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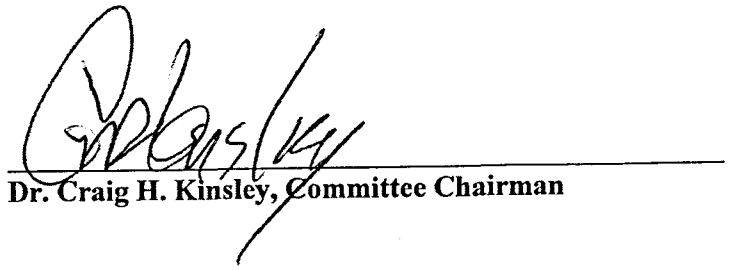
A newly maternal rat goes through many changes when she gives birth, mostly due to prolonged elevation of hormones, particularly estrogen. Estrogen has been shown to increase memory capabilities by increasing synaptic activity in the CA1 hippocampus, but exactly how is still unknown. The current project uses reproductive experience to determine whether high hormone levels experienced during pregnancy and lactation affect spatial memory and synaptophysin, a pre-synaptic protein that controls vesicle exocytosis and thus may be responsible for enhanced synaptic connectivity. We found that reproduction itself does not affect memory of a spatial task, but the presence of pups has a deleterious effect on performance in the Morris water maze. Additionally, reproduction seems to play a role in elevating levels of SYN-IR, but not during the period of lactation. These findings suggest that removal of the lactating mother from her pups can result in decreased spatial abilities and synaptic function.

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



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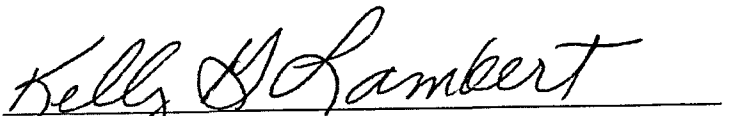

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THE PRESENCE OF PUPS AFTER BIRTH: EFFECTS ON SPATIAL MEMORY AND THE PRE-SYNAPTIC PROTEIN SYNAPTOPHYSIN

By

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The presence of pups after birth: effects on spatial memory and the pre-synaptic protein synaptophysin

For a newly maternal rat, reproductive success is measured in terms of the continuation of her genetic lineage (Russell, Douglas & Ingram, 2001); to do this she must undergo physiological and neurological changes to be able to rapidly change her behavior, exhibiting those that are beneficial to her new offspring, and suppressing those that are not. Previous research in this lab (Kinsley, Madonia, Gifford, Tureski, Griffin, Lowry, et al., 1999; Keyser-Marcus, Stafisso-Sandoz, Gerecke, Aaron, Nightingale, Lambert, et al., 2001) has shown that reproductive experience can enhance one area of behavior: spatial learning and memory. Specifically, female rats that have given birth demonstrate improved spatial memory through performance on an eight-arm radial maze (Kinsley et al., 1999). Why would new mothers need enhanced cognitive and spatial abilities? One answer is that increased cognitive capabilities help to ensure the survival of her offspring by providing her with the means to better remember the locations of food and water (Kinsley et al., 1999; Monks, Lonstein, & Breedlove, 2003; Tomizawa, Iga, Lu, Moriwaki, Matsushita, Li, Miyamoto et al., 2003). This will help to ensure normal development of offspring.

Knowing this, researchers have begun to look at the mechanisms that may be responsible for improved spatial memory in maternal females. During parturition and lactation, the number of synapses on oxytocin neurons is greatly increased (Theodosis & Poulain, 2001). Tomizawa et al. (2003) have found that oxytocin, when injected into virgin mice, improved performance on an eight-arm radial maze. Conversely, infusion of

an oxytocin antagonist into a multiparous animal (one who has born multiple litters) impaired spatial performance (Monks, Lonstein & Breedlove, 2003; Tomizawa et al., 2003). This has led to the development of the following hypothesis: delivery of pups and stimulation from suckling triggers oxytocin release, which then increases phosphorylation of cyclic-AMP response element binding protein (CREB), which in turn increases synaptic plasticity, which leads to longer lasting memory.

This hypothesis puts forth the idea that reproductive hormones may act specifically on the synapse to improve learning and memory. Past exploration of this idea has revealed that reproductive hormones - particularly estrogens - modify the most basic level of neural interactions: that of the synapse itself (Woolley, 1998; McEwen & Alves, 1999; Golding, Staff, & Spruston, 2001; McEwen, Akama, Alves, Brake, Bulloch, Lee, Li, et al., 2001; McEwen, 2002). Synapses are the points of direct communication and contact between neurons, and the sites at which neural messages travel from one neuron to another (Rosenzweig et al., 2002). Therefore, changes in synaptic behavior or function due to reproductive experience could have a direct impact on how neural messages are transmitted, perhaps influencing subsequent learning and memory.

Spines, Synapses, and Estrogen

The number and density of synapses in the brain, specifically in the CA1 region of the hippocampus (a brain region believed to be integral to learning and memory; Carlson, 1998, chap.15) undergoes a natural ebb and flow during the estrous cycle (McEwen & Woolley, 1994; Woolley, Gould, Frankfurt & McEwen, 1990; Woolley &

McEwen, 1992; Woolley, Wenzel, & Schwartzkroin, 1996). This variation occurs cyclically during the estrous cycle; that is, the number of CA1 synapses reaches a maximum on proestrus when levels of estradiol are highest, and a minimum on estrus when steroid hormone levels are at their lowest (Woolley et al., 1990; McEwen & Woolley, 1994).

Many of these synapses are located on dendritic spines, small projections from neuronal dendrites upon which are specialized sites of synaptic contact for a variety of neurons (Woolley & McEwen, 1993). These spines also vary in their number and in their density over the estrous cycle, in the exact same pattern as that described for synapses (Woolley et al., 1990; Woolley & McEwen, 1992; Woolley et al., 1996). These differences in spine density are not due to expansion and shrinking, nor to gain or loss of dendrites, but are due to differences in numbers of spines present on the CA1 dendrites. This fluctuation of both synapses and dendritic spines on neurons in the hippocampus occurs rapidly during the estrous cycle. A decrease in spine density of approximately 30% occurs within twenty-four hours (between proestrus and estrus) (Woolley et al., 1990; McEwen & Woolley, 1994).

Whereas the above results were taken from studies in which the rise and fall of synapses and dendritic spines were tested in a naturally cycling rat, there have been studies that note the effects of estradiol treatment on ovariectomized female rats. Not surprisingly, the results are similar; ovariectomy (a permanent removal of estrogen, similar to the temporary removal seen on the day of estrus) resulted in a dramatic decrease in dendritic spine density in these CA1 pyramidal cells (Gould, Woolley,

Frankfurt, & McEwen, 1990; Woolley et al., 1996; Yankova, Hart & Woolley, 2001). Treatment with estradiol as replacement for the steroid hormones lost due to the ovariectomy prevents the decrease (Gould et al., 1990; Woolley et al., 1996; Yankova et al., 2001).

Estrogen is not the only reproductive hormone that affects dendritic spines and synapses. Application of progesterone following estrogen sharply increases spine density for the first 2-6 hours, further enhancing the effects of estrogen treatment alone (Woolley & McEwen, 1993). However, this results in a much sharper decrease in spines later than is seen with estradiol application alone, and indicates that progesterone may be a particularly important factor in regulating spine and synaptic changes (Woolley & McEwen, 1993). As levels of progesterone fluctuate along with estrogen during pregnancy, progesterone may help to enhance the changes in synaptic morphology expected due to reproductive experience. Oxytocin may also alter synaptic morphology, as it has been shown to enhance spatial memory, possibly through actions on dendrites (Monks, Lonstein & Breedlove, 2003; Tomizawa et al., 2003), as well as alter synaptic transmission in magnocellular neurons of the supraoptic nucleus (Kombian, Hirasawa, Mouginot & Pittman, 2002).

Estradiol's effects are not limited to changes in the number of spines and synapses. Dendritic spines are the postsynaptic sites of excitatory input to many neurons in the mammalian brain (Yankova et al., 2001). Estradiol has the ability to increase the spines' sensitivity to excitatory input. Electrophysiological studies of the hippocampus suggest that neuronal excitability increases with greater amounts of estrogen, whether the

estrogen occurs naturally or is given artificially (Warren, Humphreys, Juraska, & Greenough, 1995). Additionally, Warren et al. found that the duration of excitatory postsynaptic potentials (EPSPs) is extended with increased exposure to estrogen, thus prolonging the postsynaptic neuron's firing capabilities and resulting in greater synaptic transmission (1995).

Estrogen has been shown to change the number and type of synaptic connections made by dendritic spines (Yankova et al., 2001). One of two types of synaptic contacts can be made; either single synapse boutons (SSBs) or multiple synapse boutons (MSBs). Yankova et al. found that estrogen application promotes an increase in MSBs proportional to SSBs, as well as an increase of synapses seen on each MSB (also reported by Woolley, 1996). Each MSB synapsed with at least two different spines; rarely were the two spines on the same neuron. This resulted in greater coupling of single presynaptic inputs to multiple postsynaptic CA1 neurons (Yankova et al., 2001). Estrogen also promotes the formation of new synaptic formations, as animals treated with estrogen were shown to have an average of 25% more synapses on CA1 dendritic spines than untreated animals (Woolley, 1996).

The previous studies provide evidence that estrogen creates the potential for a signal to be transferred to multiple post-synaptic neurons instead of merely one or two. Thus, estrogen is responsible for enhanced communication between neurons, as a single neuron is now linked with many others. Because estrogen has this ability, and due to the abundance of estrogen receptors found in the hippocampus (Kelly & Levin, 2001), researchers believe that estrogen enhances the functioning of cells in the CA1, which

provides direct evidence for a link between estrogen and maintenance of learning and memory (Sherwin, 1996). If this is the case, it seems reasonable that prolonged exposure to reproductive hormones during pregnancy and lactation (see Buckwalter, Buckwalter, Bleustein & Stanczyk, 2001, for a more in-depth discussion) could cause modification of synapses in a manner similar to that found by Yankova et al. (2001) and Woolley (1996). Reproductive hormones may enhance synapse strength in such a manner that spatial memory is increased during lactation. It is also possible that these benefits extend further, to allow the parous female to reap the benefits of reproduction later in life.

Estrogen and Synaptic Proteins

How, then, is estradiol able to affect dendritic spines and synapses to such an extent? Crispino et al. have presented evidence that estradiol coordinates the regulation of genes that code for pre-synaptic proteins that may participate in the synaptic remodeling of the CA1 region (1999). In this study, levels of mRNA for the pre-synaptic proteins synaptotagmin I (syt1), synaptotagmin IV (syt4) and synaptophysin (SYN) were analyzed in the female adult rat brain using *in situ* hybridization to explore the molecular mechanisms responsible for synaptic plasticity during the estrous cycle. Synaptotagmin I and IV are variants of synaptotagmin, a pre-synaptic protein that helps to regulate fusion of the neurotransmitter-containing synaptic vesicle to the plasma membrane (Südhoff, 1995). Synaptophysin functions as a control mechanism over release of that vesicle into the synaptic cleft (Spiwoks-Becker et al., 2001).

