Influences of pregnancy on neural and behavioral function in female rats.

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Abstract

Influences of pregnancy on neural and behavioral function in female rats.

by

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Females' memory capabilities are enhanced during pregnancy (Galea et al., 2000). One factor believed to underlie memory enhancement is an increase in dendritic spines and synapses in CA1 hippocampus (Kinsley et al., 2005). The present study examined effects of reproductive experience on spatial (object placement) and non-spatial (object recognition) memory task performance, as well as neurochemical systems that may underlie memory performance. In the first experiment, pregnant and nulliparous females were tested on the object placement task; early and late pregnant females both out-performed nulliparous females. Monoamine and metabolite levels were measured in the pre-frontal cortex, CA1 and CA3 hippocampus, and medial pre-optic area, areas important for memory and maternal behavior. Significant alterations were observed in the dopamine, serotonin and norepinephrine systems in each region; these changes may contribute to the observed spatial memory differences between pregnant and non-pregnant females.

In the second experiment, multiparous females (12 months old; 5 litters each) and age-matched nulliparous controls were tested using object recognition and placement tasks. On both tasks, multiparous females significantly out-performed nulliparous

females. Monoamines and metabolites in PFC, CA1, CA3, and olfactory bulb were measured; significant elevations in the dopamine, norepinephrine and serotonin systems were observed only in the olfactory bulb of multiparous females. Additionally, levels of BDNF protein were found to be significantly higher in the CA1 and septum of multiparous than nulliparous females. CA1 hippocampus and septum are implicated in spatial memory performance, so higher BDNF protein may have contributed to enhanced memory performance in multiparous females. In both studies, anxiety as measured on the elevated plus maze, and activity as measured on the open field, did not differ due to reproductive state. In conclusion, studies showed enhanced performance on spatial tasks during pregnancy, and after multiple pregnancies, as compared to age-matched nulliparous females. In young, pregnant females performance may be due to alterations in monoamine and metabolite levels throughout the brain. In older multiparous females, monoamines were not altered in brain areas important for cognition, but enhanced performance may be due to increased expression of BDNF.

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1. General Introduction

1.a Estrogen and spatial memory

Estrogen actions in the brain are widespread, as estrogen receptors are located in many brain areas, including the hypothalamus, the hippocampus, cerebral cortex, midbrain, and pituitary gland (Sherwin, 2003). Thus, circulating estrogens have the opportunity to affect diverse brain functions, including those that fall into the area of cognition, i.e. attention, learning, memory, problem solving, and reasoning. A large body of evidence has accumulated suggesting that estrogen may act to help maintain certain aspects of cognition in females, particularly learning and memory. In elderly women, estrogen replacement therapy has been shown to protect against declines in verbal memory, as well as reduce the risk of Alzheimer's Disease by 30-40% (Sherwin, 2003). Similarly, estrogen replacement in aged female rats improved their ability to learn a spatial task: the Morris water maze (Frick, Fernandez, & Bulinski, 2002; Markham, Pych, & Juraska, 2002). In younger rats, it has been shown that both acute (30 minutes prior to testing) and chronic administration (for 12 days prior to testing) of estrogen to ovariectomized (OVX) females enhanced spatial memory (radial arm maze), visual memory, and place memory (Luine, Jacome & MacLusky, 2003; Luine, Richards, Wu & Beck, 1998, respectively).

Spatial memory is dependent on an intact hippocampus. Rats with lesions to the hippocampus have impaired spatial learning abilities (Morris, Garrud, Rawlins & O'Keefe, 1982), and species of rodents with larger home ranges have larger hippocampi than those with smaller home territories (Jacobs, Gaulin, Sherry, & Hoffman, 1990). Gonadal steroids – particularly estrogen – have been shown to affect the morphology of hippocampal neurons. Removal of gonadal steroid hormones by ovariectomy profoundly

decreased the density of dendritic spines on pyramidal cells in the CA1 region; replacement of estradiol to these animals prevented the decrease (Gould, Woolley, Frankfurt, & McEwen, 1990). A similar change in spine density was also shown to occur naturally in the female rat hippocampus; density differed across the four-day estrous cycle, with the highest density on the afternoon of proestrus when levels of estradiol are highest, and a 30% lower density on day of estrus when levels of estradiol are lowest (Woolley, Gould, Frankfurt, & McEwen, 1990). In a similar manner, the density of synapses on hippocampal dendritic spines decreased by 32% on day of estrus, almost exactly the same degree that numbers of spines decreased from proestrus to estrus (Woolley and McEwen, 1992; McEwen and Woolley, 1994). This result indicated that the differences in spine density seen across the estrous cycle may have resulted in an altered pattern of synaptic connectivity between these cells (Woolley & McEwen, 1992). More recently, it was shown that the density of CA1 pyramidal spine synapses is markedly increased very shortly after exogenous administration of both 17α- and 17β- estradiol (MacLusky, Luine, Hajszan & Leranth, 2005).

Given the evidence that the hippocampus is critical for spatial memory (Morris et al., 1982), particularly intact CA1 hippocampus (Potvin et al., 2006), presumably these morphological changes would influence the ability of a female to remember spatial information. Unfortunately, experiments addressing spatial performance relative to natural fluctuations in hormone levels have not produced clear results. Much of the research has found little or no relationship between the estrous cycle and performance on a spatial task. Stackman, Blasberg, Langan & Clark (1997) found that the day of estrous in terms of natural fluctuations in hormone level did not produce clear results. Much of the research

found little or no relationship between estrous cycle and performance on a spatial task. Berry, McMahon & Gallager (1997) found no difference between females in proestrus or estrus in acquisition or retention of a spatial task, suggesting that naturally increased estrogen may not affect spatial learning.

Warren & Juraska (1997) did find that spatial learning varied across the estrous cycle in a manner consistent with the anatomical fluctuations discussed previously; improved spatial learning on a cued version of the Morris water maze occurred on proestrus, when the maximum number of dendritic spines and synapses were seen. The proestrous females did not show improved learning on the place version of the task, which is more difficult, and therefore potentially more stressful, than the cued version of the water maze. Interestingly, ovariectomized rats treated with 17β-estradiol, who were subsequently trained on the Morris water maze, did not demonstrate an increase in hippocampal spine and synapse density, while those not trained on the task did exhibit an increase, similar to previous studies (Frick, Fernandez, Bennett, Prange-Kiel, MacLusky & Leranth, 2004). Therefore, it is possible that training on this specific spatial task (Morris water maze), which is known to be stressful to rats (see Frick et al., 2004) is likely to have been responsible for the failure of proestrous females to outperform estrous females, possibly by blocking the effects of estrogen on hippocampal spine synapse density. Additionally, Viau & Meaney (1991) found that females examined on proestrus exhibited a significant increase in levels of corticosterone and ACTH when restrained as compared to estrous females; an increase in these hormones is indicative of higher stress levels due to the restraint. Combined, these studies may explain why proestrus females failed to outperform estrous females on the Morris water maze.

Although elevated estrogen levels on proestrus did not seem to be linked to improved spatial memory, treatment with estrogen has reliably enhanced learning and memory. Daniel et al (1997) found that estrogen-treated females outperformed control females during acquisition of the radial arm maze. Additionally, females whose treatment ceased after acquisition of the task continued to outperform control females during the next 24 days of task performance (Daniel et al., 1997). Intrahippocampal injections of estrogen immediately following training in the Morris water maze led to enhanced memory 24 hours later (Packard, 1998; Markham, Pych & Juraska, 2002), while systemic treatment with estrogen led to a similar, more rapid effect, with females demonstrating enhanced memory during the same trial (Packard, 1998). Frick, Fernandez & Bulinski (2002) found that daily treatment with estrogen for five days prior to and during testing significantly improved performance in the Morris water maze as compared to ovariectomized females. Finally, twelve days of estrogen treatment prior to testing increased spatial performance as compared to ovariectomized females (Luine et al., 1998); it was later found that estrogen treatment thirty minutes prior to testing could also increase spatial performance (Luine, Jacome & MacLusky, 2003). Recent evidence indicates that estrogen may act on CA1 to enhance spatial memory, as injections of 17α– estradiol increased the density of CA1 pyramidal spine synapses by 44% above ovariectomized controls within 30 minutes of injection (MacLusky et al., 2005).

Observations that estrogen treatment alone enhanced ovariectomized females' spatial memory, whereas endogenous estrogen in intact females did not, indicated the possible contribution of another gonadal hormone, progesterone. Ovariectomized females treated only with estrogen as a replacement are missing progesterone, which is also

elevated on proestrus (Figure 1B). Progesterone may also affect hippocampal plasticity and memory functioning. Woolley and McEwen (1993) found that while proestrus was associated with an increased density of dendritic spines, subsequent elevation in progesterone late in proestrus decreased dendritic spine density. Decreased spine density due to presence of progesterone (after or in concert with estrogen) may underlie decreased working memory by female rats in proestrus (Chesler and Juraska, 2000). Additionally, acute administration of estrogen and progesterone impaired ovariectomized females' acquisition of the place (spatial) version of the Morris water maze (Chesler and Juraska, 2000). These studies provide experimental evidence in support of the hypothesis that progesterone may impair acquisition of a spatial task. Additionally, it helps to explain why females in proestrus demonstrated decreased working memory (Stackman et al., 1997), as progesterone is elevated late in the day of proestrus.

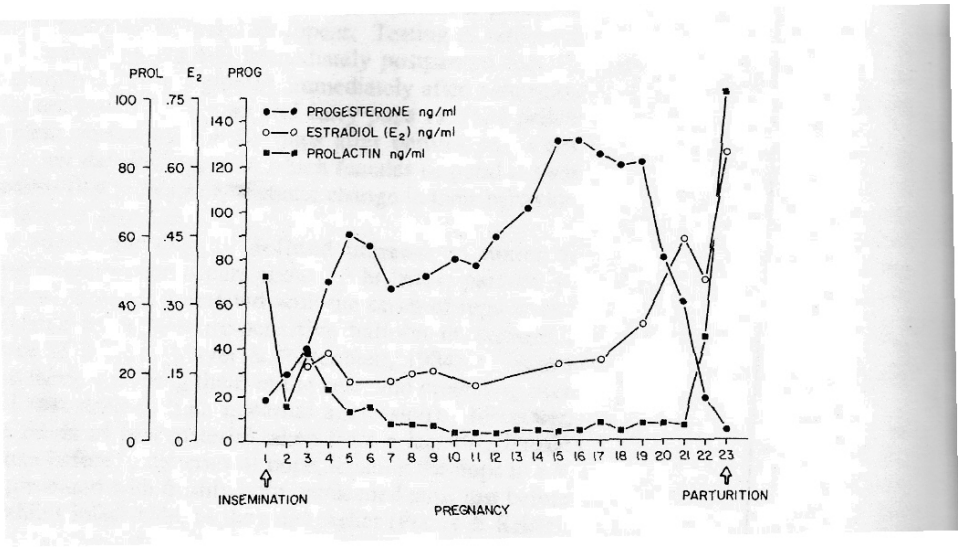
1.b Gonadal hormones during pregnancy

Based on the growing body of evidence that estrogen enhances the memory capabilities of females, prolonged exposure to elevated estrogen levels during pregnancy could have effects on spatial memory performance. A stable, prolonged elevation and subsequent decrease, first in progesterone and then in estrogen, occurs during pregnancy (Figure 1A). In primates (Reyes, Winter, Faiman, & Hobson, 1975) and rats (Rosenblatt, Meyer, & Giordano, 1988), pregnancy is marked by first an elevation of progesterone (throughout the first and second trimester), followed by an elevation of estrogen (beginning in the second trimester, with a peak just prior to labor; Figure 1A). Elevation of estrogen and progesterone during pregnancy occurs for a much longer duration than the

elevation on proestrus during the estrous cycle (Figure 1B). The rat estrous cycle consists of four days (proestrus, estrus, diestrus I, diestrus II); elevations of estrogen, progesterone and prolactin are seen only on proestrus, with a return to baseline 24 hours later on estrus (Figure 1B). During pregnancy, as compared to before, estrogen levels are elevated for approximately seven days, progesterone levels for almost 14 days, and prolactin levels for 3 days (Figure 1A). As these hormones remain high for a much longer duration than during the estrous cycle, pregnancy provides an opportunity to examine whether prolonged exposure to estrogen, progesterone and/or prolactin may influence memory.

Much of the existing research about behavioral changes in the mother during pregnancy and lactation has focused on the development and display of maternal behaviors (i.e. licking, grooming, and pup retrieval), which seem to arise from the hormonal changes occurring during pregnancy and postpartum periods (Rosenblatt, Mayer & Giordano, 1988). These elevations in hormones result in permanent neural changes in the new mother's brain, with the new 'maternal circuit' responsible for the newly acquired behaviors (Fleming, O'Day & Kraemer, 1999). Specifically, pregnancy and pregnancy-like steroid treatment with an estrogen and progesterone regimen resulted in larger soma, more basal dendrites, and increased dendritic length for neurons in the medial pre-optic area (Keyser-Marcus et al., 2001), an area known to be influential in the onset of maternal behavior.

A



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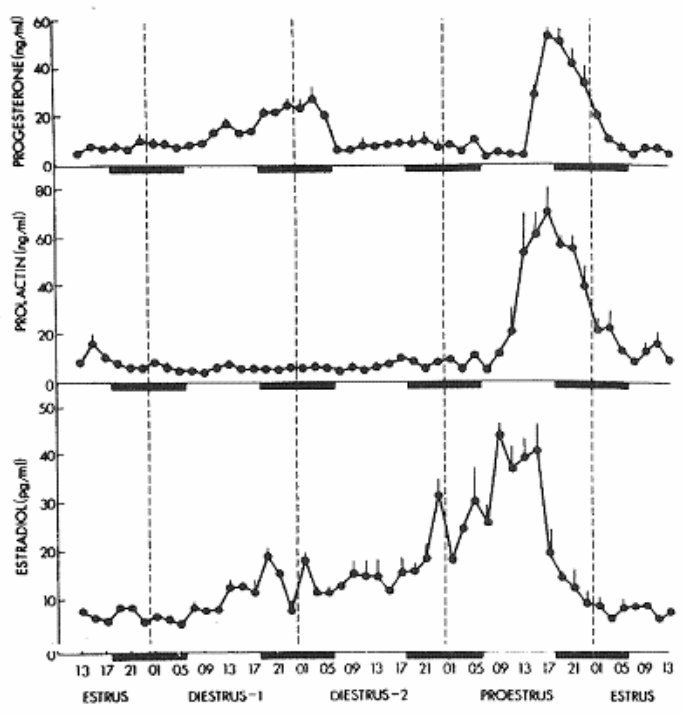

Figure 1: Estrogen, progesterone & prolactin levels during pregnancy and estrous cycle of the rat. A. Levels of estrogen, progesterone and prolactin during the 21 days of pregnancy in the rat. Figure from Rosenblatt, Mayer & Giordano (1988). **B.** Estrogen, progesterone and prolactin concentration in peripheral plasma during the 4-day rat estrous cycle. Figure from Smith, Freeman & Neill (1975).

The medial amygdala also seems to influence maternal behavior, particularly recognition of offspring (Keller et al., 2004). Two nuclei in particular (posterodorsal nucleus of medial amygdala: MePD and medial nucleus of amygdala: MeAD) are affected by pregnancy, with postpartum females displaying more dendritic spines in the MeAD and fewer dendritic spines in the MePD than virgin females (Rasia-Filho, Fabian, Rigoti & Achaval, 2004). As the medial amygdala inhibits maternal behavior in virgin females (Sheehan et al., 2001), growth in these nuclei could counter the inhibition, and help to turn on maternal behavior in the new mom.

While estrogen and progesterone are elevated during pregnancy (Figure 1A), other hormones are elevated during parturition and lactation, particularly oxytocin and prolactin, both of which appear to aid in display of normal maternal behavior (Young & Insel, in Becker, Breedlove, Crews & McCarthy, 2002). Oxytocin, a neuropeptide, is produced in the hypothalamus, and released into the bloodstream by the posterior pituitary (Young & Insel, in Becker et al., 2002). Vaginal stimulation causes peripheral release of oxytocin whereupon it induces normal labor and lactation in the new mother (Argiolas & Gessa, 1991; Bielsky & Young, 2004). During the postpartum period oxytocin also contributes to the initiation and regulation of maternal behavior (Pederson & Boccia, 2003). Postpartum females given intracerebroventricular infusion of an oxytocin antagonist for 2 hours demonstrated significantly decreased pup licking, increased self-grooming, decreased frequency of upright posture over pups, and increased prone posture over pups (Pederson & Boccia, 2003). Similarly, when compared to nulliparous wild-type mice, nulliparous oxytocin knockout mice (lacking the oxyotocin gene) demonstrated significantly fewer pup retrievals and pup licking behaviors, demonstrating decreased maternal behaviors

(Pederson, Vadlamudi, Boccia & Amico, 2006). Thus, oxytocin appears necessary for shifting females' grooming from self to her pups, retrieval of pups back to the nest, and posturing over pups for nursing and protection (Pederson & Boccia, 2003).

Prolactin levels increase dramatically on the day of parturition (Figure 1B). The rise in prolactin seems to be critical for onset of maternal behavior, but only after priming with estrogen and progesterone during pregnancy (Young & Insel, in Becker et al., 2002). Throughout the brain, and particularly in the mPOA, prolactin receptor mRNA expression was significantly higher two hours postpartum than during diestrus, pregnancy or lactation, and was significantly higher during late pregnancy than during lactation (Mann & Bridges, 2002). Thus, prolactin levels were highest just around parturition, consistent with previous studies indicating that estrogen (also increased substantially at end of gestation) increases prolactin receptor gene transcription (Mann & Bridges, 2002).

Exogenous prolactin administration to the lateral ventricles or mPOA stimulated maternal behavior in ovariectomized, nulliparous females with prior estrogen and progesterone priming, mimicking the hormonal environment during pregnancy (Bridges, Numan, Ronsheim, Mann & Lupini, 1990; Bridges & Freemark, 1995). Similarly, infusion of a prolactin receptor antagonist to estrogen and progesterone primed nulliparous females significantly delayed the onset of maternal behavior (Bridges, Rigero, Byrnes, Yang & Walker, 2001). Interestingly, as compared to virgins, primiparous females eight weeks post weaning had higher levels of prolactin receptor mRNA in the mPOA, indicating that reproductive experience increased central prolactin generation and responsiveness (Anderson, Grattan, van den Ancker & Bridges, 2006). Thus, it appears as

if pregnancy and lactation enhanced prolactin responsiveness, possibly accounting for retention of maternal behavior observed in primiparous females (Bridges, 1975).

Recent evidence suggests that prolactin triggers the onset of maternal behavior through increased activity in the olfactory bulb. Olfaction and olfactory discrimination are important cues in offspring recognition and rearing (Kendrick et al., 1997); recent evidence indicated that cell proliferation occurs during pregnancy in the forebrain subventricular zone, which gives rise to olfactory bulb interneurons (Shingo, Gregg, Enwere, Fujikawa, Hassam, Geary et al., 2003). The number of new neurons (as labeled by BrdU staining) in the forebrain subventricular zone was increased by 65% in pregnant females (day 7) as compared to virgins (Shingo et al., 2003). Additionally, significantly more of the BrdU stained neurons of pregnant females were found in the olfactory bulb four weeks later than in virgins (Shingo et al., 2003). Interestingly, infusion of prolactin into virgins for six days increased BrdU-labeled cells to equal those found in pregnant females, indicating that the creation of new cells is regulated by the presence of prolactin (Shingo et al., 2003). Thus, prolactin appears to trigger maternal behavior by enhancing females' ability to recognize their offspring (Bridges & Grattan, 2003). In summary, increased levels of estrogen and progesterone during pregnancy appear to prime females for subsequent exposure to oxytocin and prolactin, which most directly influence the expression of maternal behaviors.

1.c Pregnancy influences on anxiety

In addition to the 'traditional' maternal behaviors listed above, reproductive experience also seems to enhance certain behaviors and promote a physiological state that

may be associated with aiding a new mother in rearing her offspring. Recent research has shown that females who have had reproductive experience had a lowered stress response in comparison to controls (da Costa, Wood, Ingram & Lightman, 1996; Wartella, Amory, Macbeth, McNamara, Stevens, Lambert et al., 2003). Compared with virgin females, females in late pregnancy (days 19-21) or early lactation (days 3-4 post partum) demonstrated significantly decreased neuronal activity as measured by c-fos mRNA expression in areas associated with stress activation and regulation, including the hypothalamus, medial amygdala and lateral septum (da Costa et al., 1996). Similarly, females with one litter (primiparous) and females who had given birth to two litters (multiparous) showed a significantly decreased stress response after restraint than did virgin females (Wartella et al., 2003). Multiparous and primiparous females also had significantly fewer c-fos immunoreactive neurons in CA3 hippocampus and basolateral amygdala as compared to virgin females, indicating decreased neuronal activity due to prior reproductive experience in two brain regions typically activated by stressful stimuli (Wartella et al., 2003).

Additionally, females currently experiencing their first pregnancy (primigravid) or their second pregnancy (multigravid), as well as primiparous and multiparous females, displayed a decreased stress response after exposure to open field than did nulliparous females (Wartella et al., 2003). Stress was measured by rearing onto the hind legs, freezing (increased numbers of both are indicators of higher stress level) and number of grid crossings (increased numbers is an indicator of lower stress level, and greater willingness to explore the environment). Both parous groups displayed significantly fewer rearings than either of the other two groups; both gravid groups had significantly fewer

rearings than did nulliparous females. Nulliparous females had significantly more freezes and fewer grid crossings than did either of the four reproductive groups (Wartella et al., 2003).

A commonly applied procedure for measuring anxiety is the elevated plus maze (EPM; see Table 1 for list of common abbreviations used throughout this paper), which consists of two open arms and two closed arms. Rats like enclosed spaces; exploring an open space is an indicator of decreased anxiety (Pellow et al., 1985). Using increased duration of time spent on the open arms as an indicator of decreased anxiety, anxiety is significantly reduced in Long-Evans females during the first week postpartum, but only if they are exposed to pups (Lonstein, 2005). Additionally, at 10 and 14 months of age, primiparous (1 pregnancy and litter) and multiparous (2 pregnancies and litters) females spent a significantly greater amount of time in the open arm of the EPM as compared to nulliparous females (Love et al., 2005). Taken together, the previous studies indicate that reproductive experience can decrease the stress response typically shown by a rat in response to either restraint or exposure to an open area (whether in the open field or on the EPM).

1.d Pregnancy influences on memory

In addition to decreasing the stress response, recent work has demonstrated that the prolonged presence of estrogen and progesterone in the female brain during the reproductive period alters the hippocampus, an area not typically thought of as being involved in maternal behaviors (Kinsley et al., 2006). Specifically, physiological changes in the CA1 hippocampus occur during pregnancy in a manner similar to those occurring

during the estrus cycle (Woolley et al., 1990). Late pregnant and lactating rats both demonstrated increased spine density in the CA1 hippocampus as compared to virgin females (Kinsley et al., 2006). Additionally, treating ovariectomized females with a hormone regimen similar to that seen during pregnancy resulted in greater numbers of CA1 dendritic spines than in untreated ovariectomized females (Kinsley et al., 2006). Thus, exposure to elevated reproductive hormones during pregnancy, or hormonal treatment similar to pregnancy, affects the morphology of the CA1 hippocampus.

An important issue is whether these physiological changes in the hippocampus affect the new mother, possibly by enhancing her spatial abilities. Enhanced spatial memory could be of biological importance to the female, as parturition has been shown to facilitate learning of novel food preferences, in addition to enhancing onset of maternal behaviors (Fleming, Kuchera & Lee, 1994). More recently, Lambert et al (2005) have found that primiparous females exposed to pups had shorter latencies to reach food (i.e. increased foraging ability) than did primiparous females not exposed to pups. Therefore, enhanced spatial abilities could provide the mother with greater ability to forage for her pups.

Indeed, pregnancy and subsequent lactation have reliably enhanced females' spatial memory abilities. Primiparous females made significantly fewer working memory errors (repeated entries into baited arms) and reference memory errors (entries into non-baited arms) on the radial arm maze than did nulliparous females (Pawluski, Walker & Galea, 2006). Additionally, enhancement of spatial working memory appears soon after onset of pregnancy, as rats between the first and second trimesters had shorter latencies and path lengths to escape on the Morris water maze than did non-pregnant females (Galea et al.,

2000). In a recent, similar study, no differences were seen between pregnant and virgin females in ability to acquire, consolidate, or recall platform location in the Morris water maze over the three weeks of pregnancy (Bodensteiner, Cain, Ray & Hamula, 2006). However, throughout testing pregnant females were significantly faster, as compared to virgin females, to locate the hidden platform when moved to a new location, indicating that pregnant females had better spatial memory of their environment than virgin females (Bodensteiner et al., 2006).

As mentioned above, the peptide oxytocin regulates maternal behavior. It also appears necessary for social memory, both for mates and offspring (Bielsky & Young, 2004). Oxytocin knockout mice display complete deficits in social recognition. Male and female oxytocin knockout mice are unable to distinguish between a mate and a novel stimulus animal of the opposite sex (Ferguson, Young, Hearn, Matzuk, Insel & Winslow, 2000; Bielsky & Young, 2004; Winslow & Insel, 2004). The deficits do not represent impaired sensory processing, as knockout and wildtype mice do not differ in their ability to locate hidden food, navigate the Morris water maze, or habituate to acoustic startle (Ferguson et al., 2000; Winslow & Insel, 2004). In sheep, oxytocin has been shown to facilitate offspring recognition by inhibiting aversive responses of the ewe to amniotic fluid odors (Kendrick, da Costa, Broad, Ohkura, Guevara, Levy et al., 1997), most likely by mediating release of GABA in the olfactory bulb (in Bielsky & Young, 2004).

Finally, oxytocin has recently been found to enhance long-term spatial memory through activation of the MAP kinase pathway (Tomizawa, Iga, Lu, Moriwaki, Matsushita, Li, et al., 2003); blockage of this pathway consistently impaired learning and memory (Bi, Foy, Vouimba, Thompson & Baudry, 2001). Compared to control multiparous females,

multiparous females injected with an oxytocin antagonist demonstrated impaired reference memory on the radial arm maze (Tomizawa et al., 2003). Therefore, oxytocin may contribute to the overall effect of reproductive experience on learning capability (Monks, Lonstein, & Breedlove, 2003). In sum, oxytocin is necessary for display of full maternal behaviors, and appears to enhance the spatial ability of females with reproductive experience.

Thus, all aspects of maternal experience (pregnancy, birth, lactation, hormonal environment and pup exposure) seem to combine to allow the mother to obtain the resources necessary to promote the survival of her offspring, with particular relevance to enhancing the mother's ability to forage and find food (Lambert et al., 2005).

1.e Estrogen changes throughout female lifespan

As females age, ovarian hormone levels change. Female rats begin to experience reproductive senescence (estropause) beginning around 9-12 months of age, when estrous cycles become irregular or may cease altogether (Warren and Juraska, 2000). At this time, females enter a persistent estrus or pseudopregnancy/persistent diestrus period. Females in either group have estrogen levels that are roughly comparable to normally cycling females in estrus, but lower than females in proestrus (in Warren and Juraska, 2000). Senescence is also often characterized by a decline in spatial memory, but most of the aging studies investigating this decline have been performed solely in males (Warren & Juraska, 2000). Due to the changing pattern of hormonal secretion in aging females, researchers have questioned whether or not spatial memory decline in females may follow a different

pattern than that of males. Additionally, researchers are interested in whether or not hormone replacement might reverse cognitive decline.

Warren and Juraska (2000) examined spatial memory decline in females. Aging female rats in persistent estrus or pseudopregnancy/ persistent diestrus were tested on the spatial version of the Morris water maze. While radioimmunoassay confirmed similar levels of circulating estradiol between the two groups, the females in persistent estrus performed slightly better in the water maze compared to both pseudopregnant females and aged males (Warren and Juraska, 2000). As stated above, aged females in persistent estrus have estrogen levels similar to that of cycling females in estrus. This indicates that slightly elevated levels of estrogen (as compared to females in persistent diestrus) may be beneficial in maintaining spatial memory in aged female rats. However, the presence of similar estradiol levels in the persistent estrus and pseudopregnant groups indicated that other factors may play a role in decreased performance in the pseudopregnant females; as stated above and below, some evidence points to the hormone progesterone.

Similar to their younger, normally cycling counterparts, aged females in persistent estrus also have low levels of progesterone. Similarly, aged females in persistent diestrus have high levels of progesterone, approximating those found in young, normally cycling proestrus females (in Warren and Juraska, 2000). Thus, one hypothesis for these results is that circulating estrogen in the persistent estrus group may have helped to slow down the cognitive decline, but higher levels of circulating progesterone may have countered estrogen's helpful effects in the persistent diestrous females (Warren and Juraska, 2000). This idea is identical to that proposed for why normally cycling females in proestrus may

perform worse than females in estrus (Warren and Juraska, 1997; Stackman et al., 1997; Chesler and Juraska, 2000).

Other studies have demonstrated that maintaining estrogen levels in aged females aids in prevention of forgetting during acquisition of spatial tasks. Chronic replacement of estrogen or estrogen plus progesterone beginning immediately or 3 months after ovariectomy significantly enhanced acquisition of a delayed-match-to-position task (Gibbs, 2000). Acute and chronic estrogen treatment, as well as chronic replacement of estrogen and progesterone, enhanced acquisition of the Morris water maze (Markham, Pych & Juraska, 2002). Specifically, all hormone replacement regimens prevented forgetting from the first to the second day of testing (Markham, Pych & Juraska, 2002). Thus, estrogen replacement may be most beneficial in terms of aiding females' initial learning of a spatial task (acquisition), rather than enhancement of spatial abilities during task performance (memory).

Interestingly, in both of the above studies (Gibbs, 2000; Markham et al., 2002), chronic replacement of estrogen plus progesterone was just as effective at preventing forgetting as was chronic replacement of estrogen alone. The role of progesterone in hippocampal plasticity and functioning has been investigated less than estrogen. Administration of estrogen alone enhances females' performance on spatial tasks (Luine et al., 1998; Luine, Jacome & MacLusky, 2003), and there is evidence that progesterone alone is responsible for impaired performance on spatial tasks (Warren and Juraska, 1997; Stackman et al., 1997; Warren and Juraska, 2000). Co-administration of estrogen with progesterone may act to counter the deficits produced by progesterone alone.

The exact mechanism through which estrogen may aid in acquisition or performance on a spatial task, even in the presence of progesterone, remains unknown. One hypothesis put forth is that estrogen is neuroprotective in aged females; however, studies of aged males have found no appreciable loss of hippocampal neurons during normal rat aging, indicating that neuronal loss is not a likely candidate for spatial memory decline due to aging (in Markham, Pych & Juraska, 2002). Additionally, in this study, acute estrogen replacement was found to be just as effective as chronic replacement in aiding acquisition of the water maze task (Markham, Pych & Juraska, 2002). Acute injections do not maintain estrogen levels long enough for the hormone to exert any neuroprotective effects (i.e. prevention of neural death, maintenance of dendritic arbors; see Manthey & Bell, 2006 for review); therefore, when administered acutely, estrogen must have a positive impact on spatial memory during rat female aging by some other mechanism.

Another hypothesis that has been put forth as a mechanism for estrogen to enhance acquisition of a spatial task involves the effects of estrogen on long-term potentiation (LTP), a long-lasting form of synaptic plasticity in which the synapse remains sensitized for a long period; LTP is a mechanism through which memory acquisition may occur (Warren, Humphreys, Juraska and Greenough, 1995). In addition to increasing dendritic spine and synapse density in young females (Woolley and McEwen, 1992; McEwen and Woolley, 1994), naturally higher levels of estrogen during proestrus were associated with the induction of LTP in the CA1 hippocampus (Warren et al., 1995). It has been proposed that this elevation in estrogen results in heightened synaptic activity to the extent that too much ‘noise’ is added to the system; this over-activity may be detrimental to acquisition of

a spatial task (Markham, Pych and Juraska, 2002). As induction of LTP gets more difficult in aging animals, elevating estrogen may again boost synaptic activity, but this time to a level that is beneficial for maintaining normal hippocampal functioning (Markham, Pych and Juraska, 2002). Thus, estrogen replacement may be beneficial for performance on spatial tasks in older females. Despite lack of knowledge on the exact mechanism, the available literature does indicate that administration of estrogen alone, or estrogen plus progesterone, aids in memory maintenance as female rats age.

