, sham OVX (n=8) and OVX (n=8) subjects. Dashed line at 0.5 indicates chance performance of task (same amount of time spent exploring the old and new object). Two-way ANOVA of ratios (group x week) showed only a significant group effect ($F(1,6)=18.98$, $p<0.00003$) indicating that OVX was associated with a decline in performance of OR following surgery. Exploration times at old and new objects were also analyzed by repeated measures ANOVA (group x object x week.) and a significant group x object interaction ($F(1,14)=12.75$, $p<0.0004$) was present. Post hoc testing by paired t-test determined whether subjects significantly discriminated between the old and new objects at each week. Neither group discriminated between objects 1 week after surgery ($p< 0.05$). At weeks 2,3,5,6,7 after surgery, OVX subjects did not significantly discriminate between the objects while the intact, sham OVX subjects significantly discriminated at each week ($p<0.05$)

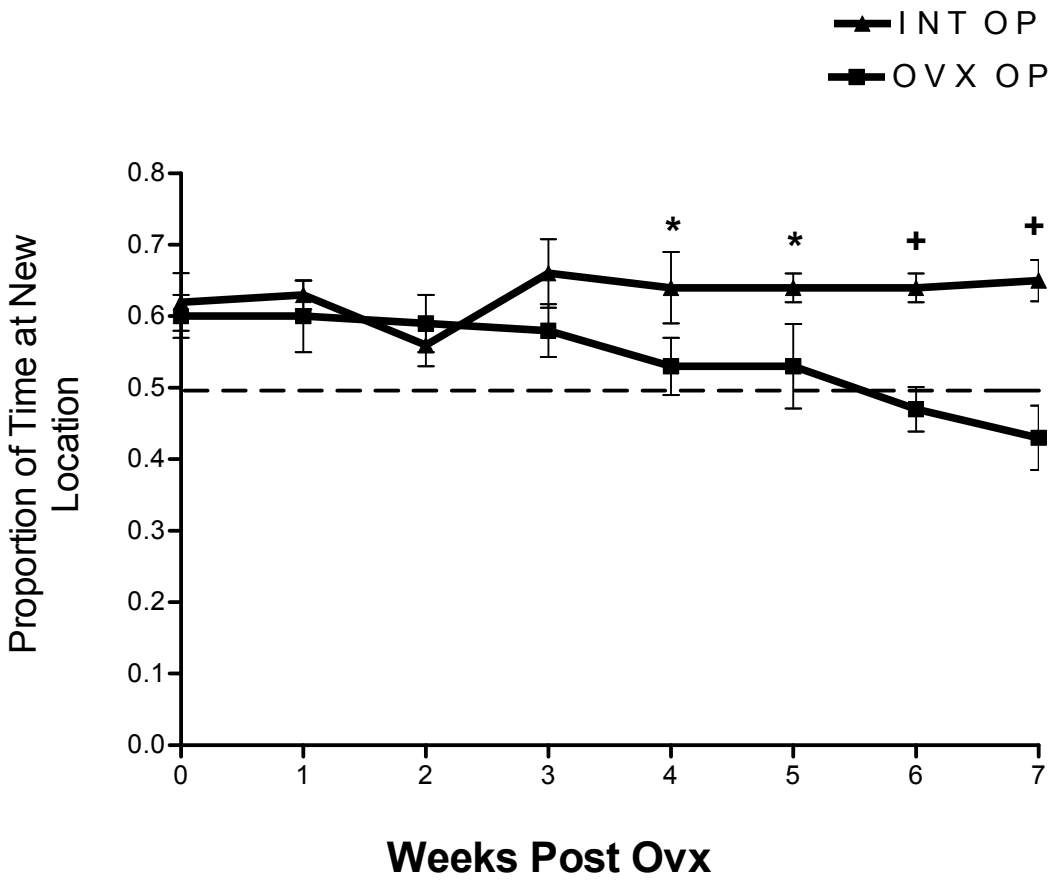

Fig 4. The effect of OVX on Object Placement
The exploration ratio ± SEM (time at new location/time at old + time at new location) is shown prior to OVX (0 week) and for 7 weeks post OVX for Intact, sham OVX (n=8) and OVX (n=8) subjects. Dashed line at 0.5 indicates chance performance of task (same amount of time spent exploring the objects at old and new locations). Two-way ANOVA (group x week) of ratios showed no significant group or week effects, but a significant group x week effect interaction ($F(1,6) = 2.22$, $p < 0.047$). Post hoc testing by T test indicates a significant difference between groups post surgically at weeks 4 ($p<0.03$), 5 ($p<0.02$), 6 ($p<0.001$) and 7 ($p<0.001$). Exploration times at old and new locations were also analyzed by repeated measures ANOVA (group x object x week). Significant effects on the group x location interaction ($F(1,14)=20.11$, $p<0.0005$) and the group x location x week interaction ($F(6,14)=2.30$, $p<0.04$) were found. Ability to discriminate between old and new locations was tested by paired T-test and showed that both groups could significantly discriminate between old and new locations at weeks 1 and 2 after surgery ($p<0.05$). From weeks 3-7 post-surgery, however, OVX animals could no longer significantly discriminate between locations ($p>0.05$), while intact, sham OVX subjects retained the ability to significantly discriminate between the old and new locations ($p<0.05$). * = significant at $p<0.05$, + = significant at $p<0.001$

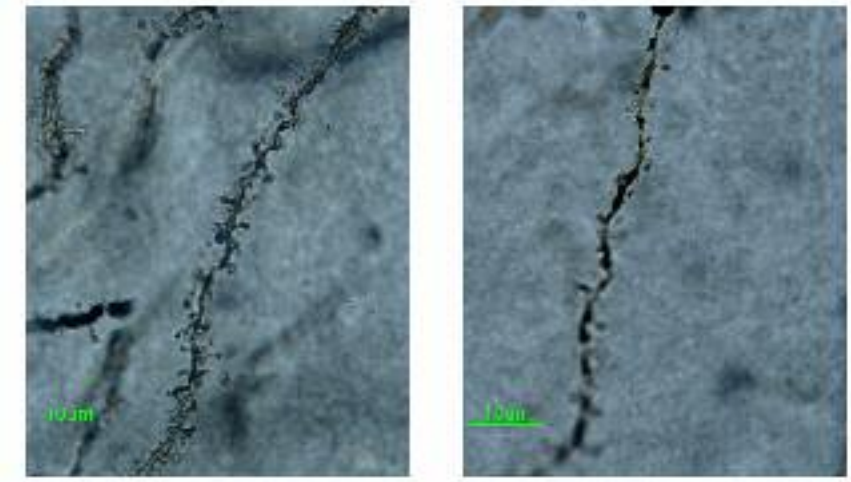

Fig.5A Fig.5B

Fig. 5. Photomicrographs of dendrites from Intact and Ovariectomized rat Photomicrographs (100X) of secondary basal dendrites from pyramidal cells of medial prefrontal cortex (Layer II/III). A.Gonadally intact female rat B.OVX female rat

Table 1

Spine density in PFC, CA1 and CA3 pyramidal neurons in young OVX and intact rats

Group	PFC Apical	PFC Basal	CA1 Apical	CA1 Basal	CA3 Apical	CA3 Basal
Intact (Sham OVX)	4.47 ±.49	5.91 ±1.19	8.97 ±.65	8.16 ±.46.5	5.20 ±.64	4.47 ±.42
OVX	3.15** ±.22	2.8** ±.32	7.43* ±.50	4.27*** ±.36	5.81 ±.63	4.87 ±.44

Entries are the mean number of spines/10μm ±SEM for intact and OVX subjects. T-test showed significant differences in dendritic spine density between the groups for PFC Apical ($t= -2.49$, $p<0.02$)**, PFC Basal ($t=-2.52$, $p<0.02$)**, CA1 Apical ($t=1.99$, $p<0.04$)* and CA1 Basal ($t= 6.65$, $p<0.0001$)***.

Discussion

These results demonstrate decreases in performance on memory tasks following OVX. Specifically, the non-spatial memory task, OR, and the spatial memory task, OP, declined in OVX as compared to intact females following surgery. Thus, the results provide additional support for the hypothesis that ovarian hormones contribute to maintenance of memory function in adult, female rats. Interestingly, OVX subjects showed a lower novel object exploration ratio during OR trials than intact subjects beginning in the 1st week following surgery, and this difference was maintained for the duration of the seven week testing period. In contrast, the ratio for exploration at the new location for OP trials in OVX rats was not significantly lower than intact rats until four weeks post OVX. Surgery is a stressful, and stress enhances performance of OP and several other spatial memory tasks in female rats (Beck 2002, Bowman 2003, Luine 2002). For example, Yang et al (2003) found that stress experience enhanced spatial memory retrieval on a water maze task in young (under 13 weeks), male Wistar rats (Yang et al, 2003). In the current experiment, although subjects were female, the stress experience of the surgery may have resulted in a similar enhancement to that seen by Yang et al (2003) while completing the Object Placement task in the first few weeks after surgery. In contrast, OR performance is not altered by stress in females (Beck 2002, Bowman 2003). Thus, it is possible that post surgical stress enhancements on OP may have overridden the loss of ovarian hormones in the weeks just after the OVX surgery.

The better performance of the intact females relative to OVX females on the two memory tasks is consistent with some prior studies with young, OVX rats. It has

been found that hormone replacement to young, OVX rats results in better acquisition of spatial memory tasks when compared to groups not receiving treatment as well as better working memory (Dohanich, 2003),Gibbs 2004, Luine 1998). Beneficial effects of hormone replacement on cognitive function in young animals have also been observed in mice (Frick et al, 2006, Li et al, 2004). Few studies have examined effects of OVX on memory tasks, but Singh et al found that OVX rats performed worse than intact rats in active avoidance behavior (Singh, 1994)**.** In this case, impaired performance was shown at 5 weeks post OVX. Additionally, in a study using mice, OVX has been associated with decreases in spatial memory performance (Heikkinen, 2004). Specifically, mice ovariectomized at 5 months old showed impaired maze learning ability on the T-maze when tested 2 months later, at age 7 months, compared to sham operated mice.