The levels of syt1 mRNA did vary cyclically in the CA3 region of the hippocampus throughout the estrous cycle; both syt4 and SYN were relatively invariant and did not show fluctuations during the estrous cycle. However, as CA3 pyramidal neurons make synaptic contacts with the CA1 region, modulation of syt1 in the CA3 may participate in the synaptic fluctuations for CA1 pyramidal neurons discussed above (Crispino et al., 1999). Additionally, SYN mRNA was lower on proestrus than on any other day of the cycle in the entorhinal cortex layer II. The entorhinal cortex sends axons to the CA3 field of the hippocampus, and the CA3 pyramidal cells synapse with neurons in the CA1 region, which provides the primary output of information from the hippocampus (Carlson, 1998, chap. 14). This suggests that gonadal hormones affect upstream projections to the hippocampus,

Brake et al. (2001) performed a study similar to those described above for dendritic spines and synapses. They found that treatment with estradiol-benzoate produced a 20-30% increase in the amount of immunoreactivity of the pre-synaptic proteins synaptophysin, syntaxin, and spinophilin in the CA1 region of the hippocampus (also see McEwen et al., 2001). Syntaxin forms a high-affinity binding site with SNAP-25 (another pre-synaptic vesicle protein), thus preparing the site for vesicle docking (Südhof, 1995). Spinophilin has recently been found to play an important role in spine homeostasis (Feng, Yan, Ferreira, Tomizawa, Liauw, Zhuo, et al., 2000). It therefore seems likely that there are multiple pre-synaptic proteins mediated by estrogens.

One such protein is synaptophysin, which is thought to be a reliable marker for synaptogenesis due to its role in vesicle docking and release (Südhof, 1995), and was also

found to be affected by levels of estradiol (see Crispino et al., 1999; Brake et al., 2001; McEwen et al., 2001). It also functions as a marker for presynaptic synapse formation, as its mRNA can be found in the growth cone of the budding synapse (McEwen et al., 2001).

Synaptophysin has also been chosen for this study due to its role in controlling vesicle exocytosis – the release of neurotransmitter-containing vesicles from the pre-synaptic neuron (Südhoff, 1995; Spiwoks-Becker et al., 2001). While other proteins mentioned in the studies above were affected by estradiol, and others – such as SNAP-25, synaptotagmins, and synaptoporin – are also held to be pre-synaptic markers, only synaptophysin appears to be vital for vesicular docking, fusion, and release (for further discussion, see below). Thus, synaptophysin has been selected as the most likely candidate for alterations due to hormone changes and reproductive experience (please see Table 1 for a summary of all presynaptic proteins and functions mentioned in this section).

Synaptophysin and Vesicle Exocytosis

Synaptophysin is a 38-kDa protein embedded in the membranes of small presynaptic vesicles by means of four transmembrane regions (Mullany & Lynch, 1998; Spiwoks-Becker, Vollrath, Seeliger, Jaissle, Eshkind & Leube, 2001; King & Arendash, 2002). The amino and carboxyl termini of the protein protrude into the cytoplasm. The carboxyl – terminus contains multiple phosphorylation sites that, when activated, allows the protein to be incorporated into the lipid bilayers of the cell membrane (Südhoff, 1995;

Spiwoks-Becker et al., 2001). The result is the formation of a fusion pore (open channel) in the vesicle membrane through which neurotransmitter release can occur (Mullany & Lynch, 1998; Spiwoks-Becker et al., 2001).

Synaptophysin often forms a highly specific binding complex with synaptobrevin, another pre-synaptic vesicle membrane protein (Edelmann, Hanson, Chapman & Jahn, 1995). Synaptobrevin is one of the most prominent components of small synaptic vesicles and is necessary for vesicle exocytosis (Südhof, 1995; Washbourne, Schiavo, & Montecucco, 1995; Becher et al., 1999). Once synaptophysin forms this bond with synaptobrevin, the synaptobrevin is unable to bind to any other membrane complexes or proteins, and allows synaptophysin to act as a control protein over vesicle exocytosis (Edelmann et al., 1995; Becher, Drenckhahn, Pahner, Margittai, Jahn & Ahnert-Hilger, 1999; Spiwoks-Becker et al., 2001; see Appendix A).

As important as synaptophysin is for exocytosis, one must remember that without the Ca^{2+} spike seen during an action potential, exocytosis cannot occur. Even with the Ca^{2+} trigger, not every action potential is able to lead to vesicle exocytosis – in fact, only about one in every three to ten action potentials do (Südhof, 1995; Spiwoks-Becker et al., 2001). However, alterations in synaptophysin expression have been shown to affect Ca^{2+} -dependent vesicle exocytosis (Spiwoks-Becker et al., 2001), although the mechanisms by which it does this are still unclear. As estrogen treatment in ovariectomized rats produces facilitation of synaptic transmission in CA1 neurons, leading to the enrichment of voltage-gated Ca^{2+} currents (McEwen & Alves, 1999), this paper proposes that an increase in synaptophysin in the CA1 hippocampus, due to prolonged estrogen during

pregnancy, has the ability to prolong voltage-gated Ca^{2+} currents (see McEwen et al., 2001). This would result in increased likelihood of depolarization and signaling, which could result in enhancement of spatial learning abilities.

Estrogen and Long-term Potentiation

How exactly is the CA1 able to help in memory formation? And what role does synaptophysin play in memory consolidation? Based on the research stated previously it is known that the CA1 hippocampus is an area that displays great amounts of synaptic plasticity and plays a role in memory formation (Carlson, 1998, chap. 14). Additionally, the effects of estradiol on dendrite and synapse formations seem fairly specific to the CA1. Although there is agreement that the induction of plasticity in these neurons is dependent upon the Ca^{2+} influx noted previously (Antonova, Arancio, Trillat, Wang, Zablow, Hiroshi et al., 2001), researchers have yet to agree on the actual function(s) of this plasticity.

One thought comes from evidence that these CA1 pyramidal neurons exhibit long-term potentiation (LTP); a long lasting form of synaptic plasticity that may regulate the expression of certain forms of long-term memory in the mammalian brain (Bliss & Collingridge, 1993; Warren et al., 1995). LTP is triggered by receptors for the excitatory neurotransmitter glutamate (Bliss & Collingridge, 1993). Glutamate synapses in the hippocampus are prone to long-term changes in function and strength and provide a potential cellular explanation for memory formation (Bliss & Collingridge, 1993).

In this region, LTP can be produced at the same synapses that have been shown to fluctuate with the estrous cycle - the apical dendritic spine synapses discussed previously (Warren et al., 1995; Mullany & Lynch, 1998). After inducing LTP in CA1 post-synaptic neurons, growth of entirely new dendritic spines was seen (Engert & Bonhoeffer, 1999). Furthermore, high-frequency synaptic stimulation was shown to induce protrusions on dendrites near the stimulation for a period of at least 30 minutes (Maletic-Savatic, Malinow, & Svoboda, 1999). It is therefore not unreasonable to assume that changes in hippocampal dendritic spines and synapses due to the estrous cycle could also affect LTP.

The available literature supports this viewpoint. Warren et al. (1995) found that female rats exhibited significantly more LTP on the afternoon of proestrous than on the afternoon of estrous or diestrous, an effect that may be due to the expected increase in dendritic synapses. Woolley et al. (1996) found evidence that the threshold for induction of LTP fluctuates during the estrous cycle; it is the lowest on proestrous when the number of spines and synapses are at their highest. The magnitude of LTP has also been associated with changes in estrogen levels during the estrous cycle (Bi, Foy, Vouimba, Thompson & Baudry, 2001). Specifically, increased estrogen levels (such as those seen during proestrous) have been shown to produce an increase in the magnitude of LTP in hippocampal sections through activation of the MAP kinase pathway (Bi et al., 2001).

Synaptophysin and Long-term Potentiation

Antonova et al. (2001) found evidence that LTP may involve presynaptic mechanisms. More specifically, they found that an increase of synaptophysin-

immunoreactive (SYN-IR) puncta (pre-fabricated packets of synaptic proteins) was seen during LTP with duration of at least 2 hours, when new synaptic growth occurred. Maintenance of LTP is functionally linked to glutamate release (Bliss & Collingridge, 1993; Xiao, Niu, Dozmorov, & Wingström, 2001; Jia, Lu, Agopyan, & Roder, 2001), and as synaptophysin and glutamate are often co-localized in cultures of CA1 (Antonova et al., 2001), synaptophysin may participate in the activation of functional glutamatergic synapses, which were noted previously to trigger LTP when activated (Bliss & Collingridge, 1993).

Additional evidence by Mullany & Lynch (1998) demonstrated that LTP might alter synaptophysin. They found an increase in protein synthesis in the entorhinal cortex through immunoprecipitation 45 minutes after LTP induction. Additional increases in synaptophysin and other vesicle proteins in the dentate gyrus were seen up to 3 hours after LTP. Thus, there may be a role for synaptophysin in the generation of LTP.

A number of researchers believe that LTP plays a role in learning and memory, specifically the involvement of synaptic LTP (Warren & Juraska, 1997; Berry, McMahan & Gallagher, 1997). The research noted above supports the idea that the estrous cycle affects LTP; what researchers have not yet agreed upon is whether the estrous cycle affects learning and memory, and what role – if any – synaptophysin plays.

Estrous Cycle and Spatial Memory

In looking at links between the estrous cycle and learning and memory the findings are obviously in conflict. Bi et al. (2001) have shown that estrogen fluctuations

affect the activity of a signaling pathway known as the MAP kinase pathway, and that blockage of this pathway has consistently has produced impairments in learning and memory. Interestingly, oxytocin enhances long-term memory through activating the same pathway, providing a role for motherhood in improved spatial memory (Tomizawa et al., 2003). Warren & Juraska (1997) found that spatial learning varied across the estrous cycle in a manner consistent with the anatomical and electro-physiological fluctuations discussed previously, with improved learning on proestrous, when the maximum number of dendritic spines and synapses are seen. However, performance on each day of the cycle was task-dependent: estrous female rats performed better on a place task, but proestrous female rats performed better on a cue task.

Due to its relative difficulty, the place task is more stressful to a rat than the cue task (Warren & Juraska, 1997); therefore, stress will have a significant effect on performance of the task. Viau & Meaney (1991) found that females in proestrous showed significantly more stress when restrained than estrous females did, as measured by a significant increase in levels of corticosterone and adrenocorticotrophin hormone (in Warren & Juraska, 1997) . This may be the reason why proestrous females gave results contrary to the hypothesis.

However, recent research has shown that females who have gone through pregnancy and reproductive experience (and thus higher levels of continuous estrogen) have a lowered stress response in comparison to controls (Wartella, Amory, Macbeth, McNamara, Stevens, Lambert et al., in press). Therefore, the explanation given by

Warren & Juraska may not be sufficient as a reason for their unexpected results, and other mechanisms may be responsible.

Other researchers have found little or no relationship between the estrous cycle and spatial task performance. Stackman, Blasberg, Langan & Clark (1997) found that day of estrous cycle affected neither acquisition of a spatial task nor performance on the first day of testing, but on subsequent days of testing rats performed significantly more slowly on proestrous than on any other day of their cycle. They suggest that this worsened performance was not due to an altered stress response, but that the neural network was compensating for the steroid hormone-dependent alterations in synaptic connectivity discussed above, possibly to the point of over-compensation. Berry et al. (1997) found even more extreme results. They showed no difference between proestrous and estrous rats in acquisition or retention of the spatial task, which suggested that spine density fluctuations (that coordinate with the estrous cycle) do not alter spatial memory performance.

Although the aforementioned research reveals inconsistent findings with respect to the role of the estrous cycle in learning and memory, there is a great deal of evidence supporting the idea that treatment with estrogen enhances learning and memory. Estrogen treatment of ovariectomized rats has been reported to improve acquisition and accuracy on a radial maze task, especially when treatment with estradiol was extended (Luine, Richards, Wu & Beck, 1998; McEwen & Alves, 1999). Intrahippocampal injections of estrogen immediately following training in the Morris water maze led to enhanced memory 24 hours later (McEwen & Alves, 1999), and systemic treatment with

estrogen led to a similar, more rapid effect, with females demonstrating enhanced memory in the maze (McEwen & Alves, 1999).