1.f Long-term effects of multiple pregnancies

Recently, researchers have begun to look at whether reproductive experience might affect cognitive aging in a manner similar to estrogen replacement, due to prolonged exposure to estrogen and progesterone while pregnant. While little research has investigated the long-term effects of reproductive experience on aging, the extant evidence indicates that multiple pregnancies are beneficial in maintaining memory capabilities, while the females are young and as they age (Kinsley et al., 1999; Gatewood et al., 2005).

Multiparous females (2 pregnancies and litters, followed by full 21 days of maternal care until weaning) demonstrated enhanced working memory on the radial arm maze as compared to nulliparous rats (Kinsley et al., 1999). A similar study found that multiparous females had a reduced number of working memory errors in the radial arm maze; a non-significant trend existed towards enhanced spatial memory performance as compared to nulliparous females, as demonstrated by a decrease in total errors on the maze (Pawluski, Walker & Galea, 2006). When tested on a different spatial task (a dry-land version of the water maze), multiparous females had significantly shorter latencies to find a

baited food well than nulliparous females (Love et al., 2005). These three studies indicate that experiencing more than one pregnancy is beneficial to performance on a spatial task, at least while the mother is still young (in both studies, the females were tested at approximately 4-5 months of age).

A recent study indicated that early pregnancy experience may be beneficial for maintaining memory in females throughout their lifespan (Gatewood et al, 2005). Multiparous females learned a dry-land version of the Morris water maze at 6 months of age significantly faster than did primiparous (1 pregnancy and litter) or nulliparous (virgin) females. Interestingly, on three successive tests (at 12, 18, and 24 months of age), this pattern continued: multiparous females consistently outperformed the other two groups; however, at these three later testing periods primiparous females also outperformed the nulliparous group (Gatewood et al., 2005).

This behavioral difference may be due to the multiparous females displaying significantly fewer deposits of amyloid precursor protein (APP) throughout the CA1 hippocampus and dentate gyrus (Gatewood et al, 2005). As the presence of APP is a marker for neurodegeneration and age-related cognitive decline (see Chapman et al., 1999), it is possible that reproductive experience may help to maintain memory as female rats age through this mechanism of inhibiting APP deposits. Although not examined in the Gatewood (2005) study, it is also possible that memory maintenance in the multiparous females may be due to the presence of new neurons, as it was previously found that aged male animals (23 months) that exhibited unimpaired spatial memory had a higher level of cell proliferation and a higher number of new neurons throughout the hippocampus and dentate gyrus, as compared to aged rats with spatial memory impairments (Drapeau et al.,

2003). Thus, multiple reproductive experiences may have a beneficial effect on spatial memory by maintaining neural functioning, particularly in the hippocampal formation.

1.g Mechanism for pregnancy effects on cognition: monoaminergic activity

Any observed behavioral differences between virgins and females with reproductive experience are likely to be accompanied by neuroanatomical differences in specific brain areas. Brain regions that make up the 'maternal circuit', particularly the medial preoptic area (mPOA), have high levels of monoaminergic neurotransmitters, including dopamine (DA), norepinephrine (NE), and serotonin (5HT) (Lonstein et al., 2003). Estrogen appears to regulate monoamine levels, particularly dopamine and norepinephrine. During the estrous cycle, concentrations of NE and DA significantly changed in a number of brain nuclei, including the mPOA, a region believed to be integral in female ovulation and mating behavior (Crowley, O'Donohue, & Jacobowitz, 1978). Additionally, the levels of NE, DA, 5HT and their metabolites were significantly altered during proestrus, as compared to diestrus, in areas implicated in the descending circuit for lordosis regulation (medial preoptic area, ventromedial nucleus of hypothalamus, midbrain central gray; Luine, 1993). Thus, areas of the brain contributing to sexual solicitation and receptivity behaviors in female rats demonstrated significant changes in monoaminergic activity during proestrous.

The onset of maternal behaviors in new mothers may also depend upon changes in monoamine systems. In the mPOA, a region known to be important for promoting maternal behaviors, dopamine levels are high in virgin and early pregnant females, then decrease to be lowest on day of parturition, and then become very high during lactation;

DOPAC levels (a dopamine metabolite) also followed this pattern (Lonstein et al., 2003). In the doroslateral striatum (containing the nucleus accumbens), dopamine turnover was highest during late pregnancy and day of parturition (Lonstein et al., 2003). Additionally, multigravid rats (second pregnancy) had significantly higher levels of dopamine in the striatum and hypothalamus than primigravid (first pregnancy) rats (Felicio et al., 1996), and dopamine uptake is increased in the striatum during pup-licking by the dam (Hansen, Bergvall & Nyiredi, 1993). Thus, dopamine levels increased significantly throughout the brain during lactation, indicating that dopamine could influence the onset of maternal behaviors.

In addition to the areas mentioned above, the concentration of certain monoamines is also altered in the hippocampus during pregnancy. The concentration and turnover rate of norepinephrine in the hippocampus is greatly depressed during pregnancy, then rises through late pregnancy and sharply at parturition (Smolen, Smolen & van de Kamp, 1987; Desan, Woodmansee, Ryan, Smock & Maier, 1988). Similarly, females in late pregnancy (day 20) had significantly higher levels of the norepinephrine metabolite MHPG, the ratio of MHPG to norepinephrine, and the serotonin metabolite 5-HIAA levels as compared to females in mid pregnancy (Desan et al., 1988; Glaser, Russell & Taljaard, 1992). NE is modulatory, aiding in synaptic efficacy (Woodward et al., 1991), and with its metabolites appears to modulate cognitive processes such as vigilance, attention, and memory (Lapiz & Morilak, 2006). Elevation of NE levels in the prefrontal cortex aided male rats' performance on an attention-based task (Lapiz & Morilak, 2006). NE levels may thus be elevated at late pregnancy and parturition in order to enhance females' ability to pay attention to her offspring.

NE may also act to reduce stress around parturition. A decrease in noradrenergic activity occurs in late pregnancy in rats (day 20), which may contribute to the reduced responsiveness of the HPA to stress during late pregnancy (Douglas, Meddle, Toschi, Bosch & Neumann, 2005). Evidence in support of this idea exists in human women as well. Reduction in NE is associated with a relaxed state during pregnancy (Teixeira, Martin, Prendiville & Glover, 2005), and levels of MHPG in cerebrospinal fluid were significantly decreased in women just prior to parturition (Altemus, Fong, Yang, Damast, Luine & Ferguson, 2004). Thus, activity in the NE system may affect the females' stress response to aid in ability to mother her offspring.

Also in humans, depletion of serotonin throughout the brain impaired performance on a verbal task, and depletion of dopamine throughout the brain impaired performance on a spatial memory task (Harrison, Olver, Norman, Burrows, Wesnes & Nathan, 2004). Thus, presence of specific monoamines seems important for attention, stress response, and performance on memory tasks in both humans and rats. As the hippocampus is important for both spatial memory and recognition memory in rats (Broadbent, Squire, & Clark, 2004), it is possible that changes in norepinephrine, dopamine, and serotonin levels in the hippocampus due to pregnancy could affect memory performance.

1.h Mechanisms for pregnancy effects on cognition: BDNF

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin class (also included are nerve growth factor and neurotrophin 3; see Sohrabji, Miranda & Toran-Allerand, 1995). Of the neurotrophins , BDNF in particular appears to be important to neuronal maintenance into adulthood (Allen & Dawbarn, 2006). It stimulates cell

proliferation in a variety of areas (including basal forebrain and cortex), plays a role in modulating synaptic strength, regulates dendritic and axonal growth, and most importantly for this research, facilitates synaptic transmission at excitatory hippocampal synapses (see Allen & Dawbarn, 2006 for review).

Estrogen regulates BDNF protein levels and mRNA expression within the hippocampus (see Sohrabji et al., 1995). Significant fluctuations in levels of BDNF mRNA in the CA1 and CA3 hippocampal regions were detected across the estrous cycle of gonadally intact female rats: on proestrus and estrus females showed significantly higher levels of BDNF protein than any other day of the estrous cycle (Scharfman et al., 2003). Twenty-eight weeks post ovariectomy, females with estradiol replacement for 25 weeks had significantly elevated BDNF mRNA levels in CA3, CA4 and dentate gyrus hippocampal subregions as compared to ovariectomized females given sham treatment (Singh, Meyer & Simpkins, 1995). In a similar study, treatment with 17β-estradiol for only 14 days resulted in increased BDNF mRNA in medial and basomedial amygdala, as well as CA1 and CA3 regions of hippocampus (Zhou, Zhang, Cohen & Pandey, 2005).

Acute hormone treatment (single injection of 10μg 17β estradiol) also enhanced BDNF mRNA and total protein levels (Gibbs 1998, 1999). Animals sacrificed 53 hours post treatment (the time point at which natural hormone levels would have returned to baseline after elevation on day of proestrus, Yoshinaga, Hawkins & Stocker, 1969) showed significant increases in BDNF mRNA in the CA1 region (28% above controls) and CA3 region (77% above controls; Gibbs 1998), as well as significant increases in BDNF protein (detected by enzyme-linked immunosorbent assay (ELISA)) in the septum, but not the whole hippocampus (Gibbs, 1999). As the septum contains the cell bodies of

magnocellular cholinergic neurons which project to the hippocampus, and these neurons have been shown to retrogradely-transport BDNF from the hippocampus to the medial septum (in Gibbs, 1999), it appears as if estradiol treatment may affect BDNF expression and transport throughout the hippocampal formation (also in Sohrabji, 1995).

During pregnancy, estrogen remains elevated (as compared to cycling females) for approximately 7 days, with levels rising sharply just prior to parturition (Figure 1A); it is possible that elevated estrogen levels during pregnancy could increase BDNF expression in a manner similar to those described above, after exogenous estrogen treatment (Singh et al., 1995; Gibbs, 1998; Zhou et al., 2005). However, treatment with estrogen plus progesterone has a different effect than estrogen treatment alone, with levels of hippocampal BDNF no different from those seen in ovariectomized controls (Bimonte-Nelson et al., 2004), or significantly lowered as compared to controls (Gibbs, 1999). Decreased levels of BDNF expression in the presence of progesterone corresponded with previously mentioned results that progesterone decreased dendritic spines and synapses, and may be responsible for impaired spatial memory performance. As progesterone levels are also significantly elevated during pregnancy, and remain high for approximately 14 days (Figure 1), it is possible that any positive effects of estrogen on BDNF expression due to pregnancy could be mitigated by the presence of high levels of progesterone.

As estrogen treatment appears to enhance memory abilities (particularly hippocampally-dependent spatial memory; see Luine et al., 1998; 2003), and estrogen increases BDNF expression, BDNF may be implicated in performance on memory tasks. Recent evidence indicates that BDNF is important for short-term and long-term memory, as bilateral infusions of anti-BNDF antibody into CA1 hippocampus impaired short-term

and long-term memory retention scores on a one trial fear-motivated learning task (Alonso et al., 2002a; Alonso et al., 2005). Additionally, infusion of BDNF for 7 days prior to chronic immobilization stress protected against stress-induced impairments on the Morris water maze (Radecki, Brown, Martinez & Teyler, 2005); in other words, normal levels of BDNF maintained memory that normally decreases after chronic stress. Thus, the presence BDNF protein seems to be beneficial for maintaining spatial memory; it is possible that BDNF may be a protein that mediates the positive effects of reproductive experience on spatial memory.

2. Specific aims of the study

Pregnancy appears to be associated with behavioral changes, particularly in spatial memory and anxiety. Additionally, neural changes occur during pregnancy, particularly in the hippocampal region. Research also exists indicating that multiple pregnancies early in life may be beneficial in maintaining memory throughout the female's lifespan. The current study will examine spatial memory, recognition memory, and anxiety in relation to pregnancy and reproductive experience, as well as examine possible neural systems that may be affected by parity.

2.a Specific Aim 1: Determine the effects of pregnancy state on spatial memory and anxiety.

The possible relationship between pregnancy state and cognitive function and anxiety will be examined. The hypothesis is that late pregnant (LP) females will demonstrate better memory on a spatial task as compared to early pregnant (EP) and nulliparous (NP) females and that LP females will demonstrate decreased anxiety as tested on the elevated plus maze in comparison to EP and NP females. This hypothesis is based on previously published results and because gonadal hormones influence these behaviors. The spatial memory task, object placement, has not been previously investigated in relation to pregnancy state.

2.b Specific Aim 2: Determine effects of multiple reproductive experiences on memory and anxiety.

The possible relationship between multiple reproductive experiences and alterations in cognitive function and anxiety will be examined. The performance of multiparous (MP; female with at least 5 litters) and age-matched nulliparous (NP) females will be compared

on recognition and spatial memory tasks; it is hypothesized that MP females will out-perform NP females on both tasks and will display a reduced anxiety response on the EPM as compared to age-matched NP females. This hypothesis is based on previously published results indicating that parity can maintain spatial memory throughout the lifespan and can decrease anxiety after parturition. Object placement and object recognition (a non-spatial memory task) have not been previously investigated in relation to parity.

2.c Specific Aim 3: Determine effects of reproductive experience on levels of monoamines and metabolites in brain areas which contribute to memory.

Monamine-containing neural systems contribute to memory function. Thus, a possible mechanism underlying performance on memory tasks is pregnancy-dependent effects on monoaminergic systems in specific brain regions that contribute to memory and maternal behavior: CA1, CA3 hippocampus, pre-frontal cortex, and mPOA. It is hypothesized that significant differences in levels and activities of monoamines will be observed between NP, EP & LP females, particularly in DA and NE concentration. Similarly, it is hypothesized that long-term alterations in monoamine systems due to multiple reproductive experiences will be seen, with significant differences in levels and activities of monoamines observed between MP and NP females in four brain areas: CA1, CA3 hippocampus, pre-frontal cortex, and olfactory bulb.

2.d Specific Aim 4: Determine effects of reproductive experience on BDNF expression in brain areas which contribute to memory.

Another mechanism that may underlie differences in performance on memory tasks is BDNF expression in the hippocampal formation. It is hypothesized that MP females

will have greater BDNF expression in both hippocampus and septum than age-matched NP females. This hypothesis is based on the fact that exposure to estrogen (such as that experienced by MP females) upregulates BDNF expression in the hippocampus and septum, and that BDNF is implicated in memory performance. BDNF has not previously been investigated in relation to parity.

Table 1. List of common words and their abbreviations throughout this thesis.

Term	Abbreviation
dopamine	DA
early pregnant	EP
elevated plus maze	EPM
late pregnant	LP
medial preoptic area	mPOA
multiparous	MP
norepinephrine	NE
nulliparous	NP
object recognition	OR
object placement	OP
serotonin	5HT

3. Effects of pregnancy state on spatial memory and anxiety

Influences of pregnancy state on spatial memory were assessed using the object placement (OP) task, a test of spatial memory (Luine et al., 2002). The task was given at a 2 hour ITD, in which object location can be discriminated by control (ovariectomized and gonadally-intact, untreated females) and hormone-treated females (Luine et al., 1998; 2003), and a 4 hour ITD, which is more difficult for female rats. Performance after both delays was analyzed in females in two states of pregnancy (early: days 7-8 and late: days 16-17 of pregnancy), and nulliparous females, in order to examine whether pregnancy state might affect performance on a spatial memory task. It was hypothesized that nulliparous (NP: control), early pregnant (EP) and late pregnant (LP) females would all discriminate object location after the 2 hour ITD, indicating spatial memory ability at that delay, as previous studies have indicated that gonadally intact and hormone-treated females can discriminate at this delay (Luine, 2002). In contrast, it was hypothesized that LP females would outperform NP females, and possibly EP females, after the 4 hour ITD, as higher levels of estradiol treatment are typically needed to display spatial memory at the longer delay (Luine, 2002).

Influences of pregnancy state on anxiety were assessed using the elevated plus maze (EPM; Pellow et al., 1985). Based upon previous research (Wartella, 2003; Lonstein, 2005), it was hypothesized that pregnant females would have more entries into, and spend more time in, the open arm of the EPM (reliable measures of decreased anxiety; see Lonstein, 2005), as compared to nulliparous females. Post behavior testing, monoamine concentrations in PFC, CA1 and CA3 hippocampus, and mPOA were measured in NP, EP and LP states, to determine whether pregnancy state altered

monoamine expression in regions underlying memory performance (PFC, CA1, CA3) and expression of maternal behaviors (mPOA; see section 3.b for these results).

3.a Method

Subjects

Sixteen two-month-old (60 days) intact female Sprague-Dawley rats (210-230g) were obtained from Harlan Inc., and double-housed under a 12:12 light:dark cycle (lights on at 5:00 h) with water and food (Purina Rat Chow) available ad libitum. Experiments began after a two-week acclimation period to their home cage, during which all subjects were handled daily by the experimenter. Animals were randomly assigned to either the control (virgin: n = 8) or the pregnant group (n = 8). All testing occurred between 1000-1500 hours. All procedures used were approved by the IACUC of Hunter College of the City University of New York.

Behavior Testing: Object Placement

Tests were conducted as previously described (Bisagno, Ferguson & Luine, 2002; Luine, Jacome & MacLusky, 2003); habituation to the OP task consisted of acclimation to the object recognition (OR) task, followed by the OP task. Each task consisted of a sample trial (T1) and a recognition trial (T2), separated by a 2 or 4 hour ITD. In T1, two identical objects were placed at one end of an open field consisting of a 3 x 3 grid (70 x 70cm, enclosing walls 30cm high); the amount of time spent exploring the two objects over a 3 min period was recorded. Exploration of the objects was defined as any time in which the subject sniffed at, whisked at, or looked at the objects from no more than 2 cm away.

In the OR task, one of the identical objects was replaced by a new object for the duration of T2; the time spent exploring the old (familiar) and the new (novel) object was recorded for a 3 min period. In the OP task, one of the identical objects was moved to a new location in the open field for the duration of T2, and the time spent exploring the objects at the old (familiar) and the new (novel) locations was recorded for 3 minutes. OR tasks used a variety of bottles, cans, and containers. OP tasks used more intricate objects such as funnels, candlesticks, and figurines to encourage longer exploration times. The positions of the old objects, novel objects, and left/right placement of the objects in the field were counterbalanced across both groups. After each trial, the objects and grid were cleaned with a disinfectant.

Acclimation to the OR task consisted of four trials with progressively larger ITDs (1 min, 10 min, 1 hour, 2 hour) between T1 and T2. Acclimation to the object placement task consisted of four trials with progressively larger ITDs (10 min, 40 min, 1 hour, and 2 hour); due to familiarity with the testing apparatus from acclimation to the OR task, it was unnecessary to begin acclimation at the 1 minute delay. After acclimation, animals were tested on the OP task at a 2 hour and a 4 hour ITD, in two pregnancy states: early (days 7-8 of pregnancy) and late (days 16-17 of pregnancy).

Behavior Testing: Elevated Plus Maze

On the day subsequent to OP testing (day 9 for early pregnancy; day 18 for late pregnancy), performance on the elevated plus maze (EPM) was recorded to monitor anxiety. The plus maze (Pellow et al., 1985) was constructed of plywood, painted gray and consisted of two open arms (50cm long x 10cm wide) and two closed arms (50cm long x

10 cm wide x 40cm high). The walls extended from a central neutral area (10 x 10cm); the entire maze was elevated 50cm above the floor. Subjects were placed in the center of the four arms (neutral area), facing one of the open arms and given five minutes to explore the maze at will. The total time spent in the open and the closed arms was recorded, as was the number of entries into open and closed arms. A total of three paws inside of an arm were used as criteria for entry. Time spent in the central, neutral area was not recorded.

Mating and Testing Schedule

Females began the acclimation procedure described above at approximately day 70 of age. Post acclimation, all subjects were given OP tests at two different intervals: a 2 hour ITD followed by a 4 hour ITD on the following day (approx. days 90-91 of age), to ensure that all animals had sufficiently acclimated to the task. Based upon post-acclimation performance, subjects were counter-balanced (evenly split with best, moderate and worst performance) into two groups: nulliparous (n = 8) and pregnant (n = 8). Pilot data (not shown) suggested that in nulliparous females, day of estrous cycle did not affect performance on OR and OP tasks; therefore, nulliparous females were not smeared in this experiment.

Mating began for the pregnant group immediately upon completion of the acclimation and testing period. Three month old male Sprague Dawley rats (~ 350g) were used for mating; each mating session consisted of placing one male and two females into a new, clean cage. After confirmation of pregnancy (determined by presence of a copulatory plug and a noticeable weight gain during the week after first mating), the females were

removed from the male and placed singly into new cages for the duration of the experiment.

At days 7-8 of pregnancy (end of first trimester) nulliparous and pregnant females were tested on the OP tasks (2 hour ITD on day 7; 4 hour ITD on day 8); methods were the same as described above. On the following day (day 9 of pregnancy), nulliparous and pregnant females were tested for anxiety on the EPM (methods as described previously). At days 16-17 of pregnancy (beginning of third trimester), nulliparous and pregnant females were tested again on the OP task at both durations (2 hour ITD on day 16; 4 hour ITD on day 17); methods were the same as described above. On the following day (day 18 of pregnancy), nulliparous and pregnant females were again tested for anxiety on the EPM. Immediately post EPM testing, all 16 females were sacrificed by decapitation (using light anesthesia by carbon dioxide). Trunk blood was collected from all animals, spun for 10 minutes at 13,000rpm to collect supernatant, and then stored at 4^{o}C until later analysis of estradiol (E2), progesterone, and testosterone levels by radioimmunoassay (RIA; described below). The brains were then removed, frozen on dry ice, and stored at -80^{o}C for future analysis of brain neurochemistry.

Additional subjects

As the pregnant females were tested twice, it was not possible to collect blood and brains during early pregnancy. In order to compare neurochemistry and hormone levels between EP, LP and NP females, an additional eight females were impregnated and sacrificed on day 9 of pregnancy, without undergoing any behavioral testing. Trunk blood was collected in the same manner as described above for analysis of serum estrogen,

progesterone, and testosterone levels; the brains from these animals were also removed, frozen on dry ice, and stored at -80^{o}C for later neurochemical analysis.

Radioimmunoassay (RIA)

Serum hormone levels were analyzed as described in Sharfman et al (2003, 2005). Briefly, serum testosterone (T) and progesterone (P) levels were measured utilizing Coat-a-Count kits from Diagnostic Products Corporation (testosterone, catalog #TKTT1; progesterone, catalog #TKPG1), following modified instructions from the kit. The standards provided were used; for testosterone, 0-1600 pg/tube; for progesterone, 0-40pg/tube. For both hormones, serum sample volumes were 50µl. To each sample, 1ml of ^{125}I-testosterone or ^{125}I-progesterone was added, vortexed briefly, then incubated overnight at 4^{o}C. The following day, the liquid was poured off and bound isotope was counted in a Wizard 1470 Automatic Gamma Counter (PerkinElmer Life Sciences (Wellesley, MA). Amount of testosterone and progesterone present was calculated by comparison with the standard curve using log-logit transformation of the data.

Serum estradiol (E) levels were measured independently at two different laboratories. Both laboratories used a I^{125} estradiol Coat-a-Count kit from DPC (catalog #TKE21), which according to the manufacturer's instructions is designed for use with human samples. High levels of estradiol binding protein present in rat serum can result in artificially high measurement of estradiol levels when the standards present in the kit are used (unpublished observations). This interference can be minimized by two different methods. In one laboratory, E levels were measured in the original 16 animals (NP: n = 8; LP: n = 8) using a #TKE21 kit with ether extraction (see Scharfman et al., 2003), which

uses organic solvent extraction to separate estradiol from the binding proteins present in rat serum. Aliquots (100 μl) of serum were extracted with 2 x 1ml anhydrous ethyl ether from a freshly opened can of anesthetic ether; the extracts were transferred to 12 x 75 mm glass tubes and dried under a stream of air. Equal volumes of serum from the kit standards were extracted in parallel, to correct for procedural losses. The dry extracts from both samples and standards were redissolved by addition of 1ml of the kit I^{125} estradiol solution to each tube, and vortexed thoroughly. The mixtures of I^{125} estradiol and the redissolved extracts were poured across into the plastic Coat-a-Count antibody-coated tubes provided in the kit. The remainder of the assay was performed as described above (incubation overnight, followed by bound isotope measurement on the following day).

Serum E levels from the eight additional subjects sacrificed during early pregnancy (day 8) were measured using a #TKE21 kit. The correction for high E levels was achieved by making new standards to minimize the presence of estrogen binding protein in rat serum. These standards were made by serially diluting the highest estradiol standard from the kit (3600pg/ml) with rat serum collected from ovariectomized female rats. Thus, both standards and samples contained the same amount of binding protein; calculations from the binding curve thus provided an accurate measurement of estradiol. The remainder of the RIA was carried out as described above for testosterone and progesterone.

Monoamine analysis

Concentrations of monoamines and metabolites were obtained through high performance liquid chromatography (HPLC) with electrochemical detection: dopamine (DA) and two of its metabolites 3,4-dihydroxy-phenylacetic acid (DOPAC) and

homovanillic acid (HVA); norepinephrine (NE) and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG); and serotonin (5HT) and its metabolite 5-hydroxy indole acetic acid (5-HIAA). Monoamines and metabolites were measured in four brain regions: prefrontal cortex (PFC), CA1 hippocampus, CA3 hippocampus, and medial preoptic area (mPOA). PFC, CA1 and CA3 hippocampus were analyzed due to their role in spatial working memory tasks (Jones & Wilson, 2005; Sloan, Good & Dunnett, 2006); the mPOA was analyzed due to its role in expression of maternal behaviors (Lonstein et al., 2003).

The procedure was as described previously (Bisagno, Ferguson & Luine, 2002). Briefly, the brain was blocked at the PFC (removed and frozen on dry ice) and the cerebellum (discarded); the remaining brain was sliced into 300μm sections using a microtome cryostat at -4^{o}C. Sections containing the entire hippocampus and mPOA were put on microscope slides and kept frozen on dry ice. The frozen PFC and hippocampal sections were sampled with a 500μm-diameter tissue punch (4-6 tissue punches from each animal in the PFC and mPOA, and 10-12 tissue punches from each animal in CA1 and CA3) under a dissecting microscope, on a microscope stage maintained at -4^{o}C.

To each tissue sample, 60μl sodium acetate buffer (pH 5.0) with α-methyl-dopamine as an internal standard was added. The samples were frozen and thawed, then centrifuged, the supernatant drawn off and the pellet re-suspended in 100μl (PFC: 4-6 tissue punches) or 200μl (CA1/CA3: 10-12 tissue punches) of 2.0N NaOH for protein analysis using Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA). The supernatant containing the monoamines and metabolites was measured in a Waters Associates chromatographic system (Waters 2690), consisting of an automated refrigerated injector, pump, C-18 reverse-phase column (Novapak three micron), and an ESA Coulochem III

detector (-150mV to 0.50V potential). The mobile phase, described elsewhere (Luine and Hearns, 1990), contained 3% acetonitrile and peak sharpness was increased by the addition of 100% methanol (99.5% mobile: 0.5% methanol). Millennium software (Waters Associates) was used to run the chromatography system, in which concentrations of transmitters and metabolites were calculated by reference to standards using peak integration. Monoamine concentrations are expressed as pg/μg total protein.

Statistical Analysis

Data from the sample trial (T1) was analyzed separately from each pregnancy state; comparisons were thus made between NP and EP females, or NP and LP females. Data was analyzed with a 2 (group) x 2 (delay) ANOVA to identify any possible differences in overall exploration between the two groups at each test delay (2 and 4 hour ITD).

Data from the retention trial (T2) was analyzed with a 3 (pregnancy state: NP, EP, LP) x 2 (delay: 2 hour OP, 4 hour OP) ANOVA to identify any differences between the groups in exploration ratio (time in new location/total exploration time) at the two testing delays. To further examine possible group differences, planned comparisons between NP and EP state, NP and LP state, and EP and LP states, were carried out using 2 (group) x 2 (delay) ANOVAs.

For the EPM, a one way (pregnancy state: NP, EP, LP) MANOVA was carried out to identify any differences between the groups in two measures of anxiety: entries into open arms and time in open arms. To further examine possible group differences, planned comparisons between NP and EP state, NP and LP state, and EP and LP states, were carried out using 2 (group) x 2 (entries into open arm, time spent in open arm) ANOVA

design. Post-hoc analysis was performed using Tukey's LSD test to analyze individual differences between the three groups, and a *p* value of less than .05 was set for significance.

Data from the RIA was analyzed with a one way (pregnancy state: NP, EP, LP) ANOVA for each hormone measured (estradiol, progesterone, testosterone). Post-hoc analysis was conducted using LSD test to identify specific group differences in serum hormone levels.

Data from HPLC analysis of brain monoamine levels was analyzed with a one-way (group: NP, EP, LP) MANOVA, analyzing seven monoamine concentrations in each of four brain regions (CA1, CA3, PFC, mPOA). Turnover rates of the primary monoamines (DA, NE, 5HT) into their metabolites (DOPAC, HVA, MHPG, 5-HIAA, respectively) were also analyzed with a one-way (group: NP, EP, LP) MANOVA on the four turnover ratios in each of four brain regions (CA1, CA3, PFC, mPOA). Post-hoc analysis was carried out using LSD test; a p value $< .05$ was set for significance.

3.b Results

Object Placement: Overall performance

Exploration was examined on the sample trial (T1) at early and late pregnancy. An ANOVA revealed no difference in object exploration time between NP and EP females, $F(1,17) = 0.88$, $p > 0.05$. (Figure 2A), or between NP and LP females, $F(1,17) = 3.94$, $p < 0.05$ (Figure 2B).

Performance on the retention trial (T2) was analyzed by exploration ratio (time spent in the new object location/total exploration time). The exploration ratio of all three pregnant states (NP, EP, and LP) at both ITDs (2 and 4 hour) was analyzed with an ANOVA, which revealed a significant effect of reproductive experience: $F(6,82) = 4.64$, $p < 0.001$; data not shown. No significant effect of delay or an interaction between group and delay was observed. Post-hoc analysis of the overall group effect revealed that NP females had significantly lower exploration ratios over both ITDs (0.47) than either EP (0.64) or LP (0.69) females ($p <0$ .001); data not shown. To further examine differences between pregnant and non-pregnant females, the data was analyzed at each test time (early and late pregnancy).

Object Placement: Early pregnancy

Planned comparison was made between NP and EP females on the OP task at 2- and 4-hour delays. Analysis of the retention trial (T2) revealed an overall significant effect of group: $F(1,24) = 5.361$, $p < 0.05$. Averaged over both ITDs, the EP exploration ratio (time in new location/total exploration time) was significantly higher than the NP exploration ratio, $p < 0.05$ (Figure 3A). Overall, the EP group showed a ratio 28% higher

than NP females. In addition, NP females performed at chance (50% of their time in the new object location), while EP females performed at greater than chance (64% of time in the new location). There were no significant effects of delay or an interaction between group and delay.

Object Placement: Late pregnancy

Planned comparison was made between NP and LP females on the OP task at 2- and 4-hour delays. Analysis of the retention trial (T2) revealed an overall significant effect of group: $F(1,24) = 7.485$, $p < 0.05$. Averaged over both ITDs, the LP exploration ratio (time in new location/total exploration time) was significantly higher than the NP ratio, $p < 0.05$ (Figure 3B). Overall, the LP group showed a ratio 26% higher than NP females. In addition, NP females performed at just above chance (54% of time with the new object), while LP females performed at levels significantly greater than chance (69% of time in the new location). There were no significant effects of delay or an interaction between group and delay.