The decrease observed in the spine density of CA1 but not CA3 hippocampal pyramidal neurons is similar to results of a previous study following short term OVX. Gould et al (1990) found a 47% decrease in CA1 apical spine density and a 30% decrease in CA1 basal spine density in rats OVXed for 1 week as compared to intact subjects. Like the previous study, no OVX-dependent changes were found in the CA3 areas of the hippocampus. The current results show a 17% lower apical spine density in CA1 and 48% lower basal spine in CA1 for long term OVX as compared to intacts. Interestingly the percent decreases following short versus long term OVX are different, with long term OVX demonstrating smaller changes apically and larger changes basally. This pattern suggests further remodeling of CA1 neurons with longer hormonal deprivation. Our finding of decreases in prefrontal cortex pyramidal

neuron spine density following OVX are novel, but are consistent with Tang et al, (2004) who reported lower numbers of spinophilin-immunoreactive spines in the prefrontal cortex of female rhesus monkeys were OVX compared to those receiving estradiol treatment.

Losses in spine density in the cortex and hippocampus were similar, with both areas showing decreased spine density in OVX subjects relative to intact subjects. Interestingly, the OVX subjects also demonstrated poorer performance on both Object Recognition (non-spatial memory) and Object Placement (spatial memory), suggesting an association between spine density and cognitive function. In a study using young (less than 55 days old) male, white footed mice (*P. leucopus*), Pyter et al (2005) observed better performance on the Morris water maze with higher dendritic spine density in area CA1 of the hippocampus. As spines potentially form synapses, one possibility for the association between dendritic spine density and cognitive function is the increase in the number of connections that may be made between neurons with higher spine density. The increase in the number of connections may lead to more efficient signaling between neurons (Pyter et al, 2005) which may enhance cognitive function. Therefore, in the current experiment, the decreased memory performance seen in rats with lower dendritic spine density may be the result of less efficient signaling between neurons.

The underlying mechanism for changes in dendritic spine density in the prefrontal cortex and hippocampus is unknown, but both areas contain nuclear estrogen receptors. A small amount of estrogen receptor alpha (ERα) is present in the hippocampus (Zhao, 2004) and both areas contain the recently identified estrogen

receptor beta (ERβ) (Zhao, 2004, Kritzer, 2006). Therefore, it is possible that the estrogen being produced by the intact rats was interacting with nuclear estrogen receptors and, consequently, leading to the production of more neurotrophics (Gibbs, 1999). The increased production of neurotrophics would, in turn, increase dendritic spine density. Receptors in the membranes of neurons have also been reported (Bulayeva, 2005). Thus, possible estrogenic interactions with membrane receptors may be responsible for regulating dendritic spines.

Stewart and Kolb (1994) reported an increase in total length of dendrites per neuron for pyramidal cells in layers II and III of the parietal cortex following OVX in rats. Dendritic lengths were not measured in the current study so it is unknown whether medial prefrontal pyramidal neurons also show this change after OVX. Increased lengths could account for the decreased spine density reported here. However, this possibility appears unlikely since the decreases in spine density observed in the present experiment (30-50%) were greater than the dendritic lengthening observed by Stewart and Kolb (1994)(14-19%), and the dendritic change they observed was found at 4 months post OVX, as opposed to 7 weeks in the current study. Further, a recent study showed that dendritic length in basal forebrain cholinergic neurons was decreased following OVX (Saenz, 2005), which would actually result in an increase in spine density.

Dendritic spines in CA1 region of the hippocampus have been implicated in memory function. Studies have shown that estradiol increases spine or spine synapse density in this region (Leranth, 2004,Woolley 1998). Moreover, estradiol enhances memory function at the same doses which increase spine density in rats using the

water maze (Sandstrom, 2001) or in mice using object placement (Li 2004). McLaughlin et al (2005) also found that stress increased CA1 spines with heads, and this increase was significantly correlated with performance on the Y-Maze, a spatial memory task (McLaughlin 2005). Here, we demonstrate, in the same subjects, a decline in performance of memory tasks and in spine density in hippocampus and prefrontal cortex pyramidal neurons following OVX. It would be interesting to measure spine densities at the earlier time points after OVX in the current study when OR, but not OP, was impaired in OVX subjects.

In conclusion, long term OVX results in a decline in performance of both a spatial and a non-spatial memory task when compared to intact rats. The decline in performance following OVX was also associated with a decrease in dendritic spine density in both apical and basal dendritic branches of pyramidal cells in the prefrontal cortex and CA1, but not in CA3. This effect supports the idea that ovarian hormones play a role in the maintenance of neuronal morphology in brain structures important for cognitive ability and that long-term gonadal hormone deprivation results in decreased memory function, which may be mediated by these morphological changes.

Specific Aim 2

The effect of age on memory and neuronal morphology

A portion of this specific aim has been published (Wallace et al, 2006). To assess the role of the aging process on memory in females, intact young and aged female rats will be compared on the same visual and spatial memory tasks as the intact and ovariectomized rats as the previous specific aim. As aging is associated with decreased gonadal hormone production (Cranton, 1997, Bowman et al, 2006), it is hypothesized that aged rats will show behavioral and morphological changes similar to the chronically hormone deprived, young female rats in specific aim 1. The golgi impregnation procedure will be applied to determine whether morphological changes of brain structures that occur with aging are similar to those that occur as a result of ovarian hormone deprivation.

Subjects

Eighteen female Fisher 344 rats, 10 aged 19 months on arrival and 8 aged 2 months on arrival, all gonadally intact, were used in this experiment. Animals were cared for in accordance with the NIH Guide for Care and Use of Laboratory Animals and the experiment was approved by the Institutional Animal Care and Use Committee. The rats were double housed and free access to food and water was permitted for the entire duration of the experiment. Animals were maintained in a 12-hour light/dark cycle (lights off 7:00pm).

Procedures

Animals were provided with a one week acclimation period, which included daily handling by the experimenter, after arriving at the laboratory. Following acclimation, the same open field paradigm described previously was utilized to allow animals to habituate to the testing field.

Non-Spatial recognition ability was then examined using the Object Recognition task previously described. Following the sample trial, inter-trial delays of gradually increasing length (1 minute, 10 minutes, 1 hour) on consecutive days were enforced. After the delay, one of the objects was replaced with a new object and the rat was returned to the testing field. Three minutes exploration time was again provided (T2) and the time the animal spent exploring each object was recorded separately. Animals that did not explore during the sample and/or retention trial were excluded from the data for that particular trial. Spatial memory was then examined using the Object Placement described previously. Following the sample trial, inter-trial delays of gradually increasing length were enforced (10 minutes, 1 hour, 1.5 hours, 2 hours). At the end of the delay, the position of one of the objects was changed and the rat was returned to the testing field. Three minutes exploration time was again provided (T2) and the time spent exploring each location was recorded separately, and rats that did not explore during the sample trial and/or retention trial were excluded from the data for that particular trial.

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Golgi Impregnation

After completion of behavioral testing, one week later, animals were sacrificed by rapid decapitation and the brains were removed for golgi impregnation. Tissue was blocked, sliced, impregnated, dehydrated and cleared following the same procedure described in specific aim 1.

RIA Analysis

At the time of sacrifice, trunk blood samples were collected to analyze levels of gonadal hormones. Trunk blood was centrifuged and the serum was removed for analysis. Analysis was carried out using commercially available radio immunoassay procedure kits (Coat-A-Count) purchased from Diagnostic Products Corporation (Los Angeles, CA) for both estrogen and testosterone. The kits contained propylene tubes coated with antibodies to the hormone being tested, a radioactive, synthetic version of the hormone being measured and serum based standards to construct a calibration curve. The tubes were incubated at room temperature to allow the hormone present in the serum to compete with the radio active labeled hormone for antibody binding sites. Decanting separated the bound from free. Samples were counted in a gamma counter (PerkinElmer Wizard 1470 Automatic Gamma Counter, Model 1470-015), and the concentration of hormones calculated by comparison to the calibration curve.

Mortality

During the course of the experiment, one aged animal died prior to the collection of all data.

Statistics

Exploration ratios (time with new object/time with new object + time with old object) for Object Recognition and Placement data were analyzed using a two-way ANOVA (group x delay). Additional analysis of the exploration time (actual time in seconds spent exploring new and old objects) was completed using a three-way ANOVA (group x object x delay). Post hoc comparisons were done by paired-t test. Analysis of exploration time during the sample trial for both Object Recognition and Object placement was completed by two-sample t-test. Analysis of R.I.A. results was also completed by two-sample t-test. Analysis of dendritic spine density was completed by two-way ANOVA (group x brain area) with post hoc analysis by two-sample t-test. All statistics were completed using Number Cruncher Statistical Systems (NCSS) 2000 software (©1999 Jerry Hintze).