Gibbs (2000) found that treatment with estrogen or estrogen plus progesterone for eight to twelve months after ovariectomy resulted in significantly enhanced acquisition of a delayed matching-to-position spatial memory task. Frick, Fernandez & Bulinski (2002) found that daily treatment with estrogen for five days prior to and during testing significantly improved spatial learning and memory in the Morris water maze as compared to control animals. Finally, Markham, Pych & Juraska (2002) found that both acute and chronic estrogen treatment results in maintained overnight memory of the Morris water maze (see Table 2 for a summary of estrogen and its effects on spatial memory). Overall, this data seems to indicate that although the fluctuating hormone levels during the estrous cycle do not always lead to improved memory for spatial tasks, giving estrogen to these animals does lead to memory enhancement.

Most important to the current work are the findings that the duration of estrogen administration may be what is necessary to see the enhancing action of estrogen on spatial maze performance (Luine et al., 1998; Gibbs, 2000; Markham, Pych, & Juraska, 2002). Based on this growing body of evidence, there is reason to believe that prolonged exposure to elevated estrogen levels during pregnancy and lactation will result in similar enhanced memory functioning on the Morris water maze.

Synaptophysin and Spatial Memory

Unfortunately, little data exist describing the role that synaptophysin plays in learning and memory. As it does appear to play a role in LTP, as well as synaptic connectivity in the hippocampus (as described previously), it is believed that synaptophysin is vital to learning and memory. One study shows that rats with a reduction in synaptophysin displayed spatial learning deficits in both young controls and age-matched rats (Smith, Adams, Gallagher, Morrison & Rapp, 2000), thus lending credence to the idea that synaptophysin maintenance is necessary for spatial learning to occur.

A later study by King & Arendash (2002) revealed a conflicting role for synaptophysin, as impaired acquisition and spatial reference processing in the hippocampus on both a beam task and the Morris-Paul water maze was significantly correlated to maintained levels of synaptophysin immunoreactivity (SYN-IR). In other words, high levels of SYN-IR result in a delay in acquisition and memory of the maze task (King & Arendash, 2002). The impairment seen in this study may have occurred due to a compensatory synaptic response that contributes to altered synaptic function, as in Stackman et al. (1997). Also, these results may not be entirely applicable to the current project as this study involves aged (10-16 months) Alzheimer's disease knockout mice, not younger (~4-6 months) female rats, as were used here. Overall, the data seem to indicate a link between the estrous cycle, synaptophysin, and LTP in spatial learning and memory.

Current Work

Previous studies have shown three things; first, that changing levels of estradiol affect synaptic plasticity during the estrous cycle, most likely by acting upon synaptophysin, a protein that has the ability to almost single-handedly control vesicle exocytosis and subsequent neurotransmitter release, thereby affecting synaptic connectivity. Second, the most important reason for this synaptic plasticity is likely the production of LTP, which is affected by the estrous cycle and synaptophysin. Third, LTP is the most likely model for how learning and memory occur. Unfortunately, no consensus exists for whether learning and memory are affected by estradiol.

This project attempts to show that reproductive hormones can play a role in modulating learning and memory (most likely by acting on synaptophysin). Previous studies have looked only at natural fluctuations of the estrous cycle, or on estrogen replacement after ovariectomy, on the behaviors and biological systems described above. Here, however, the attempt will be to show that maintained hormone levels due to reproduction will have the same effects on pre-synaptic dendritic spine quantity – and therefore, on quantity of a specific pre-synaptic protein - as well as on learning and memory. Previous research has already shown that motherhood enhances neuronal activity and cognitive ability (Kinsley et al., 1999); this project may provide additional evidence that motherhood affects learning and memory, specifically through hormonal effects on synaptophysin.

Method

Experiment 1

The objective in the first experiment was to establish if the amounts of synaptophysin-immunoreactivity (SYN-IR) present in the CA1 hippocampus were altered by reproductive experience. Twenty-four age-matched (3-5 months of age) female Sprague-Dawley rats obtained from Harlan Laboratories were used. All animals were double-housed in 20 x 45 x 25 cm polypropylene cages, the floors of which were covered with a layer of 1/8" corncob bedding. Food (rat chow) and water were available *ad libitum* in light - (14 hr light/10 hr dark; lights on at 0500 hours) and temperature - controlled rooms. Animals were maintained in accordance with the guidelines put forth by the University of Richmond Institutional Animal Care and Use Committee (IACUC), and all of the following procedures also met with the committee's approval.

The twenty-four animals were split into four groups: six remained *virgin* (nulliparous – no reproductive experience), six were *late pregnant* (defined by day 20 or 21 of pregnancy), and twelve were lactating (these animals were sacrificed at day 5-6 of lactation). Due to problems with freezing tissue, one brain was lost from the virgin and from the late pregnant group (leaving twenty-two animals in the experiment). In the lactating group, six females were allowed to keep their pups and six females had all pups removed immediately after parturition.

All were killed by anesthesia (exposure to carbon dioxide) followed by decapitation; the brains were removed immediately and blocked into a smaller section containing the hippocampus. This section was snap-frozen on dry ice for approximately

10 minutes. Those brains not sectioned immediately were stored at –80°C until needed. For staining procedures, each brain was sectioned at 20 μm through the hippocampus and mounted on pre-subbed slides. All brains were sectioned on a Microm cryostat (model HM500) from Zeiss, Inc. at –20°C.

Staining Procedure

Each section was stained for synaptophysin using immunohistochemistry (IHC). This is the most appropriate procedure for quantification purposes, as it will stain only for synaptophysin using a specific primary and secondary antibody; therefore, it is acceptable for use as a quantitative tool. The Chemicon International, Inc. (Temecula, Ca) mouse-to-mouse IHC detection kit (Chemicon #2702) was used due to its increased sensitivity for staining mouse and rat tissue, as compared to other techniques and kits.

Briefly, sections were rehydrated in a series of descending alcohol washes (2 x 2 min, 100%; 2 min 95%; 2 min 70%; 2 min 50%), then washed in 3% H_2O_2 + dH_2O for 10 minutes to block endogenous peroxidase action. Pap pen was applied to the slides to maintain the liquid on the sections. To prepare the slides for application of the primary antibody, the slides were incubated in a pre-antibody solution (Chemicon #2702a) in a humid chamber for 60 minutes, followed by 2 x 5 minute washes in TBS. The sections were then incubated in mouse anti-synaptophysin (Chemicon #MAB5258-20UG) diluted in 10% NGS + TBS for 60 minutes in the same humid chamber, and then washed again in TBS for 2 x 5 minutes. Post-antibody blocking solution (Chemicon #2702b) was applied for 10 minutes; the slides were again rinsed in TBS for 2 x 5 minutes.

A secondary antibody (Poly-HRP-Anti-Mouse/Rabbit IgG; Chemicon #2702c) was applied for 10 minutes, followed by a final wash of TBS for 2 x 5 minutes. Diaminobenzidine [a solution of DAB-A (Chemicon #2702d), DAB-B (Chemicon #2702e) and dH_2O] was applied to each section for at least 5 minutes; once the sections changed to a dark brown the slides were rinsed twice with dH_2O. The slides were then dehydrated in a series of alcohol washes (2 min 50%; 2 min 70%; 2 x 2 min 95%; 2 x 4 min 100%), cleared for 4 minutes with xylene, cover slipped using CitraMount (Polysciences, Inc; #24214) and allowed to dry overnight.

The slides were analyzed using the BioQuant Nova image analysis system version 5.00.8 designed by R & M Biometrics, Inc. (Nashville, Tn). The program is designed to allow the user to view and analyze an assortment of neurological morphology at a variety of magnifications. In this experiment, the area of interest is the peak of the CA1 hippocampus. As synaptophysin is found throughout the entire area, and is found on the pre-synaptic side of every synapse, a magnification of 20X was used, as that was deemed to allow the largest amount of CA1 to be analyzed while still providing enough magnification to be sure the staining seen was actually synaptophysin.

Four slides (eight sections) were taken from each brain. As there were two sections per slide, a total of four area measurements (in μm^2) were obtained per slide, one from the left and right hippocampus of each section. The areas for each slide were summed to provide a total area measurement for that brain. While analyzing, the experimenter was blind as to which slides were in which group. Statistical analysis was

then carried out using a one-way ANOVA, with reproductive status as the independent variable, and area of CA1 containing SYN-IR as the dependent variable.

Experiment 2

Based on the results from experiment 1 (see following results section), the effect of giving birth was considered of greater interest than that of pregnancy, so the groups used in the behavioral task were slightly different from that in experiment one. Sixteen age-matched Sprague Dawley female rats (age 5-6 months) were trained to navigate the Morris water maze. Two main groups were used, virgins and lactating females (day five of lactation). Each group started containing eight animals; however, after two matings, one animal in the lactating group had still failed to get pregnant so she was excluded from testing, leaving seven animals in the lactating group.

Additionally, two smaller groups consisting of four animals each were tested (for a total of twenty four animals); post-partum females (currently lactating) with pups removed immediately after birth, and primiparous females whose pups had been removed or weaned at least two weeks prior to testing (and were therefore no longer lactating). These two groups were included in order to further illuminate the effects of reproduction on spatial learning, specifically by determining whether or not the presence (or absence) of pups affects spatial learning in new mothers.

The purpose of this experiment was twofold: (1) to determine if reproductive status affected the speed with which a spatial maze could be navigated after sufficient

training in said maze, and (2) to determine if elevated levels of synaptophysin-IR in the CA1 impacts learning and memory in the Morris water maze.

Training

All rats were trained for 8 trials per day for the three days prior to testing (please see Appendix B for a schedule of testing) in a 5'(diameter) x 2'(height) galvanized steel tub. The tub was broken up into four quadrants, with the escape platform remaining in the same location in quadrant four throughout the training and testing of all animals (please see Appendix C for pictorial representation). The tub was filled to 18.2 inches with tap water at room temperature (ranging from 20°C – 24.5°C) so that the escape platform was just below water level (at 18 inches). To occlude the water, non-toxic white shoe polish was added to the tub after filling until the platform could no longer be seen, and the water was opaque. Due to the fact that the rats repeatedly brushed the platform with some portion of their anatomy, and yet did not climb onto the platform, it was believed that the rats were unable to see the platform from water level despite the fact that it could be seen from above.

Each rat was placed into the water facing the wall at one of three training locations (please see Appendix C) for all (24) training sessions. The starting location for each rat was chosen randomly, and all rats did not start from the same location. Although chosen randomly, the locations were chosen with the intention of spreading all 23 rats evenly over the three starting locations. Each rat was given 60 sec to locate the platform; if she did not do so within that time period the experimenter guided her there and allowed

her to remain on the hidden platform for 15 sec. She was then placed back into the water at the exact same starting location for the next trial. At the end of the eight training sessions each rat was dried off, allowed to remain in a warm, dry holding tank for the duration of the next animal's session in the maze, and then placed back into her original cage. Between each testing session, fecal boli and any other waste material in the maze were removed with a small net, and the temperature was measured to insure that all rats were tested in approximately the same environment.

Training for all rats began at approximately the same time of day (beginning between 1200 and 1300 hours), except for two, which were tested beginning at 1500 hours. Although activity for rats is different in the afternoon than in the morning, the data from these two rats were not greater than two standard deviations from the mean so data from all rats was included in the results. Training on days two and three, and testing on day four, began within half an hour of the original starting time on day one.

Testing

Each rat received 8 testing trials in which the platform remained at the same location as during the training sessions; however for testing memory the rats were placed into the tub in a random sequence over the three testing locations, beginning with the location at which they had been trained during the last three days. No two rats were given the same sequences of testing positions (see Table 3). Except for differences in initial location, methodology was identical to that of the training sessions (procedures

adopted from Morris, 1981; Morris, Garrud, Rawlins, & O'Keefe, 1982; Warren & Juraska, 1995; Berry et al., 1997; Smith et al., 2000).

Immediately after testing on the last day, each rat was killed in the same manner as in experiment one, the only difference being that all brains were sectioned on the same day, so it was not necessary to store any brains at –80°C. The synaptophysin of one virgin female's brain could not be analyzed, as the brain snapped in half before sectioning (leaving seven virgin brains, the same number as lactating). The brains were again sectioned through the hippocampus at 20μm, with eight sections (four slides) analyzed per brain. All sections were analyzed in the same manner as those in experiment one. Statistical analysis was also carried out in the same manner.