Object Placement: Comparison between pregnancy states

To determine possible differences in spatial memory due to pregnancy state, the performance of EP and LP females was compared on the OP task at a 2 and 4 hour ITD. An ANOVA revealed no significant effect of group: $F(1,32) = 1.79$; $p > 0.05$. There was also no significant effect of delay; however, there was a significant interaction between group and delay: $F(1,32) = 5.72$, $p < 0.05$. At a 2 hour ITD, performance did not significantly differ between females in early and late pregnancy, $p > 0.05$ (Figure 4A). In

contrast, at a 4 hour ITD, LP females had a significantly higher exploration ratio than EP females; $p < 0.01$ (Figure 4B). Overall, with a 4 hour ITD, LP females showed an exploration ratio 25% higher than EP females.

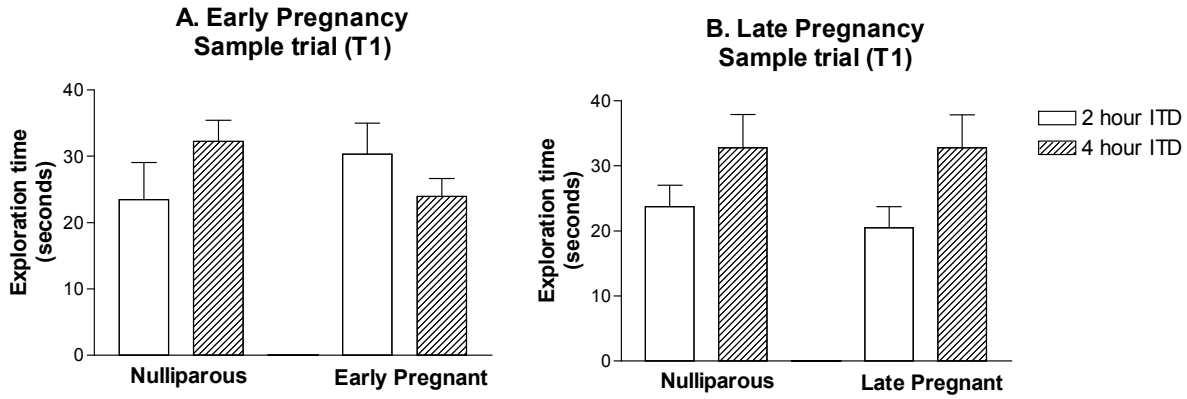

Figure 2. Effect of pregnancy on exploration during OP sample trial (T1). (A) Time spent exploring both objects during the sample trial for NP and EP (days 8 & 9) females at 2 and 4 hour ITD. There were no differences between groups by one-way ANOVA. **(B)** Time spent exploring both objects during the sample trial for NP and LP (days 16 & 17) females at 2 and 4 hour ITD. There were no differences between groups by one-way ANOVA. Entries are (mean $\pm$ SEM) for NP (n = 8) and pregnant (n = 8).

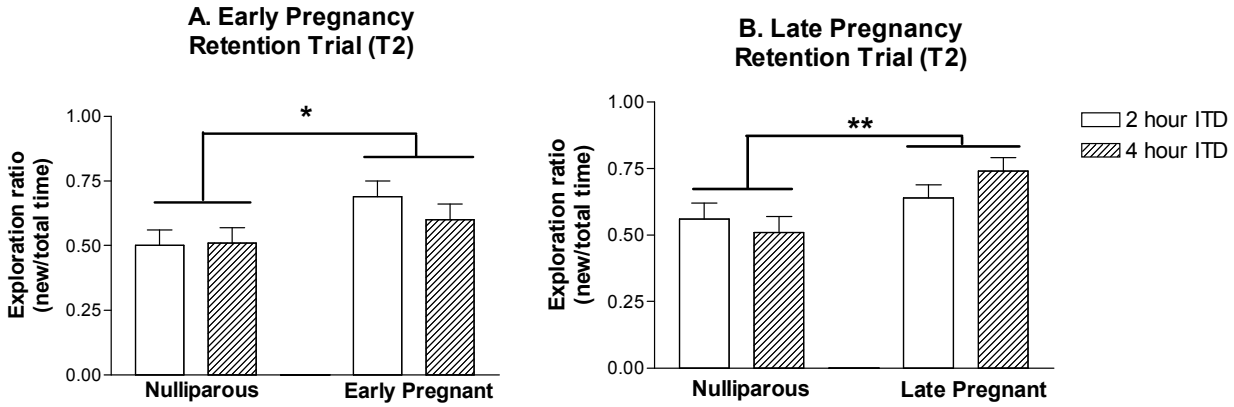

Figure 3. Effect of pregnancy on exploration during OP retention trial (T2).
(A) The exploration ratio (time in new location/total exploration time) for NP and EP females is shown at both a 2 and 4 hour ITD. Data were analyzed by two-way ANOVA (group x delay), where $F(1,24) = 5.361$, $p < 0.05$ for group; * $p < 0.05$. (**B)** The exploration ratio (time in new location/total exploration time) for NP and LP females is shown at both a 2 and 4 hour ITD. Data were analyzed by two-way ANOVA (group x delay), where $F(1,24) = 7.485$, $p < 0.05$ for group; **$p < 0.01$.
Entries are (mean $\pm$ SEM) for NP (n = 8) and pregnant (n = 8).

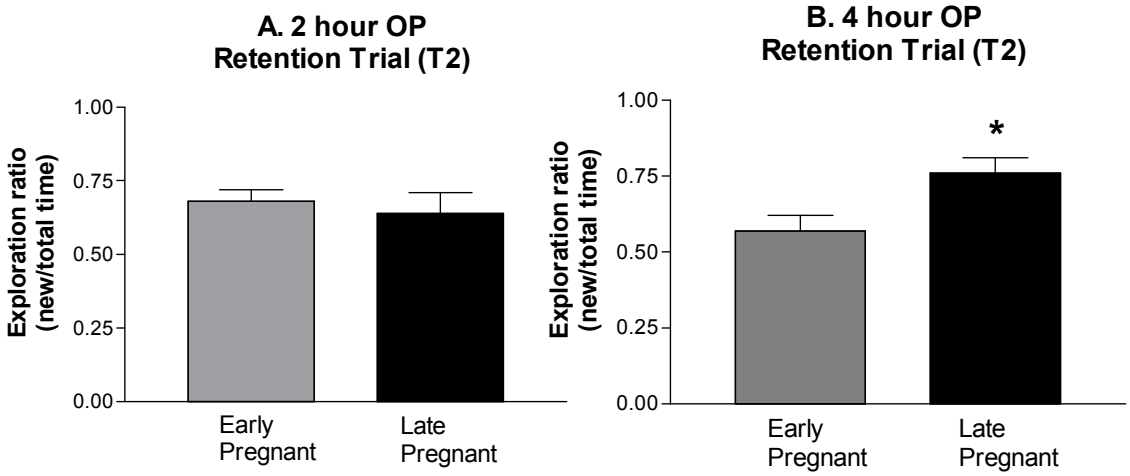

Figure 4. Effect of pregnancy state on object placement performance. The exploration ratio (time in new location/total exploration time) for EP and LP females is shown at a 2 hour ITD (**A**) and 4 hour ITD (**B**). Data were analyzed by a two-way ANOVA (group x delay), where $F(1,32) = 5.72$, $p < 0.05$ for interaction. EP and LP females significantly differed in exploration only at the 4 hour ITD (**B**). Entries are (mean $\pm$ SEM) for EP and LP (total N = 8); * $p < 0.05$

Elevated Plus Maze: Overall performance

To examine possible effects of pregnancy state on anxiety, performance of nulliparous and pregnant females was compared on the EPM. Two behaviors were used as indicators of decreased anxiety: time spent in the open arms, and number of entries into the open arms of the EPM. A MANOVA revealed a significant effect of group: $F(8, 56) = 2.10$; $p = 0.05$. To fully examine the differences between pregnant and non-pregnant, the data was further analyzed at each test time (early and late pregnancy).

Elevated Plus Maze: Early Pregnancy

Performance of EP and NP females was compared on two measures of anxiety on the EPM. During early pregnancy, a MANOVA revealed no overall significant effects of pregnancy state: $F(4,9) = 1.824$, $p > 0.05$, and no significant interaction between pregnancy state and the number of entries into the open arm: $F(1,12) = 1.434$, $p > 0.05$ (Figure 5A). However, there was a non-significant trend towards EP females spending more time in the open arm than NP females: $F(1,12) = 3.991$, $p = 0.07$ (Figure 5B).

As the lack of differences between NP and EP could be due to overall activity in the maze, both variables were examined in terms of ratios (number of open arm entries/total arm entries; time spent in open arms/total time spent in maze), which revealed similar results: no overall significant effects of pregnancy state: $F(2,11) = 2.376$, $p > 0.05$, and no significant interaction between pregnancy state and the ratios of entries into the open arm: $F(1,12) = 0.82$, $p > 0.05$ (Figure 5C). However, there was a non-significant trend towards EP females spending a higher percentage of their time in the open arm of the maze than NP females: $F(1,12) = 4.376$, $p = 0.06$; Figure 5D). Thus, regardless of how the

data was analyzed, EP females did not demonstrate a significant decrease in anxiety as compared to NP females.

Elevated Plus Maze: Late Pregnancy

Performance of LP and NP females was compared on two measures of anxiety on the EPM. During late pregnancy, no significant effect was seen due to pregnancy state: $F(4,9) = 1.013$, $p > 0.05$, as well as no significant interaction between pregnancy state and the two measures of anxiety: $F(1,12) = 0.34$, $p > 0.05$. Thus, NP and LP females did not significantly differ in either the number of entries onto the open arm of the EPM (Figure 6A), or in the amount of time spent in the open arm of the EPM (Figure 6B).

Examination of entries into open arms and time in open arms in terms of ratios (described above) revealed similar results: during late pregnancy no significant effect was seen due to pregnancy state: $F(2,11) = 0.382$, $p > 0.05$, and no significant interactions between pregnancy state and the two anxiety ratios: $F(1,12) = 0.44$, $p > 0.05$ (Figure 6C, 6D). Thus, regardless of how the data was analyzed, LP females did not demonstrate significantly altered anxiety as compared to NP females.

Elevated Plus Maze: comparison between pregnancy states

Finally, performance on the EPM was compared between the two states of pregnancy; no overall significant effects of pregnancy state were present: $F(2,13) = 2.658$, $p > 0.05$. A non-significant interaction between pregnancy state and entries into open arms was seen: $F(1,14) = 4.18$, $p = 0.06$ (Figure 7A), indicating a tendency for EP females to have more entries into the open arms of the EPM than LP females. No significant

interaction was seen between pregnancy state and time spent in the open arms: $F(1,14) = 0.95$, $p > 0.05$ (Figure 7B).

Examining entries into open arms and time spent in open arms in terms of ratios revealed no significant effect of pregnancy state: $F(2,13) = 1.992$, $p > 0.05$. A non-significant interaction between pregnancy state and ratio of entries into open arms was seen: $F(1,14) = 3.65$, $p = 0.06$ (Figure 7C), indicating a tendency for EP females to have a higher percentage of entries into the open arms of the EPM than LP females. However, no significant interaction between pregnancy state and ratio of time spent in the open arm was seen: $F(1,14) = 0.83$, $p > 0.05$ (Figure 7D). Regardless of how the data was analyzed, anxiety did not significantly differ between states of pregnancy.

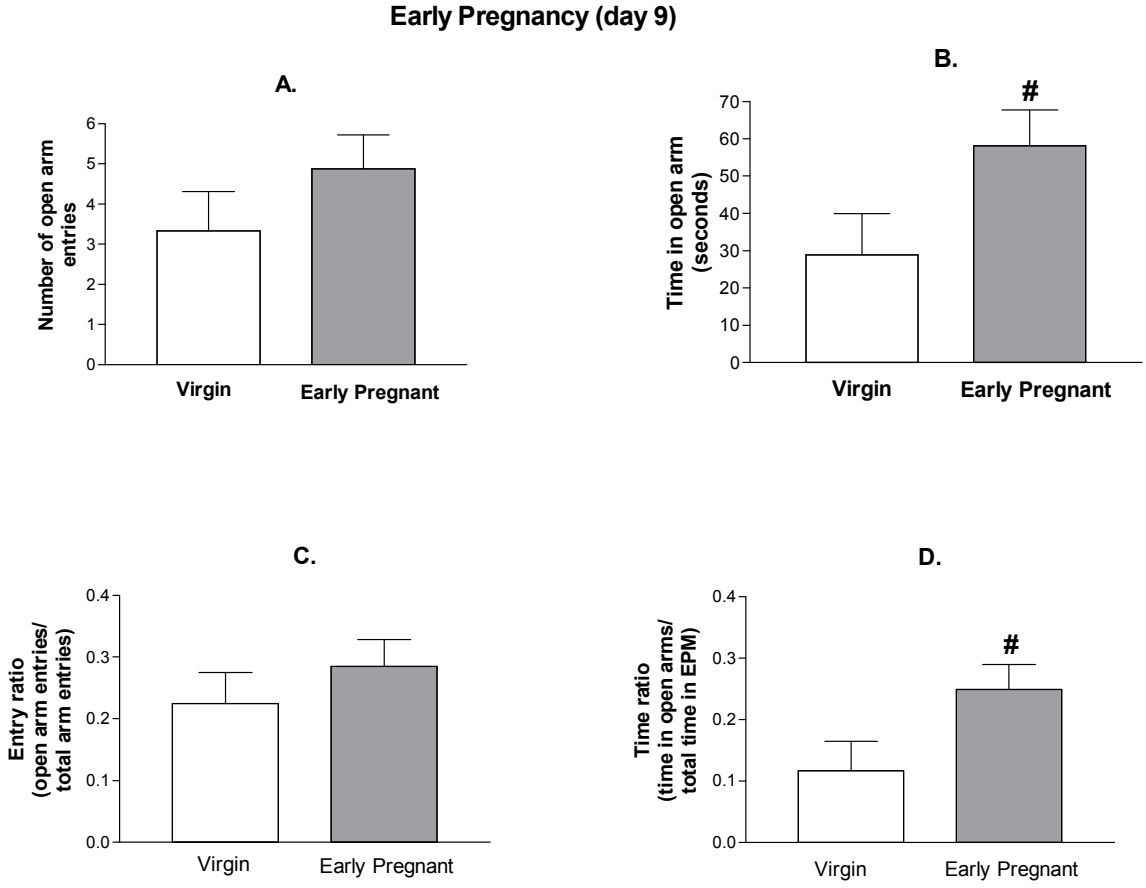

Figure 5. Performance on the elevated plus maze during early pregnancy. (A) The number of open arm entries and **(B)** amount of time spent in the open arms of the plus maze by NP and EP females. Data were analyzed by MANOVA, where $F(4,9) = 1.824$, $p > 0.05$. **(C)** The open arm entry ratio (open arm entries/total entries) and **(D)** the time ratio (time in open arm/total exploration time) for NP and EP females. Ratios were analyzed by MANOVA, where $F(2,11) = 2.376$, $p > 0.05$. A non-significant trend was present towards EP females spending more time in the open arm of the maze than NP females, $^{\#}p = 0.06$ **(B,D)**. Entries are (mean $\pm$ SEM) for virgin (n = 8) and EP (n = 8).

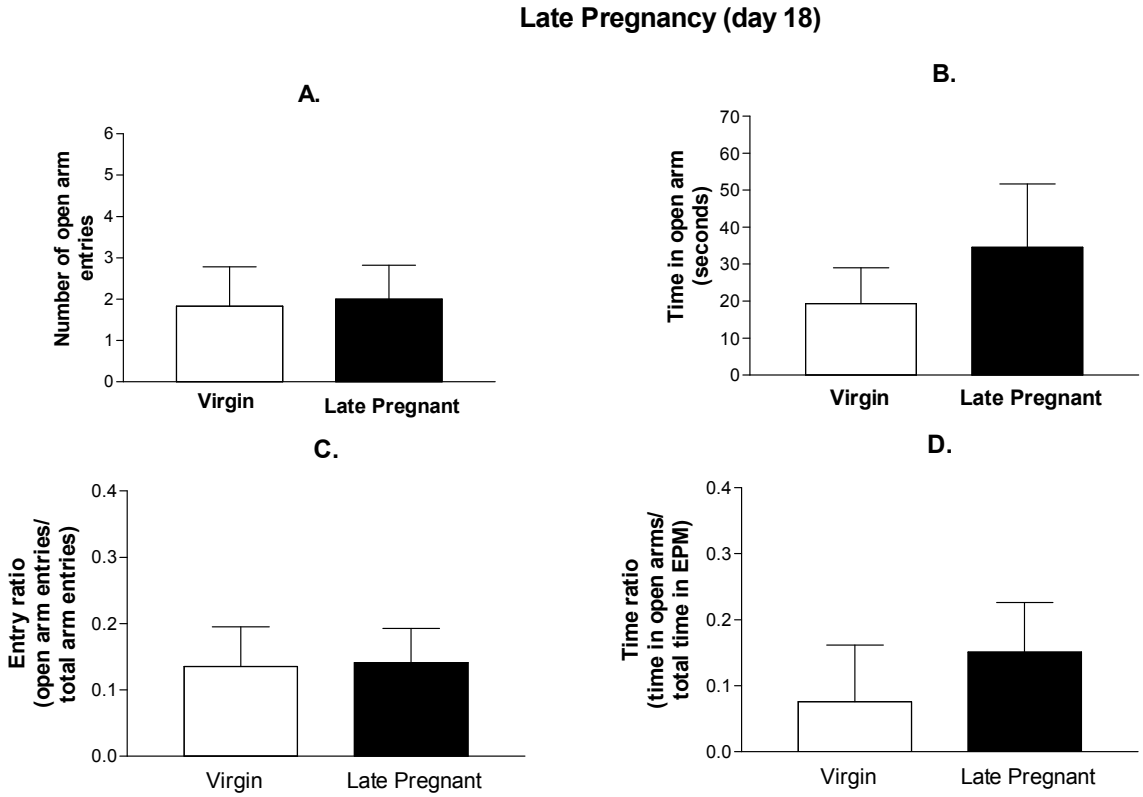

Figure 6. Performance on the elevated plus maze during late pregnancy. (A) The number of open arm entries and **(B)** amount of time spent in the open arms of the plus maze by NP and LP females. Data were analyzed by MANOVA, where $F(4,9) = 1.013$, $p > 0.05$. **(C)** The open arm entry ratio (open arm entries/total entries) and **(D)** the time ratio (time in open arm/total exploration time) for NP and LP females. Ratios were analyzed by MANOVA, where $F(2,11) = 0.382$, $p > 0.05$. Entries are (mean $\pm$ SEM) for NP (n = 8) and LP (n = 8).

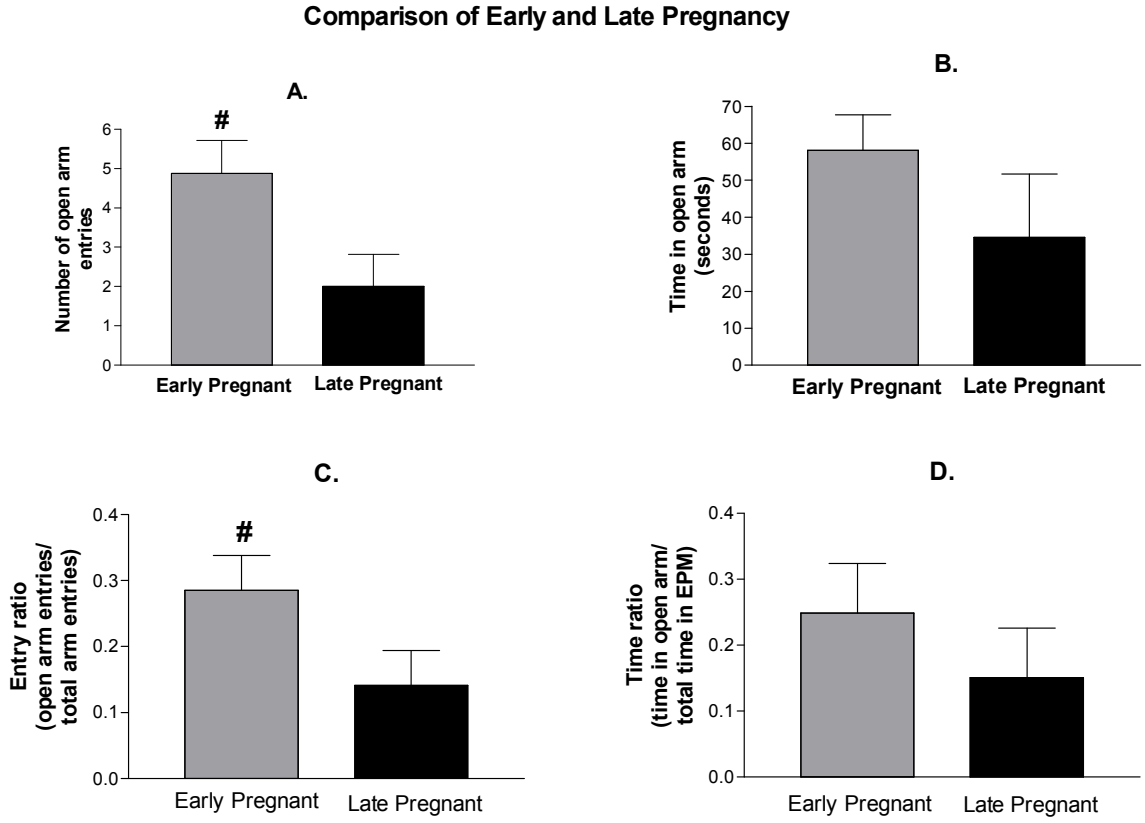

Figure 7. Performance on the elevated plus maze in different pregnancy states. **(A)** The number of open arm entries and (**B)** amount of time spent in the open arms of the plus maze by EP and LP females. Data were analyzed by MANOVA, where $F(2,13) = 2.658$, $p > 0.05$. **(C)** The open arm entry ratio (open arm entries/total entries) and **(D)** the time ratio (time in open arm/total exploration time) for EP and LP females. Ratios were analyzed by MANOVA, where $F(2,13) = 1.992$, $p > 0.05$. A non-significant trend was present towards EP females have more entries into the open arm of the maze than LP females, $^{\#}p = 0.06$ **(A,C)**. Entries are (mean $\pm$ SEM) for pregnant (n = 8).

Serum hormone levels: Comparison between nulliparous, early and late pregnant subjects

Levels of estradiol, progesterone and testosterone were measured by RIA in nulliparous females and in pregnant females in two different states: early and late. A one-way ANOVA revealed no significant differences in serum estradiol levels between the three groups: $F(2,19) = 0.14$, $p > 0.05$ (Figure 8A). Similarly, levels of serum progesterone did not differ significantly between the three groups: $F(2,19) = 3.26$, $p > 0.05$ (Figure 8B). In contrast, serum testosterone levels significantly differed between the groups: $F(2, 19) = 15.85$, $p < 0.001$ (Figure 8C). Post-hoc analysis revealed significantly higher testosterone levels in LP females than in NP or EP females (Figure 8C). A large difference existed in testosterone levels: levels in LP females were 29 fold higher than EP females, and 5 fold higher than NP females; NP and EP females did not significantly differ from each other.

As levels of testosterone were so much higher in LP females, and LP females performed the best overall on OP, a Pearson correlational analysis was run between testosterone levels and performance on OP task at 2 and 4 hour ITD by LP females. The test revealed no significant correlation between testosterone and exploration ratio at a 2 hour ITD; $R = 0.24$, $p > 0.05$ (Figure 9A). Similarly, no significant correlation was present between testosterone and exploration ratio at a 4 hour ITD; $R = 0.52$, $p > 0.05$ (Figure 9B). Testosterone levels were not significantly correlated with performance on the spatial memory task, at either delay.

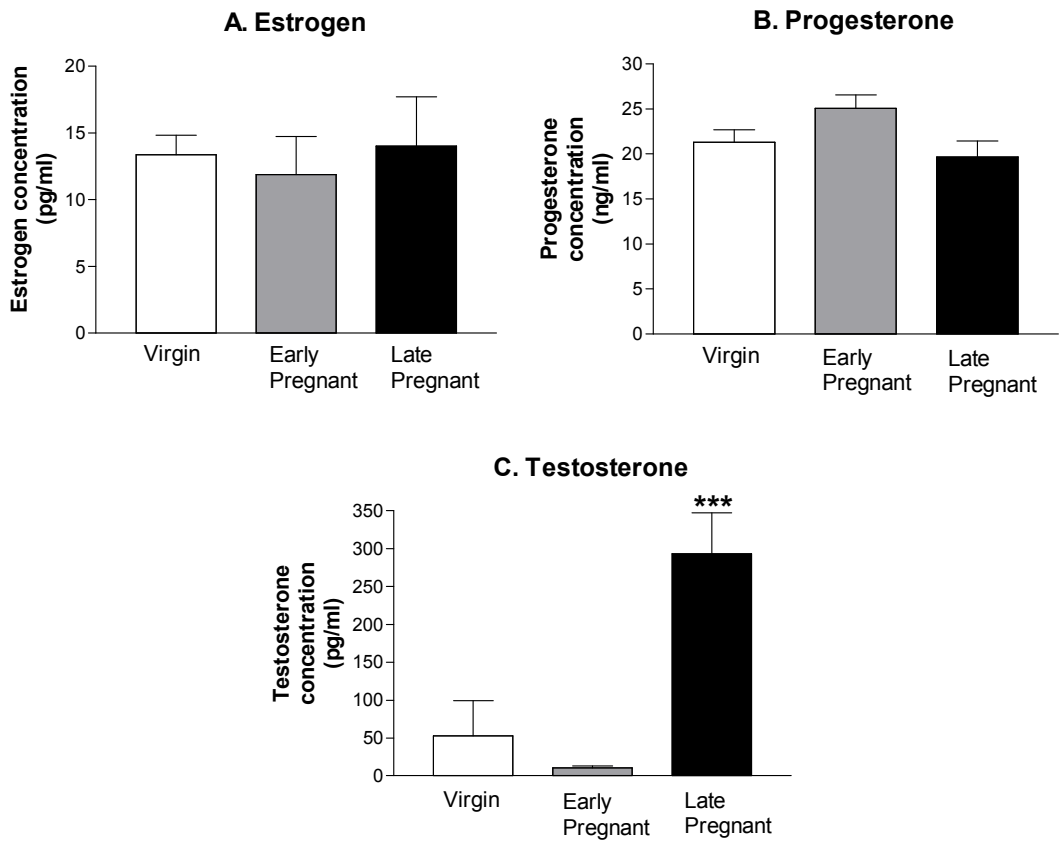

Figure 8. Effect of pregnancy state on gonadal hormone concentrations. Serum hormone levels of **(A)** estradiol, (**B)** progesterone, and (**C)** testosterone. One-way ANOVA for each hormone revealed significant differences in testosterone levels, $F(2,19) = 15.85$, $^{***}p < 0.001$. Entries are (mean $\pm$ SEM) for NP (n = 6), EP (n = 8) and LP (n = 8).

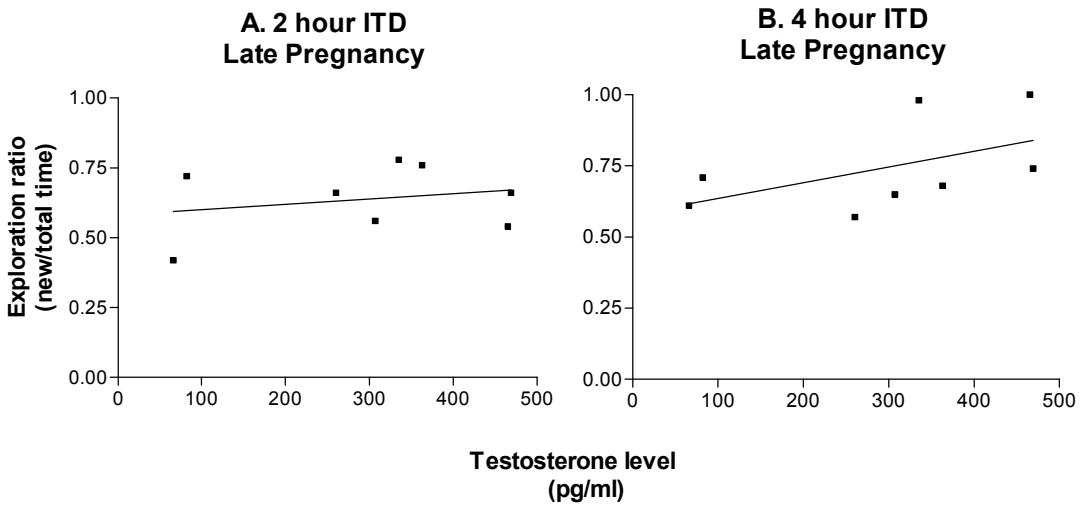

Figure 9. Correlation between testosterone level and performance on the OP task by LP females. The exploration ratio (time in new location/total exploration time) of LP females after a 2 hour ITD **(A)** and a 4 hour ITD **(B)**, plotted against measured testosterone levels. Correlations tested by Pearson correlation analysis; neither correlation was significant ($p > 0.05$). Entries are actual data obtained for LP females (n = 8).

Brain monoamines: Levels in nulliparous females, and females in EP and LP

Pre-frontal Cortex

Monoamine levels were compared in the prefrontal cortex (PFC) of NP, EP, and LP females. A MANOVA revealed a significant overall effect of pregnancy state: $F(14,16) = 7.139$, $p < 0.001$; a significant interaction was found between pregnancy state and all seven monoamines (Table 2). Post hoc analysis revealed that levels of DA were significantly higher in NP females than in EP or LP females ($p < 0.05$; Figure 10A). The same differences were seen for both metabolites of dopamine: DOPAC and HVA levels were both significantly higher in the PFC of nulliparous females than EP or LP females ($p < 0.001$; Figure 10A). For DA and its metabolites, EP and LP females did not significantly differ from one another.

Similarly, the concentration of NE was significantly higher in the PFC of NP and EP females than LP females ($p < 0.001$); NP and EP females did not differ significantly from one another (Figure 10C). The NE metabolite MHPG demonstrated the same pattern as the DA metabolites, with NP females having significantly higher concentrations of MHPG than EP or LP females ($p < 0.001$; Figure 10C). Concentrations of 5HT and its metabolite 5-HIAA were significantly higher in NP than EP or LP females ($p < 0.001$; Figure 10E). Concentrations of 5HT and 5-HIAA did not significantly differ between EP and LP females.

The turnover ratios of DA, NE, and 5HT (i.e. ratio of metabolite to monoamine) were also examined, to determine whether activities in the PFC differed due to pregnancy state. A MANOVA revealed an overall significant effect of pregnancy state: $F(8,22) = 4.093$, $p < 0.01$; a significant interaction was found between pregnancy state and three of

the four monoamine turnover ratios (Table 2). Post hoc analysis revealed no significant differences due to pregnancy state in the turnover ratio of HVA/DA (Figure 10B). NP females had a significantly higher turnover ratio of DOPAC/DA than either EP or LP females ($p < 0.05$; Figure 10B). LP females had a significantly higher turnover ratio of MHPG/NE than either NP or EP females ($p < 0.01$; Figure 10D). EP females had a significantly higher turnover ratio of 5-HIAA/5HT ratio than LP females ($p < 0.01$). NP females did not significantly differ from either of the two pregnant groups (Figure 10F).

Table 2. ANOVA results, F and p values for monoamine and metabolite measurements in four brain regions.
A *p* value of < .05 was accepted for significance; # indicates comparisons that were not significant.