Results

Results of object recognition testing are shown in Fig.6 as the exploration ratio, time spent with the new object/time spent the old + new object. Two-way ANOVA, group x delay showed a significant delay effect, $F(1,39)=4.9$, $p<0.03$), and no significant group or group x delay interaction. Additional analysis of the exploration times were conducted by 3 way ANOVA (group x object x delay). Results indicated a significant group effect, $F(1,78)= 18.47$, $p<0.00005$, a significant object effect, $F(1,78)=20.01$, $p<0.00003$, and a group x object interaction, $F(1,78)=6.65$, $p<0.01$. Post-hoc analysis by paired T test determined whether subjects

significantly discriminated between the old and new objects at each inter-trial delay used for behavior testing. Results indicated that both young and aged animals spent significantly greater time with the new object as compared to the old object at the 10-minute delay ($p<0.006$, $p<0.003$, respectively). At a 1hour inter-trial delay, young rats were able to discriminate between the new and old objects ($p<0.01$). Aged rats, however, were no longer able to distinguish between the new and old objects at this delay ($p<0.24$). Importantly, the exploration time during the sample trial (T1) was analyzed by two sample T test and showed no significant difference between young and aged animals ($p<0.13$), indicating that the findings are not the result of differences in activity level between the groups or the length of time that subjects spent exploring the objects (Fig. 7)

On the object placement task, analysis of the exploration ratio, time at new location/time at old + new location, showed a significant group effect (Two way ANOVA, group x delay, $F(1,76)=4.68$, $p<0.03$), but no effect by delay and no group x delay interaction (Fig.8), suggesting that the young rats, overall, spend a greater proportion of time exploring the novel location compared to the familiar location relative to aged rats. Additional analysis of the exploration times was conducted by 3 way ANOVA (group x object x delay). Results indicated a significant effect by group, $F(1,152)= 31.78$, $p<0.0$, an effect by object, $F(1,152)=14.19$, $p<0.0002$, a group x object interaction, $F(1,152)=6.28$, $p<0.01$, and a group x delay interaction, $F(3,152)= 3.88$, $p<0.01$. Post-hoc analysis by paired T test determined whether subjects significantly discriminated between the old and new locations at each inter-trial delay used for behavior testing. Results indicated that at a 10-minute delay, the young

animals significantly discriminated between old and new locations ($p<0.01$) but the aged animals did not ($p<0.6$). At a 1-hour delay, both young and aged animals discriminated between the old and new locations ($p<0.009$, $p<0.004$, respectively). At a 1.5 hour delay the young animals successfully discriminated between old and new locations ($p<0.04$), while the old animals did not ($p<0.54$). At a 2 hr delay, neither the young nor the aged rats could discriminate between the old and new locations ($p<0.1$, $p<0.3$, respectively). Thus, young subjects were able to successfully differentiate between old and new locations at a longer inter-trial delay than aged subjects. Additionally, the exploration time during the sample trial (T1) was analyzed for the test with the 1.5 hour inter-trial delay, the longest inter-trial delay that could be completed by young, but not aged rats, and no significant difference was found. Therefore, the results obtained are not due to differences in activity level between the groups or time spent exploring the objects by the subjects (Fig. 9).

Following behavior testing, dendritic spine density was measured in the prefrontal cortex (layers II/III) and hippocampus (CA1 and CA3) as described in specific aim 1. Spines of tertiary, apical dendritic branches and secondary basal dendritic branches of pyramidal cells were counted. Table 2 shows the average spine density per 10 μm for each area, and Fig.10 shows representative photomicrographs of spines on dendritic branches. Analysis by two way ANOVA (group x brain area) demonstrated an area effect ($F(5,60)= 14.24$, $p<0.00001$) and a group x area interaction ($F(5, 60)= 3.35$, $p<0.009$). Additional analysis by two sample t-tests indicates that aged rats had significantly lower spine density for tertiary apical branches in the prefrontal cortex and area CA1 of the hippocampus compared to aged

rats ($p<0.05$). Specifically, the mean spine density for tertiary, apical branches in the prefrontal cortex of aged rats was 16% lower than young rats and the mean spine density for tertiary, apical branches in area CA1 was 16% lower in aged rats than young rats. No significant difference in spine density was found between young and aged rats for secondary basal branches of the prefrontal cortex or area CA1 of the hippocampus. No significant differences in spine density were found between young and aged rats in area CA3 for either tertiary, apical dendritic branches or secondary, basal dendritic branches.

Trunk blood was analyzed by radio immunoassay to assess circulating levels of gonadal hormones in young and aged animals. The results indicated that both estrogen and testosterone were higher in the young animals compared to the aged animals, but two-sample t-test indicated the difference was not significant ($p<0.057$, $p<0.63$, respectively). Specifically, young rats had a mean of 13.5 ± 4.46 pg/ml of estrogen and 149.7 ± 46.82 pg/ml of testosterone. Aged rats had a mean of 7.9 ± 1.04 pg/ml of estrogen and 103.3 ± 33.83 pg/ml of testosterone.

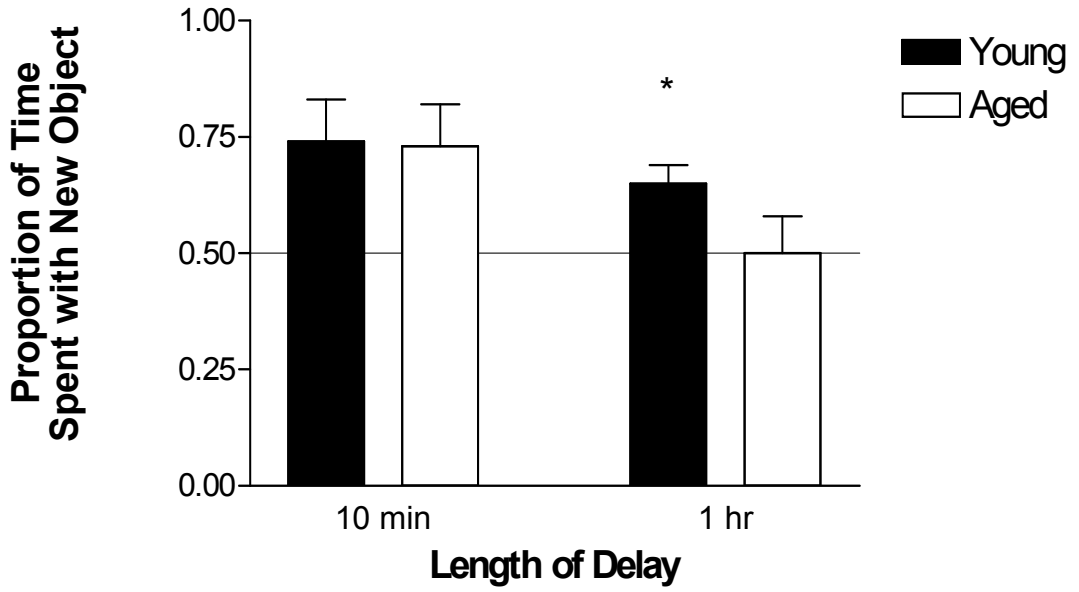

Fig. 6. Effect of Age on Object Recognition
The exploration ratio ± SEM (time with new object /time with old +new object) is shown for both a 10 minute and 1 hour inter-trial delay for young (n=8) and aged (n=10) female Fischer 344 rats. Line at 0.5 indicates chance performance of task (same proportion of time exploring old and new objects). Two way ANOVA of ratios (group x delay) indicated a significant delay effect, $F(1,39)=4.9$, $p<0.03$), and no significant group or group x delay interaction. Analysis of exploration ratios by T-test demonstrated no significant difference between groups at a 10-minute inter-trial delay. At a 1-hour inter-trial delay, the young animals had a significantly higher exploration ratio than aged animals ($p<0.04$) indicating aging is associated with a decline in performance on Object Recognition

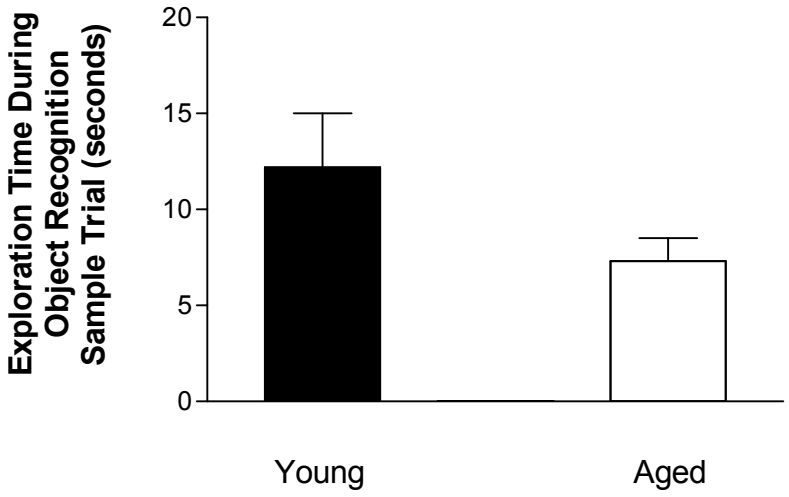

Fig. 7. Effect of Age on Exploration Time during the Sample trial for Object Recognition

Bar graph of mean exploration time ± SEM during the sample trial (T1) for Object Recognition prior to the 1 hour inter-trial delay for young (n=8) and aged (n=10) rats. The amount of time exploring the objects during T1 by young and aged rats was analyzed by two sample t test. The findings demonstrated no significant difference in the time spent exploring between the young and aged animals ($p<0.13$).