Results

Experiment 1

A one-way analysis of variance (ANOVA) was performed on the data using an alpha level of .05. Results revealed no significance for reproductive experience on amount of SYN-IR in CA1 hippocampus, $F(3, 79) = 1.693$, $p = .175$. A least-significant differences (LSD) test also resulted in no significant differences between the four reproductive groups; however, the required value for significance was almost reached between virgin and lactating females, with less SYN-IR in virgins ($M = 4723.90$, $SD = 2654.24$) than in lactating females ($M = 6695.51$, $SD = 4897.83$), $p = .058$. Additionally, significance was almost reached between lactating females with pups and lactating females whose pups were removed, with lactating females displaying greater amounts of

SYN-IR in the CA1 ($M = 6695.51$, $SD = 4897.83$) than did lactating females whose pups were removed ($M = 4863.46$, $SD = 1715.58$), $p = .054$. As significance was very nearly reached, adding just a few more animals to each group may have resulted in significant differences overall.

Even without statistical significance, there was a trend toward the highest amounts of SYN-IR in the hippocampi of lactating females (see Figure 1). As can easily be seen, the smallest area of SYN-IR is found in virgins, with an increase seen in the CA1 of late pregnant animals, and the highest amount seen in lactating females. Lactating females whose pups had been removed had much less SYN-IR in the CA1, almost the same amount as virgins. Based on this trend, it appears that the amount of SYN-IR found in the CA1 was linked to reproductive experience, and that this increase in synaptophysin due to reproduction might assist new mothers in negotiating and learning spatial tasks. Additionally, it was believed that the removal of pups after birth canceled the increase seen in lactating females, and thus, that the presence of pups may be a factor in the amount of SYN-IR seen in the CA1.

Experiment 2

A one-way ANOVA revealed that reproductive experience had a significant effect on the amount of time (in seconds) it took the females to navigate the maze, $F(3, 180) = 6.05$, $p < .05$. A LSD test revealed that virgins took significantly less time ($M = 12.89$, $SD = 10.84$) to reach the escape platform than did lactating females ($M = 21.54$, $SD = 19.86$). Lactating females whose pups were removed immediately after birth were also

significantly faster at escaping ($M = 9.81$, $SD = 9.46$) than were lactating females ($M = 21.54$, $SD = 19.86$). The same results were also seen for primiparous females, which reached the escape platform in significantly less time ($M = 12.63$, $SD = 12.69$) than did lactating females ($M = 21.54$, $SD = 19.86$). No significant differences were seen among virgin females, lactating females without pups, and primiparous females (see Figure 2).

The same ANOVA revealed that reproductive experience did not significantly affect the percentage of time each rat spent in the target quadrant, $F(3, 180) = 1.70$, $p = .17$. However, a LSD test revealed that virgins spent a significantly larger percentage of time in the target quadrant ($M = 53.97$, $SD = 19.41$) than did lactating females ($M = 46.34$, $SD = 18.15$), $p < .05$. No other pairs were significant (see Figure 3). This indicates that although the overall ANOVA was not significant, virgin females did spend a significantly larger percentage of time in the target quadrant than did lactating females, which correlates with the results on total time to navigate the maze.

Finally, the ANOVA revealed a significant effect of reproductive experience on the distance swum by each rat, $F(3, 183) = 2.73$, $p < .05$. A LSD test revealed that lactating animals swam a significantly longer distance to reach the escape platform ($M = 17.07$, $SD = 14.34$) than did either lactating females without pups ($M = 9.84$, $SD = 8.90$) or primiparous females ($M = 11.44$, $SD = 12.02$). No significant differences were seen between lactating females without pups and primiparous females, and virgin females were not significantly different from any other group (see Figure 4).

A separate one-way ANOVA yielded a significant effect of reproductive experience on the amount of SYN-IR in the CA1 for females who have learned to

navigate a spatial maze, $F(3, 84) = 4.92, p < .05$. A LSD post-hoc analysis revealed that the CA1 hippocampus in virgin females contained significantly higher levels of SYN-IR ($M = 9557.10$, $SD = 5364.38$) than did the CA1 in lactating females ($M = 6011.95$, $SD = 2846.26$). Virgin females also had significantly more SYN-IR in the CA1 ($M = 9557.10$, $SD = 5364.38$) than did lactating females without pups ($M = 6315.16$, 1959.75). Finally, primiparous females had significantly more SYN-IR in the CA1 ($M = 9096.52$, 4475.36) than did lactating females ($M = 6011.95$, $SD = 2846.26$). No differences were seen between virgins and primiparous, or between lactating and lactating without pups (see Figure 5; Appendix D). This seems to indicate that maze learning affects SYN-IR, as these results are not the same as what was found in the first experiment.

Finally, two separate Pearson's bivariate correlations were run on the data. The first determined if the three behaviors measured were correlated to one another, and if the increase in SYN-IR in the hippocampus was correlated to the results obtained for the three behavioral measures. The correlation revealed a significant positive correlation between latency to escape onto the hidden platform and the distance traveled to reach the platform, $r = .968, p < .05$. No other significant correlations were revealed (see Tables 4 and 5).

The second Pearson's bivariate correlation was used to determine whether or not performance on test day was influenced by performance on the previous three training days. A significant negative correlation was found between day of testing and latency to escape onto the hidden platform, $r = -.526, p < .05$. This means that the overall time (measured across all groups) to escape from the Morris water maze was less each on each

consecutive day of training/testing (see Table 6 for a list of means). No day was found to significantly correlate with any other day, and day of testing was not significantly correlated to reproductive group.

Discussion

The hypothesis in the present study was that pregnancy and lactation would enhance female rats' ability to navigate a spatial maze, possibly through increases in the amount of SYN-IR present in the CA1 hippocampus, an area that is known to control learning and memory. As synaptophysin is the synaptic protein that acts as a control over release of neurotransmitter-containing vesicles from the pre-synaptic neuron, this protein could play a role in consolidating memory by enhancing synaptic strength.

Analysis of the data from experiment one seemed to support this hypothesis. Although non-significant, a trend in the direction hypothesized was revealed. Virgin females, with no reproductive experience, had the lowest amount of SYN-IR in the CA1, followed by late pregnant females, and lactating females containing the most synaptophysin in the CA1. Lactating females whose pups were removed showed decreased amounts of SYN-IR, similar to that seen in the virgins (Figure 1). These data indicate that the hypothesis may be correct, and that reproduction might result in an increase in SYN-IR in the CA1 hippocampus. Furthermore, it seems as if removal of pups leads to a decrease in SYN-IR in the hippocampus, possibly due to decreased stimulation and post-partum hormone levels, particularly oxytocin. This led to experiment two, where the goal was to determine if this trend is actually significant, and

reproduction does result in an increase in SYN-IR, as well as any effect reproduction might have on learning and memory for a spatial task.

The effects of reproduction on learning and memory do not correlate with what was hypothesized after the first experiment. Lactating females currently nursing pups took the longest time to reach the escape platform in the Morris water maze (Figure 2). All other groups (virgins, lactating females without pups, and primiparous females) were significantly faster in escaping the maze than the lactating group. While at first glance this seems to indicate that reproduction does not result in enhanced spatial memory, a closer look reveals that both lactating females without pups and primiparous females, two groups who had given birth, were able to remember the task just as well as the virgin females were. The main difference between these three groups and the lactating females was the presence (or absence) of pups. None of the three groups who had the best performance in the Morris water maze were currently nursing pups (even though one group was lactating), implicating pup presence as a factor in learning this task (see Table 4).

This finding goes directly against recent findings that oxytocin, which is released due to pup stimulation during the post-partum period, enhances spatial memory (Monks, Lonstein & Breedlove, 2003; Tomizawa et al., 2003). Those females who would be experiencing the highest levels of oxytocin – lactating females currently nursing – exhibited the worst spatial memory. This indicates that other hormones or proteins may be interfering with this process, or that the stress of being removed from their pups

repeatedly during testing has completely overcome the neurological benefits of giving birth.

A second analysis of the behavioral data, using the percentage of time in the target quadrant as the dependent variable, did not reveal an overall significant effect of reproduction. However, post hoc analysis revealed that virgin females spent significantly more time in the escape quadrant than did lactating females (Figure 3). This was a planned comparison, as the difference between virgin and lactating females was the greatest area of interest in this study; therefore, these findings do seem to correlate with the escape latency results, further implying that memory for the general location of the escape platform may be hindered by the presence of pups, as seems to be the case with memory for the specific location for the platform. However, as the only groups that showed significant differences with this variable were virgin and lactating females the effect seen here may be due to reproduction alone and not be influenced by pups.

The third variable measured, the distance traveled by the females, provides more evidence that the most important variable in increased ability to consolidate memories is not reproduction itself but whether or not pups are present at the time. Lactating females had the longest average path length to the escape platform, significantly longer than either lactating without pups or primiparous females (Figure 4, Table 4). This implies that the presence of pups is again a mediating factor in spatial memory abilities. As the path length for virgin females was not statistically different from any of the other groups, the data imply that reproduction itself does not confer any particular advantages on the reproducing female, but that pups are detrimental to the learning process.

Although there is room for speculation, the results clearly implicate the presence of pups in affecting memory consolidation, most likely for the worse. Whereas this effect is fairly easily seen the cause is somewhat more difficult to determine. One possibility is that the pups cause a neurological change that interferes with memory in the Morris water maze. Analysis of SYN-IR in the CA1 hippocampus of these animals lends credence to this idea. Both virgins and primiparous females had significantly more SYN-IR than did lactating females and lactating females without pups (see Figure 4). This would indicate that lactation results in lowered levels of SYN-IR in the hippocampus, and that reproductive experience does have some influence on SYN-IR, as primiparous females had high levels. As these results were not in the same direction as those obtained in the first experiment, there is the suggestion that learning a spatial task has an influence on SYN-IR; otherwise, the results should be similar to the trend observed in experiment one.

Thome and colleagues found that acute immobilization stress for one hour significantly decreased the amount of hippocampal synaptophysin by 50% (Thome, Pesold, Baader, Hu, Gewirtz, Duman, & Henn, 2001). Although these females were not immobilized, and earlier research in this lab found that lactating females have a decreased stress response when immobilized for one hour (Wartella et al., 2003, in press), being removed from their pups for at least 20 minutes and forced to swim (a second stressor) may have affected them in a manner similar to that found by Thome, and resulted in decreased SYN-IR. This may have caused the lactating females to be less able to consolidate the information received during training into their memory for use on testing day.

This stress hypothesis goes against a recent finding that the presence of an enriched environment led aged female mice to display both enhanced memory in the Morris water maze and increased levels of hippocampal synaptophysin (Frick & Fernandez, 2003). If pups act as an enriching environment on a new mother, than this would lead to the conclusion that lactating females currently nursing their pups should show increased spatial memory and SYN-IR. As that was not found in this experiment, other factors more important than an enriching environment must be at work here; however, the idea of pups as enriching for the new mother is something that should be looked into, particularly in relation to the mother's cognitive abilities.

An alternative explanation for how reproduction can affect SYN-IR may be through mediating the glial cell protein apolipoprotein E (apoE). Glial cells function as recyclers of neuronal chemicals, as well as removers of waste materials that accumulate in the nervous system. Astrocytes (a type of glial cell) are particularly adept at this, due to the prevalence of apoE, which helps in carrying lipids across the cell membrane, particularly in response to peripheral and central nerve cell damage (Mahley, 1988 in Stone, Rozovsky, Morgan, Anderson, Jahian, & Finch, 1997). ApoE also helps to scavenge cholesterol from cellular debris for use in axonal regeneration and remyelination, which could directly affect neuronal transmission of a stimulus as well as indirectly affect synaptic plasticity and remodeling (Stone et al., 1997).