Frontal Cortex			CA1			CA3			mPOA		
Monoamine	F(2,16)	*p* value	Monoamine	F(2,15)	*p* value	Monoamine	F(2,16)	*p* value	Monoamine	F(2,16)	*p* value
DA	5.64	< .05	DA	0.83	> .05#	DA	5.64	< .05	DA	0.19	> .05#
DOPAC	29.91	< .01	DOPAC	69.87	< .001	DOPAC	29.91	< .01	DOPAC	2.86	> .05#
HVA	41.26	< .01	HVA	22.76	< .001	HVA	41.26	< .01	HVA	0.33	> .05#
NE	8.34	< .01	NE	135.87	< .001	NE	8.34	< .01	NE	7.46	< .01
MHPG	28.32	< .01	MHPG	24.06	< .001	MHPG	28.32	< .01	MHPG	15.20	< .001
5HT	69.09	< .01	5HT	17.48	< .001	5HT	69.09	< .01	5HT	0.10	> .05#
5-HIAA	93.28	< .01	5-HIAA	0.46	> .05#	5-HIAA	93.28	< .01	5-HIAA	0.35	> .05#
Turnover ratio	F(2,16)	*p* value	Turnover ratio	F(2,15)	*p* value	Turnover ratio	F(2,16)	*p* value	Turnover ratio	F(2,16)	*p* value
HVA/DA	1.59	> .05#	HVA/DA	16.11	< .001	HVA/DA	1.59	> .05#	HVA/DA	0.35	> .05#
DOPAC/DA	5.20	< .05	DOPAC/DA	12.41	= .001	DOPAC/DA	5.20	< .05	DOPAC/DA	2.02	> .05#
MHPG/NE	5.74	< .05	MHPG/NE	3.82	= .05	MHPG/NE	5.74	< .05	MHPG/NE	10.75	< .001

5-HIAA/5HT	5.74	< .05	5-HIAA/5HT	6.64	< .05	5-HIAA/5HT	5.74	< .05	5-HIAA/5HT	0.19	>.05[#]

PFC

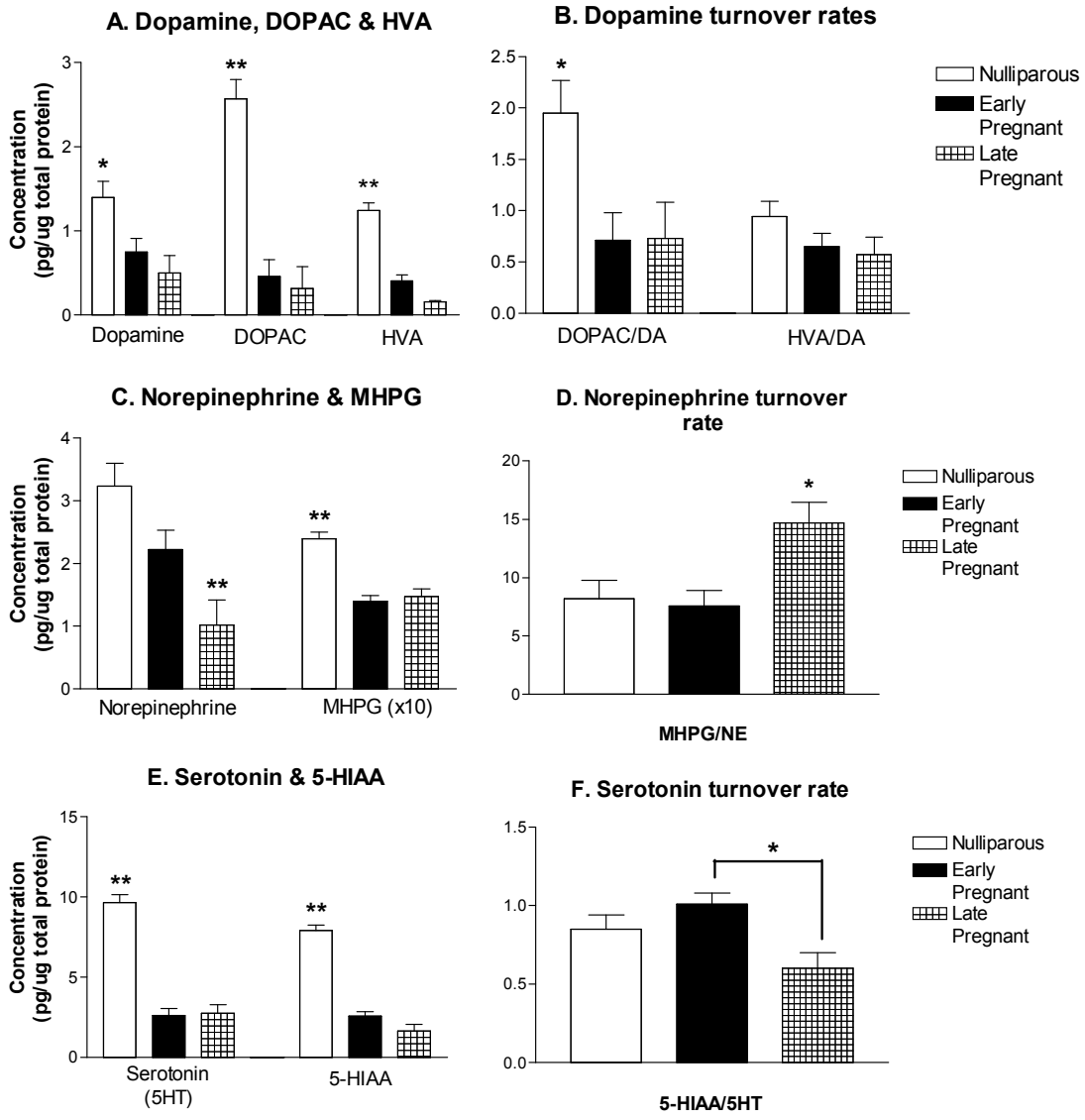

Figure 10. Effect of pregnancy state on dopamine, norepinephrine, serotonin and metabolite concentrations in prefrontal cortex. (A) Concentrations of DA and metabolites DOPAC and HVA. **(B)** Ratios of DA to DOPAC and HVA. **(C)** Concentrations of NE and metabolite MHPG. **(D)** Ratio of NE to MHPG. **(E)** Concentrations of 5HT and metabolite 5-HIAA. **(F)** Ratio of 5HT to 5-HIAA. Monoamine concentrations were analyzed by MANOVA (group x monoamines), where $F(14,16) = 7.139$, $p < 0.001$. Ratios were analyzed by MANOVA (group x ratios), where $F(8,22) = 4.093$, $p < 0.01$. Entries are (mean $\pm$ SEM) for NP (n = 5), EP (n = 7) and LP (n = 4). $*p < .05$, $**p < .01$, $***p < .001$.

CA1 Hippocampus

Monoamine levels were compared in CA1 hippocampus of NP, EP, and LP females. A MANOVA revealed a significant overall effect of pregnancy state: $F(14,14) = 37.57$, $p < .001$; a significant interaction was found between pregnancy state and five monoamines (Table 2). Post hoc analysis revealed no significant differences between the three groups in concentration of DA in CA1 hippocampus (Figure 11A); however, the interactions between pregnancy state and the two dopamine metabolites were significant (Table 2). Concentrations of DOPAC in the CA1 of NP females were significantly higher than either EP or LP females ($p < 0.001$; Figure 11A); EP and LP females did not significantly differ from one another. Concentrations of HVA in the CA1 of EP females were significantly higher than in NP or LP females ($p < 0.001$, Figure 11A); EP and LP females did not significantly differ from one another.

Post hoc analysis of the NE interaction revealed that all three groups significantly differed from one another. NP females had the lowest concentration of NE in the CA1, LP females had the highest concentration, and EP females were in the middle ($p < 0.001$ for all comparisons; Figure 11C). Concentrations of the NE metabolite MHPG were significantly higher in EP females than NP or LP females ($p < 0.001$; Figure 11C); NP and LP females did not significantly differ. In contrast, concentration of 5HT was significantly lower in EP females than in NP or LP females ($p < .01$; Figure 11E). There were no significant differences due to pregnancy state in concentration of the serotonin metabolite 5-HIAA ($p > 0.05$; Figure 11E).

The turnover ratios of DA, NE, and 5HT (ratio of metabolite to monoamine) to were also examined, to determine whether activities in the CA1 differed due to pregnancy

state. A MANOVA revealed an overall significant effect of pregnancy state: $F(8,20) = 7.03$, $p < 0.001$; a significant interaction was present between pregnancy state and all four monoamine turnover ratios (Table 2). Post hoc analysis revealed that NP females had a significantly higher turnover ratio of DOPAC/DA than EP or LP females ($p< 0.01$; Figure 11B). EP females had a significantly higher turnover ratio of HVA/DA than NP or LP females ($p < 0.001$; Figure 11B). Analysis of the MHPG/NE ratio revealed that NP and EP females both had a significantly higher turnover ratio than LP females ($p < 0.05$); NP and EP females did not significantly differ (Figure 11D). Finally, the 5-HIAA/5HT turnover ratio was significantly higher in EP females than in LP females ($p \leq 0.05$); NP females did not significantly differ from either pregnant group (Figure 11F).

CA1 Hippocampus

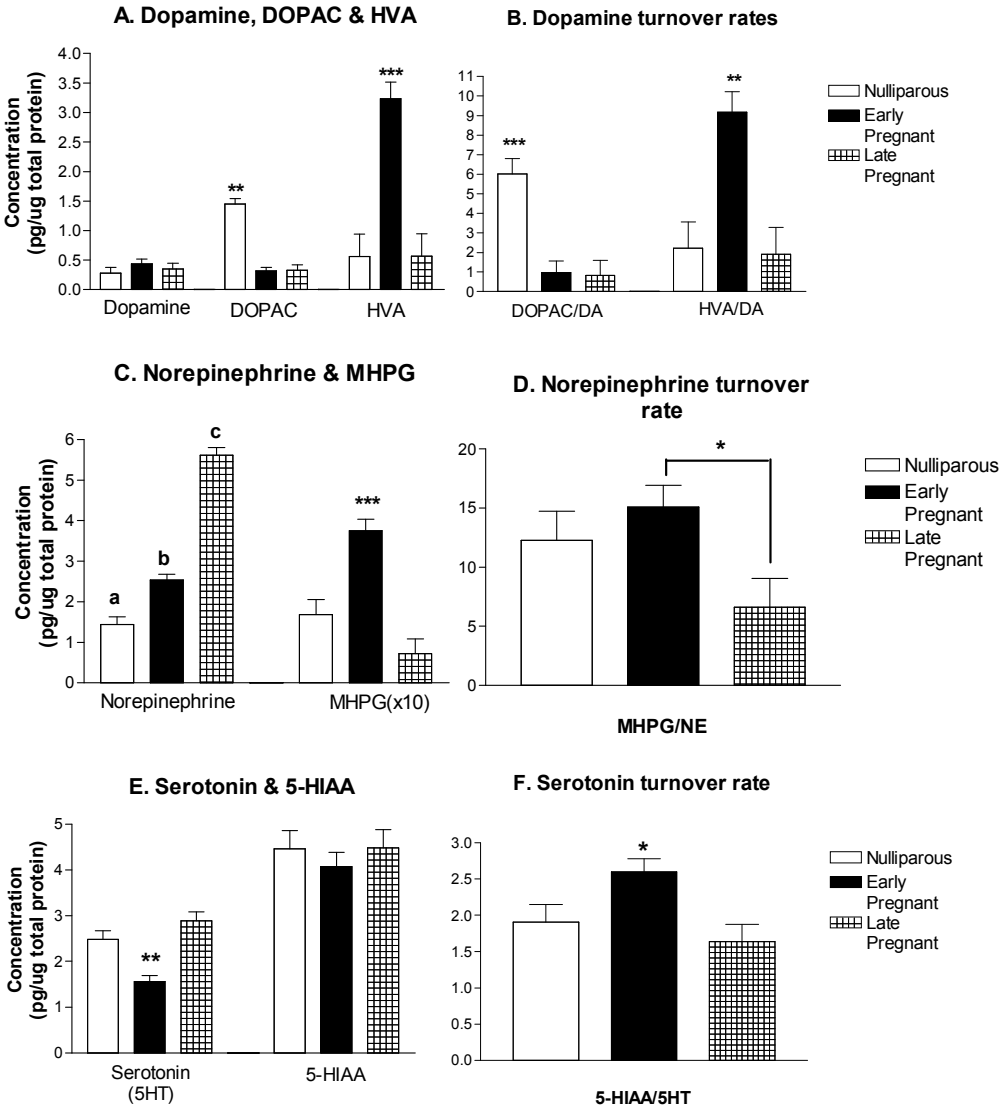

Figure 11. Effect of pregnancy state on dopamine, norepinephrine, serotonin and metabolite concentrations in CA1 hippocampus. (A) Concentrations of DA and metabolites DOPAC and HVA. **(B)** Ratios of DA to DOPAC and HVA. **(C)** Concentrations of NE and metabolite MHPG. **(D)** Ratio of NE to MHPG. **(E)** Concentrations of 5HT and metabolite 5-HIAA. **(F)** Ratio of 5HT to 5-HIAA. Monoamine concentrations were analyzed by MANOVA (group x monoamines), where $F(14,14) = 37.57$, $p < 0.001$. Ratios were analyzed by MANOVA (group x ratios), where $F(8,20) = 7.03$, $p < 0.001$. Entries are (mean $\pm$ SEM) for NP (n = 4), EP (n = 7) and LP (n = 4). *$p < .05$; **$p < .01$; ***$p < .001$.

CA3 Hippocampus

Monoamine levels were compared in CA3 hippocampus of NP, EP, and LP females. A MANOVA revealed a significant overall effect of pregnancy state: $F(14,16) = 7.09$, $p < 0.001$; a significant interaction was present between pregnancy state and four monoamines (Table 2). Post hoc analysis revealed that concentration of DA and its metabolite DOPAC did not significantly differ due to pregnancy state (Table 2; Figure 12A). In contrast, concentration of the DA metabolite HVA did significantly differ: EP females had significantly higher concentration than NP or LP females ($p < 0.001$; Figure 12A).

Similar to DA, no significant difference due to pregnancy state was present in concentration of NE (Table 2; Figure 11C). In contrast, concentration of the NE metabolite MHPG was significantly decreased in LP females as compared to NP and EP females (Figure 12C). Similarly, the concentration of 5HT was significantly decreased in LP females as compared to NP and EP females ($p < 0.01$); NP and EP females did not significantly differ (Figure 12E). Concentration of the serotonin metabolite 5-HIAA was significantly higher in EP females than in NP or LP females ($p < 0.01$; Figure 12E).

The turnover ratios of DA, NE, and 5HT (ratio of metabolite to monoamine) to were also examined, to determine whether activities in the CA3 differed due to pregnancy state. A MANOVA revealed an overall significant effect of pregnancy state: $F(8,22) = 6.33$, $p < .001$; a significant interaction was present between pregnancy state and three of the monoamine turnover ratios (Table 2). Post hoc analysis revealed no significant differences in the turnover ratio of DOPAC/DA due to pregnancy state (Table 2; Figure 12B). In contrast, the turnover ratio for HVA/DA was significantly higher in EP females

than NP or LP females ($p = 0.001$; Figure 12B). Similarly, EP females had a significantly higher turnover ratio of MHPG/NE than LP females ($p < 0.001$); NP females did not significantly differ from either pregnant group (Figure 12D). Finally, the 5-HIAA/5HT turnover ratio was significantly higher in EP and LP females than in NP females ($p < 0.01$); the two pregnant groups did not differ significantly from one another (Figure 12F).

CA3 Hippocampus

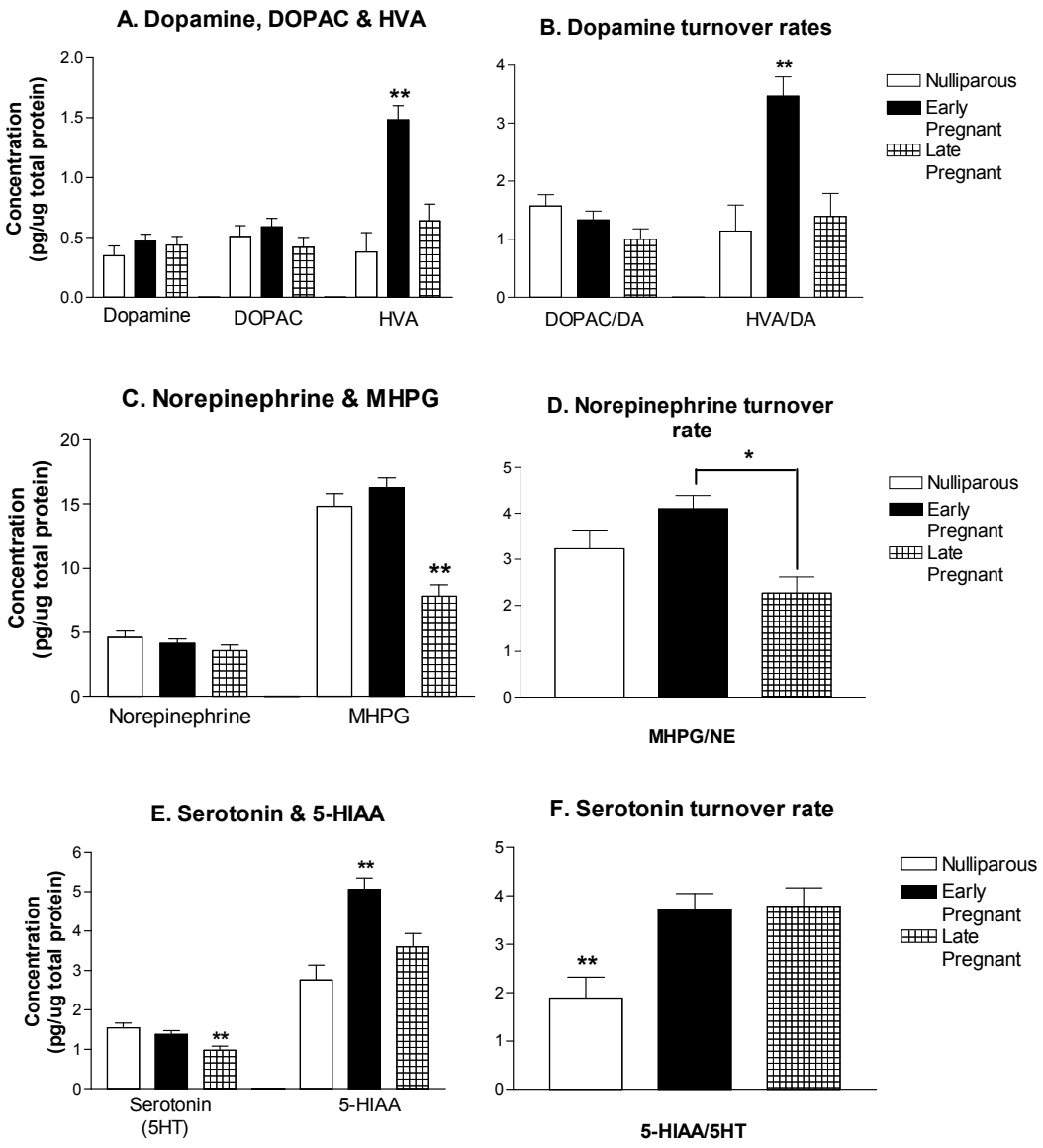

Figure 12. Effect of pregnancy state on dopamine, norepinephrine, serotonin and metabolite concentrations in CA3 hippocampus. **(A)** Concentrations of DA and metabolites DOPAC and HVA. **(B)** Ratios of DA to DOPAC and HVA. **(C)** Concentrations of NE and metabolite MHPG. **(D)** Ratio of NE to MHPG. **(E)** Concentrations of 5HT and metabolite 5-HIAA. **(F)** Ratio of 5HT to 5-HIAA. Monoamine concentrations were analyzed by MANOVA (group x monoamines), where $F(14,16) = 7.09$, $p < 0.001$. Ratios were analyzed by MANOVA (group x ratios), where $F(8,22) = 6.33$, $p < 0.001$. Entries are (mean $\pm$ SEM) for NP (n = 4), EP (n = 7) and LP (n = 5). $^{**}p < .01$; $^{***}p < .001$.

Medial preoptic area (mPOA)

Monoamine levels were compared in the mPOA of NP, EP, and LP females. A MANOVA revealed a significant overall effect of group: $F(13,16) = 4.39$, $p < 0.05$; a significant interaction was present between pregnancy state and three of the monoamines examined (Table 2). Post hoc analysis revealed that concentration of DA and its metabolite HVA did not significantly differ due to pregnancy state (Table 2; Figure 13A). In contrast, concentration of the DA metabolite DOPAC did significantly differ: LP females had significantly higher concentration than NP females ($p < 0.05$; Figure 13A); no significant differences were seen between the pregnant groups, or in the ratios of DA to metabolites DOPAC and HVA (Table 2; Figure 13B).

Significant differences were present in concentration of NE. NP females had significantly higher concentration of NE as compared to EP ($p < 0.05$) and LP females ($p < 0.01$; Figure 13C). In contrast, NP females had significantly lower concentration of the NE metabolite MHPG as compared to EP and LP females ($p < 0.001$; Figure 13C). The turnover ratio MHPG/NE followed a similar pattern, with a significantly lower ratio of MHPG/NE in NP females as compared to EP and LP females; $F(2,16) = 10.75$, $p < 0.001$ (Figure 13D). Additionally, there was a marginally significant trend towards a higher turnover ratio of MHPG/NE in LP as compared to EP females ($p = 0.055$; Figure 13D). Unlike DA, NE and metabolites, no significant differences due to pregnancy state were present in concentration of 5HT, its metabolite 5-HIAA, or ratio of 5-HIAA/5HT (Table 2; Figure 13E, 13F).

mPOA

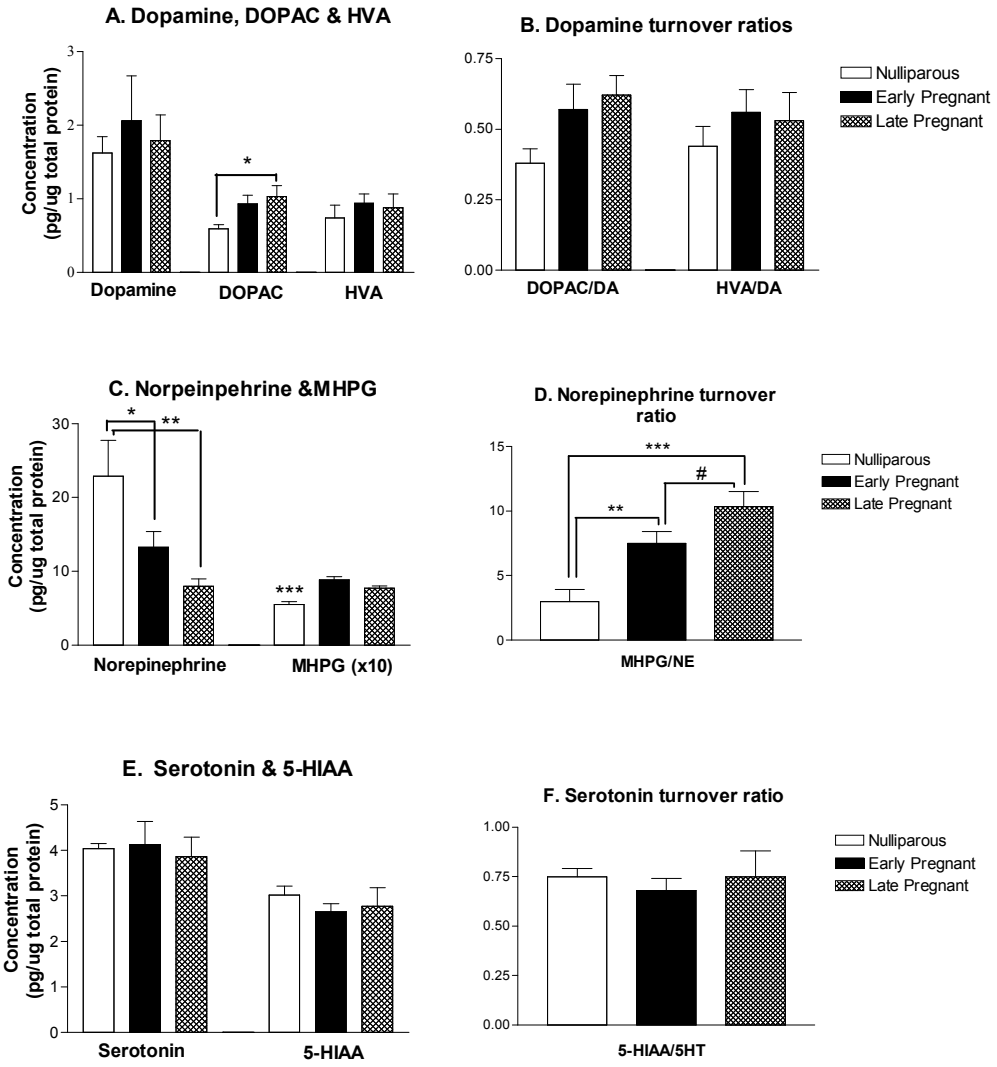

Figure 13. Effect of pregnancy state on dopamine, norepinephrine, serotonin and metabolite concentrations in mPOA. (A) Concentrations of DA and metabolites DOPAC and HVA. **(B)** Ratios of DA to DOPAC and HVA. **(C)** Concentrations of NE and metabolite MHPG. **(D)** Ratio of NE to MHPG. **(E)** Concentrations of 5HT and metabolite 5-HIAA. **(F)** Ratio of 5HT to 5-HIAA. Monoamine concentrations were analyzed by MANOVA (group x monoamines), where $F(13,16) = 4.39$, $p < 0.05$. Ratios were analyzed by MANOVA; only significant interaction was ratio of MHPG/NE: $F(2,16) = 10.75$, $p < 0.001$. Entries are (mean $\pm$ SEM) for NP (n = 4), EP (n = 7), and LP (n = 6). $*p < .05$; $**p < .01$; $***p < .0001$; $\#p = .055$.

3.c Discussion

Pregnancy state and spatial memory

The performance of females in two different pregnancy states: early (days 7-8) and late (days 16-17) and age-matched nulliparous controls was examined on a spatial memory task (object placement) to determine if pregnancy state could affect performance. It was hypothesized that LP females would significantly out-perform EP and NP females. This hypothesis was confirmed; averaged over both a 2 and 4 hour ITD, LP females significantly out-performed NP females. Interestingly, EP females also significantly out-performed NP females when averaged over both 2 and 4 hour ITDs. However, LP females significantly out-performed EP females at the 4 hour ITD. Thus, on the more difficult version of the task (a 4 hour ITD required females to remember the locations visited previously for a longer period of time), LP females out-performed EP females, indicating superior spatial memory during late pregnancy.

NP females demonstrated chance performance (exploration ratio of 0.50) at the 2 and 4 hour ITD during both test periods (early and late pregnancy), indicating lack of object discrimination. Previous work has indicated similar results in normally-cycling, control females (Luine et al., 2003); the ability to distinguish object location at delays 2 hours and greater are generally not seen without elevated estrogen and/or progesterone levels, such as those experienced throughout pregnancy (Rosenblatt et al., 1988).

The present study confirmed previous findings that females just ending the first trimester of pregnancy had significantly enhanced spatial memory as compared to nulliparous females on the Morris water maze (Galea et al, 2000). Current findings contrasted studies in which females in the third trimester did not demonstrate enhanced

spatial memory on the Morris water maze as compared to EP or NP females (Galea et al., 2000; Bodensteiner et al., 2006). Use of the Morris water maze in these studies may be a factor in contrasting findings. Male rats trained in the Morris water maze display an initial strong stress response as measured by increased corticosterone production and release in amygdala, hippocampus and hypothalamus (Aguilar-Valles et al., 2005); females generally have the same physiological response as males (Bowman, Zrull & Luine, 2002). In males restraint stress generally leads to decrements in performance on a non-stressful spatial task, the radial arm maze (Luine, Villegas, Martinez & McEwen, 1994). In contrast, females demonstrate maintained performance on radial arm maze after restraint stress (Bowman, Zrull & Luine, 2001; Luine, 2002).

While pregnant, it is less clear what impact restraint stress has on the female. In one study, females with reproductive experience given one administration of restraint stress demonstrated reduced activity in brain areas normally activated by stress (Wartella et al., 2003). More recently, pregnant females given restraint stress from day 15 through gestation demonstrated significantly impaired performance on the Morris water maze as compared to mothers not given stress, both two weeks post weaning and at 22 months of age (Lemaire et al., 2006), indicating that increased stress levels during pregnancy can impair spatial memory ability. Thus, repeated stress on a female in late pregnancy, such as that given with repeated testing in the water maze, may decrease her performance. The lack of overt stress in the OP task may account for the enhanced spatial memory observed in LP females in the current study.

Pregnancy state and anxiety

The performance of NP, EP and LP females was examined on the elevated plus maze (EPM), to examine possible effects of pregnancy state on the anxiety response. The hypothesis that LP females would demonstrate decreased anxiety as compared to NP and EP females was not confirmed; no significant differences were seen between any of the groups on either of the accepted measurements of decreased anxiety (number of entries into the open arms, and time spent in the open arms; Pellow et al., 1985; Lonstein, 2005). A non-significant trend was present for decreased anxiety in EP as compared to NP and LP females. It is possible that with larger group membership, or different testing protocol (see below), decreased anxiety during EP would be found.

A possible reason for the lack of differences between early and late pregnant females is that the same animals were tested twice. The EPM is generally given as a one-trial task, as repeated exposure to the maze can be anxiolytic (Lonstein, 2005). Indeed, it appeared as if NP females (also tested twice) decreased in both the number of entries and time spent in the open arm from testing on day 9 to testing on day 18. Thus, the late pregnant data may be confounded by the way in which testing was done. However, parous females tested on the EPM at 6, 10, 14, 18, and 22 months of age demonstrated decreased anxiety as compared to nulliparous females at 10 and 14 months of age, and these females were tested repeatedly on the same plus maze (Love et al., 2005). Thus, the behavior testing paradigm used in the current study may still account for the lack of observed differences in anxiety.

Non-significant trends were present towards EP females spending more time in the open arm of the EPM than NP females, and entering the open arm of the EPM more than

LP females. While no previous studies have examined anxiety during any stage of pregnancy, previous work indicates that pregnancy reduces anxiety-like behaviors in the open field (Wartella et al., 2003), reduces anxiety in the EPM during the first week of lactation (Lonstein, 2005) and throughout the lifespan (Love et al., 2005). The results from this study indicated that pregnancy did not decrease anxiety. Handling, habituation and testing throughout the study could be anxiolytic; however previous studies have also been handled and tested throughout, and anxiolytic effects due to testing were not observed (Lonstein et al., 2003; Love et al., 2005).

Pregnancy state and hormone levels

Levels of estradiol and progesterone did not significantly differ between NP, EP and LP females. This was a highly unexpected finding, as rats in late pregnancy have significantly increased levels of estrogen and progesterone than during early pregnancy (Rosenblatt et al., 1988). Highest levels of estrogen are seen on day 19, just after the day of sacrifice for LP females in the current study. Additionally, progesterone levels appear to drop precipitously on day 18 of pregnancy, resulting in levels not much higher than those seen on day 8 (Rosenblatt et al., 1988). Variability in day of sacrifice between studies may account for the failure to observe differences in estradiol and progesterone levels. NP females may not have differed from either pregnant group if they were sacrificed on the day of proestrus, when estrogen and progesterone levels are high. As NP females had poorer performance on the OP task as compared to EP and LP females, the lack of difference in hormone levels does indicate that the observed behavioral differences may not be due to circulating levels of estrogen or progesterone.

In contrast, LP females had significantly higher levels of testosterone than NP or EP females. During pregnancy, testosterone levels increase once the placenta begins to form and adipose tissue accumulates (see Rodriguez-Cuenca et al., 2006). Although the increase in testosterone at mid pregnancy (days 11-13) was not significantly higher than intact, control females (Rodrigez-Cuenca et al., 2006), the levels of serum hormone measured previously do correspond to those found in the current study on day 18. Thus, it appears as if testosterone levels are elevated after day 9 (measurement of EP females), and remain elevated until at least day 18 (measurement of LP females).