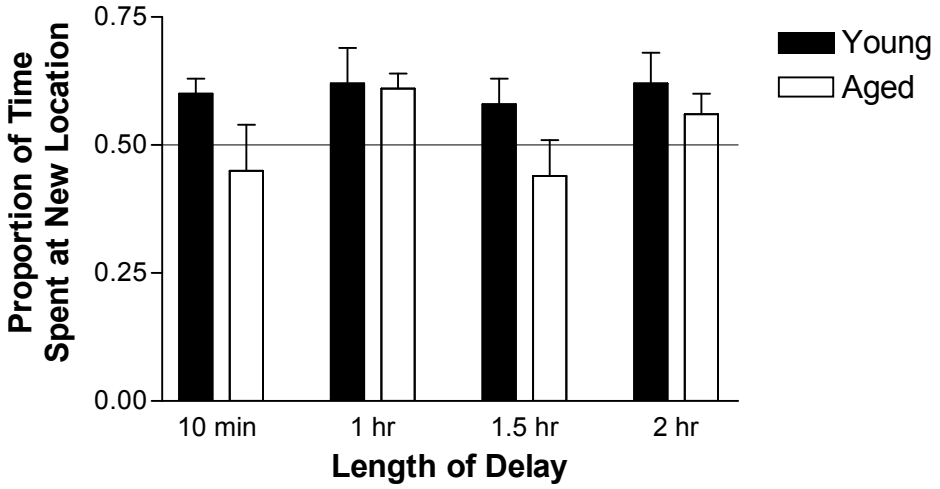

Fig.8. Effect of Age on Object Placement
The exploration ratio ± SEM (time at new location/time at old + new location) is shown for 10 minute, 1 hour, 1.5 hour and 2 hour inter-trial delays for young (n=8) and aged (n=9) female Fischer 344 rats. Line at 0.5 indicates chance performance (equal proportion of time at old and new locations). Analysis of the exploration ratios by two way ANOVA, group x delay, demonstrated a group effect, $F(1,76)=4.68$, $p<0.03$), indicating aged animals had generally poorer performance on the Object Placement task compared to young animals. Further analysis of the exploration times (actual times spent exploring the old and new locations) were conducted by 3 way ANOVA (group x location x delay) and demonstrated a significant group effect, $F(1,152)= 31.78$, $p<0.0001$, location effect, $F(1,152)=14.19$, $p<0.0002$, a group x location interaction, $F(1,152)=6.28$, $p<0.01$, and a group x delay interaction, $F(3,152)= 3.88$, $p<0.01$. Post-hoc analysis by paired t-test indicated both young and aged animals discriminated between the old and new locations ($p<0.009$, $p<0.004$, respectively) at a 1 hour delay. At a 1.5 hour delay, young animals could discriminate between old and new locations ($p<0.04$) while aged animals could not ($p<0.54$). Neither group significantly discriminated at a 2 hour delay.

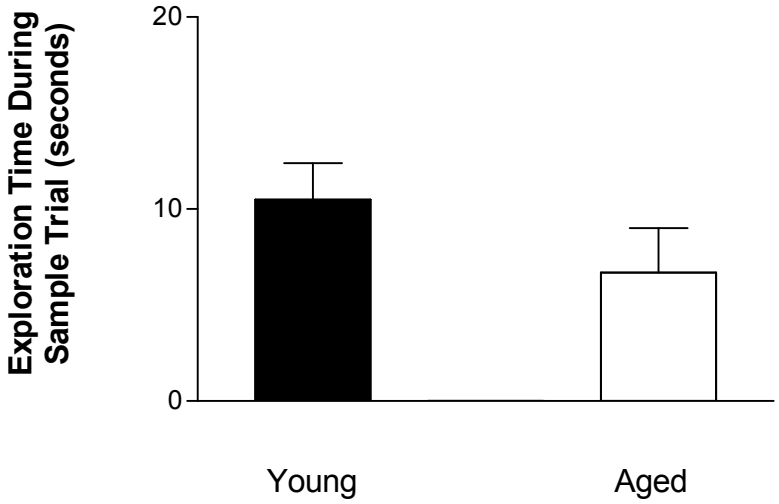

Fig. 9. Effect of Age on Exploration Time during the Sample Trial for Object Placement Bar graph of the mean exploration time ±SEM for young (n=8) and aged rats (n=9) during the sample trial (T1) for Object Placement prior to the 1.5 hour delay. The time spent exploring each location during T1 was analyzed by two sample T test. The results indicate no significant difference in the time spent exploring during the sample trial between the young and aged animals ($p<0.11$).

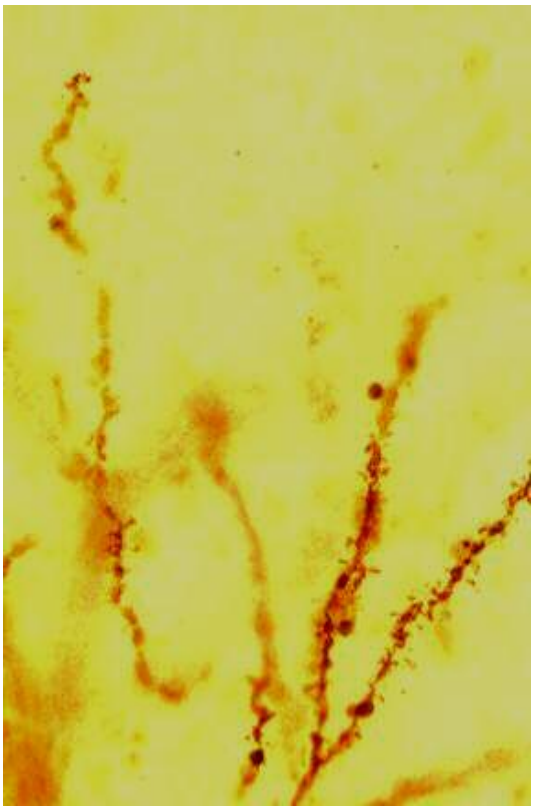

Fig. 10A.

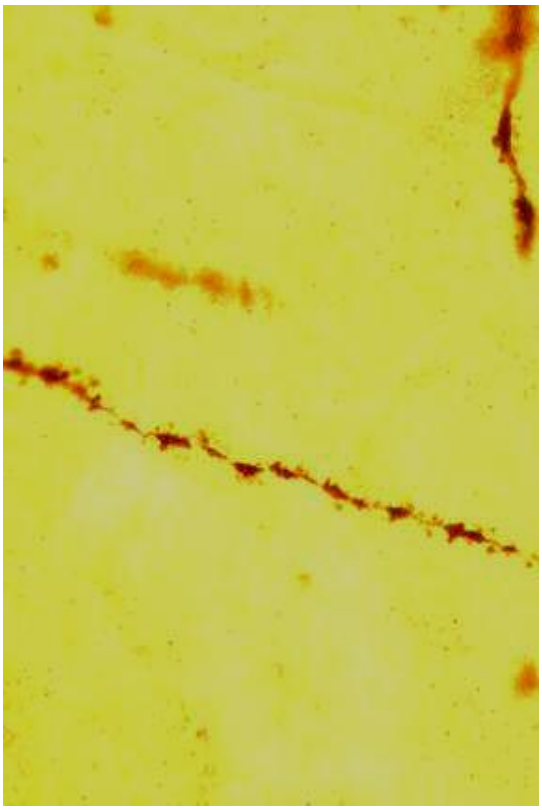

Fig. 10B.

Fig.10. Photomicrographs of tertiary, apical dendrites of pyramidal cells from medial prefrontal cortex (layers II/III). A.Young female rat B.Aged female rat

Table 2

Spine density in PFC, CA1 and CA3 pyramidal neurons in young and aged female Fischer 344 rats

Group	PFC Apical	PFC Basal	CA1 Apical	CA1 Basal	CA3 Apical	CA3 Basal
Young	8.13 ± .40	7.80 ± .41	7.87 ± .58	5.37 ±.21	5.42 ± 0.36	5.52 ± 0.29
Aged	6.86 * ± .52	7.19 ± .31	6.59* ± .39	5.96 ± .15	6.30 ± 0.39	4.79 ± 0.36

Entries are the mean number of spines/ 10μm ± SEM for young and aged female Fischer 344 rats. T-test showed significant differences in dendritic spine density between the groups for PFC apical ($t= -1.93$, $p<0.04$) and CA1 Apical ($t= -1.81$, $p<0.049$).

Discussion

The results of specific aim 2 demonstrate decreases in performance on memory tasks in association with aging. Specifically, the non-spatial memory task, Object Recognition, and the spatial memory task, Object Placement, declined in aged rats relative to young rats. Thus, the data provides support for the hypothesis that aging has a deleterious effect on memory function in adult, female rats. The better performance of young rats compared to aged rats on the memory tasks in this experiment is consistent with some previous studies on aging. For example, spatial reference memory deficits have been observed in 11 month old Fischer 344 rats compared to 4 month old rats (Frick et al, 1995) in a water maze task. Similar findings were reported by Winocur (Winocur, 1992) in a study using a matching to sample paradigm. The results showed aging was associated with performance deficits both when the stimulus was presented immediately after the sample and also when there were delays between the sample and the stimulus (Winocur, 1992). Luine and Hearns (1990) showed that 24 month old F344 female rats were impaired on the 8 arm radial maze as compared to 4 month old females (Luine and). Aged rats have also been found to have slower rates of acquisition and faster rates of forgetting for spatial tasks compared to younger rats (Barnes, C.A., McNaughton, B.L., 1985). These behavioral changes are similar to the results obtained in comparing the intact and ovariectomized rats which demonstrated a decline in performance relative to intact rats (Aim 1). However, this observation is opposite to results in young females which demonstrated the ability to discriminate at longer delays for non-spatial memory than spatial memory (Beck and Luine, 2002). The ability for aged rats to perform better on

a spatial versus non-spatial task suggests that cortical structures may have greater age related degenerative changes than the hippocampus. For example, in a study by Raz et al(1997), the effect of aging on the morphology of different brain regions was examined. The results indicated that, in males and females aged 48 to 77 years, there was a 4.9% loss of prefrontal gray matter per decade, 4.3% loss of superior parietal cortex per decade, but only 2% loss of hippocampus per decade (Raz et al, 1997). As the hippocampus is associated with spatial memory (Broadbent, 2004), the possible slower decline in hippocampal volume, compared to cortical volume, with aging, may explain the ability of the aged rats to successfully complete a longer delay for Object Placement than Object Recognition. Additionally, as previously stated, chronic stress can enhance performance of spatial memory tasks in female rats (Beck and Luine, 2002, Bowman et al, 2003, Luine, 2002), but does not appear to alter performance of the Object Recognition task (Beck et al, 2002, Bowman et al, 2003). . Therefore, it is possible that despite the time spent habituating the rats to the testing field, the aged animals were still experiencing stress. Thus, it is possible that stress enhanced performance on the Object Placement task for aged rats, enabling them to differentiate between old and new locations successfully at a longer inter-trial delay for the spatial task than the non-spatial task.