ApoE is a precursor to the protein APP, which produces the plaques and tangles characteristic of Alzheimer's disease (Stone et al., 1997; Poirier, 1996). Recently, a study by Gatewood (2002) showed that multiparous females, who had been exposed to

much higher levels of hormones over their life time than their primiparous and nulliparous counterparts, had significantly decreased levels of APP in the dentate gyrus (and those of apoE) than did the other two groups. As was noted above, one of the important functions of apoE is in scavenging cholesterol for use by neurons. A recent study found that synaptophysin is a cholesterol-binding protein, and that depleting levels of exogenous cholesterol significantly reduced the levels of synaptophysin in mouse hippocampal cultures (Mitter, Reisinger, Hinz, Hollmann, Yelamanchili, Treiber-Held, et al., 2003). Taken together, the data from these studies would indicate that reproductive females (like both lactating groups used currently) would have lower levels of APP, and therefore of apoE, which could result in decreased availability of cholesterol, which would down-regulate the availability of synaptophysin.

The above explanation does not explain why primiparous females, who have also had reproductive experience, would display such high levels of SYN-IR. One explanation for this may be that higher levels of SYN-IR do not necessarily represent greater activity, but the potential for activity. Synaptophysin is a pre-synaptic protein; therefore, the antibody used for staining synaptophysin reacts with pre-synaptic vesicles only (Chemicon International). In virgin females, which have not had reproductive experience, and primiparous females, which are further removed from giving birth than are lactating females, higher levels of SYN-IR may have been detected due to greater numbers of stored presynaptic vesicles, with fewer vesicles being released, and actually less communication between hippocampal neurons. This is merely conjecture, however, and no research has been found to support this idea.

It is therefore difficult to determine exactly what role synaptophysin plays in memory consolidation. It may be that due to the absence of pups the virgin, lactating without pups, and primiparous females were better able to remember the location of the escape platform, and that this memory enhancement was due to an increase in SYN-IR. However, lactating females without pups, who displayed very quick learning in the Morris water maze, had significantly lower amounts of SYN-IR in the CA1 than did the virgins and primiparous groups (level with that of lactating females), and no significant correlation was found between the amount of SYN-IR and improved performance (see Table 4). Moreover, it is possible that lactation results in a decrease in CA1 SYN-IR, as the two lactating groups had significantly lower levels than did primiparous or virgin females, and therefore the maze training does not affect SYN-IR at all. If this is true, it may mean that synaptophysin plays no role in enhancing spatial memory, and that other proteins or neuronal substrates may be just as, or more, important.

Collectively, the data implicate the presence of pups in worsening spatial memory consolidation in the Morris water maze. Levels of SYN-IR in the hippocampus also seem to be affected by reproductive experience, but from these results alone we cannot definitively say that synaptophysin is a mediator of spatial memory, or that spatial memory mediates SYN-IR.

Limitations

A number of limitations were present in this study that may have prevented clearer results from being obtained, the largest of these being somewhat small and uneven

group sizes. The first experiment was begun with six animals in each group but due to freezing some tissue was lost, leaving only five animals in two of the groups. Even if the groups had been even, more animals in each group would have been needed to produce definitive results. The outcome from the first experiment did indicate, however, that the principal differences in hippocampal SYN-IR existed between virgin and lactating females, so the main focus of experiment two was to flesh out the differences between these two groups. Thus, the largest groups were these two, leaving fewer animals to be placed into the other two groups. With more animals in each group, and even numbers across all groups, more definitive results may have been obtained for the second experiment.

A second limitation is that of the groups used. The intention all along was to see what effect elevated hormones due to pregnancy and reproduction have on memory, yet due to problems with controlling for swim speed late pregnant animals were not able to be used in the water maze. Pregnancy results in altered weight and buoyancy, both of which will affect the pregnant rat's ability to swim, thereby artificially affecting her latency to escape. Due to a limited number of people able to be in the testing room, and the lack of video and computers to calculate swim speed, we were unable to accurately judge what effect pregnancy had on swim speed, if any; therefore, the group was omitted. If this group could have been tested, it may be better known what the effect of reproduction is on memory, and whether or not the results from the Morris water maze really are due to lactation.

Third, it was unknown during testing as to what phase of the estrous cycle each rat was in. If the experimenter had known, it would be possible to correlate day of estrous cycle to performance, and see if performance in the maze is better on the day of proestrous. Additionally, continuous hormone could have been given to each rat to ensure that cycling was not occurring, which would ensure that fewer disparities in escape times would be seen.

Finally, synaptophysin is only one of many pre-synaptic proteins that assist in vesicle exocytosis. Though it does seem to play one of the more important roles in vesicle exocytosis, many other proteins also play important roles, and could thus be just as important for enhancing synaptic transmission. Staining for multiple pre-synaptic proteins would have been a nice way to try and determine which, if any, proteins enhance spatial memory. Replication of this study, addressing these limitations, may clear up any confusing data seen here, as well as further illuminate the role of reproduction in protein synthesis and memory consolidation.

Areas for future study

Despite these limitations, one basic outcome seen repeatedly throughout this study was that the expectation that reproductive experience would provide lactating females with better memory and increased SYN-IR than virgins was not met. Although this was indicated in experiment one, the data were not significant, and only displayed a trend in the hypothesized direction. In the second experiment the data revealed that the opposite of the hypothesis was actually true. Virgin females were able to learn to escape

more quickly from the Morris water maze and remember the quadrant the escape platform was located in better than the lactating animals. They also had higher levels of SYN-IR in the CA1 hippocampus, a region that has been repeatedly implicated in learning and memory. At first glance, this would seem to indicate that reproductive experience does not help, and may even hinder, spatial abilities. However, testing primiparous females and lactating females without pups reveals that reproductive experience can result in enhanced memory performance (seen in both groups) and increased levels of SYN-IR (seen in the primiparous group). Much future research is needed to further elucidate the mechanisms, if any, behind these interactions.

Although it is unclear as to what role synaptophysin has in memory consolidation, it is clear that reproduction and lactation have some effect on its expression. Therefore, it is quite likely that glutamate is also affected by reproductive state, as synaptophysin has been linked to glutamatergic synapses (Mullany & Lynch, 1998), which are able to change their functions and strength long-term. This is important because glutamate synapses activate N-methyl-D-aspartate (NMDA) receptors, which are implicated in mediating excitatory input to CA1 cells. Structural changes in dendritic spine and synapse morphology are paralleled by increases in synaptic excitability to NMDA receptors (Rudick & Woolley, 2001; Woolley, Weiland, McEwen & Schwartzkroin, 1997). Therefore, if SYN-IR is altered by reproductive experience, or by being trained in a spatial maze (as must be the case with virgins, who have had no reproductive experience), glutamate receptors may be altered as well. This would ultimately lead to a

change in NMDA receptor activation, which can affect how excitatory input is transmitted from neuron to neuron (Woolley & McEwen, 1994).

There is evidence of a sub-population of CA1 synapses mainly activated by NMDA receptors and involved in LTP activity (Woolley et al., 1996), linking NMDA to the process of learning and memory. Bi et al. (2001) provided further proof for this link by demonstrating that the MAP kinase pathway that activates NMDA receptors is necessary for synaptic plasticity, learning, and memory. This pathway is activated by oxytocin, which is elevated during lactating (Tomizawa et al., 2003), linking motherhood with NMDA receptor activation. As it cannot be said from this experiment that exposure to high levels of estrogen during pregnancy and lactation resulted in an increase in synaptophysin in the CA1 (as virgins demonstrated an increase as well; see Table 3), some mechanism other than estrogen may be responsible for the present increase in SYN-IR. Regardless of the mechanism responsible, it is quite likely that increased SYN-IR enhances transmission at glutamatergic synapses, which activate NMDA receptors, which in turn may help in modulating learning and memory. Future research using a design similar to the current one but looking at NMDA receptor activation would provide evidence either supporting or contrary to this idea.

All previously presented evidence given in support of the original hypothesis provided evidence that steroid hormones (like estrogen) increase spines, synapses, and excitatory transmission in the hippocampus. However, evidence exists stating that estrogen does not have this same effect in other brain regions. In the arcuate nucleus, application of anti-estradiol (which immunoneutralizes estradiol's effects) preserves the

number of synapses, which is counter to what is seen in other areas, primarily hippocampus (Naftolin, Mor, Horvath, Luqin, Fajer, Kohen & Garcia-Segura, 1996).

Even though this has not been shown to occur in the hippocampus, the blocking effect described here may contribute to the increase in SYN-IR seen in virgin females. If circulating estrogen causes a decrease in number of synapses during the estrous cycle in the arcuate nucleus (Naftolin et al., 1996) it is possible that the elevated levels of estrogen experienced by the females during lactation could lead to a similar decrease in synapses (and therefore in pre-synaptic proteins like synaptophysin) in the hippocampus. While highly speculative, this idea does warrant future study.

The final, and ultimately most important, area of future study is what impact, if any, this research has on humans. Maintenance of estrogen levels in women (through hormone replacement therapy) has been shown to enhance and/or maintain verbal abilities in women, as well as alters spatial memory, possibly as a side effect of mood elevation (Sherwin, 1996; Buckwalter et al., 2001). Women who have been pregnant have demonstrated increased performance on word-recall tasks 6 and 12 months post-partum (Buckwalter et al., 2001). However, the data on memory enhancement is highly inconsistent, particularly when it comes to spatial memory. This study allows the possibility that reproduction enhances spatial memory after weaning has already occurred, as both non-nursing (but lactating) mothers and mothers whose pups had been weaned displayed good spatial memory skills. Consequently, the beneficial effects of motherhood may not be reaped until later on in life.

Some research has shown this to be true. A recent study found that giving birth has spatial memory-enhancing effects late into the life of female rats, even more pronounced than the benefits seen just after reproduction (Gatewood, 2002). This has been shown to carry over into humans, as well. A recent study revealed increased performance in verbal learning and memory, attention, and psychomotor speed two years after delivery, in comparison with performance during the last month of pregnancy (Buckwalter et al., 2001). Reproductive experience can even decrease the amount of APP in the hippocampus, thereby decreasing the risk of Alzheimer's disease (Gatewood, 2002). In humans, maintenance of estrogen levels has been shown to result in a lower incidence of Alzheimer's disease (Sherwin, 1996; Kelly & Levin, 2001). Therefore, the possibility exists that reproduction does have memory-enhancing effects for the mother throughout her lifespan, particularly later in life when neuroprotective effects are seen, as measured by decreased levels of APP (Gatewood, 2002).

Hormonal effects, particularly those attributed to estrogen and other steroid hormones, are not limited to just one target. Multiple receptors, neurotransmitters, proteins, glia cells, and a variety of brain regions are all intertwined with the neurological and cognitive changes seen in the brain due to pregnancy and reproductive experience. Although the full meaning of these interactions is not yet known, the prevalence of studies linking all of these factors gives an excellent starting point for future study. This study provides evidence that the absence of pups may help mothers to be taught to navigate a spatial task better than lactating females currently nursing pups, but not better than virgin females. While this does show a direct effect of reproduction on memory

consolidation, the exact mechanisms of this effect are not yet known. Further study into many areas of reproduction may help in illuminating the answers to many of the questions that still exist.

References

Antonova, I., Arancio, O., Trillat, A.C., Wang, H.G., Zablow, L., Hiroshi, U., et al. (2001). Rapid increase in clusters of presynaptic proteins at onset of long-lasting potentiation. *Science, 294,* 1547-1550.

Becher, A., Drenckhahn, A., Pahner, I., Margittai, M., Reinhard, J., & Ahnert-Hilger, G. (1999). The synaptophysin-synaptobrevin complex: a hallmark of synaptic vesicle maturation. *Journal of Neuroscience, 19,* 1922-1931.

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

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.