Testosterone is associated with increased aggression in a number of species, particularly within males (Archer, 2006). However, females' aggression also increases in presence of testosterone. In wild female spotted hyenas, dominant females display significantly higher levels of testosterone in late pregnancy than subordinate females, indicating that testosterone aids in display of aggressive behavior (Dloniak, French & Holekamp, 2006). In females of different bird species, testosterone levels may increase when in the midst of an aggressive interaction (Langmore, Cockrem & Candy, 2002; Smith, Raouf, Bomberger-Brown, Wingfield, & Brown, 2005), and artificial elevation of testosterone can increase aggression towards the opposite sex (Zysling, Greives, Bruener, Casto, Demas & Ketterson, 2006). In humans, women with naturally high levels of testosterone are more sensitive to threat, and pay more attention to angry faces than women with lower testosterone levels (for review, see Archer, 2006).

Elevated testosterone levels may aid a mother in protecting her offspring. Pregnant female rats, beginning as early as day 12 of pregnancy, display a marked increase in aggression toward unfamiliar conspecifics (Albert, Jonik & Walsh, 1992). Aggression

increases throughout the last week of pregnancy, peaks around parturition and remains high during the first week of lactation (Lonstein & Gammie, 2002). The increase in aggression may be due to a change in the hormonal environment (Albert, Jonik & Walsh, 1992). Ovariectomized female rats implanted with testosterone display greater success and aggression rates in a food competition task than ovariectomized control females (Albert, Jonik & Walsh, 1990). Testosterone-implanted females also displayed higher levels of aggression towards an unfamiliar intruder than ovariectomized control females (Albert, Jonik & Walsh, 1990). Similarly, ovariectomized females implanted with estrogen and testosterone, but not progesterone, display significantly higher levels of aggression toward an unfamiliar conspecific than do females implanted with estrogen, testosterone, and progesterone (Albert, Jonik & Walsh, 1992). Thus, the presence of high progesterone throughout early and mid pregnancy (Rosenblatt et al., 1988) may moderate aggressive behaviors supported by the presence of estrogen and testosterone. Decrease in progesterone just prior to parturition may allow testosterone to facilitate maternal aggression (Albert, Jonik & Walsh, 1992). In the current study, the presence of testosterone levels 6-fold higher in LP females than NP females, and 29-fold higher than in EP females, further indicates that testosterone levels are significantly elevated near the end of the gestation period.

In addition to aiding in maternal aggression, high testosterone levels may aid in the observed memory enhancement in LP females. A small body of work indicates that testosterone is beneficial for spatial performance in women (Aleman et al., 2004); a great deal of work indicates that testosterone is beneficial for spatial performance in males (Gibbs, 2005). Little to no work has examined testosterone in relation to spatial memory

in female rats; however, treatment with testosterone propionate and dihydrotestosterone did increase CA1 spine synapse density in ovariectomized female rats (Leranth, Hajszan & MacLusky, 2004). As the CA1 plays a large role in spatial memory, it is possible that androgens may be implicated in female spatial memory by affecting the morphology of the hippocampus. Recent evidence has indicated that androgen treatment to females may be beneficial for spatial memory (Mohan, Sarmiento, Lachman, MacLusky & Luine, 2004).

In the current study, testosterone level alone could not account for EP females' performance, as levels of testosterone did not significantly differ between EP and NP females yet EP females displayed better spatial memory than NP females. However, it is possible that the difference in testosterone levels of EP and LP females could account for the increased performance by LP females on the 4 hour ITD, as compared to EP females. At the 4 hour delay, a non-significant positive correlation was seen between high testosterone levels and high percentage of time spent exploring the novel object location. It is possible that with a larger group, a significant positive correlation could be seen. In summary, these results indicate that research into the role of testosterone and other androgens in female memory performance, particularly during late pregnancy, might provide important information for understanding mechanisms for enhanced spatial memory in pregnant rats.

Pregnancy state and monoamine concentrations

Monoamine neurotransmitter systems in the hippocampus (particularly those involving dopamine and norepinephrine) are affected by pregnancy (Smolen, Smolen & van de Kamp, 1987; Glaser, Russell, & Taljaard, 1992, Lonstein et al., 2003). Most of the

previous studies have considered the role of monoamines in maternal behavior. This study is one of the first to consider monoamine effects on cognitive abilities during pregnancy. Alterations in monoamine concentration may be a factor in spatial memory enhancement, particularly in the PFC, CA1, and CA3. The PFC and hippocampus appear to work together to aid in performance on spatial working memory tasks, as both regions demonstrate enhanced correlated neuron firing in a forced-choice task that tests spatial working memory (Jones & Wilson, 2005). Additionally, lesions in the PFC impair retention of a delayed-match-to-position task (Sloan, Good & Dunnett, 2006); however, the same lesions did not impair performance on a spatial reference memory task (Morris water maze). Thus, the PFC seems to be indicated in maintained performance on spatial working memory tasks, but less so in spatial reference memory tasks.

In the current study, NP females had significantly higher concentrations of all monoamines and metabolites examined in the PFC, as compared to EP or LP females. Therefore, it seems likely that decreases in levels of DA, NE, 5HT and their metabolites in the PFC may contribute to better performance on a spatial working memory task, as EP and LP females displayed significantly better spatial memory. Traumatic brain injury, which results in working memory defecits, increased levels of DA and NE in the PFC of male rats (Kobori, Clifton & Dash, 2006). Similarly, DA can increase inhibition in the PFC of primates, which can impair working memory (Kroner, Krimer, Lewis & Barrionuevo, 2006). Thus, lower levels of DA and NE may be most beneficial for working memory performance; the current study lends support to this idea.

In the CA1, turnover ratios of DOPAC/DA and HVA/DA were significantly higher in EP females than NP or LP females. Similarly, NE levels were significantly higher in

both pregnant states as compared to NP females, while turnover ratio of MHPG/NE was significantly higher in EP than NP or LP females. Turnover ratio of 5-HIAA/5HT to was also significantly higher in EP females than nulliparous or LP females. In the CA3 hippocampus, turnover ratios of DA to HVA, NE to MHPG, and 5HT to 5-HIAA were all significantly higher in EP as compared to NP and LP females. Thus, in both CA1 and CA3, turnover ratios of DA, NE and 5HT to metabolites appeared to be significantly increased during early pregnancy, indicating increased activity in these systems.

The CA1 hippocampus is most often associated with spatial memory; for example, decreased CA1 spines has been associated with poorer spatial memory (von Bohlen und Halbach, Zacher, Gass & Unsicker, 2006). Alterations in DA, NE & 5HT were measured in the CA1 during pregnancy, particularly early pregnancy. As spatial memory was enhanced on the OP task during pregnancy, it seems likely that alterations in monoamine concentration could play a role in spatial memory performance. Recently, DA has been shown to be necessary for certain forms of hippocampal synaptic plasticity (Lemon & Manahan-Vaughan, 2006). Activation of D1 and D5 dopamine receptors lowered the threshold at which long-term potentiation and depression occurred in CA1 synapses (Lemon & Manahan-Vaughan, 2006). Similarly, administration of D1 and D5 dopamine receptor antagonists prevented long-term potentiation and depression at CA1 synapses. Long-term potentiation seems to be facilitated when in a novel, empty environment, and long-term depression facilitated through exploration of novel/familiar objects in different spatial configurations (Kemp & Manahan-Vaughan, 2004), much like the OP task administered currently. Thus, DA seems to be indicated in synaptic plasticity in CA1, which may help in performance on tasks like OP. While DA levels were not found to

differ in the CA1 of NP, EP, or LP females, increased levels of the DA metabolite HVA may also contribute to enhanced spatial memory, particularly if HVA acted at the D1 and D5 receptors.

NE also plays a role in memory formation. Rats given the noradrenalin-receptor antagonist propranolol in the basolateral amygdala failed to display preference for the novel object on the object recognition task (Roozendaal, Okuda, Van der Zee & McGaugh, 2006). Similarly, administration of the adrenoceptor antagonist yohimbine (increases NE levels in the brain) to the basolateral amygdala resulted in preference of the novel object (Roozendaal et al., 2006). Additionally, when injected into the hippocampus immediately post-training on an inhibitory-avoidance task, NE facilitated memory for the task (Bevilaqua, Ardenghi, Schroder, Bromberg, Quevedo, Schmitz et al., 1997). Thus, NE appears to aid in memory formation. In the present study, significantly higher levels of NE in EP and LP females, as compared to NP females, may also have aided in memory performance. Significantly higher NE levels in CA1 of LP females as compared to EP females may have contributed to enhanced performance on the 4 hour ITD.

Monoamine concentrations in the hippocampus did not coincide with those found previously. Here, significant increases in NE were seen in CA1, whereas previously NE was significantly decreased in the hippocampus throughout pregnancy (Smolen, Smolen & van de Kamp, 1987). Additionally, MHPG levels were previously found to be significantly lowered on day 15 of pregnancy as compared to day 20 (Glaser et al., 1992); in the current study, no significant differences were observed between EP and LP females. As the previous studies analyzed whole hippocampus, individual subfield differences in monoamine could account for different measurements. Regardless, the many alterations

in the DA and NE systems in CA1 hippocampus play some role in spatial performance during pregnancy.

Monoamine concentrations were analyzed in the mPOA, an area relevant to maternal behavior. The DA metabolite DOPAC was significantly elevated in LP as compared to NP females. This measurement was opposite to previous findings in which DOPAC concentration was significantly lower on gestational day 20 as compared to females on gestational day 10 (Lonstein et al., 2003). The same study found a similar pattern for DA concentration in the mPOA (Lonstein et al., 2003), whereas no differences in DA concentration were observed in the current study. In both the current and previous studies, no significant differences were seen in turnover rates of DA. Differences in DA and DOPAC levels could be due to day at which animals were sacrificed, or small differences in tissue sampling.

DA is a major contributor to the expression of maternal behaviors (Byrnes, Rigero & Bridges, 2002). Infusion of dopamine receptor antagonists (D1 and D2) beginning on gestational day 21 significantly disrupted some aspects of maternal behavior, particularly grooming and retrieval (Byrnes, Rigero & Bridges, 2002). Similarly, infusion of D1 receptor antagonist significantly disrupted retrieval of pups when administered into the nuclus accumbens (Keer & Stern, 1999; Numan, Numan, Pliakou, Stolzenberg, Mullins, Murphy & Smith, 2005) and into the POA (Miller & Lonstein, 2005). Thus, dopamine seems to be necessary for proper display of maternal behaviors. In the current study, the significantly higher DOPAC levels measured in LP than NP females could be higher in the mPOA to aid in expression of maternal behaviors after parturition.

No significant differences were observed due to pregnancy state in concentrations of serotonin or its metabolite 5-HIAA; previous work indicated no significant difference in serotonin or 5-HIAA levels in early or late pregnancy (Lonstein et al., 2003). In contrast, significant alterations were seen in NE and its metabolite MHPG. NE was significantly higher in NP as compared to EP and LP females, while MHPG was significantly lower in NP as compared to EP and LP females. Similarly, turnover ratios of NE to MHPG were significantly higher in both EP and LP females than NP females.

NE from brainstem nuclei is a major excitatory input to the paraventricular nucleus of the hypothalamus, and thus helps to mediate the stress response via the hypothalamic-pituitary-adrenal (HPA) axis (Pacak, K., Palkovitz, M., Kvetnansky, R., Kopin, I.J., & Goldstein, D.S., 1993). During late pregnancy (day 20), decreased noradrenaline release was observed in the paraventricular nucleus of the hypothalamus, which may contribute to the reduced responsiveness of the HPA to stress during late pregnancy (Douglas et al., 2005). Similarly, in women, reduction in NE is associated with a relaxed state during pregnancy (Teixeira et al., 2005). Thus, lower levels of NE (such as those seen in EP and LP females as compared to NP females) could help to mediate stressors during late pregnancy.

As metabolism of NE is greatly increased in the mPOA during late pregnancy, and NE levels and turnover are significantly elevated in the CA1 and CA3 of pregnant females as compared to non-pregnant females, NE could play a large role in onset of maternal behaviors, as well as enhance spatial memory. As stated above, previous studies have indicated a large role for DA in onset and maintenance of maternal behavior, as well as

modulation of object recognition memory; the current study is the first to find a similar role for NE.

4. Effects of multiple reproductive experiences on memory and anxiety

Possible long-term effects of multiple reproductive experiences (RE) on memory and anxiety were examined. The performance of middle-aged (12 month old) multiparous (MP) females, each bearing at least 5 litters, was compared to age-matched nulliparous (NP) females on two markers of memory: object recognition & object placement tasks. Based upon previous research (Gatewood et al., 2005), it was hypothesized that MP females would outperform age-matched NP females on the spatial task. The recognition task to be administered has not been previously investigated as a function of reproductive experience, but it was hypothesized that recognition memory would also be significantly better in MP as compared to NP females. Anxiety was examined using the EPM; it was hypothesized that MP females would demonstrate decreased anxiety as compared to NP females, as measured by more entries and time spent in the open arms, as demonstrated in previous research (Love et al., 2005). To examine overall locomotor abilities and anxiety-like behaviors, NP and MP females were also tested in the open field.

Two neural mechanisms underlying possible differences in cognitive abilities were investigated. Monoamine concentrations were measured in PFC, CA1, and CA3 hippocampus of NP and MP females, to determine whether parity influenced monoamine expression. BDNF levels were measured in two regions of the brain, hippocampus and septum, as these regions may underlie memory ability (Alonso et al., 2005). BDNF expression is increased with exposure to estrogen (Scharfman et al., 2003), and infusion of BDNF seems to maintain spatial abilities (Radecki et al., 2005). It was thus hypothesized that BDNF levels would be higher in the hippocampus and septum of MP as compared to NP females.

4.a Method

Subjects

Sixteen 11-month-old Fisher rats (NP = 8, 230g; MP = 8, 250g) were obtained from the NIA colony at Harlan, Inc and double-housed under a 12:12 light:dark cycle (lights on at 5:00 h) with water and food available ad libitum. Upon arrival, the MP rats were substantially heavier than the NP rats; at least a 10g weight difference (MP heavier than NP) was maintained throughout acclimation, habituation, and testing. Experiments began after a two-week acclimation period to their home cage, during which all subjects were handled daily by the experimenter. All testing occurred between 1000 and 1500 hours. All procedures used were approved by the IACUC at Hunter College of the City University of New York.

Behavior Testing

Object recognition (OR) and object placement (OP) tasks were used to measure non-spatial and spatial memory (respectively). Habituation and testing was conducted as described above in the pregnancy study (see Section 3.a; habituation consisted of four days of OR followed by four days of OP). However, after the first four days of OR habituation, neither group was capable of discriminating between the two objects; therefore, a second day of OR with a 2 hour inter-trial delay (ITD) was given before habituating to OP; at this point, both NP & MP females distinguished between old and new objects. After four days of OP habituation, MP females were capable of discriminating between new and old object locations at a 2 hour ITD, but NP females were unable to discriminate; therefore, a second day of OP with a 2 hour ITD was given. At this point, NP females were able to

discriminate between object locations; although discrimination scores were not as high as MP females, the scores did not significantly differ. Post-habituation, MP and NP females were given OR and OP tasks, first at a 2 hour ITD, followed by a 4 hour ITD. As the NP females demonstrated difficulty during habituation, statistical analysis was carried out on OR and OP tasks at both delays, in order to determine if there were differences between the MP and NP females.

Two weeks following OR and OP testing, MP and NP females' performance on the elevated plus maze (EPM) was examined. The same plus maze and protocol was used in this experiment as in the previous pregnancy experiment (see Section 3.a).

Two weeks following plus maze testing, all subjects were placed into an open field apparatus to determine any possible differences in locomotion and anxiety. The open-field consisted of a 5 x 3 grid (70 x 115cm; enclosing walls 30cm high). Each animal was placed into the center square, facing away from the experimenter, and recorded for a total of 6 minutes; the observations were divided into two 3-minute periods, to determine if locomotion and anxiety-related behaviors changed as a function of time in the open field In each time period, six behaviors were recorded: 1) the number of visits to the outside squares (adjacent to a wall); 2) the number of visits to inside squares (not wall-adjacent); 3) the number of rearings (standing on hind legs with both front legs completely off the floor); 4) wall-climbings (standing on hind legs with both front feet placed upon the outside wall); 5) grooming; and 6) number of fecal boli. These behaviors are standard indicators of locomotion and anxiety (Bisagno, Ferguson & Luine, 2002; Wartella et al., 2003; Hiroi & Neumaier, 2005). The open field was cleaned with a disinfectant between trials.

Two days following completion of all behavior tasks, a nulliparous female (13 months old) died and was not available for hormonal or neurochemical analysis. The remaining females (also 13 months old) were sacrificed by decapitation two days after completion of all testing (following light anesthesia by carbon dioxide). Trunk blood was collected for analysis of estradiol, progesterone and testosterone by RIA (see previous experiment for methods, section 3.a). Brains were removed and the frontal cortex was immediately removed and stored at –70^{o}C until HPLC analysis. The remaining brain was stored at -70^{o}C; the brain was later sliced in half, with one side stored at –70^{o}C until later HPLC analysis of monoamine content in the hippocampus (see below). One hippocampus and septum were dissected out of the other half and stored separately at –70^{o}C for analysis of BDNF through enzyme-linked immunosorbent assay (ELISA; see below).

Monoamine measurement

Concentrations of seven monoamines were obtained through high performance liquid chromatography (HPLC) with E.C.: dopamine (DA) and two of its metabolites 3,4-dihydroxy-phenylacetic acid (DOPAC) and homovanillic acid (HVA); norepinephrine (NE) and its metabolite 3-Methoxy-4-Hydroxyphenylglycol (MHPG); and serotonin (5HT) and its metabolite 5-hydroxy indole acetic acid (5-HIAA). The procedure was as described previously (Bisagno, Ferguson & Luine, 2002; see section 3.a).

BDNF measurement

Levels of BDNF protein were measured by enzyme-linked immunosorbent assay (ELISA). First, the brain regions of interest from one hemisphere of each subject's brain

(hippocampus and septum) were dissected out by hand. The tissue was weighed and placed into 1ml of Lysis buffer (Promega). The solution was homogenized using a mechanical homogenizer from IKA (Eurostar power-control 6000). Following centrifugation for 30 minutes at 13,000rpm, the supernatant was removed (pellet was discarded). Seven serially-diluted standards were made from the BDNF standard in the Elisa kit (Chemicon, Intl #CYT306) ranging from 0-500 pg/ml. Directions call for 100µl of each standard and sample to be added to the microplate; however, previous work has indicated that adding 25µl of each sample with 75µl of diluant yields smaller errors and a tighter standard curve, so this amount was used (MacLusky, unpublished observations). Each standard and sample was measured in duplicate. After addition to the microplate, the samples were incubated overnight at 4^{o}C.

On the second day, the wells were washed 4 times with wash buffer, followed by addition of 100µl of diluted (1:1000) biotinylated mouse anti-BDNF antibody. Following incubation for 2.5 hours at room temperature, samples were washed with buffer 4 times, followed by addition of 100µl of diluted (1:1000) streptavidin-HRP conjugate, and incubation for 1 hour at room temperature. Samples were washed 4 times, followed by application of 100µl of TMB/E solution (at room temperature) to each well and incubation at room temperature for 15 minutes. 100µl of stop solution was added to each well (samples turned yellow); the microplate was immediately placed into microplate reader (Bio-Tek EL312, interfaced with Dell Optiplex GS computer running KC Junior software) and measured at 450nm, as color fades quickly. Optical density units were converted to nanograms of BDNF using the curve generated from the standards. Concentrations are expressed as ng/g.

Statistical Analysis

Exploration times from the sample trial (T1) was analyzed utilizing a 2 (group) x 2 (delay) ANOVA for each task (OR and OP). Exploration times (in seconds and an exploration ratio) from the recognition/retention trial (T2) was analyzed with a 2 (group) x 2 (delay) MANOVA for each task (OR and OP) to identify any possible differences between the groups on time spent with the old object or location, time spent with the new object or location, and the exploration ratio (time with new object or location/total exploration time). To further examine possible differences between NP and MP females, planned comparisons were made between the two groups at each test delay (2 hour OR, 4 hour OR, 2 hour OP, 4 hour OP), using t-tests, on each of the three variables mentioned above.

Data from the EPM was analyzed with a one-way (group) MANOVA to identify any differences between NP and MP females in two measures of anxiety: entries into open arms and time in open arms. A paired samples t-test analyzed the within group differences; i.e. to determine whether MP females spent more time in the open or closed arms, or made more entries into the open or closed arms. Data from the open field task was analyzed using a 2 (group) x 2 (time period) MANOVA on each of 6 locomotor behaviors recorded: outside visits, rearing, wall climbing, grooming, defecation, inside visits.

Monoamine and metabolite concentrations in each brain region (CA1, CA3, PFC) were analyzed via a one-way (group) MANOVA analyzing seven monoamine concentrations in each brain region. Turnover rates of the primary monoamines (DA, NE, 5HT) into their metabolites (DOPAC, HVA, MHPG, 5-HIAA, respectively) were also

analyzed with a 2-way (group) MANOVA on the four turnover ratios in each of three brain regions (CA1, CA3, PFC). Post-hoc analysis was carried out using LSD test; a p value < .05 was set for significance. Monoamine analysis was carried out on data from seven MP and six NP females, as data from one animal in each group were statistical outliers (over 2 standard deviations from the mean) and therefore excluded.

Analysis of BDNF concentration from the hippocampus and septum was analyzed using a t-test to identify possible differences between the two groups in each of the two brain regions. Data from each RIA was analyzed using a t-test for each hormone measured (estradiol, progesterone and testosterone).

4.b Results

Object Recognition: Overall performance

In order to examine whether object exploration time differed between NP and MP females, exploration time from the OR sample trial (T1) at both ITDs was analyzed with an ANOVA, which revealed no difference in overall object exploration time between the NP and MP females either on the 2 hour ITD (Figure 14A), or the 4 hour ITD (Figure 14B). There was no significant effect of delay.

A MANOVA revealed an overall significant effect of parity on performance on the OR recognition trial (T2): $F(3,26) = 3.36$, $p < 0.05$. Over both delays, MP females had a significantly greater exploration ratio (time with new object/total exploration time) than did NP females: $F(1,28) = 6.80$, $p < .05$ (Figure 15A). There were no significant effects of delay, or an interaction between group and delay. To further examine differences in memory between NP and MP females, t-tests were conducted within each group on time with old object, time with new object, and exploration ratio at each test delay.

Object Recognition: Effect of parity

On the recognition trial (T2), MP females spent significantly more time exploring the new as compared to the old object at a 2 hour ITD; $t = 4.90$, $p < 0.001$ (Figure 16, top panel). No significant differences were present in exploration of old and new objects for the NP females at a 2 hour ITD; $t = 1.05$, $p > 0.05$.. At a 4 hour ITD neither group successfully discriminated between the old and new objects; $t = 0.57$ for NP, $t = 1.27$ for MP, $p > 0.05$ (Figure 16, top panel).

Examining the data in terms of exploration ratios confirmed that at the 2 hour ITD, MP females had significantly higher exploration ratios than did NP females; $t = 4.23$, $p < 0.001$ (Figure 16, bottom panel). In contrast, at the 4 hour ITD, MP and NP females did not significantly differ in exploration ratios: $t = 1.10$, $p > 0.05$ (Figure 16, bottom panel).

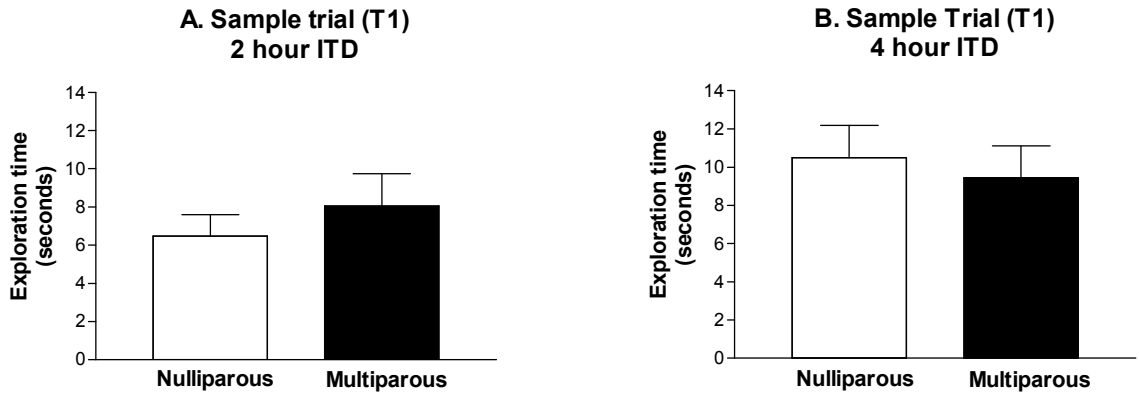

Figure 14. Effect of parity on exploration during OR sample trial (T1). (A) Time spent exploring both objects during the sample trial for NP and MP females at 2 hour ITD. **(B)** Time spent exploring both objects during the sample trial for NP and MP females at 4 hour ITD. There were no differences between groups by one-way ANOVA. Entries are (mean $\pm$ SEM) for NP (n = 8) and MP (n = 8); $p < 0.05$ used for significance.

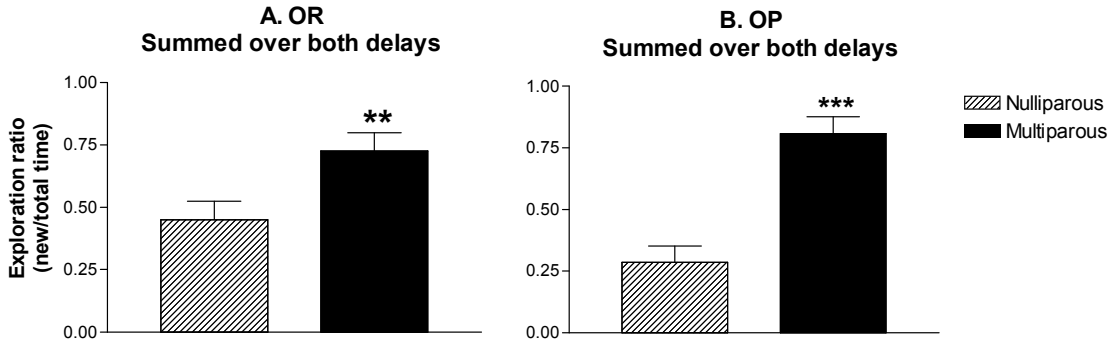

Figure 15. Exploration ratios summed over both test delays. (A) Exploration ratio of NP and MP females summed over both test delays (2 and 4 hour). Data was analyzed by MANOVA; $F(1,28) = 6.80$, $p < 0.05$. **(B)** Exploration ratio of NP and MP females summed over both test delays (2 and 4 hour). Data was analyzed by MANOVA: $F(1,28) = 30.69$, $p < 0.001$. Entries are (mean $\pm$ SEM) for NP (n = 8) and MP (n = 8). $^{**}p < 0.01$, $^{***}p < 0.001$.

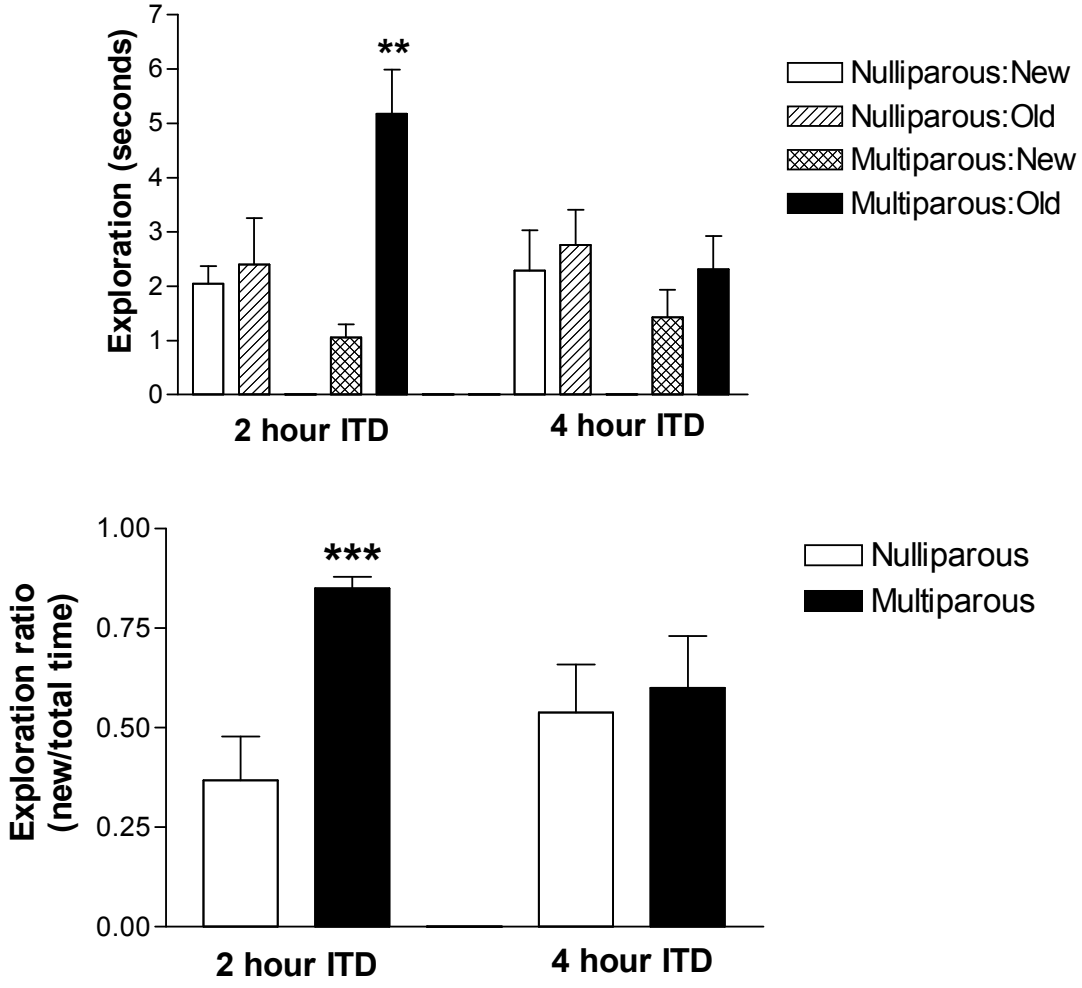

Figure 16. Effect of parity on exploration during OR retention trial (T2).
Top panel: Time exploring old and new objects for NP and MP females at 2 and 4 hour ITDs. Data were analyzed by t-test; t = 4.90, $p < 0.001$ for MP females at the 2 hour ITD. *Bottom panel:* Exploration ratio (time in new location/total exploration time) for NP and MP females at 2 and 4 hour ITDs. Data were analyzed by t-test; t = 4.25, $p = 0.001$ at the 2 hour ITD. Entries are (mean $\pm$ SEM) for NP (n = 8) and MP (n = 8); $^{**}p < 0.01$, $^{***}p < 0.001$.

Object Placement: Overall performance

In order to examine whether object exploration time differed between NP and MP females, exploration time from the OP sample trial (T1) at both ITDs was analyzed with an ANOVA, which revealed no difference in overall object exploration time between the NP and MP females either on the 2 hour ITD (Figure 17A), or the 4 hour ITD (Figure 17B). There was no significant effect of delay.

A MANOVA revealed an overall significant effect of parity on performance on the OP retention trial (T2): $F(3,26) = 9.78$, $p < 0.001$. Over both delays, MP females had significantly higher exploration ratios than NP females: $F(1,28) = 30.69$, $p < 0.001$ (Figure 15B). There were no significant effects of delay, or an interaction between group and delay. To further examine differences in memory between NP and MP females, t-tests were conducted within each group on time with old object, time with new object, and exploration ratio at each test delay.

Object Recognition: Effect of parity

On the recognition trial (T2), MP females spent significantly more time exploring the new as compared to the old object at a 2 hour ITD; $t = 3.90$, $p < 0.01$ (Figure 18, top panel). No significant differences were present in exploration of old and new objects for the NP females at a 2 hour ITD; $t = 1.10$, $p > 0.05$. At a 4 hour ITD, MP females spent significantly more time exploring the new object location than old; $t = 2.54$, $p < 0.05$. NP females did not significantly differ in exploration of old and new objects; $t = 0.85$, $p > 0.05$ (Figure 18, top panel).