The decrease in dendritic spine density of pyramidal cells in the prefrontal cortex of aged rats compared to young rats is similar to prior findings on age related changes in dendritic spine density. Duan et al (2003) examined age related morphological changes in layer III pyramidal cells forming corticocortical projections from the superior temporal cortex to prefrontal area 46 in both male and female

primates (long tailed macaques and rhesus monkeys). A 25% decrease in both apical and basal dendritic spine density was observed in aged animals, aged 24-25 years, compared to younger animals, aged 10-12 years. Further, Peters et al (1998) found a reduction in the number of synapses in layer 1 of area 46 of the prefrontal cortex in rhesus monkeys and an associated decrease in the number of profiles of post-synaptic dendrites and spines, implying that spiny, apical dendrites of pyramidal cells are degenerating with age. Similarly, the current study found a 16% decrease in tertiary, apical spine density of pyramidal cells in aged rats compared to young rats, suggesting the rats experienced age related neural degeneration similar to that reported in primates. The spine density for secondary, basal branches was also 7% lower in aged animals compared to young animals. Interestingly, spines "contain neurotransmitter receptors, organelles, and signaling systems essential for synaptic function and plasticity" (Nimchinsky et al, 2002). Therefore, the decrease in spine density seen in the aged rats in the current experiment may have resulted in decreased synapse function. Less efficient functioning of synapses with age, in turn, may have lead to the observed cognitive decline.

In addition to the prefrontal cortex, lower dendritic spine density was also found in tertiary, apical dendritic branches of area CA1 of the hippocampus. Specifically, the dendritic spine density for this area was 16% lower in aged compared to young rats. While few studies have been conducted on the influence of aging on dendritic spine density in the hippocampus, research has shown that the level of circulating gonadal hormones influences spine density in this structure (Gould et al, 1990, Woolley, 1990). As aging is associated with a decline in the

production of gonadal hormones (Markowska, 1999, McEwen, 1999), and RIA analysis detected lower levels of estradiol and testosterone in the aged rats relative to young rats, it is possible that the decrease in hormone levels in aged rats resulted in decreased dendritic spine density in the hippocampus. As previously mentioned, the lower spine density may have caused reduced synapse function (Nimchinsky et al, 2002) and, consequently, a decline in memory.

In combination with the decrease in circulating gonadal hormones, age related changes in the expression of estrogen receptors may be an important factor in the observed decrease in spine density in the aged rats. For example, Chakraborty et al (2003) reported a decrease in cells expressing the estrogen receptor β (ER β) in middle aged (10-12 months) and old (24-26 months) rats compared to young rats (3-4 months) (Chakraborty et al, 2003) in the anteroventral periventricular nucleus of the hypothalamus. Both the prefrontal cortex and the hippocampus, the structures examined in the current study, contain estrogen receptors. Therefore, these areas may experience a decrease in estrogen receptor expression similar to that observed in the hypothalamus by Chakraborty et al (2003). This suggests that the decrease in spine density seen in aged animals may be the combined result of smaller amounts of estrogen having fewer receptors with which to interact.

In conclusion, aging was associated with a decrease in performance of both a spatial and non-spatial memory task in aged as compared to young rats. This decline was also associated with a decrease in dendritic spine density of pyramidal cells in the prefrontal cortex and hippocampus. There was also an associated decrease in the production of gonadal hormones in aged compared to young rats. These findings

suggest that aging influences neuronal morphology, possibly as a result of lower hormone levels, which results in decreased memory function.

Specific Aim 3

The effect of estrogen treatment on memory and neuronal morphology in aged rats

Effects of estrogen treatment on memory function in aged rats were assessed using the same visual and spatial memory task described previously, and golgi impregnation was also applied. It was hypothesized that treatment of aged female rats with estrogen would be associated with an increase in dendritic spine density and better ability to discriminate on the memory tasks compared to the untreated aged rats based on the findings of specific aims 1 and 2. Similar to the OVX rats in aim 1, the aged rats in aim 2 showed a decrease in memory function and a decrease in spine density of pyramidal cell neurons compared to the young rats. As the aged rats had lower estrogen levels in aim 2, compared to the young rats, it was predicted that by providing estrogen replacement, there would be an increase in dendritic spine density (Gould et al, 1990, Woolley, 1998) and an improvement in memory function (Sandstrom, 2001). However, some reports have indicated that hormone deprivation does not always impair memory function. For example, Markowska and Savonenko (2002) found that reference memory in Fischer 344 rats, tested using a place discrimination task and split-stem T-maze, was not affected by ovariectomy (Markowska and Savonenko, 2002). Further, some evidence suggests that hormone replacement to older subjects that have experienced hormone deprivation does not enhance cognitive function (Daniel et al, 2006). Therefore, the results of this specific aim will further the understanding of the role of gonadal hormones and the aging

process on memory through the changes induced in the morphology of memory related brain structures.

Subjects

Twelve female Fisher344 rats, aged 19 months on arrival, were used in this experiment. Animals were cared for in accordance with the NIH Guide for Care and Use of Laboratory Animals and the experiment was approved by the Institutional Animal Care and Use Committee. The rats were double housed and free access to food and water was permitted for the entire duration of the experiment. Animals were maintained in a 12 hour light/dark cycle (lights off 7:00pm).

Procedures

Animals were provided with a one week acclimation period, which included daily handling by the experimenter, after arriving at the laboratory. Following acclimation, the same open field paradigm described in specific aim 1 and 2 was utilized to allow animals to habituate to the testing field. Non-Spatial recognition ability was then examined using the Object Recognition task described in the previous 2 experiments. In this experiment, a within subjects design was utilized. The rats were acclimated to the task while untreated and inter-trial delays of gradually increasing length (1 minute, 10 minutes, 1 hour) were given on consecutive days until the rats could no longer discriminate between old and new objects. Once the animals could no longer make this distinction, a sub-chronic treatment paradigm, according to the procedures established by Jacome et al (Jacome et al, 2005, Jacome et al, 2006) was implemented. Using a within subjects design, half of the animals were treated

with estradiol benzoate (EB) dissolved in corn oil, beginning at a dose of 50 μg/kg, the dose found effective in young animals by Jacome et al (Jacome et al, 2005), while the other half received corn oil. Two treatments, given 24 hours apart, were administered. Forty-eight hours after the last treatment was given, the Object Recognition task was repeated. One week later, the treatment paradigm was repeated with the treatment condition switched. If no difference in performance was observed using a dose of 50 μg/kg EB, the same treatment paradigm at 100 μg/kg EB was implemented.

Spatial memory was then examined using the Object Placement task described in the previous experiments. As with Object Recognition, rats were acclimated to the task by gradually increasing the inter-trial delay until the rats could no longer discriminate between old and new locations. Once the animals could no longer distinguish between old and new locations, the same sub-chronic treatment paradigm used for the Object Recognition task was implemented. The Object Placement task was repeated 48 hours after the last treatment and one week later the treatment paradigm was repeated with the treatment groups switched.

Golgi Impregnation

One week after behavior testing was completed, half of the rats were administered corn oil vehicle and half of the rats were administered 100 μg/kg EB, 2 doses, 24 hours apart, replicating the treatment conditions for testing. Animals were sacrificed by rapid decapitation at 48 hours after the last dose and the brains were removed for golgi impregnation. Brains were blocked in the same manner outlined in

specific aim 1 and the same procedures were followed for staining and sectioning. Sections were rinsed, buffered, impregnated, dehydrated and cleared as previously described.

Results

During habituation to the Object Recognition task, analysis by paired T test showed the aged rats were able to discriminate between old and new objects at a 10-minute inter-trial delay ($p<0.02$) without any treatment (data not shown). At a 1 hour inter-trial delay, the animals were unable to differentiate between old and new objects while untreated ($p<0.08$) (data not shown). A within subjects, subchronic treatment paradigm using 50 μg/kg of estradiol benzoate (EB) and a 1 hour inter-trial delay was then implemented, and analysis of the exploration ratio (time with new/time with old+ time with new) by paired T test demonstrated no significant difference in the proportion of time spent with the new versus the old object during treatment as compared to receiving vehicle (corn oil) ($p<0.72$). Treatment at a higher dose, 100 μg/kg EB, was then given and the results indicated that, like the previous dose, there was also no significant difference in the ratio of time spent with the new versus the old object ($p<0.22$) during treatment. Further analysis of the exploration ratio for each treatment condition by repeated measures ANOVA (dose) showed an effect by dose ($F(2, 40)=4.94$, $p<0.017$), and post-hoc analysis by Neuman-Keuls showed all treatment conditions to be significantly different from each other, with the highest exploration ratio (0.63) found in the 100 μg/kg condition (Fig. 11). Analysis of the exploration time (time in seconds spent exploring old object and time in seconds

spent exploring new object) by paired t-test determined whether subjects were able to significantly discriminate between old and new objects in each treatment condition (corn oil, 50 μg/kg EB, 100 μg/kg EB). The results indicated that the rats could not significantly discriminate between old and new objects when given corn oil or 50 μg/kg EB. When given 100 μg/kg, however, the rats were able to significantly discriminate between old and new objects ($p<0.0001$)(Fig. 12). These results suggest that a dose of 100 μg/kg EB was more effective at enhancing Object Recognition performance than 50 μg/kg EB.