Brake, W.G., Alves, S.E., Dunlop, J.C., Lee, S.J., Bulloch, K., Allen, P.B, et al. (2001). Novel target sites for estrogen action in the dorsal hippocampus: an examination of synaptic proteins. *Endocrinology, 142,* 1284-1289.

Buckwalter, J.G., Buckwalter, D.K., Bluestein, B.W. & Stanczyk, F.Z. (2001). Pregnancy and post partum: changes in cognition and mood. *Progress in Brain Research, 133,* 303-319.

Carlson, N. (1998). Physiology of Behavior, 6th Ed. Boston: Allyn and Bacon.

Crispino, M., Stone, D.J., Wei, M., Anderson, C.P., Tocco, G., Finch, C.E., et al. (1999). Variations of synaptotagmin I, synaptotagmin IV, and synaptophysin mRNA levels in rat hippocampus during the estrus cycle. *Experimental Neurology, 159,* 574-583.

Edelmann, L., Hanson, P.I., Chapman, E.R., & Jahn, R. (1995). Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. *EMBO Journal, 14,* 224-231.

Engert, F. & Bonhoeffer, T. (1999). Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Science, 399,* 66-70.

Feng, J., Yan, Z., Ferreira, A., Tomizawa, K., Liauw, J.A., Zhuo, M., et al.(2000). Spinophilin regulates the formation and function of dendritic spines. *Proceedings of the National Academy of Sciences of the United States of America, 97,* 9287-9292.

Frick, K.M. & Fernandez, S.M. (2003). Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. *Neurobiology of Aging, 24,* 615-626.

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.

Gatewood, J.D. (2002). *Reproductive experience and aging: Possible neuroprotective effects of motherhood.* Unpublished masters' thesis, University of Richmond, Virginia.

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

Golding, N.L., Staff, N.P. & Spruston, N. (2002). Dendritic spikes as a mechanism for cooperative long-term potentiation. *Nature, 418,* 326-331.

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.

Jia, Z., Lu, Y.M., Agopyan, N. & Roder, J. (2001). Gene targeting reveals a role for the glutamate receptors mGluR5 and GluR2 in learning and memory. *Physiology & Behavior, 73,* 793-802.

Kelly, M.J. & Levin, E.R. (2001). Rapid actions of plasma membrane estrogen receptors. *Trends in Endocrinology & Metabolism, 12,* 152-156.

Keyser-Marcus, L., Stafisso-Sandoz, G., Gerecke, K., Aaron, J., 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.

King, D.L. & Arendash, G.W. (2002). Maintained synaptophysin immunoreactivity in Tg2576 transgenic mice during aging: correlations with cognitive impairment. *Brain Research, 926,* 56-68.

Kinsley, C.H., Madonia, L., Gifford, G.W., Yureski, K., Griffin, G.R., Lowry, C., et al. (1999). Motherhood improves learning and memory: Neural activity in rats is enhanced by pregnancy and the demands of rearing offspring. *Nature, 402,* 137-138.

Kombian, S.B., Hirasawa, M., Mouginot, D., & Pittman, Q.J. (2002). Modulation of synaptic transmission by oxytocin and vasopressin in the supraoptic nucleus. *Progress in Brain Research, 139,* 235-246.

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 and Behavior, 34,* 149-162.

Maletic-Savatic, M., Malinow, R., & Svoboda, K. (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science, 283,* 1923-1927.

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. (2002). Estrogen actions throughout the brain. *Recent Progress in Hormone Research, 57,* 357-385.

McEwen, B.S., Akama, K., Alves, S., Brake, W.G., Bulloch, K., Lee, S., et al. (2001). Tracking the estrogen receptor in neurons: Implications for estrogen-induced synapse formation. *Proceedings of the National Academy of Sciences, 98,* 7093-7100.

McEwen, B.S. & Alves, S.E. (1999). Estrogen actions in the central nervous system. *Endocrine Review, 20,* 279-307.

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.

Mitter, D., Reisinger, C., Hinz, B., Hollmann, S., Yelamanchili, S.V., Treiber-Held, S., et al. (2003). The synaptophysin/synaptobrevin interaction critically depends on the cholesterol content. *Journal of Neurochemistry, 84,* 35-42.

Morris, R.G.M. (1981). Spatial localization does not require the presence of local cues. *Learning and Motivation, 12,* 239-260.

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.

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

Mullany, P.M., & Lynch, M.A. (1998). Evidence for a role for synaptophysin in expression of long-term potentiation in rat dentate gyrus. *Neuro Report, 9,* 2489-2494.

Naftolin, F., Mor, G., Horvath, T.L., Luquin, S., Fajer, A.B., Kohen, F, et al. (1996). Synaptic remodeling in the arcuate nucleus during the estrus cycle is induced by estrogen and precedes the preovulatory gonadotropin surge. *Endocrinology, 137,* 5576-5580.

Poirier, J. (1996). Apolipoprotein E in the brain and its role in Alzheimer's disease. *Journal of Psychiatry & Neuroscience, 21,* 128-134.

Rosenzweig, M.R., Breedlove, S.M., & Leiman, A.L. (2002) *Behavioral Psychology, 3rd ed* (pp. 31). Sunderland, Mass: Sinauer Associations, Inc.

Rudick, C.N. & Woolley, C.S. (2001). Estrogen regulates functional inhibition of hippocampal CA1 pyramidal cells in the adult female rat. *Journal of Neuroscience, 21,* 6532-6543.

Russell, J.A., Douglas, A.J., & Ingram, C.D. (2001). Brain preparations for maternity – brain changes in behavioral and neuroendocrine systems during pregnancy and lactation. An overview. *Progress in Brain Research, 133,* 1-38.

Sherwin, B.B. (1996). Estrogen, the brain, and memory. *Menopause: The Journal of The North American Menopause Society, 3,* 97-105.

Smith, T.D., Adams, M.M., Gallagher, M., Morrison, J.H., & Rapp, P.R. (2000). Circuit-specific alterations in hippocampal synaptophysin immunoreactivity predict spatial learning impairments in aged rats. *Journal of Neuroscience, 20,* 6587-6593.

Spiwocks-Becker, I., Vollrath, L., Seeliger, M.W., Jaissle, G., Eshkind, L.G., Leube, R.E. (2001). Synaptic vesicle alterations in rod photoreceptors of synaptophysin-deficient mice. *Neuroscience, 107,* 127-142.

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.

Stone, D.J., Rozovsky, I., Morgan, T.E., Anderson, C.P., Hajian, H., & Finch, C.E. (1997). Astrocytes and microglia respond to estrogen with increased apoE mRNA in Vivo and in Vitro. *Experimental Neurology, 143,* 313-318.

Südhof, T.C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature, 375,* 645-653.

Theodosis, D.T. & Poulain, D.A. (2001). Maternity leads to morphological synaptic plasticity in the oxytocin system. *Progress in Brain Research, 133,* 49-58.

Thome, J., Pesold, B., Baader, M., Hu, M., Gewirtz, J.D., Duman, R. S. et al. (2001). Stress differentially regulates synaptophysin and synaptotagmin expression in hippocampus. *Biological Psychiatry, 50,* 809-812.

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.

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.

Wartella, J., Amory, E., Macbeth, A., McNamara, I., Stevens, L., Lambert, K.G., et al. (2003). Single or multiple reproductive experiences attenuate neurobehavioral stress and fear responses in the female rat. *Physiology & Behavior, in press.*

Washbourne, P., Schiavo, G., & Montecucco, C. (1995). Vesicle-associated membrane protein-2 (synaptobrevin-2) forms a complex with synaptophysin. *Biochemical Journal, 305,* 721-724.

Woolley, C.S. (1998). Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus. *Hormones and Behavior, 34,* 140-148.

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. *The Journal of Comparative Neurology, 336,* 293-306.

Woolley, C.S., & McEwen, B.S. (1994). Estradiol regulates hippocampal dendritic spine density via an N-Methyl-D-Aspartate receptor-dependent mechanism. *The Journal of Neuroscience, 14,* 7680-7687.

Woolley, C.S., Weiland, N.G., McEwen, B.S. & Schwartzkroin, P.A. (1997). Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: Correlation with dendritic spine density. *The Journal of Neuroscience, 17,* 1848-1859.

Woolley, C.S., Wenzel, H.J., & Schwartzkroin, P.A. (1996). Estradiol increases the frequency of multiple synapse boutons in the hippocampal CA1 region of the adult female rat. *The Journal of Comparative Neurology, 373,* 108-117.

Xiao, M.Y., Niu, Y.P., Dozmorov, M. & Wigström, H. (2001). Comparing fluctuations of synaptic responses mediated via AMPA and NMDA receptor channels – implications for synaptic plasticity. *Biosystems, 62,* 45-56.

Yankova, M., Hart, S.A., & Woolley, C.S. (2001). Estrogen increases synaptic connectivity between single presynaptic inputs and multiple postsynaptic CA1 pyramidal cells: A serial electron-microscopic study. *Proceedings of the National Academy of Science, 98,* 3525-3530.

Table 1

Properties of Various Pre-Synaptic Vesicle Proteins Involved in Vesicle Exocytosis

Pre-synaptic protein	Location	Function	Reference
Synaptotagmins	Synaptic vesicle membrane	Ca^{2+} sensitive sensors for final step of fusing small vesicles to the pre-synaptic membrane just prior to vesicle exocytosis	Südhof, 1995
Synaptophysin	Synaptic vesicle membrane	Regulates vesicle exocytosis; controls binding of synaptobrevin to SNARE complex and subsequent vesicle docking; marker for synaptic vesicles and terminals	Becher et al., 1999 Edelmann et al., 1995 Spiwoks-Becker et al., 2001 Südhof, 1995
Syntaxin	Pre-synaptic plasma membrane	Binds with SNAP-25 to provide a highly specific binding site (SNARE) for vesicles; marker for synaptogenesis	Brake et al., 2001 Südhof, 1995
Spinophilin	Dendritic spines	Regulates spine homeostasis	Brake et al., 2001 Feng et al., 2000
SNAP-25	Pre-synaptic plasma membrane	Binds with syntaxin to form to provide a highly specific binding site (SNARE) for vesicles and synaptobrevin	Becher et al., 1999 Südhof, 1995
Synaptoporin	Synaptic vesicle membrane	Closely related isoform of synaptophysin; marker of pre-synaptic vesicles	Becher et al., 1999 Südhof, 1995
Synaptobrevin (VAMP)	Synaptic vesicle membrane	Bound to synaptophysin until released to bind with SNARE complex to complete vesicle docking at the pre-synaptic membrane; acts as a vesicle receptor in the process of vesicle docking	Becher et al., 1999 Edelmann et al., 1995 Washbourne et al., 1995

Table 2

Summary of the Effects of the Estrous Cycle and Estrogen Treatment on Spatial Memory

Author	Estrous Cycle or Estrogen Treatment	Spatial Task	Performance
Berry, McMahan, & Gallagher (1997)	Estrous cycle	Morris water maze	Not affected by estrous cycle
Stackman, Blasberg, Langan & Clark (1997)	Estrous cycle	Delayed non-match-to-sample version of radial arm maze	No effect on acquistion or performance on the first day On subsequent days, rats performed more slowly on proestrous
Warren & Juraska (1997)	Estrous cycle	Morris water maze	Increased during proestrous on a cue task Increased during estrous on a place task
Luine, Richards, Wu & Beck (1998)	Estrogen treatment	Radial arm maze	Improved spatial memory
McEwen & Alves (1999)	Estrogen treatment	Morris water maze	Improved spatial memory 24 hours later
Gibbs (2000)	Estrogen treatment	Delayed matching-to-position	Improved acquisition as compared to OVX animals
Frick, Fernandez & Bulinski (2002)	Estrogen treatment	Morris water maze	Improved spatial learning and memory
Markham, Pych & Juraska (2002)	Estrogen treatment	Morris water maze	Improved spatial memory with both acute and chronic doses

Table 3

Quadrant Placement for Each Rat on Testing Day (Day 4)