Examining the data in terms of exploration ratios confirmed that at the 2 hour ITD MP females had significantly higher exploration ratios than did NP females; $t = 5.61$, $p < 0.001$ (Figure 18, bottom panel). Similarly, at the 4 hour ITD, MP females spent a significantly greater portion of time in the new location than NP females; $t = 3.01$, $p < .01$ (Figure 18, bottom panel). Thus, MP females demonstrated significantly better spatial memory than NP females.

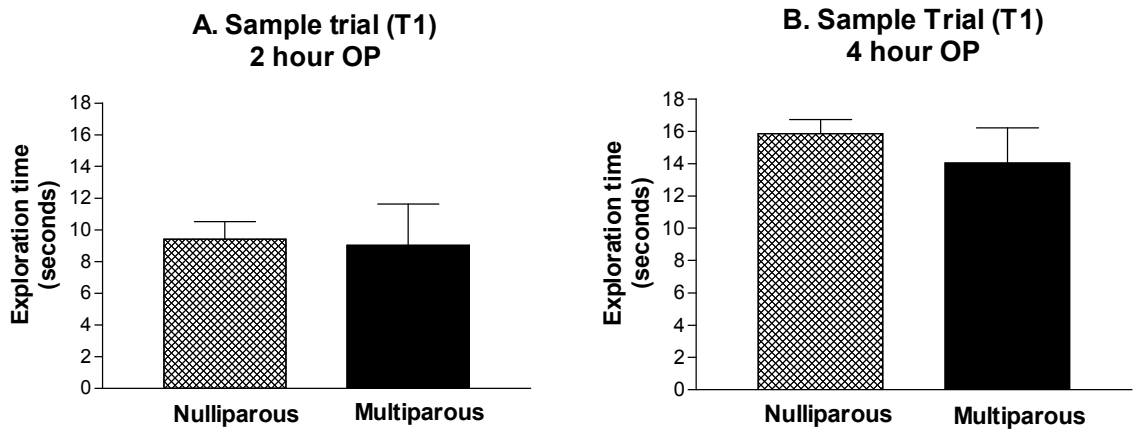

Figure 17. Effect of parity on performance during OP sample trial (T1). (A) Time spent exploring both objects during the sample trial for NP and MP females at 2 hour ITD. There were no differences between groups by one-way ANOVA. **(B)** Time spent exploring both objects during the sample trial for NP and MP females at 4 hour ITD. There were no differences between groups by one-way ANOVA. Entries are (mean $\pm$ SEM) for NP (n = 8) and MP (n = 8); $p < .05$ used for significance.

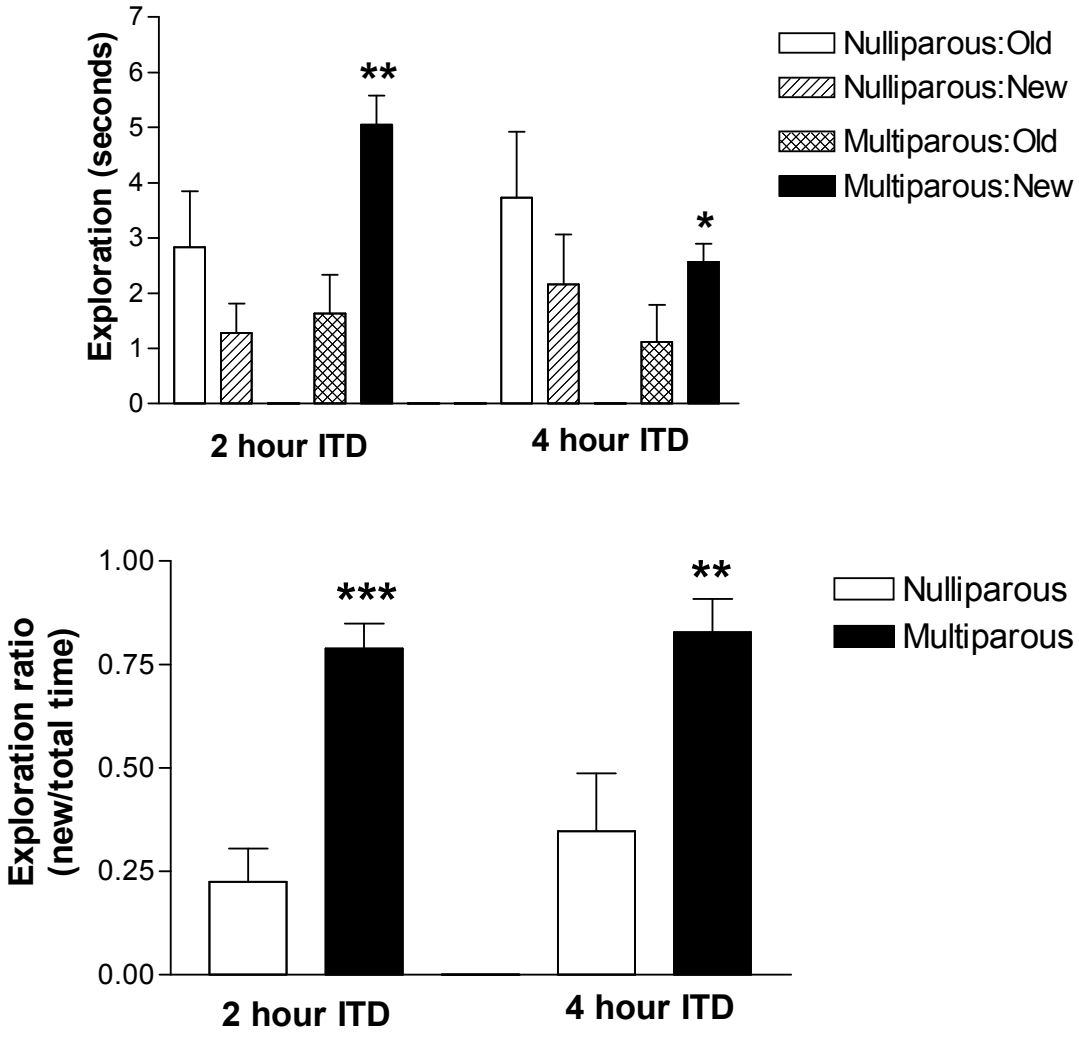

Figure 18. Effect of parity on exploration during OP retention trial (T2).
Top panel: Time exploring old and new objects for NP and MP at 2 and 4 hour ITDs. Data were analyzed by t-test; $t = 3.90$, $p < 0.01$ for MP females (left) and $t = 2.54$, $p < 0.05$ for MP females (right). *Bottom panel:* Exploration ratio (time in new location/total exploration time) for NP and MP females at 2 and 4 ITDs. Data were analyzed by t-test; $t = 5.61$, $p < 0.001$ (left) and $t = 3.01$, $p < 0.01$ (right). Entries are (mean $\pm$ SEM) for NP ($n = 8$) and MP ($n = 8$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Elevated Plus Maze

Performance of NP and MP females was compared on the elevated plus maze for entries into and time spent in the open arms of the maze. A MANOVA revealed no significant differences between NP and MP females in the two measured anxiety-related behaviors: $F(4,11) = 2.12$, $p > 0.05$ (Figure 19).

Open Field

Locomotor and anxiety-related behaviors of NP and MP females were compared on the open field. A MANOVA revealed no significant overall effect of group: $F(2,77) = 0.91$, $p > 0.05$. There were no significant interactions between group and measured behaviors: $F(10, 156) = 0.686$, $p > 0.05$. Additionally, no significant interactions were present in measured behaviors (rearing, wall climbing, grooming, defecation, outside grid crossing, inside grid crossing) during the first three minutes of exploration: $F(5,78) = 0.53$, $p > 0.05$ or the second three minutes of exploration: $F(5,78) = 1.03$, $p > 0.05$ (Table 3).

Radioimmunoassay Results

No significant differences were present between NP and MP females in serum estradiol levels: $t = 0.37$, $p > .05$; in serum progesterone levels: $t = 1.29$, $p > .05$; or in serum testosterone levels: $t = 1.13$, $p > .05$ (Figure 20).

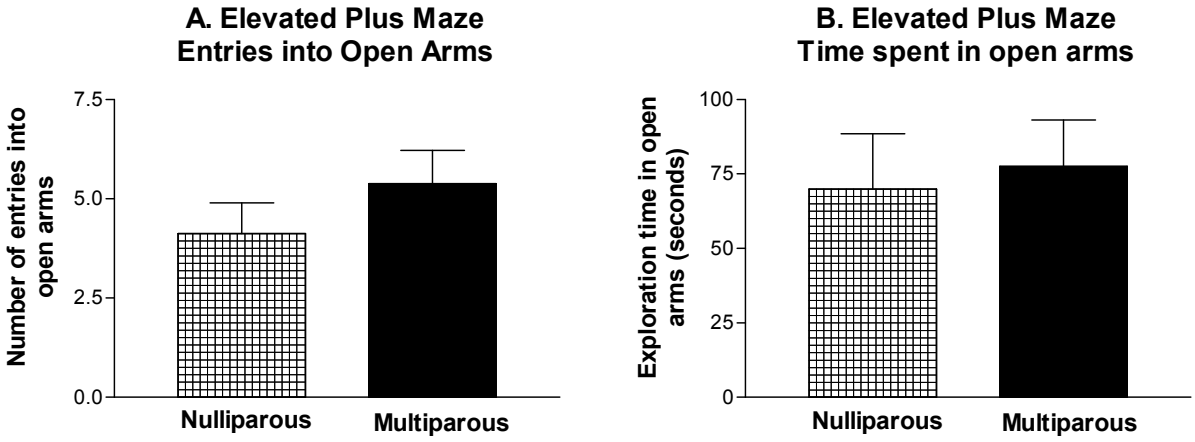

Figure 19. Effect of parity on performance in the elevated plus maze. (A) Number of open arm entries and **(B)** time spent in open arms of the EPM by NP and MP females. Data wee analyzed by MANOVA, $F(4,11) = 2.118$, $p > 0.05$. Entries are (mean $\pm$ SEM) for NP (n = 8) and MP (n = 8).

Table 3. Performance of NP and MP females on the open field.
No significant differences were found between NP and MP females on any of the six indicators of locomotor ability and anxiety-like behaviors measured. Entries are (mean ± SEM) for NP (n = 8) and MP (n = 8).

Activity	Group	Time 1	Time 2
Rearing	Nulliparous	0.00 ± 0.21	0.14 ± 0.17
	Multiparous	0.50 ± 0.20	0.38 ± 0.16
Wall Climbs	Nulliparous	1.57 ± 0.61	3.00 ± 0.68
	Multiparous	3.00 ± 0.57	3.75 ± 0.63
Grooming	Nulliparous	3.86 ± 0.72	3.29 ± 0.72
	Multiparous	4.38 ± 0.67	2.63 ± 0.67
Defecation	Nulliparous	0.29 ± 0.16	0.43 ± 0.23
	Multiparous	0.13 ± 0.15	0.13 ± 0.21
Outside Grid Crossings	Nulliparous	24.00 ± 4.19	20.00 ± 4.21
	Multiparous	28.38 ± 3.92	26.00 ± 3.94
Inner Grid Crossings	Nulliparous	2.57 ± 0.78	2.00 ± 0.74
	Multiparous	2.00 ± 0.73	2.00 ± 0.69

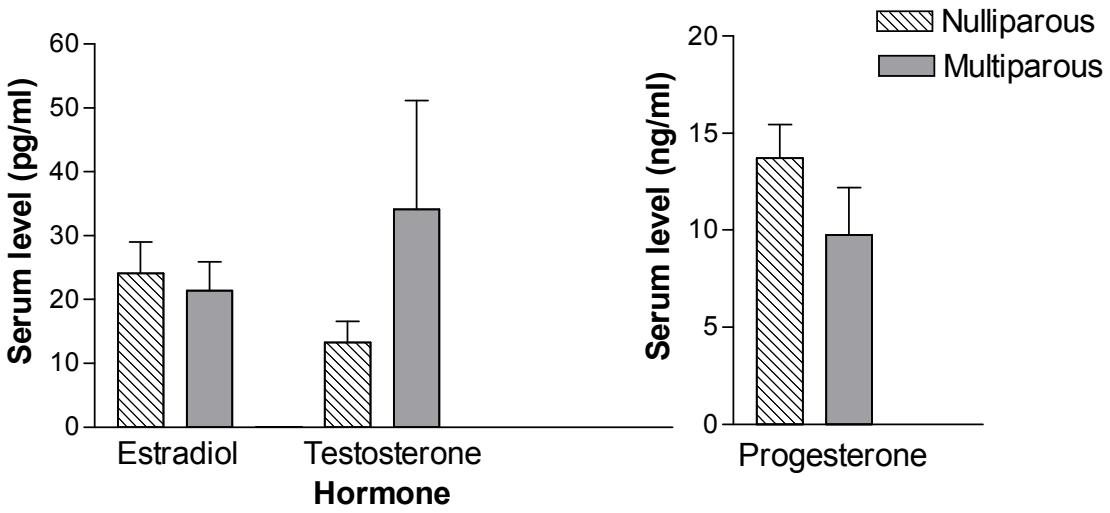

Figure 20. Effect of parity on gonadal hormone concentrations. Serum hormone levels of **(A)** estradiol, (**B)** progesterone, and (**C)** testosterone. T-tests for each hormone revealed no significant differences in levels of estradiol (t = 0.37), progesterone (t = 1.29) and testosterone (t = 1.13) levels, $p > 0.05$. Entries are (mean $\pm$ SEM) for NP (n = 7) and MP (n = 8).

Brain monoamines and metabolites

Monoamine concentrations were measured in three brain regions: CA1, CA3, and PFC, in order to determine whether long-term changes due to reproductive experience would be observed in three monoamine systems (DA and metabolites DOPAC and HVA, NE and metabolite MHPG, 5HT and metabolite 5-HIAA). In each brain region, a MANOVA was carried out on the data. In the CA1, the MANOVA revealed no overall significant effect of group: $F(1,11) = 12.30$, $p > .05$. Identical results were obtained in the CA3: $F(1,11) = 1.07$, $p > .05$, and in the PFC: $F(2, 10) = 1.03$, $p > .05$. In all three of the above regions, there were no significant interactions between group (NP and MP females) and any monoamine, metabolite, or turnover ratios (Table 4).

BDNF: Expression in nulliparous and multiparous females

Concentrations of BDNF were analyzed in the hippocampus and septum of NP and MP females. No significant differences were present in BDNF expression in the hippocampus of NP and MP females: $t = 0.558$, $p > 0.05$ (Figure 21, left). In contrast, MP females, as compared to NP females, had significantly greater BDNF expression in the septum: $t = 2.185$, $p < 0.05$ (Figure 21, right).

Table 4. ANOVA results, F and p values for monoamine and metabolite measurements in three brain regions.
No significant differences were measured between NP and MP females for any monoamine, metabolite, or turnover ratio measured in CA1, CA3, and PFC. Concentrations given are in pg/μg total protein. Entries are (mean ± SEM) for NP (n = 6) and MP = 7)

	Monoamine Concentrations (pg/μg)							
Region		DA	DOPAC	HVA	NE	MHPG	5HT	5-HIAA
CA1	NP	0.64 ± 0.54	0.51 ± 0.20	1.36 ± 0.47	2.54 ± 0.52	15.35 ± 1.76	2.77 ± 0.88	4.21 ± 0.75
	MP	0.35 ± 0.13	0.26 ± 0.06	0.72 ± 0.18	2.84 ± 0.34	17.05 ± 4.00	2.64 ± 0.43	4.54 ± 0.69
CA3	NP	0.96 ± 0.27	0.92 ± 0.27	1.40 ± 0.44	6.09 ± 0.94	6.55 ± 0.98	3.54 ± 0.33	4.90 ± 0.33
	MP	0.87 ± 0.10	0.75 ± 0.09	1.29 ± 0.21	6.10 ± 0.54	4.97 ± 0.83	3.14 ± 0.24	4.92 ± 0.28
PFC	NP	1.32 ± 0.13	0.82 ± 0.10	0.72 ± 0.09	3.42 ± 0.46	4.39 ± 0.90	5.44 ± 0.71	5.50 ± 0.83
	MP	1.10 ± 0.15	0.77 ± 0.13	0.71 ± 0.12	3.54 ± 0.39	3.00 ± 0.38	4.87 ± 0.63	6.32 ± 1.40

	Turnover Ratios (metabolite/monoamine)				
Region		DOPAC/DA	HVA/DA	MHPG/NE	5-HIAA/5HT
CA1	NP	0.84 ± 0.30	2.14 ± 0.94	8.95 ± 2.15	1.29 ± 0.23
	MP	1.07 ± 0.27	3.16 ± 0.87	5.89 ± 1.20	1.74 ± 0.21
CA3	NP	1.05 ± 0.14	1.43 ± 0.18	1.33 ± 0.32	1.41 ± 0.10
	MP	0.97 ± 0.13	1.51 ± 0.17	0.77 ± 0.29	1.55 ± 0.09
PFC	NP	0.63 ± 0.10	0.61 ± 0.15	1.33 ± 0.21	1.02 ± 0.13
	MP	0.74 ± 0.09	0.70 ± 0.13	0.90 ± 0.20	1.27 ± 0.12

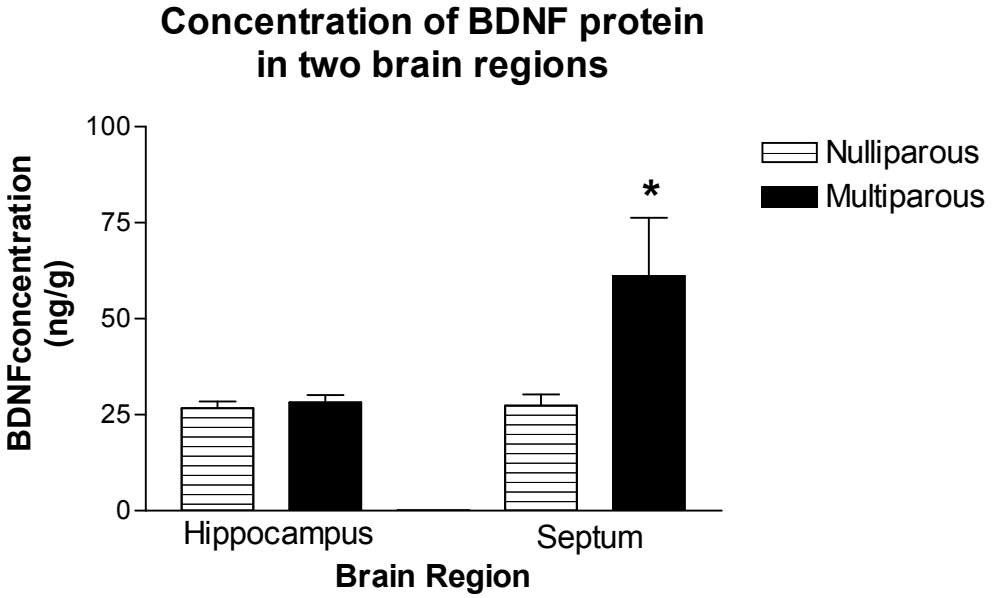

Figure 21. Effect of parity on BDNF expression in hippocampus and septum. *Left side*: BDNF in hippocampus of NP and MP females. Data were analyzed by t-test; t = 0.558, $p > .05$. *Right side*: Expression of BDNF in septum of NP and MP females. Data were analyzed by t-test; t = 2.185, $p < 0.05$. Entries are (mean $\pm$ SEM) for NP (n = 7) and MP (n = 8); * $p < 0.05$.

4.c Discussion

Reproductive experience and spatial memory performance

In the current study, non-spatial and spatial memory were examined in12 month old multiparous (MP) and age-matched nulliparous (NP) females. On both tests of memory, MP females as compared to NP females, demonstrated significantly greater memory ability for recognition of new object (non-spatial) and recognition of new location (spatial). The current findings of better performance by MP as compared to NP females are new in relation to these tasks, and suggest that parity can significantly enhance spatial and non-spatial memory.

These findings corroborate previous work in which MP females demonstrated fewer working memory errors than NP females on the radial arm maze (Kinsley et al., 1999; Pawluski et al., 2006). Additionally, at 13 months of age (similar to the age at which testing took place in the current study), primiparous females (a single pregnancy and litter at 3 months of age, followed by full 21 days of maternal care) had significantly shorter latencies to find a baited food in a dry-land maze well than nulliparous females (Love et al., 2005). A single reproductive experience may therefore be sufficient to enhance spatial memory, even months after the pregnancy. However, both radial arm and dry-land maze require food deprivation, which can place stress on the female and thus alter the results. It may be beneficial to repeat the current study utilizing a third group (age-matched primiparous females), in order to corroborate the findings that a single reproductive experience can also enhance spatial memory months after pregnancy (Love et al., 2005) without using a spatial task requiring food deprivation.

Reproductive experience and anxiety

A significant increase in number of entries into, and time spent in, the open arm of the EPM is used to indicate decreased anxiety (Pellow et al., 1985; Lonstein, 2005). NP and MP females did not significantly differ in either measure of anxiety. Previous work indicated that MP females (2 pregnancies) demonstrated decreased anxiety at 10 and 14 months of age as compared to NP females (Love et al., 2005). Current EPM testing was conducted in a manner similar to that by Love et al (2005), yet results differed. In both previous and current studies, animals were handled in the laboratory, parous females were mated, and other cognitive tasks were administered prior to EPM testing. However, significantly decreased anxiety was not observed in parous females until five or nine months post weaning; performance on EPM at 6 months of age, only one month post weaning, did not significantly differ between NP, primiparous and MP females (Love et al., 2005). In the present study, MP females were approximately three months post last weaning; time elapsed between maternal experience and anxiety measure may be a factor in expression of the behavior.

Performance of MP and NP females was examined on the open field maze to identify possible differences in overall locomotor activity, as well as anxiety-related behaviors. No significant differences were present between MP and NP females on measures of locomotion and anxiety-related behaviors (grid crossings, grooming, rearing, defecation, wall climbing) on the open field apparatus. Lack of group differences in locomotion indicated that the observed increase in exploration of new objects/locations during the memory tasks was not due to locomotor differences between NP and MP females. Additionally, as behaviors related to anxiety (exploration of open arm on EPM

and inner grid crossings in the open field) did not significantly differ between MP and NP females, the current study indicates that differences in anxiety did not contribute to differences in memory performance. A recent study has indicated that in the EPM and open field, middle-aged (10-11 month old) primiparous females demonstrated an increase in expression of anxiety-related behaviors as compared to age-matched nulliparous females, and young (3-4 month old) primiparous females (Byrnes & Bridges, 2006). Thus, despite reproductive experience, anxiety did not seem to be reduced in older females; opposite effects were observed in young females, where primiparous females demonstrated a decrease in anxiety-related behaviors (Byrnes & Bridges, 2006). However, results by Love et al. (2005) indicate that early reproductive experience can reduce anxiety as females age, particularly around 10-14 months. Thus, the relationship between parity and anxiety warrants further exploration.

Reproductive experience and hormone state

Levels of estradiol, testosterone, and progesterone did not differ significantly between nulliparous and multiparous females. Additionally, at time of sacrifice, analysis of vaginal smears showed that the majority of females in both groups had entered a persistent estrus state (data not shown). Beginning around 9-12 months of age, female rats begin to experience irregular estrous cycles, and eventually stop cycling all together, entering a persistent estrous or persistent diestrus/pseudopregnant state (Warren & Juraska, 2000). At this point, females typically experience estrogen levels equivalent to those seen in the diestrous/estrous stage of the cycle. Estradiol levels in NP and MP females obtained in the current experiment correspond well to those seen in females in a

diestrous state or entering day of estrus (Rosenblatt, 1988), and confirmed that NP and MP females in the current study did not significantly differ in hormonal levels. Therefore, enhanced performance by MP females on the OR and OP tasks was not due to differences in circulating gonadal hormone levels.

Reproductive experience and monoamine concentration

Monoamine concentrations in CA1, CA3 hippocampus and PFC did not differ significantly between NP and MP females. These are brain regions known to underlie performance on non-spatial and spatial memory tasks (Broadbent, Squire & Clark, 2004; Jones & Wilson, 2005). A number of studies have examined concentrations of DA, NE, 5HT and their metabolites in male and female rats as they age (Luine, Bowling & Hearns, 1990; Tanila et al., 1994; Miguez et al., 1999; Lee et al., 2001), and have found significant alterations in these monoamine systems.

As compared to 4 month old females, decreased levels of DA, NE and 5HT were observed in forebrain nuclei, entorhinal cortex, and hippocampus of aged (25-26 month) female rats (Luine, Bowling & Hearns, 1990). In both male and female aged (27-31 month) rats, age-related decreases in NE, DA, 5-HT and turnover ratios into metabolites were observed in the PFC, striatum and hippocampus (Tanila et al., 1994). By 24 months of age, significant decreases in hippocampal DA and DOPAC were observed as compared to young (3 month) and middle-aged (12 month) rats (Miguez et al., 1999). Finally, as compared to 6 month old males, 24 month old males demonstrated significant decreases in concentration of DA, DOPAC, 5-HT and 5-HIAA in the cerebral cortex and plasma (Lee et al., 2001). Thus, in both male and female rats, marked decreases in

concentration of DA, NE, 5-HT and metabolites can be observed throughout the brain. Therefore, it is possible that both NP and MP females demonstrated alterations in monoamine systems as compared to younger females, but not as compared to one another. Future studies should investigate monoamine concentrations between aged NP and MP females, and young NP and MP females, in order to determine whether there are differences in CA1, CA3 and PFC due to aging, as the literature suggests, and whether multiple reproductive experiences may influence monoamine systems in young females.

From this study it appears as if parity did not elevate concentrations of DA, NE, 5HT or metabolites in the three brain regions (PFC, CA1, CA3) examined of middle-aged females. Therefore, the observed behavior differences on the OR and OP tasks cannot be ascribed to alterations in these monoamine systems. However, in section 3 of this thesis, (see 3.b for results, 3.c for discussion), concentrations of DA, NE, 5HT and metabolites were significantly altered in the hippocampus during pregnancy. In particular, activity of these systems, as measured by turnover ratios, was significantly increased in early pregnant as compared to late pregnant and NP females in CA1 and CA3 (see section 3.b). Thus, beneficial effects of pregnancy on spatial memory appeared to be mediated, at least in part, by concentrations and activity of DA, NE and 5HT in the hippocampus.

In this study, lactating females, or new dams whose pups had been weaned, were not examined for monoaminergic activity. However, few differences in monoaminergic activity in the hippocampus existed between late pregnant and NP females (see section 3.b) Thus, effects of pregnancy/reproductive experience on monoamine concentration and activity may occur early in pregnancy (within the first week), and then slowly revert to pre-pregnancy levels. While the MP females examined currently were older than the

pregnant females in section 3 (13 months and 4 months, respectively), the MP females were approximately 3-4 months past last pregnancy. It is possible that alterations in hippocampal monoamine systems could have occurred when pregnant, but by time of sacrifice had returned to pre-pregnancy levels. Future studies should examine monoamine concentrations in females with and without reproductive experience at different time periods (during pregnancy, lactation, and after weaning) to fully determine when differences in hippocampal monoamines due to reproductive experience are present.

Although differences in monoamine concentration and activity were not observed in the PFC and hippocampus, it is possible that multiple reproductive experiences could affect monoamine systems in other brain regions. While the PFC and hippocampus are mainly involved with spatial ability and higher-order cognitive processes (Broadbent, Squire & Clark, 2004; Jones & Wilson, 2005), the olfactory bulb (OB) is involved in both memory processes (Guan & Dluzen, 1994) and recognition of offspring (Fleming & Rosenblatt, 1974). Monoaminergic systems, particularly the NE system, play a large role in both olfactory memory and offspring recognition (Dluzen, 1996; Dickinson & Keverne, 1988). The effect of multiple reproductive experiences on NE levels, or any other monoamine, in the OB has yet to be examined. To do so, levels of DA, NE, 5-HT and metabolites will be measured in the OB of middle-aged (13 month) multiparous and nulliparous females, to determine whether reproductive-related changes in these metabolites are observed in the OB post-weaning. This experiment is in the next section.

Reproductive experience and BDNF concentration

One factor that may underlie the significant differences in memory performance between 13 month-old NP and MP females is BDNF. BDNF levels were significantly higher in the septum of MP females relative to NP females, although levels were not higher in the hippocampus. These results show a similar pattern to those found by Gibbs (1999), in which acute treatment with estradiol produced a decrease in BDNF protein levels in the hippocampus, but increased BDNF protein in the septum. Stimulation of medial septum neurons upregulated BDNF gene expression throughout the hippocampus four hours later (Lindefors, Ernfors, Falkenberg & Persson, 1992), and complete lesion of the medial septum reduced baseline BDNF mRNA expression throughout the hippocampus (Berchtold, Kesslak & Cotman, 2002). Thus, input from the septum seems to be necessary for BDNF expression in the hippocampus.

The lack of observed differences between NP and MP females in hippocampal BDNF expression may be due to increased retrograde transport of BDNF from the hippocampus to the septum. Treatment with estradiol and progesterone decreased hippocampal BDNF protein, but increased hippocampal BDNF mRNA (Gibbs, 1999). These results suggest either an increase in BDNF protein degradation or transport away from hippocampus. The medial septum contains neurons which are the major source of cholinergic enervation to the hippocampus and are responsible for retrograde transport of BDNF protein (through TrkB receptors) from hippocampus to septum (Sobreviela, Pagcatipunan, Kroin & Mufson, 1996). Estrogen appears to maintain expression of TrkB receptor, as female serotonin/BDNF knockout mice did not demonstrate the TrkB decrease found in male serotonin/BDNF knockout mice (Ren-Patterson, Cochran,

Holmes, Lesch, Lu & Murphy, 2006). Thus, repeated exposure to estrogen during pregnancies could have enhanced expression of TrkB, resulting in increased activity of the septum cholinergic neurons, and retrograde transport of hippocampal BDNF protein.

However, it is also possible that lack of differences in hippocampal BDNF between NP and MP females may be due to the sampling methods used. The entire hippocampus was removed, homogenized, and analyzed for levels of BDNF; recent evidence suggests that in the hippocampus, BDNF expression is higher in the CA3 and dentate gyrus regions (Bora et al., 2005). Specifically, BDNF expression is higher in the granule cell layer of the dentate gyrus, and the mossy fiber terminals of the CA3 (Conner, Lauterborn, Yan, Gall & Varon, 1997; Smith, Zhang, Lyons & Mamounas, 1997; Danzer & McNamara, 2004). By homogenizing the entire hippocampus, different effects in sub-regions of the hippocampus may be obscured. In the following study, BDNF expression in 3 subregions of the hippocampus (CA1, CA3 and DG) will be examined in a second cohort of NP and MP females (section 5).

5. Effects of multiple reproductive experiences on monoamine and BDNF concentration

As stated above, it seems likely that monoamine concentrations in the OB could be altered by multiple reproductive experiences. Therefore, concentrations of DA, NE, 5HT and metabolites were measured in the OB of NP and MP females. Based on prior research (Pissonier, Thiery, Fabre-Nys, Poindron & Keverne, 1985; Dickinson & Keverne, 1988; Levy, Gervais, Kindermann, Orgeur & Piketty, 1990; Calamandrei, Wilkinson & Keverne, 1992), it was hypothesized that MP females would have higher concentrations of monoamines and metabolites, particularly NE and its metabolite MHPG.

It also seems likely that in the previous experiment, lack of difference in BDNF concentrations in the hippocampus of NP and MP females may have been due to the methods used. Therefore, a second cohort of NP and MP females were used to examine BDNF concentrations in subregions of the hippocampus: CA1, CA3, and dentate gyrus (DG), as well as the medial septum (MS). Of all hippocampal regions, the CA1 hippocampus is particularly important for spatial memory performance (Clark, Broadbent & Squire, 2005). Therefore, it was hypothesized that if subregional differences in BDNF concentration were present, MP females would have significantly higher concentrations of BDNF in CA1 hippocampus as compared to NP females. Due to the localization of BDNF to CA3 and DG (Conner, et al., 1997; Smith et al., 1997; Danzer & McNamara, 2004), it was not believed that reproductive experience would significantly affect protein expression in these subregions. Finally, BDNF protein level as a function of reproductive experience was measured in the MS, as this region has previously been shown to mediate BDNF expression in the hippocampus (Berchtold, Kesslak & Cotman, 2002).