During habituation to the Object placement task, analysis by paired T-test showed that the aged rats were able to discriminate between old and new locations at a 10-minute inter-trial delay without any treatment ($p<0.004$). The aged rats were also able to discriminate between old and new locations at a 1 hour inter-trial delay ($p<0.03$) (data not shown). At a 1.5 hr inter-trial delay, the rats were unable to differentiate between old and new locations ($p<0.68$) while untreated (data not shown). A subchronic treatment paradigm using 50 μg/kg EB and a 1.5 hr inter-trial delay was then given and analysis of the exploration ratio (time at new location/ time at old location + new location) showed no significant difference between performance with 50 μg/kg EB compared to performance while receiving vehicle ($p<0.6$). A subchronic treatment paradigm of 100 μg/kg EB was given, and analysis of the exploration ratio showed no significant difference between performance with 100 μg/kg EB and performance while receiving vehicle ($p<0.1$), although this treatment condition showed the highest exploration ratio (0.78). Further analysis by analyzing the exploration ratio for each treatment condition by repeated measures ANOVA

showed an effect by treatment ($F(2,44)= 3.72$, $p<0.04$) and post-hoc analysis by Neuman-Keuls showed the 100 μg/kg EB condition to be significantly different from both the corn oil and 50 μg/kg EB condition (Fig. 13). Analysis by paired T test of the exploration times (time in seconds with old object and time in seconds with new object) determined whether the rats could significantly discriminate between old and new locations in each treatment condition (corn oil vehicle, 50 μg/kg EB, 100μg/kg EB). Although results showed that the rats could not significantly discriminate between old and new locations in any of the treatment conditions (Fig. 14), the highest exploration ratio was found using the 100μg/kg EB dose, indicating a trend towards improvement.

Following behavior testing, dendritic spine density was measured in the prefrontal cortex (layers II/III) and hippocampus (CA1, CA3) as described in the previous specific aims. Spines of tertiary, apical dendritic branches and secondary basal dendritic branches of pyramidal cells were counted. Table 3 shows the average spine density/10μm for each area. Analysis by 2 way ANOVA (treatment x area) showed no effect by treatment ($F(1,60)=0.0$, $p<0.9$), area ($F(5, 60)=111.82$, $p<0.12$) or treatment x area interaction ($F(5, 60)=0.66$, $p<0.65$). While there were no significant differences between groups, EB treated rats had a 4% lower spine density than vehicle treated rats for tertiary, apical branches, while vehicle treated rats had a 9% lower spine density for secondary basal branches in the prefrontal cortex. In the hippocampus, in subfield CA1, EB treated rats had a 10% lower spine density for tertiary, apical dendritic branches and an 11% lower spine density for secondary basal branches compared to vehicle treated rats. In subfield CA3, vehicle treated rats had an

11% lower spine density for tertiary apical branches and a 5% lower spine density for secondary basal branches compared to EB treated rats.

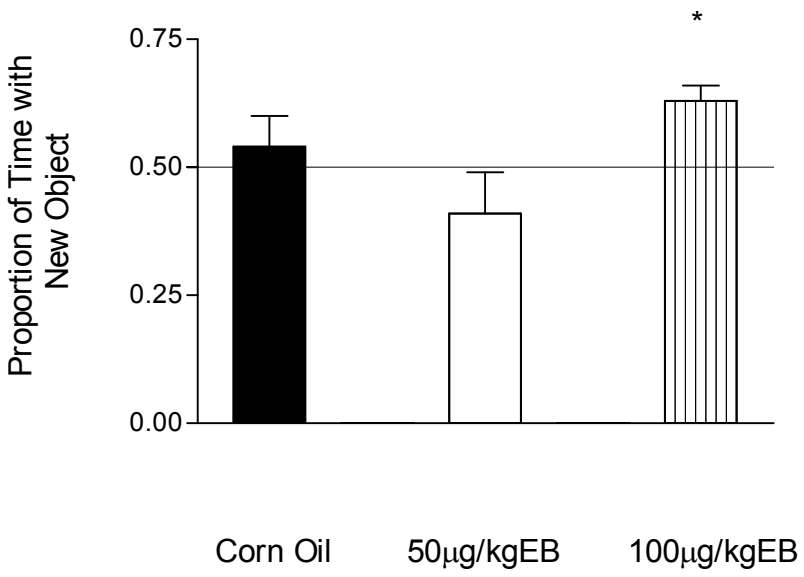

Fig. 11. Effect of dose on exploration ratio for Object Recognition.
Bars represent the mean exploration ratio (time at new/time at old + new) ±SEM for aged rats (n=12) at each dose administered (corn oil vehicle, 50 μg/kg EB, 100 μg/kg EB). Line at 0.5 represents chance performance (equal time at both old and new). Analysis of the exploration ratio by repeated measures ANOVA showed a significant effect by dose ($F(2, 40)=4.94$, $p<0.017$) and post-hoc analysis by Neuman-Keuls showed all treatments to be significantly different from each other. The highest exploration ratio (0.63) was observed in the 100 μg/kg condition. The findings indicate that the dose of 100μg/kg EB was the most effective at enhancing Object Recognition Performance.

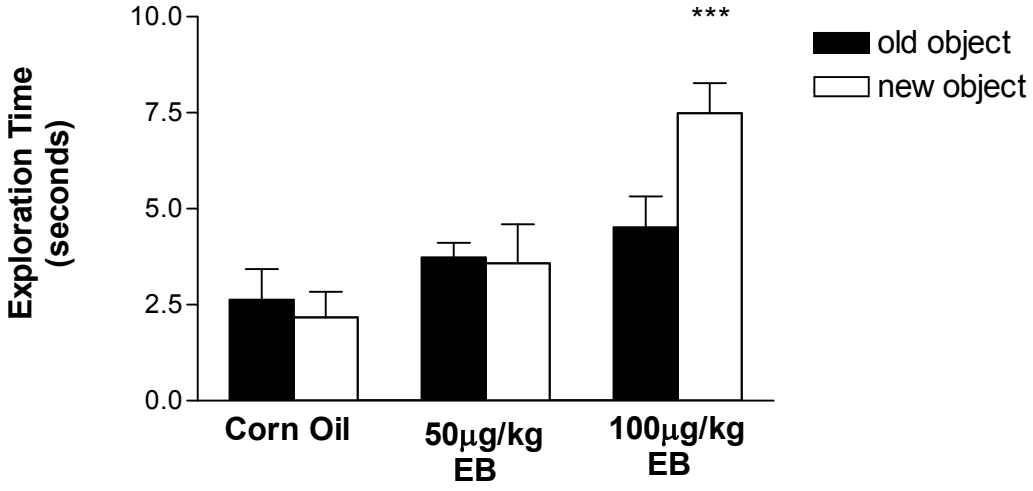

Fig. 12. Effect of treatment on time spent exploring old and new objects
Bar graph of mean time in seconds ± SEM spent exploring the old and new objects by aged rats (n=12) during retention (T2) trial for Object Recognition in each of the treatment conditions (corn oil vehicle, 50 μg/kg EB, 100 μg/kg EB). Analysis by paired t- test showed no significant difference in time spent exploring old versus new for corn oil vehicle and 50 μg/kg EB, indicating rats were unable to discriminate between the old and new objects. At 100μg/kg EB there was a significant difference in time spent exploring old versus new ($p<0.0001$), indicating at this dose the rats were able to distinguish between old and new objects.

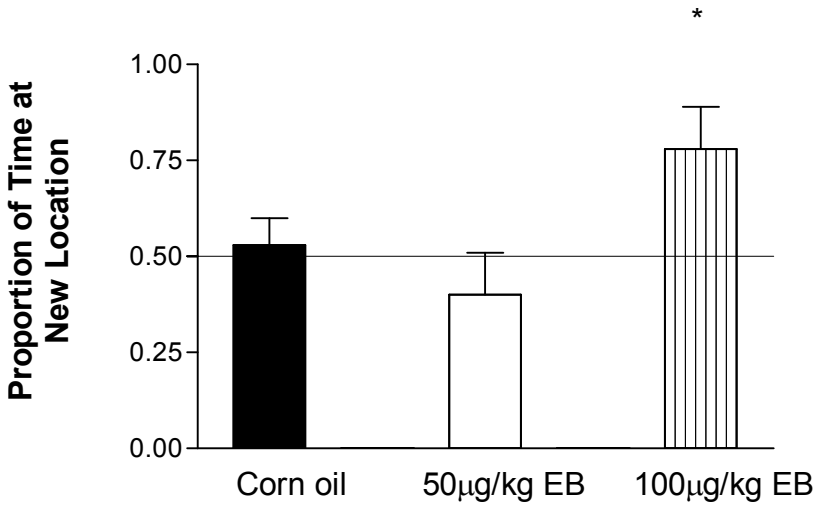

Fig. 13. Effect of dose on exploration ratio for Object Placement
Bars represent the mean exploration ratio (time at new/time at old + new) ±SEM by aged rats (n=11) for each dose administered (corn oil vehicle, 50 μg/kg EB, 100 μg/kg EB). Line at 0.5 represents chance performance (equal time at both old and new). Analysis of the exploration ratio for Object Placement by repeated measures ANOVA showed an effect by dose ($F(2,44)= 3.72$, $p<0.04$) and post-hoc analysis by Neuman-Keuls showed the 100 μg/kg EB condition to be significantly different from both the corn oil and 50 μg/kg EB condition. The highest exploration ratio (0.78) was obtained when rats were receiving 100 μg/kg EB.