Rat	Quadrant placement on testing day								Rat	Quadrant placement on testing day							
1	1	2	3	1	2	3	1	2	13	2	3	2	1	3	2	1	3
2	1	3	1	3	2	1	2	3	14	1	3	2	1	2	3	1	2
3	3	2	3	1	2	3	1	2	15	2	3	1	2	1	3	1	2
4	3	2	1	3	1	2	3	1	16	1	3	2	1	3	2	1	3
5	Not used for testing								17	3	2	3	2	1	3	2	1
6	1	2	1	3	2	1	2	3	18	1	2	3	2	1	2	3	1
7	3	1	2	3	1	2	3	1	19	1	3	1	2	3	2	1	2
8	3	1	2	1	2	3	2	3	20	2	1	3	2	1	3	2	1
9	2	1	3	1	2	1	3	2	21	3	1	3	2	1	2	3	1
10	2	3	1	3	2	3	2	1	22	2	3	2	1	3	1	2	1
11	1	3	2	3	2	1	3	1	23	2	1	2	3	1	2	1	3
12	2	1	3	1	2	3	1	2	24	3	2	1	2	1	2	3	1

Table 4

Significant Differences Between Groups For Behavior Testing Results and Synaptophysin Immunoreactivity

	Mean Time to reach escape platform in Morris water maze (sec)	Percentage of time spent in target quadrant	Distance traveled to reach escape platform (feet)	Mean area of CA1 hippocampus containing synaptophysin - IR
Lactating	**21.54	*46.34	*85.35	*6011.95[2]
Virgin	12.89	*53.97	66.25	*9557.10[1]
Lactating without pups	9.81	48.78	*49.20[1]	*6315.16[2]
Primiparous	12.63	49.78	*57.20[1]	*9096.52[1]

** = significantly different from all others
* = significantly different from each other
[1] = not significantly different from each other
[2] = not significantly different from each other

Significance: $p < .05$

Table 5

Correlations Between Behavioral Results and SYN – IR Over All Reproductive Groups

		Latency to Escape	Percentage of Time in Target Quadrant	Path Length	SYN-IR
Latency to Escape	*r*	1.0			
	p	0.0			
Percentage of Time in Target Quadrant	*r*	-.544	1.0		
	p	.456	0.0		
Path Length	*r*	.968	-.327	1.0	
	p	.032*	.673	0.0	
SYN-IR	*r*	-.390	.853	-.240	1.0
	p	.610	.147	.760	0.0

* = significance; $p < .05$

Table 6

Mean Latency to Escape (in Seconds) From the Morris Water Maze on each Day of Testing Over All Reproductive Groups.

Day of Testing	Latency to Escape onto a Hidden Platform
1	46.87 sec
2	27.83 sec
3	19.15 sec
4	14.94 sec

Figure Captions

Figure 1. Synaptophysin immunoreactivity (μm^2) in the peak of the CA1 hippocampus as a function of reproductive experience.

Figure 2. Latency to escape (in seconds) onto a hidden platform in the Morris water maze on day of testing as a function of reproductive experience.

Figure 3. Percentage of time spent in the target quadrant (containing the hidden platform) of the Morris water maze on day of testing as a function of reproductive experience.

Figure 4. Distance traveled in feet (averaged across all trials on day of testing for each animal) to reach the hidden platform in the Morris water maze as a function of reproductive experience

Figure 5. Synaptophysin immunoreactivity (μm^2) in the peak of the CA1 hippocampus in animals trained to escape onto a hidden platform in the Morris water maze

Figure 1

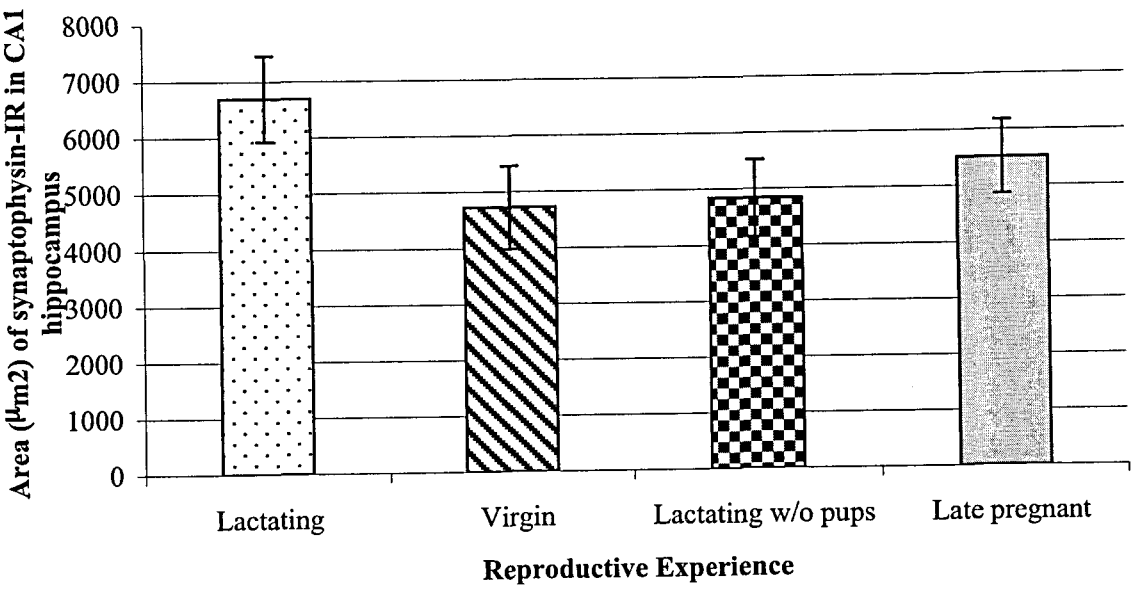

Area (μm2) of synaptophysin-IR in CA1 hippocampus
8000
7000
6000
5000
4000
3000
2000
1000
0
Lactating
Virgin
Lactating w/o pups
Late pregnant
Reproductive Experience

Figure 2

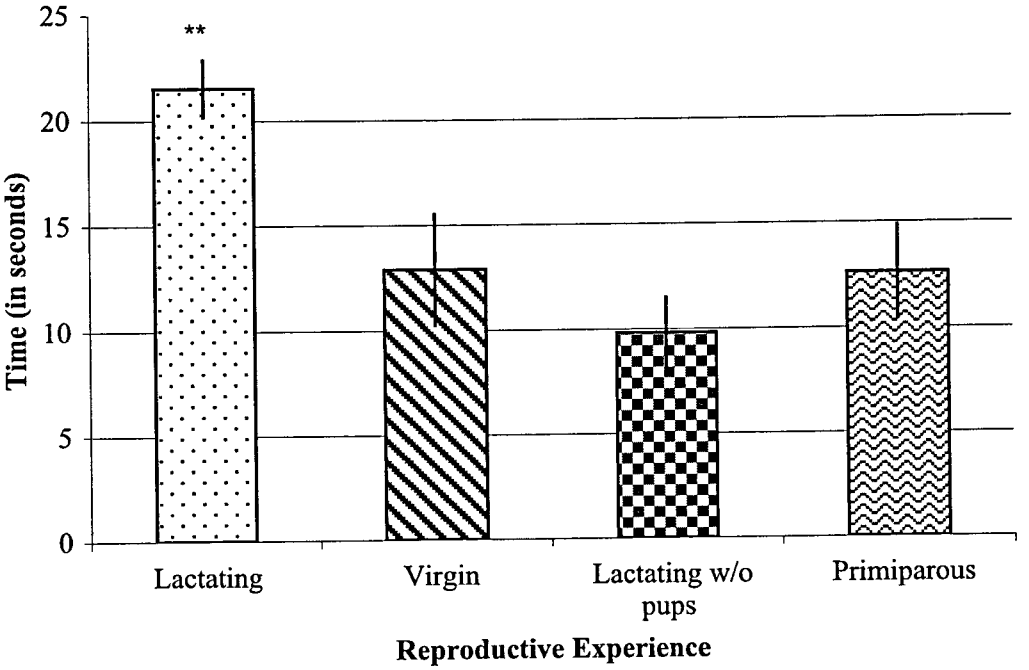

25
20
15
10
5
0
**
Time (in seconds)
Lactating
Virgin
Lactating w/o pups
Primiparous
Reproductive Experience

Figure 3

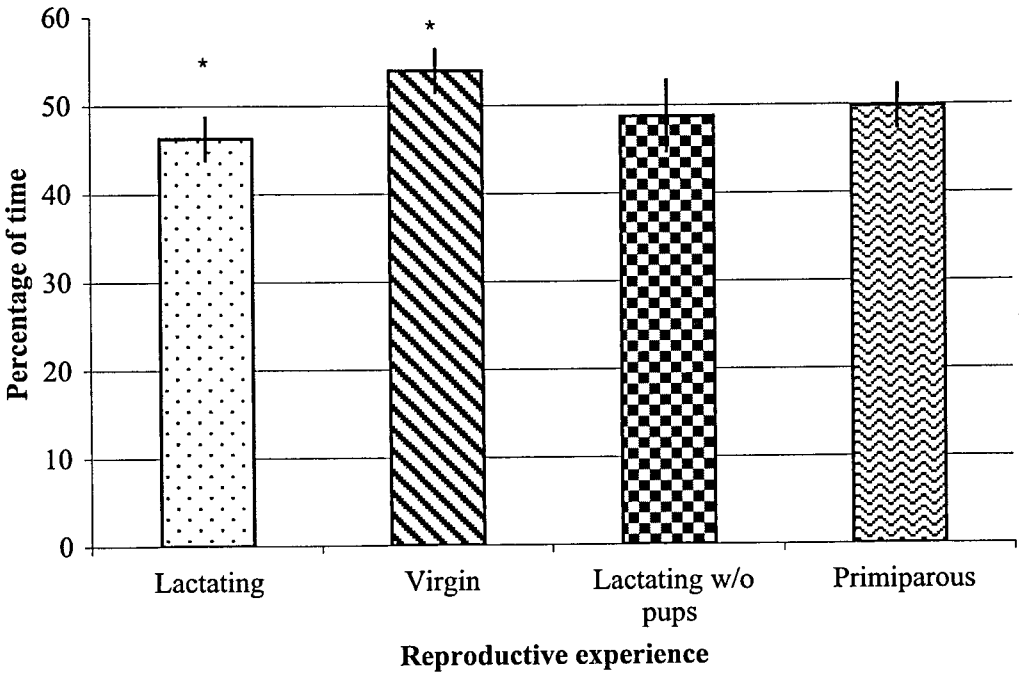

60
50
40
30
20
10
0
Percentage of time
*
*
Lactating
Virgin
Lactating w/o pups
Primiparous
Reproductive experience