5.a Method

Subjects

Fourteen 12-month-old animals (NP = 6; MP = 8) were a kind gift from the rat colony at Helen Hayes Hospital (West Haverstraw, NY), maintained by Dr. Helen Scharfman. Prior to use in this study, the MP females had been used solely for breeding (4 with four litters, 2 with 3 litters, and 2 with two litters), and the NP females were housed in pairs and aged (no history of behavioral use). All subjects were sacrificed by decapitation following light anesthesia by carbon dioxide. Brains were immediately removed and stored at -70^{o}C; each brain was then divided into 6-7 thick sections based upon anatomical markings (sections made at olfactory bulb (OB), prefrontal cortex, anterior to optic chiasm, posterior to optic chiasm, just anterior to the hypothalamus, and just posterior to the hypothalamus; see Luine et al., 1974).

Monoamine analysis

The brain sections containing the OB were sampled with a 500μm-diameter cannula; 4-6 tissue punches were obtained from each animal on a frozen stage at -8^{o}C. Concentrations of three monoamines (DA, NE, 5HT) and four metabolites (DOPAC, HVA, MHPG and 5-HIAA) were obtained through the same HPLC technique utilized in the previous sections (3 and 4). Monoamine and metabolite concentrations were expressed in pg/μg total protein.

BDNF analysis

A 500μm-diameter cannula was used to punch out three subregions of the hippocampal formation: CA1, CA3, dentate gyrus (DG), as well as the medial septum (MS). Tissue punches were taken under a dissecting microscope, on a microscope stage maintained at -4^{o}C. Each of these regions was then analyzed for levels of BDNF levels using the ELISA kit and procedure described in section 4.a, with minor modifications. Because the tissue punches through CA1, CA3, DG and MS were much smaller than those used previously (entire hippocampus), the tissue was homogenized in only 250μl of Lysis buffer (Promega), in order to prevent dilution of tissue samples. Similarly, the full 100μl of sample specified in the instructions from the kit was added to each well to ensure sufficient protein for analysis (as opposed to only 25μl with the larger tissue sample). As the tissue samples were too small to weigh, proper converstion from optical density to nanograms of BDNF using the curve generated from the standards was accomplished by normalizing the raw data to total protein (as measured by Bradford assay; see de Silva & Arruda, 2006). Concentrations are expressed as ng/g.

Statistical Analysis

Monoamine concentrations in the OB were analyzed via a one-way MANOVA analyzing three monoamines and four turnover ratios of the primary monoamines (DA, NE, 5HT) into metabolites (DOPAC, HVA, MHPG, 5-HIAA, respectively). A p value < .05 was set for significance. BDNF concentration from each region examined (CA1, CA3, DG, MS) was analyzed with a one-way MANOVA.

5.b Results

Brain monoamines: Levels in OB of nulliparous and multiparous females

Monoamine, metabolites and turnover ratios were compared in the OB of nulliparous (NP) and multiparous (MP) females. A MANOVA revealed a significant overall effect of group: $F(2,11) = 58.62$, $p < 0.05$. Five monoamines and metabolites differed significantly between NP and MP females. Concentrations of DA and its metabolite DOPAC were significantly higher in MP as compared to NP females (Figure 22A). Similarly, concentrations of NE and its metabolite MHPG were significantly higher in MP as compared to NP females (Figure 22C). Concentrations of serotonin did not significantly differ between MP and NP females, but the serotonin metabolite 5-HIAA was significantly higher in MP as compared to NP females (Figure 22E).

The turnover ratios (metabolite/monoamine) were examined by MANOVA, which revealed a significant overall effect of group: $F(2,11) = 58.62$, $p < 0.05$. Specifically, the turnover ratios of DOPAC/DA and 5-HIAA/5HT were significantly higher in MP as compared to NP females (Figure 22B, 22F); in contrast, the HVA/DA ratio was significantly lower in MP as compared to NP females (Figure 22B). Combined, results indicated that the monoamines DA and NE, the metabolites DOPAC, MHPG and 5-HIAA, as well as three turnover ratios, are significantly enhanced in the OB of females with prior reproductive experience.

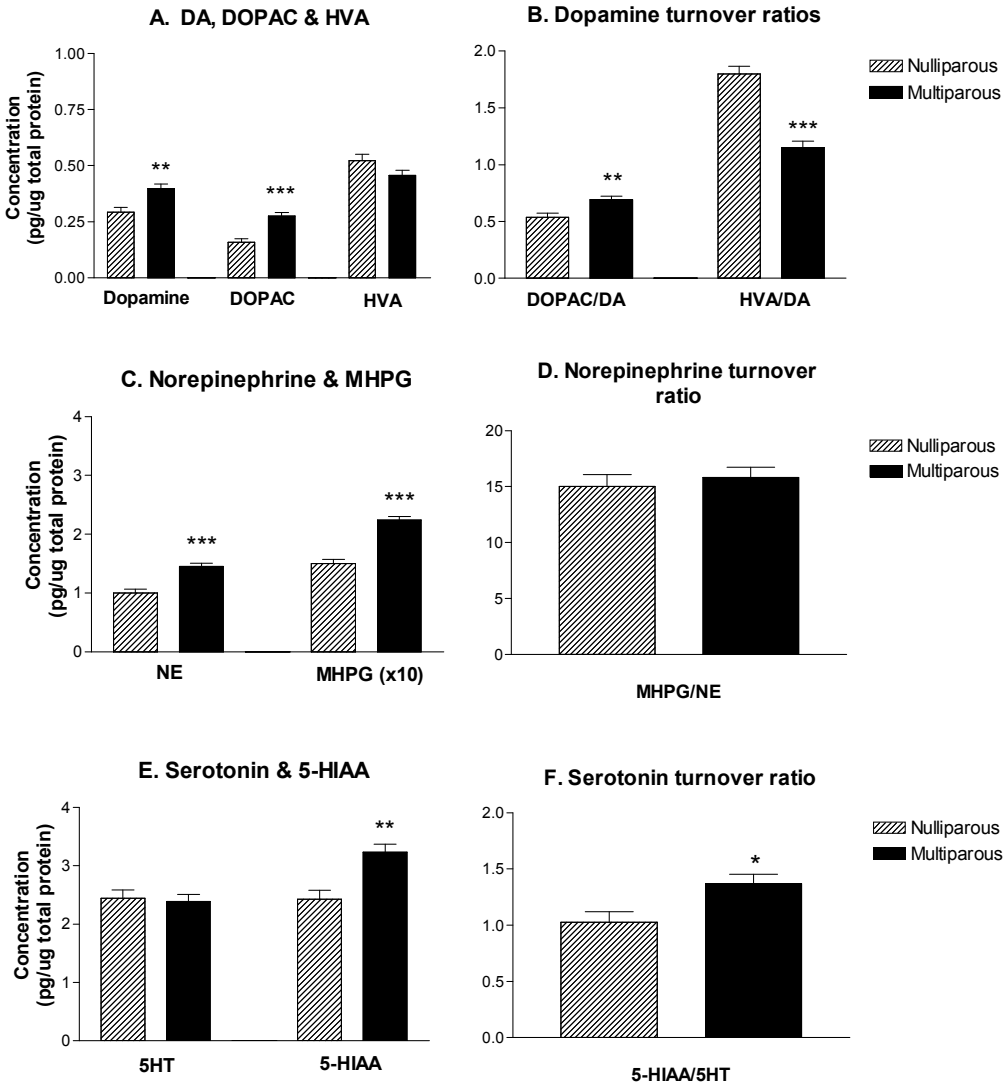

Figure 22. Effect of parity on DA, NE and 5HT concentrations in the OB of NP and MP females. **(A)** Concentrations of DA and metabolites DOPAC and HVA. **(B)** Turnover ratios of DA to DOPAC and HVA. **(C)** Concentrations of NE and metabolite MHPG. **(D)** Turnover ratio of NE to MHPG. **(E)** Concentrations of 5HT and metabolite 5-HIAA. **(F)** Turnover ratio of 5HT to 5-HIAA. Monoamine concentrations were analyzed by MANOVA (group x monoamines), where $F(2,11) = 58.62$, $p < 0.05$. Turnover rates were analyzed by MANOVA (group x ratios), where $F(2,11) = 58.62$, $p < 0.05$. Entries are (mean $\pm$ SEM) for NP (n = 6) and MP (n = 8). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

BDNF: Levels in subregions of the hippocampus and septum of nulliparous and multiparous females

Concentrations of BDNF were measured in three subregions of the hippocampus (CA1, CA3, DG) and in the MS of NP and MP females. A MANOVA revealed no overall significant effect of reproductive experience: $F(4,9) = 1.73$, $p > .05$. However, there was a significant interaction between group and CA1 subregion: $F(1,12) = 4.88$, $p < 0.05$. Specifically, BDNF levels were significantly higher in the CA1 subfield of MP as compared to NP females (Figure 23A). BDNF concentrations did not significantly differ in any of the other regions examined (CA3, DG, MS; Figure 23B, C, D, respectively).

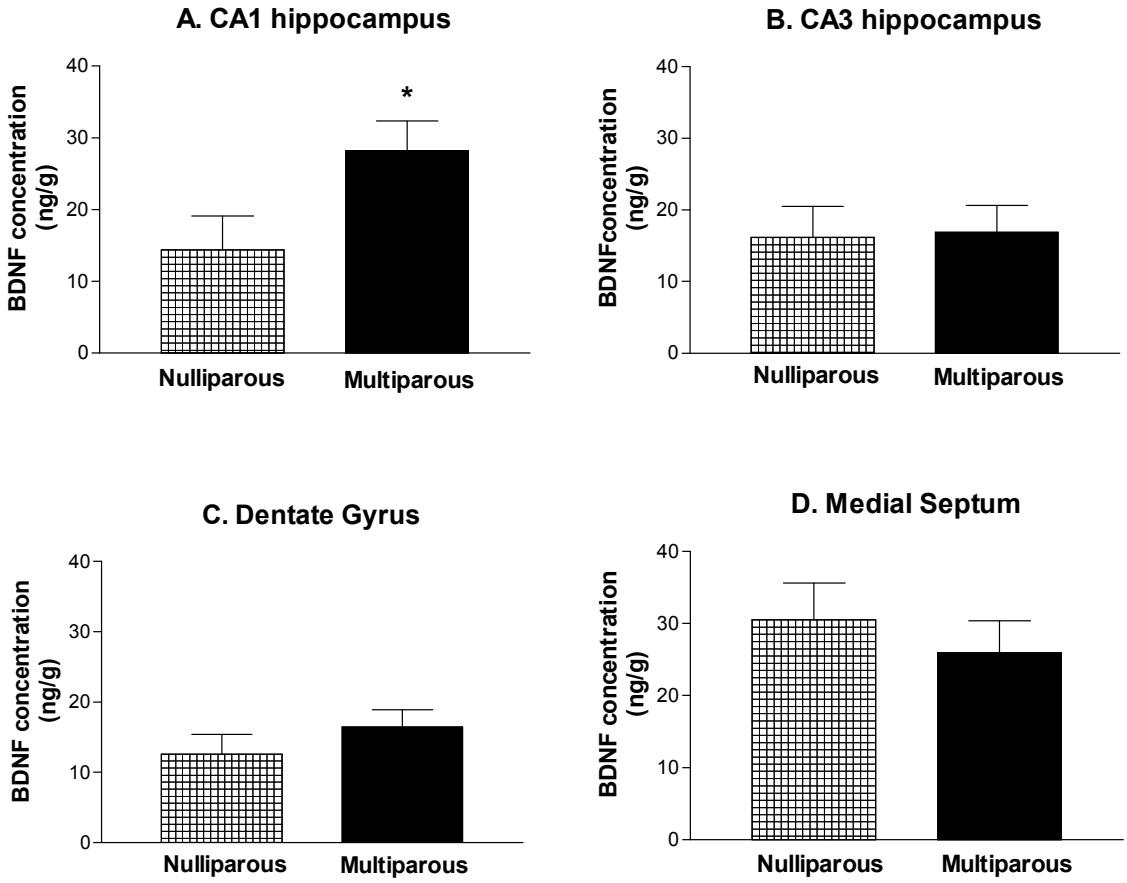

Figure 23. Effect of parity on BDNF expression in subregions of hippocampus and septum of NP and MP females. A. In the CA1, MP females had significantly higher concentrations of BDNF than NP females. **B.** BDNF concentration did not significantly differ in the CA3. **C.** BDNF concentration did not significantly differ in the DG. **D.** BDNF concentration did not significantly differ in the medial septum. Data were analyzed by MANOVA, which revealed a significant group x CA1 interaction: $F(1,12) = 4.88$, $p < 0.05$. Entries are (mean $\pm$ SEM) for NP (n = 6) and MP (n = 8). $^*p < 0.05$

5.c Discussion

Reproductive experience and monoamine concentration in the OB

Compared to NP females, MP females demonstrated significant changes in the DA, NE, and 5HT monoamine systems in the OB. Specifically, an elevation of 36% in DA levels, 75% in DOPAC levels, 45% in NE levels, 40% in MHPG levels, and 33% in 5-HIAA levels were observed in MP as compared to NP females. Additionally, turnover ratios of DOPAC/DA were elevated by 17% in MP females, and turnover ratios of 5-HIAA/5HT were elevated by 34% in MP females, indicating increased activity in the DA and 5-HT systems. Thus, multiple reproductive experiences greatly enhanced expression of specific monoamine systems in the OB.

As male and female rats age, levels of DA, NE and 5HT decrease throughout the brain: as compared to young rats (3-6 months old), aging rats display lower monoamine levels in striatum, entorhinal cortex, hippocampus and frontal cortex (Luine, Bowling & Hearns, 1990; Tanila et al., 1994; Miquez et al., 1999), as well as throughout the cerebral cortex (Lee et al., 2001). Decreased concentrations of monoamine metabolites DOPAC, MHPG and 5-HIAA were all significantly decreased by 15 months of age in male rats, as compared to 5 month-old rats (Dluzen, 1996). In rats of the same age, significant decreases in olfactory-based memory have been observed (Guan & Dluzen, 1994), implying a role for monoamines in olfactory ability. Indeed, male and female rat pups (post-natal day 6) given propranolol (NE antagonist) in the OB failed to acquire learned odor preference (Sullivan, Zyzak, Skierkowski & Wilson, 1992). Thus, monoamines and metabolites appear to aid in regulation of olfactory memory throughout the lifespan.

In the current study, significant increases were seen in levels of DA, NE, and metabolites DOPAC, MHPG and 5-HIAA in the OB of MP female rats as compared to NP females. Thus, multiple reproductive experiences appeared to maintain monoamine concentrations in the OB that ordinarily decrease with age. To be able to make this assertion, however, concentration of monoamines in the OB of middle-aged females with and without reproductive experience should be compared with young (3-6 month old) females with and without reproductive experience.

A functional reason for elevated monoamine levels in MP females' OB could be an enhanced need for the new mother to identify her offspring, particularly through her sense of smell (in Shingo et al., 2003). The olfactory bulb is critical for recognition of offspring (Fleming & Rosenblatt, 1974; Brennan & Keverne, 1997). In the presence of prolactin significant increases in neurogenesis were observed in the OB of pregnant females (Shingo et al., 2003). During adulthood, exposure to an odor-enriched environment resulted in significantly greater neurogenesis in the OB of male rats and improved olfactory memory (Rochefort, Gheusi, Vincent & Lledo, 2002). Pup exposure after parturition provides the new dam with an odor-rich environment, which could result in enhanced neurogenesis and sense of smell.

Offspring recognition seems to be particularly controlled by the NE system in the OB. In sheep, lesion of the OB, or infusion of propranolol to the OB, prevented olfactory recognition of offspring (Pissonier et al., 1985; Levy et al., 1990). In mice, lesions to the NE projections in the OB prior to parturition resulted in increased cannibalism of offspring (Dickinson & Keverne, 1988). Similarly, depletion of NE in mothers with prior exposure to their own pups significantly impaired olfactory recognition of the same pups

(Calamandrei, Wilkinson & Keverne, 1992). Thus, NE seems very necessary for the mother to recognize the specific olfactory cues given by her own offspring. The measured elevations in monoamines and metabolites in the OB of parous females, particularly the increase in NE and MHPG, could combine with neurogenesis to significantly enhance the new mother's sense of smell.

Reproductive experience and BDNF concentration in hippocampal and septal subregions

BDNF protein was 97% higher in the CA1 region of MP females as compared to NP females. No significant differences in BDNF protein level were found in CA3 or DG. Expression of BDNF in CA1 is typically much lower than in CA3 or DG (Conner et al., 1997; Yan, Rosenfeld, Matheson, Hawkins, Lopez, Bennett, & Welcher, 1997). Here, MP females demonstrated BDNF protein in CA1 equivalent to that found in CA3 or DG, indicating significant effects of reproductive experience on BDNF expression in CA1 hippocampus. Elevated BDNF protein levels in CA1 may have aided in enhanced spatial memory in MP as compared to NP females. Lesions to the dorsal hippocampus (containing CA1 and septal regions) resulted in impairments in performance on three different versions of the Morris water maze (Clark, Broadbent & Squire, 2005). Additionally, the observed impairments in spatial memory performance due to lesion of dorsal hippocampus were as robust as the observed impairments due to lesion of the entire hippocampus (Clark, Broadbent & Squire, 2005). It thus appears as if the CA1 is particularly important for performance on spatial memory tasks. Because BDNF protein levels were significantly higher in CA1 of MP females as compared to NP females, it is

likely that BDNF contributes to the enhanced spatial memory performance observed in the OP task.

Lack of differences in BDNF protein in CA3 and DG of MP and NP females may have been due to the method utilized. In the hippocampus, BDNF is mainly localized to the CA3 and dentate gyrus (DG) regions (Cotman & Berchtold, 2002; Bora et al., 2006; Franklin & Perrot-Sinal, 2006), particularly the granule cell layer of the dentate gyrus, and the mossy fiber terminals of the CA3 (Conner et al., 1997; Smith et al., 1997; Danzer & McNamara, 2004). Based upon location of BDNF immunoreactivity, BDNF protein appears to be synthesized in the granule layer of the DG and transported in anterograde direction to the CA3 pyramidal neurons (Smith et al., 1997). Thus, by taking separate punches from the CA3 and DG, these axons could have been cut, resulting in decreased BDNF presence in the assay. To correct for this, tissue sections containing the entire CA3 and DG should be analyzed for BDNF in NP and MP females.

The medial septum was also examined for differences in BDNF protein. Unlike the previous experiment, in which the entire septum was analyzed for BDNF protein, no significant differences were observed in BDNF levels in the medial septum of NP and MP females. Both medial and lateral septum play a role in BDNF expression in the hippocampus. BDNF immunoreactivity has been found localized to the lateral septum of intact females, while the medial septum appeared devoid of BDNF protein in cell bodies or terminals (Yan et al., 1997; Bora et al., 2006). Increased neuronal firing has been demonstrated in the lateral septum in relation to direction of movement and location within a spatial task (Zhou, Tamura, Kuriwaki & Ono, 1999), indicating that the lateral septum is involved in performance on spatial tasks. However, previous studies have also

indicated that the medial septum is necessary for BDNF protein expression in the hippocampus (Lindefors et al., 1992; Berchtold, Kesslak & Cotman, 2002). In its entirety, the septum is an important component in BDNF expression in the hippocampus (Lindefors et al., 1992); what remains unclear is which portion of the septum is most critical. As MP females had significantly greater BDNF protein expression in whole septum, but not medial septum, it is possible that BDNF levels could be significantly higher in the lateral septum of MP as compared to NP females. To determine this, future studies should make a systematic examination of all areas within the septum of NP and MP females.

In summary, MP females had significantly higher levels of BDNF protein in the CA1 hippocampus and septum as compared to NP females. As both the lateral septum and CA1 are indicated in spatial memory performance (Zhou et al., 1999 and Clark, Broadbent & Squire, 2005 respectively), it therefore seems likely that elevated BDNF protein in these two regions contribute to enhanced memory performance in MP females.

Both of the above experiments investigated the neural mechanisms underlying memory performance in middle-aged MP females as compared to age-matched NP females. Combined, the two studies indicate that multiple reproductive experiences enhanced/maintained dopamine, norepinephrine and serotonin levels in the olfactory bulb, most likely due to the mother's need to recognize her offspring by scent. Reproductive experience did not seem to alter the same monoamine systems in the hippocampus, and therefore were most likely not involved in memory performance. In contrast, multiple reproductive experiences appeared to increase BDNF protein in both CA1 and the septum (most likely in the lateral septum), both regions indicated in spatial

memory performance. Parity therefore could enhance spatial memory by acting on expression of BDNF.

6. General Discussion

The current study indicated that pregnancy and reproductive experience enhanced performance on two types of memory tasks: object recognition and object placement. Early (days 7-8 of pregnancy) and late (days 16-17) pregnant females significantly out-performed nulliparous females on the object placement task when performance was averaged over two different inter-trial delays: 2 hour and 4 hour. Thus, both stages of pregnancy enhanced performance on a spatial memory task as compared to NP females, corroborating previous results in which enhanced spatial memory was observed after the first and second trimesters (Galea et al., 2000).

In contrast, pregnancy did not appear to significantly decrease anxiety (as measured on the EPM), particularly in late pregnant females. While there was a non-significant trend towards early pregnant females spending more time in the open arm of the plus maze than nulliparous females, this study did not find any overall significant differences in anxiety between EP and LP females, or between pregnant and non-pregnant females. These findings did not support previous studies indicating that pregnancy experience decreased anxiety (Wartella et al., 2003; Lonstein, 2005), although these studies did not actually examine anxiety while the female was pregnant. Thus, it appears as if anxiolytic effects of reproduction on the mother are not demonstrable until after parturition.

A similar pattern of results was found when comparing spatial memory and anxiety between middle-aged multiparous and nulliparous females. Multiparous females outperformed nulliparous females on both object recognition and object placement tasks, indicating that, as in previous studies, repeated reproductive experience enhanced

memory abilities in female rats (Kinsley et al., 1999; Gatewood et al., 2005). In contrast, no significant differences were observed in performance on the EPM, or in the anxiety-related behaviors examined on the open field task, indicating that multiparty did not significantly influence anxiety or overall exploratory behavior. Thus, it appears that reproductive experience exerts long-lasting, permanent changes in spatial memory ability.

Possible neural mechanisms responsible for enhanced memory in pregnant and parous females were examined. In young pregnant females, as compared to NP females, significant alterations were seen in DA, NE and 5HT systems in the CA1 and CA3 regions of the hippocampus. It seems likely that increased monoamine expression in the hippocampus of pregnant females, and increased activity as measured through turnover ratios, has a role to play in the observed memory differences between pregnant and non-pregnant females. In contrast, middle aged multiparous females did not significantly differ from nulliparous females in the dopamine, norepinephrine and serotonin systems in CA1 or CA3 regions of the hippocampus. Thus, in older females regardless of reproductive experience, differences in memory performance did not seem to rely upon concentrations of DA, NE, 5HT or metabolites.

One implication of the above results is that elevations in specific monoamines occurred during pregnancy but do not remain elevated long-term. Indeed, many studies have demonstrated that monoamine systems, particularly DA and NE, are altered during pregnancy. Additionally, as male and female rats age the levels of DA, NE and 5HT decrease throughout the brain as compared to younger females (Luine, Bowling & Hearns, 1990; Tanila et al., 1994; Lee et al., 2001). This study did not compare

monoamine systems between young (3-4 month) and middle aged (13-14 month) females, but alterations present during pregnancy and the lack of changes in middle aged multiparous females indicated that young pregnant females should have significantly higher levels of the above monoamines than their older counterparts with reproductive experience. Future studies should examine these possible differences, in order to determine exactly how the DA, NE and 5HT systems are influenced by reproductive experience, and what impact that may have on memory.

The current study indicated that a different neural mechanism played a role in memory enhancement in middle-aged MP females: BDNF. Significantly higher levels of BDNF protein were found in the CA1 hippocampus and septum of multiparous females as compared to nulliparous females. As both of these regions are indicated in spatial memory performance (Zhou et al., 1999; Clark, Broadbent & Squire, 2005), it appears as if BDNF could be responsible for the enhanced memory abilities demonstrated by multiparous females. Exactly when levels of BDNF protein are elevated due to reproductive experience remains unknown, as BDNF levels were not measured in young pregnant females. A future study could examine BDNF levels in pregnant females, young females with one, two, or three litters, and older multiparous females to determine when reproductive experience elevates BDNF.

BDNF plays a prominent role in neuroprotection and synaptic plasticity. BDNF promotes neuronal survival throughout adulthood (Lindsay, 1996) and aids in synaptic plasticity, allowing an animal to learn about the environment (Allen & Dawbarn, 2006). BDNF enhances excitatory transmission in hippocampal neurons (Lessmann, Gottmann & Heumann, 1994), and plays a role in hippocampally-dependent cognitive functions,

such as spatial memory. Significant deficits in spatial learning and memory were observed on the radial arm maze with disruption of BDNF expression through antisense treatment (Mizuno, Yamada, Olariu, Nawa & Nabeshima, 2000). Similarly, deficits in learning and performance on the Morris water maze were observed in BDNF-knockouts (Linnarsson, Bjorklund & Emfors, 1997), and with anti-BDNF antibody administration (Mu, Li, Yao & Zhou, 1999). These studies eliminated BDNF from the entire brain; infusion of anti-BDNF into CA1 hippocampus only has also been shown to reduce memory ability on a fear-motivated learning task (Alonso et al., 2002a; Alonso et al., 2005). Thus, increased BDNF expression in the CA1 hippocampus of MP females could very easily explain the enhanced spatial memory ability (on object placement task) as compared to NP females. As BDNF has been shown to contribute to non-spatial memory tasks as well (Alonso et al., 2002a), elevated BDNF in CA1 and septum could have also contributed to enhanced performance on the non-spatial task (object recognition) by MP as compared to NP females.

Why BDNF should be elevated in MP as compared to NP females is unclear. One possibility is the physical aspect of rearing pups. New dams display a variety of physical behaviors, such as nest building, crouching over pups, and pup retrieval, that were not displayed before (Rosenblatt, Mayer & Giordano, 1988). Exercise in female rats (several days of voluntary wheel-running) significantly increased levels of BDNF mRNA and protein in the hippocampus, particularly in the CA3 and DG (Berchtold, Kesslak, Pike, Adlard & Cotman, 2001; Berchtold, Kesslak & Cotman, 2002; Cotman & Berchtold, 2002). Recent evidence suggests that exercise and the subsequent increase in BDNF protein act together to enhance spatial memory, as measured on the Morris water maze

(Vaynman, Ying & Gomez-Pinilla, 2004). The increase in physical activity by a new dam may increase BDNF levels in the hippocampus to the point that it is beneficial to her spatial memory. Multiple reproductive experiences may have resulted in permanent alteration in BDNF protein in the CA1 hippocampus. Little work has been done examining long-term effects of reproductive experience on either brain or behaviors. This study is one of the first to indicate a large enhancing effect of multiple reproductive experiences later in the females' lives on both behavior and neural mechanisms.

In sum, it appears as if pregnancy and the various aspects of reproductive experience – birth, lactation, pup exposure, maternal behaviors – act together to enhance females' memory abilities, which may aid in caring for her offspring (i.e. to better navigate through the environment in search of food; Lambert et al., 2005). A variety of neural systems have been shown to be affected by pregnancy and/or reproductive experience: increases in dendritic spines in CA1 hippocampus during pregnancy (Kinsley et al., 2005); enhances in LTP and MAP-kinase pathway in the presence of oxytocin (Tomizawa et al., 2003), which is increased during lactation; enhanced neurogenesis in the olfactory bulb during pregnancy in response to increases in the hormone prolactin (Shingo et al., 2003), possibly to aid in enhanced recognition of offspring; alterations in monoamine systems in the mPOA of pregnant females (Lonstein, 2005). The current study indicated that monoamine systems in the CA1 and CA3 hippocampus, as well as BDNF protein in CA1 hippocampus and the septum, are two further neural mechanisms that are affected by pregnancy and reproductive experience. All of the systems described above most likely work together to give the mother the resources she needs to care for her offspring.

7. References

Aguilar-Valles, A., Sanchez, E., de Gortari, P., Balderas, I., Ramirez-Amaya, V., Bermudez-Rattoni, F., & Joseph-Bravo, P. (2005). Analysis of the stress response in rats trained in the water maze: differential expression of corticotrophin-releasing hormone, CRH-R1, glucocorticoid receptors and brain-derived neurotrophic factor in limbic regions. *Neuroendocrinology, 82,* 306-319.

Albert, D.J., Jonik, R.H. & Walsh, M.L. (1990). Aggression of ovariectomized female rats with testosterone implants: Competitive experience activates aggression toward unfamiliar females. *Phsyiology & Behavior, 47,* 699-703.

Albert, D.J., Jonik, R.H. & Walsh, M.L. (1992). Hormone-dependent aggression in male and female rats: Experiential, hormonal and neural foundations. *Neuroscience and Biobehavioral Reviews, 16,* 177-192.

Albert, D.J., Jonik, R.H. & Walsh, M.L. (1992). Interaction of estradiol, testosterone and progesterone in the modulation of hormone-dependent aggression in the female rat. *Physiology & Behavior, 52,* 773-779.

Aleman, A., Bronk, E., Kessels, R.P., Koppeschaar, H.P., & van Honk, J. (2004). A single administration of testosterone improves visuospatial ability in young women. *Psychoneuroendocrinology, 29,* 612-617.

Allen, S.J. & Dawbarn, D. (2006). Clinical relevance of the neurotrophins and their receptors. *Clinical Science, 110,* 175-191.

Alonso, M., Bekinschtein, P., Cammarota, M., Vianna, M.R., Izquierdo, I., & Medina, J.H. (2005). Endogenous BDNF is required for long-term memory formation in the rat parietal cortex. *Learning & Memory, 12,*504-510.

Alonso, M., Vianna, M.R., Depino, A.M., Mello e Souza, T., Pereira, P., Szapiro, G. et al. (2002). BDNF-triggered events in the rat hippocampus are required for both short- and long-term memory formation. *Hippocampus, 12,* 551-560.

Altemus, M., Fong, J., Yang, R., Damast, S., Luine, V. & Ferguson, D. (2004). Changes in cerebrospinal fluid neurochemistry during pregnancy. *Biological Psychiatry, 56,* 386-392.

Anderson, G.M., Grattan, D.R., van den Ancker, W., & Bridges, R.S. (2006). Reproductive experience increases prolactin responsiveness in the medial preoptic area and arcuate nucleus of female rats. *Endocrinology, e-publication ahead of print.*

Archer, J. Testosterone and human aggression: an evaluation of the challenge hypothesis. *Neuroscience and Biobehavioral Review, 30,* 319-345.

Berchtold, N.C., Kesslak, J.P. & Cotman, C.W. (2002). Hippocampal brain-derived neurotrophic factor gene regulation by exercise and the medial septum. *Journal of Neuroscience Research, 68,* 511-521.

Berchtold, N.C., Kesslak, J.P., Pike, C.J., Adlard, P.A. & Cotman, C.W. (2001). Estrogen and exercise interact to regulate brain-derived neurotrophic factor mRNA and protein expression in the hippocampus. *European Journal of Neuroscience, 14,* 1992-2002.

Berry, B., McMahan, R., & Gallagher, M. (1997). Spatial learning and memory at defined points of the estrous cycle: Effects on performance of a hippocampal-dependent task. *Behavioral Neuroscience, 111,* 267-274.

Bevilaqua, L., Ardenghi, P., Schroder, N., Bromberg, E., Quevedo, J., Schmitz, P.K. et al. (1997). Agents that affect cAMP levels of protein kinase A activity modulate memory consolidation when injected into rat hippocampus but not amygdala. *Brazilian Journal of Medical and Biological Research, 30,* 967-970.