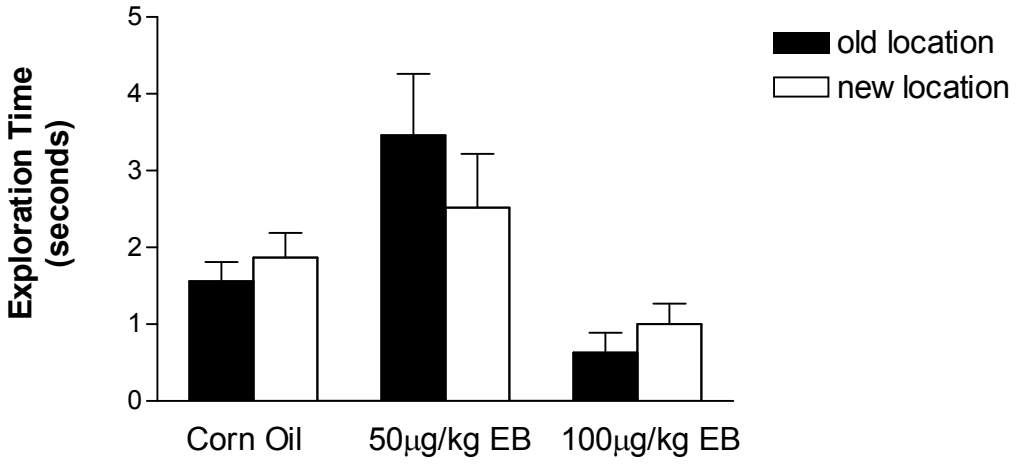

Fig. 14. Effect of treatment on time spent at old and new locations
Bar graph of mean time in seconds ± SEM spent exploring the old and new locations by aged rats (n=11) during the retention trial (T2) in each of the treatment conditions (corn oil vehicle, 50 μg/kg EB, 100 μg/kg EB). Analysis by paired T test showed no significant difference in time spent exploring old versus new locations in any of the treatment conditions, indicating rats were unable to significantly discriminate between old and new locations with any of the treatments administered.

Table 3.
Spine density in PFC, CA1 and CA3 pyramidal neurons in aged female Fischer 344 rats that received corn oil vehicle or 100 μg/kg EB prior to sacrifice

Group	PFC Apical	PFC Basal	CA1 Apical	CA1 Basal	CA3 Apical	CA3 Basal
Corn oil vehicle	7.17 ± 0.1	6.69 ± 0.54	7.9 ± 0.41	6.54 ± 1.06	7.03 ± 0.63	6.06 ± 0.25
100 μg/kg EB	6.89 ± 0.72	7.32 ± 0.51	7.13 ± 0.79	5.84 ± 0.22	7.93 ± 0.89	6.40 ± 0.39

Entries are the mean number of spines/ 10μm ± SEM for aged female Fischer 344 rats that received either corn oil vehicle or 100 μg/kg EB prior to sacrifice. Analysis by 2 way ANOVA (treatment x area) showed no effect by treatment ($F(1,60)=0.0$, $p<0.9$), area ($F(5, 60)=111.82$, $p<0.12$) or treatment x area interaction ($F(5,60)=0.66$, $p<0.65$).

Discussion

The results of specific aim 3 demonstrate improvement of Object Recognition performance in aged female Fischer 344 rats after receiving estradiol benzoate (EB) using a within subjects, subchronic treatment paradigm. The improved performance observed on the Object Recognition task used in this experiment when rats were receiving EB is consistent with previous studies on hormone replacement in young subjects. For example, Jacome et al (2005) found a dose of 50 μg/kg EB to be effective at enhancing both Object Recognition and Object Placement performance, the identical tasks used in the current experiment, in young rats (Jacome et al, 2005). However, in the current experiment, a dose of 100 ug/kg was necessary to improve object recognition performance, and on the Object Placement task, aged rats still could not discriminate between new and old locations at either the 50 or 100 ug/kg dose, although the exploration ratio improved with EB treatment at 100 μg/kg. Interestingly, in a study involving the effect of estrogen on memory, Leuner et al (2004) found that associative memory formation, measured via eye-blink conditioning, was enhanced in adult female rats, 250-300 grams, when 2 injections of 40 μg were provided 24 hours apart but not in animals receiving lower doses of 10 and 20 μg (Leuner et al., 2004). However, the dose used in the study by Leuner et al (2004), 40 μg for a 250-300 gram rat, is higher than the dose administered in the current study, which suggests the aged rats used in the present experiment may have shown greater improvement on the Object Placement task with a higher dose of EB. Therefore, estrogen treatment appears to benefit cognitive function in aged rats, but larger doses are needed to achieve the same results observed with younger animals.

Studies using aged animals have also found hormone treatment to be beneficial for cognitive function. Markham et al (2002) found ovarian hormone replacement to be beneficial for spatial ability in female rats that were 16 months old at testing. Specifically, ovariectomized animals that received no treatment displayed significant forgetting during acquisition of a spatial version of the Morris water maze, while this forgetting was prevented in animals that received either acute (2 day) or chronic (28 day) estrogen replacement (Markham et al, 2002). Improvement in cognitive function using hormone replacement has also been shown in primates (Rapp et al, 2003). Results from female rhesus monkeys averaging 22 years old, considered comparable to a 55-65 year old human, showed that ovariectomized animals injected with estradiol (100 μg/1ml peanut oil) every 3 weeks, creating a cyclic pattern of hormone fluctuation, scored significantly better than vehicle treated animals on a delayed response task as increasingly longer retention intervals were imposed (Rapp et al, 2003). The manner in which hormone replacement is administered, then, may be an important factor in the ability of hormone replacement to improve age related cognitive decline. Therefore, greater enhancement of memory function may have been observed in the current experiment if the cyclical type of hormone fluctuation seen in the 4 to 5 day estrus cycle of young rats had been recreated, as opposed to using sub-chronic treatment.

In addition to the method of treatment, the effectiveness of hormone replacement may also depend on the amount of time the animal has been hormone deprived. For example, Gibbs (2000) found that continuous estrogen replacement was not as effective at enhancing cognitive function in rats that had been hormone

deprived for longer amounts of time. Specifically, rats demonstrated enhanced performance on a delayed matching to position task when provided hormone replacement beginning 3 months after an ovariectomy compared to rats that were not provided hormone replacement until 10 months after ovariectomy (Gibbs, 2000). Similarly, Daniel et al (2006) found that rats that received estradiol implants immediately after an ovariectomy performed better on an 8 arm radial arm maze task than rats that experienced a 5 month period of deprivation before receiving any hormone replacement (Daniel et al, 2006). Further, Lewis and Frick (2006) found phasic hormone therapy resulted in no improvement in spatial reference memory in young mice (4 months), and, in fact, some impairment in older mice (17 and 24 months), suggesting the age at which treatment is initiated is an important factor in the outcome of treatment. As the average age of estropause in a rat is approximately 12 months (Chakraborty and Gore, 2004), the rats in the current experiment (19 months old) had experienced low levels of circulating estradiol for approximately 7 months before receiving any hormone replacement. Therefore, it is possible the reason the estradiol treatment was less effective in enabling the rats to significantly discriminate between old and new locations on the Object Placement task, compared to the Object Recognition task, is because they had been hormone deprived for too long. However, the estradiol treatment was effective at improving performance on the Object Recognition task at a dose of 100 μg/kg. As the Object Placement task is hippocampal dependent (Broadbent et al, 2004) and the Object Recognition task involves the prefrontal cortex (Runyan et al, 2004)), it is also possible that the Hippocampus is more sensitive to the effects of prolonged withdrawal from gonadal

hormones than the prefrontal cortex. On the other hand, Raz et al (1997) observed greater age related degeneration in the prefrontal cortex of humans compared to the hippocampus. Perhaps the prefrontal cortex, while showing degeneration with aging, is better able to respond to estradiol than the hippocampus. In addition, Chakraborty et al (2003) reported a decrease in estrogen receptors in aged female rats (24-26 months) compared to younger rats (3-4 months and 10-12 months) (Chakraborty et al, 2003). Therefore, aged rats may require higher doses of estradiol to achieve comparable cognitive effects to those seen in young rats because less receptors are available to utilize the hormone.

Morphologically, the current study found no significant differences in tertiary, apical dendritic spine density or secondary, basal dendritic spine density in either the prefrontal cortex or the hippocampus between rats that received vehicle or EB. Many studies have examined hormonal influences on neuronal morphology using young animals (Gould et al, 1990, Woolley et al, 1990, Woolley, 1998) but very little data exists using aged animals. For example, a study by Woolley et al (1997), examined dendritic spine density in young adult rats. The results indicated that the apical dendritic branches of pyramidal cells in area CA1 of the hipppocampus had a significantly higher density, 22%, for ovariectomized rats treated with 10 μg estradiol benzoate compared to vehicle treated animals (Woolley et al, 1997). The present experiment found no significant difference in spine density, and, in fact, a 10% decrease in spine density for apical dendritic branches in area CA1 for EB treated animals. Also, Woolley et al (1997) only examined spine density in the hippocampus. The current study also examined the prefrontal cortex and found a small, though

insignificant, increase in spine density for secondary, basal dendritic branches of pyramidal cell neurons in animals treated with EB compared to corn oil. As Raz et al (1997) found greater age-related degeneration in the pre-frontal cortex than the hippocampus, and estrogen levels are known to decline with age (Morrison et al, 2006), these findings suggest that, similar to the improvement seen with EB treatment in the Object Recognition task, the morphology of the prefrontal cortex may be more sensitive to the presence of gonadal hormones compared to the hippocampus. Other morphological studies support this suggestion. For example, in a study examining the prefrontal cortex using aged female rhesus monkeys with an average age of 22 years, it was found that long term treatment (2-3 years) of intramuscular injections of estradiol cypionate (100 μg/ml peanut oil) every 3 weeks was effective in increasing spine density of pyramidal cells in layer III of the prefrontal cortex (Hao et al, 2006), demonstrating responsiveness to the presence of estradiol. However, the aged hippocampus does not appear to show the same plasticity. Adams et al (2001) found a decrease in axo-spinous synapse density in aged compared to young rats in area CA1 of the hippocampus. Further, providing the aged rats with chronic estrogen treatment (silastic implants, 10% 17-β estradiol/90% cholesterol) did not reverse this decline (Adams et al, 2001), implying the aged hippocampus does not demonstrate the estradiol induced changes in morphology seen in studies using young animals (Gould et al, 1990, Woolley et al, 1990).