Bi, R., Foy, M.R., Vouimba, R.M., Thompson, R.F. & Baudry, M. (2001). Cyclic changes in estradiol regulate synaptic plasticity through the MAP kinase pathway. *Proceedings of the National Academy of Sciences of the United States of America, 98,* 13391-13395.

Bielsky, I.F. & Young, L.J. (2004). Oxyotocin, vasopressin and social recognition in mammals. *Peptides, 25,* 1565-1574.

Bimonte-Nelson, H.A., Nelson, M.E., & Granholm, A.E. (2004). Progesterone counteracts estrogen-induced increases in neurotrophins in the aged female rat brain. *Neuroreport, 15,* 2659-2663.

Bisagno, V., Ferguson, D., & Luine, V.N. (2002). Short toxic methamphetamine schedule impairs object recognition task in male rats. *Brain Research, 940,* 95-101.

Blurton-Jones, M., Kuan, P.N., & Tuszynski, M.H. (2004). Anatomical evidence for transsynaptic influences of estrogen on brain-derived neurotrophic factor expression. *Journal of Comparative Neurology, 468,* 347-360.

Bodenstiner, K.J., Cain, P., Ray, A.S., & Hamula, L.A. (2006). Effects of pregnancy on spatial cognition in female hooded Long-Evans rats. *Hormones and Behavior, 49,* 303-314.

Bora, S.H., Liu, Z., Kecojevic, A., Merchenthaler, I., & Koliatsos, V.E. (2005). Direct, complex effects of estrogens on basal forebrain cholinergic neurons. *Experimental Neurology, 194,* 506-522.

Bowman, R.E., Zrull, M.C. & Luine, V.N. (2002). Effects of chronic restraint stress and estradiol on open field activity, spatial memory, and monoaminergic neurotransmitters in ovariectomized rats. *Brain Research, 904,* 279-289.

Brennan, P.A. & Keverne, E.B. (1997). Neural mechanisms of mammalian olfactory learning. *Progress in Neurobiology, 51,* 457-481.

Bridges, R.S. (1975). Long-term effects of pregnancy and parturition upon maternal responsiveness in the rat. *Physiology & Behavior, 14,* 245-249.

Bridges, R.S. & Freemark, M.S. (1995). Human placental lactogen infusions into the medial preoptic area stimulate maternal behavior in steroid-primed, nulliparous female mice. *Hormones & Behavior, 29,* 216-226.

Bridges, R.S. & Grattan, D.R. (2003). Prolactin-induced neurogenesis in the maternal brain. *TRENDS in Endocrinology and Metabolism, 14,* 199-201.

Bridges, R.S., Numan, M., Ronsheim, P.M., Mann, P.E. & Walker, A.M. (1990). Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous females. *Proceedings of the National Academy of Science, 87,* 8003-8007.

Bridges, R.S., Rigero, B.A., Byrnes, E.M., Yang, L., & Walker, A.M. (2001). Central infusions of the recombinant human prolactin receptor antagonist, S179D-PRL, delay the onset of maternal behavior in steroid-primed, nulliparous female rats. *Endocrinology, 142,* 730-739.

Broadbent, N.J., Squire, L.R., & Clark, R.E. (2004). Spatial memory, recognition memory, and the hippocampus. *Proceedings of the National Academy of Sciences, 101,* 14515-14520.

Byrnes, E.M. & Bridges, R.S. (2006). Reproductive experience alters anxiety-like behavior in the female rat. *Hormones and Behavior, 50,* 70-76.

Byrnes, E.M., Rigero, B.A. & Bridges, R.S. (2002). Dopoamine antagonists during parturition disrupt maternal care and the retention of maternal behavior in rats. *Pharmacology, Biochemistry and Behavior, 73,* 869-875.

Calamandrei, G., Wilkinson, L.S. & Keverne, E.B. (1992). Olfactory recognition of infants in laboratory mice: role of noradrenergic mechanisms. *Physiology & Behavior, 52,* 901-907.

Clark, R.E., Broadbent, N.J. & Squire, L.R. (2005). Hippocampus and remote spatial memory in rats. *Hippocampus, 15,* 260-272.

Chapman, P.F., White, G.L., Jones, M.W., Cooper-Blacketer, D., Marshall, V.J., Irizarry, M., et al. (1999). Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nature Neuroscience, 2,* 271-276.

Chesler, E.J. & Juraska, J.M. (2000). Acute administration of estrogen and progesterone impairs the acquisition of the spatial Morris water maze in ovariectomized rats. *Hormones and Behavior, 38,* 234-242.

Conner, J.M., Lauterborn, J.C., Yan, Q., Gall, C.M. & Varon, S. (1997). Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: Evidence for anterograde axonal transport. *The Journal of Neuroscience, 17,* 2295-2313.

Cotman, C.W. & Berchtold, N.C. (2002). Exercise: a behavioral intervention to enhance brain health and plasticity. *TRENDS in Neuroscience, 25,* 295-301.

Crowley, W.R., O'Donohue, T.L. & Jacobowitz, D.M. (1978). Changes in catecholamine content in discrete brain nuclei during the estrous cycle of the rat. *Brain Research, 147,* 315-326.

da Costa, A.P.C., Wood, S., Ingram, C.D., & Lightman, S.L. (1996). Region-specific reduction in stress-induced c-fos mRNA expression during pregnancy and lactation. *Brain Research, 742,* 177-184.

Daniel, J.M., Fader, A.J., Spencer, A.L., & Dohanich, G.P. (1997). Estrogen enhances performance of female rats during acquisition of a radial arm maze. *Hormones and Behavior, 32,* 217-225.

Danzer, S.C. & McNamara, J.O. (2004). Localization of brain-derived neurotrophic factor to distinct terminals of mossy fiber axons implies regulation of both excitation and feedforward inhibition of CA3 pyramidal cells. *The Journal of Neuroscience, 24,* 11346-11355.

de Silva, M.A. & Arruda, M.A. (2006). Mechanization of the Bradford reaction for the spectrophotometric determination of total proteins. *Analytical Biochemistry, 351,* 155-157.

Desan, P.H., Woodmansee, W.W., Ryan, S.R., Smock, T.K. & Maier, S.F. (1988). Monoamine neurotransmitters and metabolites during the estrous cycle, pregnancy, and the postpartum period. *Pharmacology, Biochemistry & Behavior, 30,* 563-568.

Dloniak, S.M., French, J.A. & Holekamp, K.E. (2006). Rank-related maternal effects of androgens on behaviour in wild spotted hyaenas. *Nature, 440,* 1190-1193.

Dluzen, D.E. (1996). Age-related changes in monoamines within the olfactory bulbs of the Fisher 344 male rat. *Mechanisms of ageing and development, 91,* 37-45.

Dickinson, C. & Keverne, E.B. (1988). Importance of noradrenergic mechanisms in the olfactory bulbs for the maternal behaviour of mice. *Phsysiology & Behavior, 43,* 313-316.

Douglas, A.J., Meddle, S.L., Toschi, N., Bosch, O.J. & Neumann, I.D. (2005). Reduced activity of the noradrenergic system in the paraventricular nucleus at the end of pregnancy: Implications for stress hyporesponsiveness. *Journal of Neuroendocrinology, 17,* 40-48.

Drapeau, E., Mayo, W., Aurousseau, C., Le Moal, M., Piazza, P.V., & Abrous, D.N. (2003). Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. *Proceedings of the National Academy of Sciences, USA, 100,* 14385-14390.

Felicio, L.F., Florio, J.C., Sider, L.H., Cruz-Casallas, P.E., & Bridges, R.S. (1996). Reproductive experience increases striatal and hypothalamic dopamine levels in pregnant rats. *Brain Research Bulletin, 40,* 253-256.

Ferguson, J.N., Young, L.J., Hearn, E.F., Matzuk, M.M., Insel, T.R., Winslow, J.T. (2000). Social amnesia in mice lacking the oxytocin gene. *Nature Genetics, 25,* 284-288.

Fleming, A.S., Kuchera, C., & Lee, A. (1994). Olfactory-based social learning varies as function of parity in female rats. *Psychobiology, 22,* 37-43.

Fleming, A.S., O'Day, D.H., & Kraemer, G.W. (1999). Neurobiology of mother-infant interactions: experience and central nervous system plasticity across development and generations. *Neuroscience & Biobehavioral Reviews, 23,* 273-285.

Fleming, A.S. & Rosenblatt, J.S. (1974). Olfactory regulation of maternal behavior in rats: Effects of olfactory bulb removal in experienced lactating and cycling females. *Journal of Comparative Physiology & Psychology, 86,* 221-232.

Franklin, T.B. & Perrot-Sinal, T.S. (2006). Sex and ovarian steroids modulate brain-derived neurotrophic factor (BDNF) protein levels in rat hippocampus under stressful and non-stressful conditions. *Psychoneuroendocrinology, 31,* 38-48.

Frick, K.M., Fernandez, S.M., Bennett, J.C., Prange-Kiel, J., MacLusky, N.J. & Leranth, C. (2004). Behavioral training interferes with the ability of gonadal hormones to increase CA1 spine synapse density in ovariectomized female rats. *European Journal of Neuroscience, 19,* 3026-3032.

Frick, K.M., Fernandez, S.M., & Bulinski, S.C. (2002). Estrogen replacement improves spatial reference memory and increases hippocampal synaptophysin in aged female mice. *Neuroscience, 115,* 547-558.

Galea, L.A.M., Ormerod, B.K., Sampath, S., Kostaras, X., Wilkie, D.M., & Phelps, M.T. (2000). Spatial working memory and hippocampal size across pregnancy in rats. *Hormones and Behavior, 37,* 86-95.

Gatewood, J.D., Morgan, M.D., Eaton, M., McNamara, I.M., Stevens, L.F., Macbeth, A.H. et al. (2005). Motherhood mitigates aging-related decrements in learning and memory and positively affects brain aging in the rat. *Brain Research Bulletin, 66,* 91-98.

Gibbs, R.B. (1998). Levels of trkA and BDNF mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. *Brain Research, 787,* 259-268.

Gibbs, R.B. (1999). Treatment with estrogen and progesterone affects relative levels of brain-derived neurotrophic factor mRNA and protein in different regions of the adult rat brain. *Brain Research, 844,* 20-27.

Gibbs, R.B. (2000). Long-term treatment with estrogen and progesterone enhances acquisition of a spatial task by ovariectomized aged rats. *Neurobiology of Aging, 21,* 107-116.

Gibbs, R.B. (2005). Testosterone and estradiol produce different effects on cognitive performance in male rats. *Hormones and Behavior, 48,* 268-277.

Glaser, J., Russell, V.A., & Taljaard, J.J. (1992). Rat brain hypothalamic and hippocampal monoamine and hippocampal beta-adrenergic receptor changes during pregnancy. *Brain Research, 577,* 293-299.

Gould, E., Woolley, C.S., Frankfurt, M., & McEwen, B.S. (1990). Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *Journal of Neuroscience, 10,* 1286-1291.

Guan, X.B. & Dluzen, D.E. (1994). Age related changes of social memory/recognition in male Fischer 344 rats. *Behavioral Brain Research, 61,* 87-90.

Hansen, S., Bergvall, A.H., & Nyiredi, S. (1993). Interaction with pups enhances dopamine release in the ventral striatum of maternal rats: a microdialysis study. *Pharmacology, Biochemistry and Behavior, 45,* 673-676.

Harrison, B.J., Olver, J.S., Norman, T.R., Burrows, G.D., Wesnes, K.A. & Nathan, P.J. (2004). Selective effects of acute serotonin and catecholamine depletion on memory in healthy women. *Journal of Psychophamracology, 18,* 32-40.

Jacobs, L.F., Gaulin, S.J.C., Sherry, D.F., & Hoffman, G.E. (1990). Evolution of spatial cognition: Sex-specific patterns of spatial behavior predict hippocampal size. *Proceedings of the National Academy of Science, 87*, 6349-6352.

Jezierski, M.K. and Sohrabji, F. (2001). Neurotrophin expression in the reproductively senescent forebrain is refractory to estrogen stimulation. *Neurobiology of Aging, 22,* 311-321.

Jones, M.W. & Wilson, M.A. (2005). Theta rhythms coordinate hippocampal-prefrontal interactions in a spatial memory task. *PLoS Biology, 3,* 402-412.

Keer, S.E. & Stern, J.M. (1999). Dopamine receptor blockade in teh nucleus accumbens inhibits maternal retrieval and licking, but enhances nursing behavior in lactating rats. *Physiology & Behavior, 67,* 659-669.

Kemp, A. & Manahan-Vaughan, D. (2004). Hippocampal long-term depression and long-term potentiation encode different aspects of novelty acquisition. *Proceedings of the National Academy of Sciences of the U.S.A., 101,* 8192-8197.

Kendrick, K.M., da Costa, A.P.C., Broad, K.D., Ohkura, S., Guevara, R., Levy, F., et al. (1997). Neural control of maternal behaviors and olfactory recognition of offspring. *Brain Research Bulletin, 44,* 383-395.

Keyser-Marcus, L., Stafisso-Sandoz, G., Gerecke, K., Jasnow, A., Nightingale, L., Lambert, K.G et al. (2001). Alterations of medial preoptic area neurons following pregnancy and pregnancy-like steroidal treatment in the rat. *Brain Research Bulletin, 55,* 737-745.

Kinsley, C.H., Madonia, L., Gifford, G.W., Tureski, K., Griffin, G.R., Lowry, C. et al. (1999). Motherhood improves learning and memory. *Nature, 402,* 137-138.

Kinsley, C.H., Trainer, R., Stafisso-Sandoz, G., Quadros, P., Keyser-Marcus, L., Hearon, C., et al. (2006). Motherhood and the hormones of pregnancy modify concentrations of hippocampal neuronal dendritic spines. *Hormones and Behavior, 49,* 131-142.

Kobori, N., Clifton, G.L. & Dash, P.K. (2006). Enhanced catecholamine synthesis in the prefrontal cortex after traumatic brain injury: implications for prefrontal dysfunction. *Journal of Neurotrauma, 23,* 1094-1102.

Kroner, S., Krimer, L.S., Lewis, D.A. & Barrionuevo, G. (2006). Dopamine increases inhibition in the monkey dorsolateral prefrontal cortex through cell type-specific modulation of interneurons. *Cerebral Cortex, epublication*

Lambert, K.G., Berry, A.E., Griffins, G., Amory-Meyers, E., Madonia-Lomas, L., Love, G., & Kinsley, C.H. (2005). Pup exposure differentially enhances foraging ability in primiparous and nulliparous rats. *Physiology & Behavior, 84,* 799-806.

Langmore, N.E., Cockrem, J.F. & Candy, E.J. (2002). Competition for male reproductive investment elevates testosterone levels in female dunnocks, Prunella modularis. *Proceedings of The Royal Society of Biological Sciences, 269,* 2473-2478.

Lapiz, M.D.S. & Morilak, D.A. (2006). Noradrenergic modulation of cognitive function in rat medial prefrontal cortex as measured by attentional set shiftin capability. *Neuroscience, 137,* 1039-1049.

Lee, J.J., Chang, C.K., Liu, I.M., Chi, T.C., Yu, H.J. & Cheng, J.T. (2001). Changes in endogenous monoamines in aged rats. *Clinical and Experimental Pharmacology and Physiology, 28,* 285-289.

Lemaire, V., Billard, J.-M., Dutar, P., George, O., Piazza, P.V., Epelbaum, J., et al. (2006). Motherhood-induced memory improvement persists across lifespan in rats but is abolished by a gestational stress. *European Journal of Neuroscience, 23,* 3368-3374.

Lemon, N. & Manahan-Vaughan, D. (2006). Dopamine D1/D5 receptors gate the acquisition of novel information through hippocampal long-term potentiation and long-term depression. *Journal of Neuroscience, 26,* 7723-7729.

Leranth, C., Hajszan, T., & MacLusky, N.J. (2004). Androgens increase spine synapse density in the CA1 hippocampal subfield of ovariectomized female rats. *Journal of Neuroscience, 24,* 495-499.

Levy, F., Gervais, R., Kindermann, U., Orgeur, P. & Piketty, V. (1990). Importance of beta-noradrenergic receptors in the olfactory bulb of sheep for recognition of lambs. *Behavioral Neuroscience, 104,* 464-469.

Lindefors, N., Ernfors, P., Falkenberg, T. & Persson, H. (1992). Septal cholinergic afferents regulate expresion of brain-derived neurotrophic factor and beta-nerve growth factor mRNA in rat hippocampus. *Experimental Brain Research, 88,* 78-90.

Lindsay, R.M. (1996). Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons: an overview. *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences, 351,* 376-373.

Linnarsson, S., Bjorklund, A. & Emfors, P. (1997). Learning deficit in BDNF mutant mice. *European Journal of Neuroscience, 9,* 2581-2587.

Lonstein, J.S. (2005). Reduced anxiety in postpartum rats requires recent physical interactions with pups, but is independent of suckling and peripheral sources of hormones. *Hormones and Behavior, 47,* 241-255.

Lonstein, J.S., Dominguez, J.M., Putnam, S.K., De Vries, G.J., & Hull, E.M. (2003). Intracellular preoptic and striatal monoamines in pregnant and lactating rats: possible role in maternal behavior. *Brain Research, 970,* 149-158.

Lonstein, J.S. & Gammie, S.C. (2002). Sensory, hormonal, and neural control of maternal aggression in laboratory rodents. *Neuroscience & Biobehavioral Reviews, 26,* 869-888.

Love, G., Torrey, N., McNamara, I., Morgan, M., Banks, M., Hester, N. et al (2005). Maternal experience produces long-lasting behavioral modifications in the rat. *Behavioral Neuroscience, 119,* 1084-1096.

Luine, V.N. (1993). Serotonin, catecholamines and metabolites in discrete brain areas in relation to lordotic responding on proestrus. *Neuroendocrinology, 57,* 946-954.

Luine, V. (2002). Sex differences in chronic stress effects on memory in rats. *Stress, 5,* 205-216.

Luine, V., Bowling, D., & Hearns, M. (1990). Spatial memory deficits in aged rats: contributions of monoaminergic systems. *Brain Research, 537,* 271-278.

Luine, V.N., Jacome, L.F., & MacLusky, N.J. (2003). Rapid enhancement of visual and place memory by estrogens in rats. *Endocrinology, 14,* 2836-2844.

Luine, V.N., Richards, S.T., Wu, V.Y., & Beck, K.D. (1998). Estradiol enhances learning and memory in a spatial memory task and effects levels of monoaminergic neurotransmitters. *Hormones & Behavior, 34,* 149-162.

Luine, V.N., Villegas, M., Martinez, C., McEwen, B.S. (1994). Repeated stress causes reversible impairments of spatial memory performance. *Brain Research, 639,* 167-170.

MacLusky, N.J., Luine, V.N. Hajszan, T., & Leranth, C. (2005). The 17α and 17β isomers of estradiol both induce rapid spine synapse formation in the CA1 hippocampal subfield of ovariectomized female rats. *Endocrinology, 146,* 287-293.

Manthey, D. & Behl, C. (2006). From structural biochemistry to expression profiling: neuroprotective activities of estrogen. *Neuroscience, 138,* 845-850.

Mann, P.E. & Bridges, R.S. (2002). Prolactin receptor gene expression in the forebrain of pregnant and lactating rats. *Molecular Brain Research, 105,* 136-145.

Marcondes, F.K., Miguel, K.J., Melo, L.L., & Spadari-Bratfisch, R.C. (2001). Estrous cycle influences the response of female rats in the elevated plus maze test. *Physiology & Behavior, 74,* 435-440.

Markham, J.A., Pych, J.C., & Juraska, J.M. (2002). Ovarian hormone replacement to aged ovariectomized female rats benefits acquisition of the Morris water maze. *Hormones & Behavior, 42,* 284-293.

McEwen, B.S., & Woolley, C.S. (1994). Estradiol and progesterone regulate neuronal structure and synaptic connectivity in adult as well as developing brain. *Experimental Gerontology, 29,* 431-436.

Miller, S.M. & Lonstein, J.S. (2005). Dopamine d1 and d2 receptor antagonism in the preoptic area produces different effects on maternal behavior in lactating rats. *Behavioral Neuroscience, 119,* 1072-1083.

Miquez, J.M., Aldegunde, M., Paz-Valinas, L., Recio, J., & Sanchez-Barcelo, E. (1999). Selective changes in the contents of noradrenaline, dopamine and serotonin in rat brain areas during aging. *Journal of neural transmission, 106,* 1089-1098.

Mizuno, M., Yamada, K., Olariu, A., Nawa, H. & Nabeshima, T. (2000). Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in radial arm maze test in rats. *Journal of Neuroscience, 20,* 7116-7121.

Mohan, G., Sarmiento, Y., Lachman, V., MacLusky, N. & Luine, V. (2004). DHEA enhances spatial but not visual recognition memory in female rats. *Society for Neuroscience annual meeting, published abstract*

Monks, D.A., Lonstein, J.S. & Breedlove, S.M. (2003). Got milk? Oxytocin triggers hippocampal plasticity. *Nature Neuroscience, 6,* 327-328.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P., & O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. *Nature, 29*, 681-683.

Mu, J.S., Li, W.P., Yao, Z.B. & Zhou, X.F. (1999). Deprivation of endogenous brain-derived neurotrophic factor results in impairment of spatial learning and memory in adult rats. *Brain Research, 835,* 259-265.

Numan, M., Numan, M.H., Pliakou, N., Stolzenberg, D.S., Mullins, O.J., Murphy, J.M. & Smith, C.D. (2005). The effects of D1 or D2 dopamine receptor antagonism in the medial preoptic area, ventral pallidum, or nucleus accumbens on the maternal retrieval response and other aspects of maternal behavior in rats. *Behavioral Neuroscience, 119,* 1588-1604.

Pacak, K., Palkovits, M., Kvetnansky, R., Kopin, I.J. & Goldstein, D.S. (1993). Stress—induced norepinephrine release in the paraventricular nucleus of rats with brainstem hemisections: a microdialysis study. *Neuroendocrinology, 58,* 196-201.

Packard, M.G. (1998). Posttraining estrogen and memory modulation. *Hormones and Behavior, 34,* 126-139.

Pawluski, J.L., Walker, S.K., & Galea, L.A.M. (2006). Reproductive experience differentially affects spatial reference and working memory performance in the mother. *Hormones and Behavior, 49,* 143-149.

Pederson, C.A. & Boccia, M.L. (2003). Oxytocin antagonism alters rat dams' oral grooming and upright posturing over pups. *Phsyiology & Behavior, 80,* 233-241.

Pederson, C.A., Vadlamudi, S.V., Boccia, M.L. & Amico, J.A. (2006). Maternal behavior defecits in nulliparous oxytocin knockout mice. *Genes, Brain and Behavior, 5,* 274-281.

Pellow, S., Chopin, P., File, S.E., & Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods, 14,* 149-167.

Pissonnier, D., Thiery, J.C., Fabre-Nys, C., Poindron, P. & Keverne, E.B. (1985). The importance of olfactory bulb noradrenalin for maternal recognition in sheep. *Physiology & Behavior, 35,* 361-363.

Potvin, O., Allen, K., Thibaudeau, G., Dore, F.Y. & Goulet, S. (2006). Performance on spatial workign memory tasks after dorsal or ventral hippocampal lesions and adjacent damage to the subiculum. *Behavioral Neuroscience, 120,* 413-422.

Rasia-Filho, A.A., Fabian, C., Rigoti, K.M., & Achaval, M. (2004). Influence of sex, estrous cycle and motherhood on dendritic spine density in the rat medial amygdala revealed by the Golgi method. *Neuroscience, 126,* 839-847.

Ren-Patterson, R.F., Cochran, L.W., Holmes, A., Lesch, K-P., Lu, B. & Murphy, D.L. (2006). Gender-dependent modulation of brain monoamines and anxiety-like behaviors in mice with genetic serotonin transporter and BDNF deficiencies. *Cellular and Molecular Neurobiology, e-publication.*

Reyes, F.I., Winter, J.S., Faiman, C. & Hobson, W.C. (1975). Serial serum levels of gonadotropins, prolactin and sex steroids in the nonpregnant and pregnant chimpanzee. Endocrinology, 96, 1447-1455.

Rochefort, C., Gheusi, G., Vincent, J.D. & Lledo, P.M. (2002). Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. *Journal of Neuroscience, 22,* 2679-2689.

Rodriguez-Cuenca, S., Gianotti, M., Roca, P., & Proenza, A.M. (2006). Sex steroid receptor expression in different adipose depots is modified during midpregnancy. *Molecular and Cellular Endocrinology, 249,* 58-63.

Roozendaal, B., Okuda, S., Van der Zee, E.A. & McGaugh, J.L. (2006). Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. *Proceedings of the National Academy of Sciences, 103,* 6741-6746.

Rosenblatt, J.S., Mayer, A.D., Giordano, A.L. (1988). Hormonal basis during pregnancy for the onset of maternal behavior in the rat. *Psychoneuroendocrinology, 13*, 29-46.

Scharfman, H.E. & MacLusky, N.J. (2005). Similarities between actions of estrogen and BDNF in the hippocampus: coincidence or clue? *TRENDS in Neuroscience, 28,* 79-85.

Scharfman, H.E., Mercurio, T.C., Goodman, J.H., Wilson, M.A., & MacLusky, N.J. (2003). Hippocampal excitability increases during the estrous cycle in the rat: A potential role for brain-derived neurotrophic factor. *The Journal of Neuroscience, 23,* 11641-11652.

Sheehan, T., Paul, M., Amaral, E., Numan, M.J., & Numan, M. (2001). Evidence that the medial amygdala projects to the anterior/ventromedial hypothalamic nuclei to inhibit maternal behavior in rats. *Neuroscience, 106,* 341-356.

Sherwin, B.B. (2003). Estrogen and cognitive functioning in women. *Endocrine Reviews, 24,* 133-151.

Shingo, T., Gregg, C., Enwere, E., Fujikawa, H., Hawwam, R., Geary, C., Cross, J.C. & Weiss, S. (2003). Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. *Science, 299,* 117-120.

Singh, M., Meyer, E.M. & Simpkins, J.W. (1995). The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. *Endocrinology, 136,* 2320-2324.

Siuciak, J.A., Lewis, D.R., Wiegand, S.J. & Lindsay, R.M. (1997). Antidepressant-like effects of brain-derived neurotrophic factor (BDNF). *Pharmacology, Biochemistry, and Behavior, 56,* 131-137.

Sloan, H.L., Good, M. & Dunnett, S.B. (2006). Double dissociation between hippocampal and prefrontal lesions on an operant delayed matching task and a water maze reference memory task. *Behavior and Brain Research, 171,* 116-126.

Smith, L.C., Raouf, S.A., Bomberger-Brown, M., Wingfield, J.C. & Brown,C.R. (2005). Testosterone and group size in cliff swallows: testing the "challenge hypothesis" in a colonial bird. *Hormones & Behavior, 47,* 76-82.

Smith, M.A., Zhang, L.-X., Lyons, W.E. & Mamounas, L.A. (1997). Anterograde transport of endogenous brain-derived neurotrophic factor in hippocampal mossy fibers. *NeuroReport, 8,* 1829-1834.

Smith, M.S., Freeman, M.E. & Neill, J.D. (1975). The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology, 96,* 219-226.

Smolen, A., Smolen, T.N., & van de Kamp, J.L. (1987). Alterations in brain catecholamines during pregnancy. *Pharmacology & Biochemistry of Behavior, 26,* 613-618.

Sobreviela, T., Pagcatipunan, M., Kroin, J.S. & Mufson, E.J. (1996). Retrograde transport of brain-derived neurotrophic factor (BDNF) following infusion in neo- and limbic cortex in rat: relationship to BDNF mRNA expressing neurons. *Journal of Comparative Neurology, 375,* 417-444.

Sohrabji, F., Miranda, R.C.G., & Toran-Allerand, C.D. (1995). Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. *Proceedings of the National Academy of Science of United States of America, 92,* 11110-11114.

Solum, D.T., & Handa, R.J. (2002). Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus. *The Journal of Neuroscience, 22,* 2650-2659.

Sullivan, R.M., Zyzak, D.R., Skierkowski, P. & Wilson, D.A. (1992). The role of olfactory bulb norepinephrine in early olfactory learning. *Brain Research: Developmental brain research, 18,* 279-282.

Stackman, R.W., Blasberg, M.E., Langan, C.J., & Clark, A.S. (1997). Stability of spatial working memory across the estrous cycle of Long-Evans rats. *Neurobiology of Learning and Memory, 67,* 167-171.

Tanila, H., Taira, T., Piepponen, T.P. & Honkanen, A. (1994). Effect of sex and age on brain monoamines and spatial learning in rats. *Neurobiology of Aging, 15,* 733-741.

Teixeira, J., Martin, D., Prendiville, O. & Glover, V. (2005). The effects of acute relaxation on indices of anxiety during pregnancy. *Journal of Psychosomatic Obstetrics & Gynecology, 26,* 271-276.

Tomizawa, K., Iga, N., Lu, Y.F., Moriwaki, A., Matsushita, M., Li, S.T., et al. (2003). Oxytocin improves long-lasting spatial memory during motherhood through MAP kinase cascade. *Nature Neuroscience, 6,* 384-390.

Viau, V. & Meaney, M.J. (1991). Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology, 129*, 2503-2511.

von Bohlen und Halbach, O., Zacher, C., Gass, P., & Unsicker, K. (2006). Age-related alterations in hippocampal spines and deficiencies in spatial memory in mice. *Journal of Neuroscience Research, 83,* 525-531.

Warren, S.G., Humphreys, A.G., Juraska, J.M. & Greenough, W.T. (1995). LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. *Brain Research, 703,* 26-30.

Warren, S.G. & Juraska, J. (1997). Spatial and nonspatial learning across the rat estrous cycle. *Behavioral Neuroscience, 111,* 259-266.

Warren, S.G. & Juraska, J. (2000). Sex differences and estropausal phase effects on water maze performance in aged rats. *Neurobiology of Learning and Memory, 74,* 229-240.

Wartella, J., Amory, E., Lomas, L.M., Macbeth, A., McNamara, I., Stevens, L., et al. (2003). Single or multiple reproductive experiences attenuate neurobehavioral stress and fear responses in the female rat. *Physiology & Behavior, 6979*, 373-381.

Winslow, J.T. & Insel, T.R. (2004). Neuroendocrine basis of social recognition. *Current Opinion in Neurobiology, 14,* 248-253.

Woolley, C.S., Gould, E., Frankfurt, M., & McEwen, B.S. (1990). Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. *The Journal of Neuroscience, 10,* 4035-4039.

Woolley, C.S., & McEwen, B.S. (1992). Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *The Journal of Neuroscience, 12,* 2549-2554.

Woolley, C.S., & McEwen, B.S. (1993). Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *Journal of Comparative Neurology, 336,* 293-306.

Yan, Q., Rosenfeld, R.D., Matheson, C.R., Hawkins, N., Lopez, O.T., Bennett, L. & Welcher, A. (1997). Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. *Neuroscience, 78* 431-448.

Young, L.J. & Insel, T.R. Hormones and Parental Behavior. In Becker, J.B., Breedlove, S.M., Crews, D. & McCarthy, M.M. (2002). Behavioral Endocrinology, 2nd ed. MIT Press, Cambridge Mass.

Zhou, J., Zhang, H., Cohen, R.S., & Pandey, S.C. (2005). Effects of estrogen treatment on expression of brain-derived neurotrophic factor and cAMP response element-binding protein expression and phosphorylation in rat amygdaloid and hippocampal structures. *Neuroendocrinology, 81,* 294-310.

Zhou, T.L., Tamura, R., Kuriwaki, J., & Ono, T. (1999). Comparison of medial and lateral septal neuron activity during performance of spatial tasks in rats. *Hippocampus, 9,* 220-234.

Zysling, D.A., Greives, T.J., Bruener, C.W., Casto, J.M., Demas, G.E. & Ketterson, E.D. (2006). Behavioral and physiological responses to experimentally elevated testosterone in female dark-eyed juncos (Junco hyemalis carolinensis). *Hormones & Behavior, 50,* 200-207.