In conclusion, treatment of aged rats with 100 μg/kg EB resulted in greater improvement of Object Recognition, a task relying on the prefrontal cortex, than Object Placement, a task relying on the hippocampus. Although no significant

changes in dendritic spine density were observed with EB treatment, slight improvement in density was seen in the secondary basal dendrites of the prefrontal cortex. These findings suggest that with aging, the prefrontal cortex may remain more responsive to enhancing effects of estradiol in comparison to the hippocampus.

General Discussion

In specific aim 1, young, intact and OVX female rats were investigated and intact animals demonstrated better performance on tasks of both non-spatial and spatial memory compared to OVX animals. Further, these results are associated with significantly higher dendritic spine density of both apical and basal branches of pyramidal cell neurons in both the prefrontal cortex and subfield CA1 of the hippocampus for intact animals compared to OVX animals.

These effects support the idea that ovarian hormones in young animals play a role in the maintenance of neuronal morphology in brain structures important for cognitive ability and that long-term gonadal hormone deprivation results in decreased memory function, which may be mediated by these morphological changes. An underlying mechanism for the results is that both the prefrontal cortex and hippocampus contain nuclear estrogen receptors. Estrogen receptor alpha (ERα) is found in the hippocampus (Zhao, 2004) and both the prefrontal cortex and hippocampus contain estrogen receptor beta (ERβ) (Zhao, 2004, Kritzer, 2006). Neurons have also been found to have membrane receptors for estrogen (Bulayeva, 2005). Therefore, it is possible that estrogenic interactions with either nuclear or membrane bound receptors, or both, affect dendritic spine density. Additionally, dendritic spines have the potential to form synapses, suggesting that neurons with higher dendritic spine density can form more connections, allowing for more efficient signaling (Pyter et al, 2005). Therefore, the association between impaired cognitive function and lower dendritic spine density observed in young OVX rats compared to

intact rats in the current experiment may be due to decreased efficiency of signaling between neurons as a result of the presence of fewer spines.

In specific aim 2, young and aged female rats were compared and results indicated that the young animals demonstrated better performance on tasks of both non-spatial and spatial memory relative to aged animals. Additionally, these behavioral findings were associated with significantly higher dendritic spine density for apical branches of pyramidal cell neurons in the prefrontal cortex and area CA1 of the hippocampus for the young rats compared to aged rats, a pattern similar to the results obtained comparing young, intact rats to OVX rats.

These results support the idea that the aging process, similar to ovarian hormone levels, has a role in the maintenance of neuronal morphology in areas of the brain important in cognitive function. It is difficult to determine the extent that the aging process itself and declines in hormone levels contribute to the deterioration in performance of memory tasks. One possibility is the decrease in gonadal hormone levels that occurs with age (Markowska, 1999, McEwen, 1999). Further, decreased gonadal hormone levels have been linked to decreased memory function and changes in neuronal morphology (Wallace et al, 2006, Hao et al, 2006, Rapp et al, 2003). The structures examined in this study, the prefrontal cortex and hippocampus, are known to have estrogen receptors (Bulayeva, 2005, Kritzer, 2006, Zhao, 2004). However, other structures involved in memory do not contain estrogen receptors and thus may not be influenced by gonadal hormone levels. There is also evidence for age associated degeneration of the hippocampus and prefrontal cortex (Raz et al, 1997) and the estrogen receptors experience age associated decrements as well (Adams et

al, 2002). Loss of prefrontal cortex and hippocampus dendritic spines with age, therefore, suggests the presence of fewer estrogen receptors, as well as deterioration in function of existing receptors. It is possible, then, that age related cognitive impairment is the result of lower levels of estrogen interacting with receptors, or a decrease in the number and quality of receptors available, that influence spine density and, consequently, cognitive function.

Although the current study measured circulating estradiol, which was shown to be lower in aged rats compared to young rats, the potential effects of progesterone, another important gonadal hormone, on cognition and neural morphology must also be considered. A known effect of estrogen is an increase in transcription of neurotrophics (Gibbs, 1999) which, with respect to this thesis, could potentially increase dendritic spine density and benefit cognitive function. The hormone progesterone, however, has been shown to reverse both estrogen induced enhancement of memory (Bimonte-Nelson et al, 2006) as well as estrogen induced increases in neurotrophics (Bimonte-Nelson et al, 2004). The possible role of progesterone in cognition function has received little attention and is an area which should be further investigated. Nonetheless, as the young and aged rats in this specific aim were gonadally intact, the actions of progesterone may also have contributed to the observed results.

Specific aim 3 compared performance of aged female rats while untreated and while receiving estradiol on tasks of non-spatial and spatial memory. Specifically, the performance of the aged rats showed improvement on the Object Recognition task while receiving a dose of 100 μg/kg EB compared to performance while receiving

corn oil vehicle or 50 μg/kg EB and the highest exploration ratio for Object Placement was also found with a dose of 100 μg/kg EB. In contrast, there were no significant changes in dendritic spine density in either the prefrontal cortex or the hippocampus in rats treated with 100μg/kg EB when compared to rats that received the same volume of corn oil vehicle prior to sacrifice. Thus, unlike the relationship between spine density and behavior seen in the young intact and ovariectomized rats, a relationship between spine density and behavior was not seen in treated versus untreated aged rats. Therefore, young and aged rats may respond differently to the presence of ovarian hormones. As the current study with aged subjects used 100 μg/kg EB, compared to 50 μg/kg EB in prior experiments with young rats (Jacome et al, 2005, Jacome et al, 2006), the aged animals required a higher dose than young animals to achieve similar effects. As there is a reduction in both number (Chakraborty et al, 2003) and quality (Adams et al, 2002) of estrogen receptors with aging, the ability to utilize estrogen efficiently may decline with age, making larger doses necessary.

The duration of hormone deprivation experienced may also be an important contributing factor to the effectiveness of treatment. Research has shown that female rats and mice subjected to long term ovarian hormone deprivation do not respond as favorably to replacement therapy as animals deprived for a shorter interval (Gibbs, 2000, Daniel et al, 2006, Lewis and Frick, 2006). The rats in the present study, at 19 months old on arrival, had experienced low levels of circulating estradiol for several months before receiving estrogen replacement. Therefore, greater enhancement of

cognitive function and dendritic spine density may have been observed if the length of deprivation prior to treatment had been shorter.

Results obtained in this study have important implications for aging in humans, specifically females. Age related cognitive deficits in females may be the result of changes in neuronal morphology in brain structures relevant to memory function and these morphological changes appear to be, at least partially, mediated by changes in circulating gonadal hormone levels and the responsiveness of estrogen receptors. The current results show that a hormonal replacement regimen which is effective in restoring memory function and neuronal morphology in young rats (Jacome et al, 2005) is not effective in aged rats. Thus, different hormonal treatment strategies must be developed for aged female rats and, presumably, aged primates as well. Other data suggests that by initiating hormone replacement therapy immediately after endogenous hormone production declines at menopause, age associated cognitive decline and morphological changes may be more successfully prevented or treated.

Future experiments

As significant behavioral and morphological changes were observed comparing young intact and long term ovariectomized rats in specific aim 1, future studies might examine the relationship between these variables after a short-term ovariectomy. Short-term ovariectomy has been shown to result in significant morphological changes in the hippocampus (Gould et al, 1990, Woolley et al, 1997) but behavior has not been analyzed. Thus, it would be interesting determine whether

the morphological changes observed with short-term ovariectomy are also associated with changes in cognitive function. Also, the current data predict that dendritic spine density in the hippocampus may decline slower than in the prefrontal cortex because declines in performance of the non-spatial memory task were found before declines in the spatial memory task..

In the present study, significant decreases were seen in memory function and dendritic spine density in aged rats compared to young rats. However, estradiol administration was not sufficient to reverse the age–related morphological changes in the aged Fischer 344 rats but did improve memory performance. Studies of young rats, including those used in this thesis and by Jacome et al (2005), used the Sprague-Dawley strain whereas aged experiments, including this thesis, used rats of the Fischer 344 strain which are provided through the National Institute of Aging. Therefore, it is important to determine if the same dose of estradiol would prove effective at increasing dendritic spine density and improving memory performance in the young Fischer 344 rats. Similar effects of estrogen treatment in the young Fisher 344 and Sprague-Dawley rats would suggest that the young animals are more sensitive to the presence of ovarian hormones and that aged rats are less sensitive. Thus, aged rats may need larger doses and/or longer term treatment compared to young rats to obtain the same effects.

A subchronic treatment paradigm of two doses, 24 hours apart, was used to treat the aged rats in the current experiment. However, this replacement paradigm did not effectively reverse the decreases in dendritic spine density associated with aging. Therefore, for future experiments it would be interesting to determine if longer term

treatment would be more effective at reversing age related morphological changes compared to the subchronic treatment used in the present study. Significant findings would have implications for treating humans. In particular, it would suggest that to reverse age-related morphological changes with estrogen replacement requires longer-term treatment.

In conclusion, results of morphological and behavioral experiments in this thesis have shown that estradiol exerts critical influences on prefrontal cortical and hippocampal neuronal functions. Moreover, declines in circulating estradiol during aging also impact these functions. More importantly, the results indicate that replacement regimens which are effective in restoring functions in young female rats are not as effective in aged rats. Thus, these results have important implications for designing hormonal treatment paradigms for women during the perimenopausal and post menopausal period.

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