Figure 4

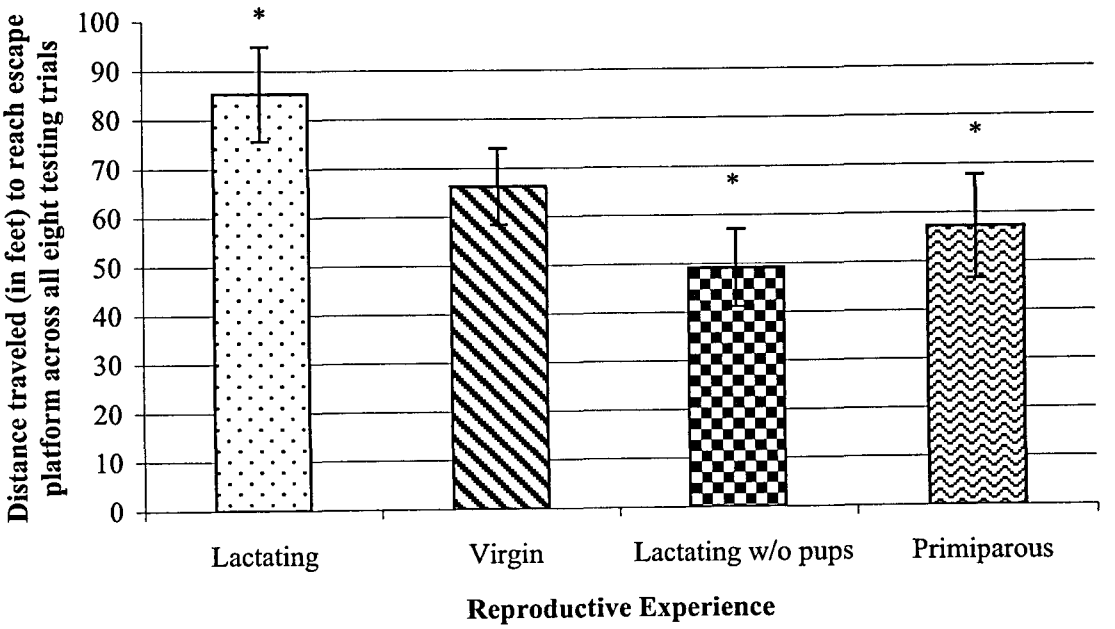
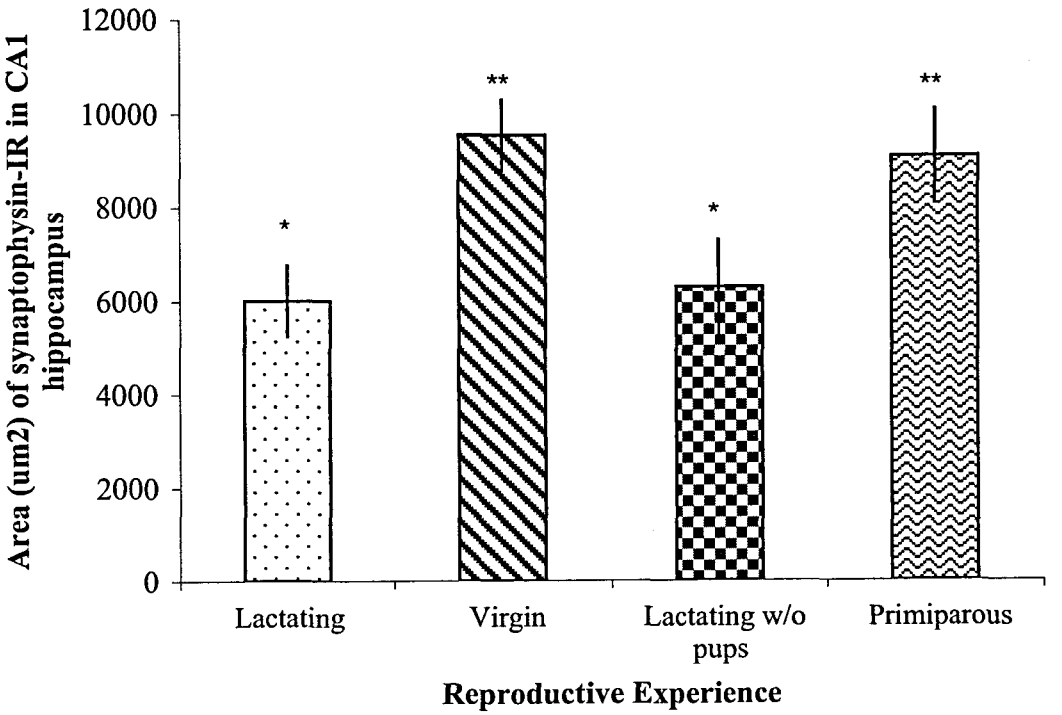
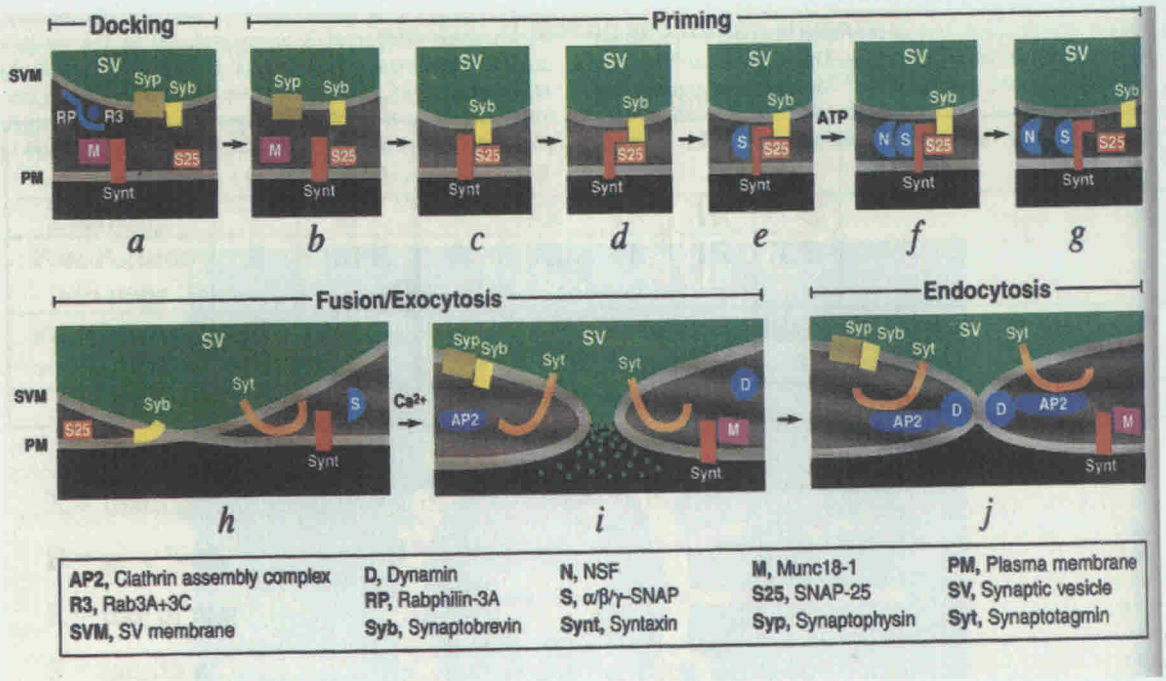

Distance traveled (in feet) to reach escape platform across all eight testing trials
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Lactating
Virgin
Lactating w/o pups
Primiparous
Reproductive Experience

Figure 5


Area (um2) of synaptophysin-IR in CA1 hippocampus
12000
10000
8000
6000
4000
2000
0
*
**
*
**
Lactating
Virgin
Lactating w/o pups
Primiparous
Reproductive Experience

Appendix A

Binding relationship between synaptophysin and synaptobrevin



From Südhof, 1995

Appendix B

Schedule of mating and testing animals

	Gestation		Lactation								
Reproductive Group	Day 0	Day 20-21	Day 1	Day 2	Day 3	Day 4	Day 5	Two week interval	Day 1	Day 2	Day 3	Day 4
Lactating	X	B	R	TR	TR	TR	T/S					
Post Partum w/o pups	X	B/PR	R	TR	TR	TR	T/S					
Primiparous	X	B	Pups were kept during this time					PR	TR	TR	TR	T/S
Virgin									TR	TR	TR	T/S

X = mating

B = give birth

R = day of rest

S = sacrifice

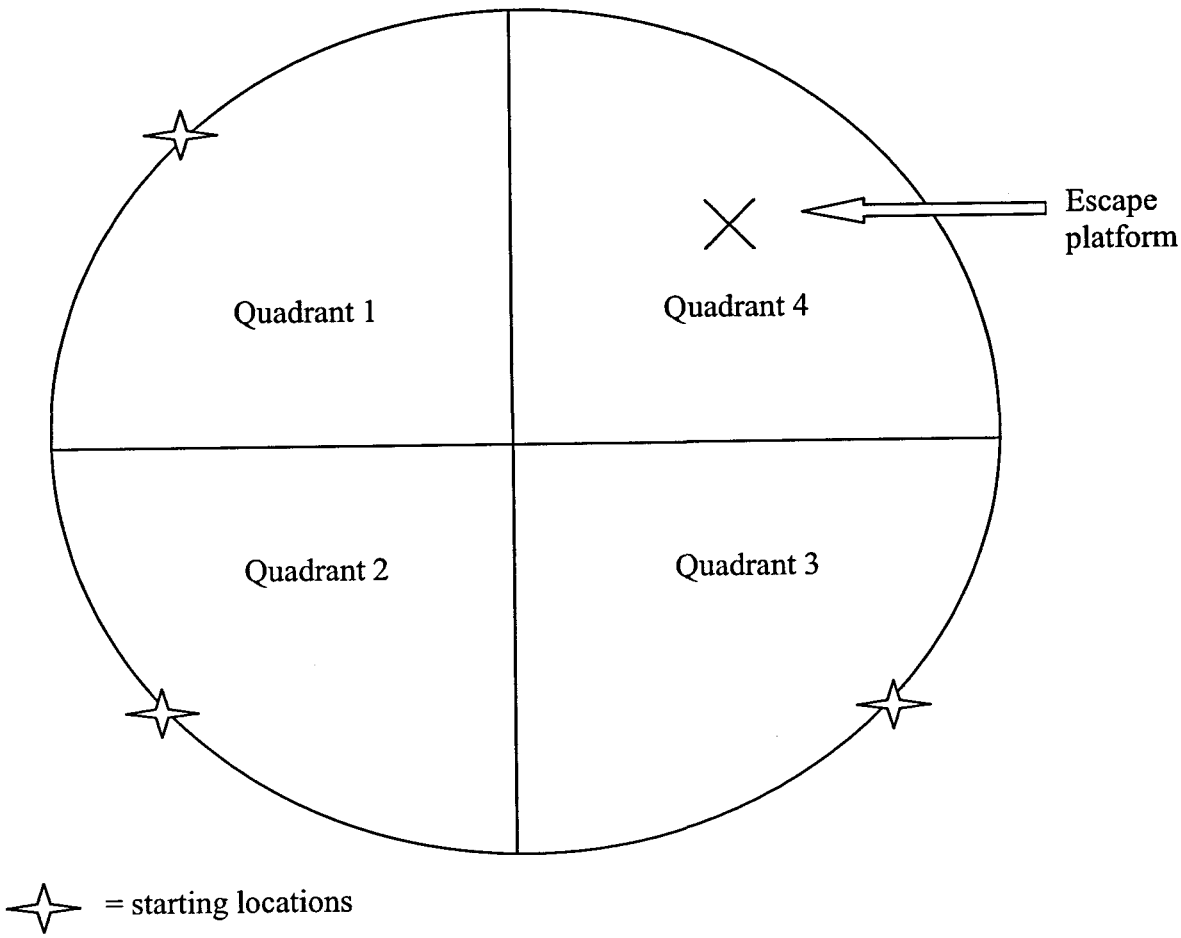
T = day of testing

TR = training in maze

PR = pups removed immediately

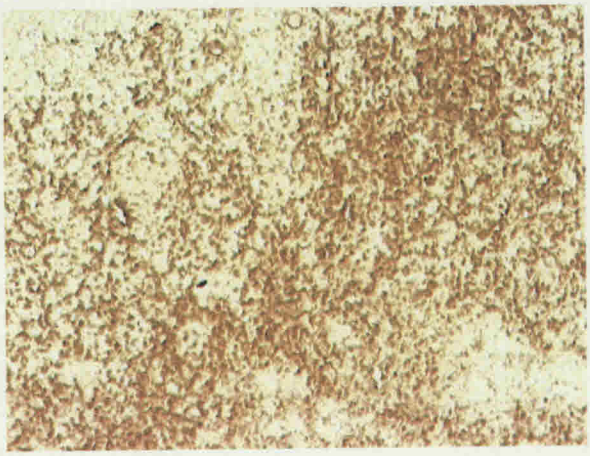
Appendix C

Diagram of Morris water maze and pertinent locations



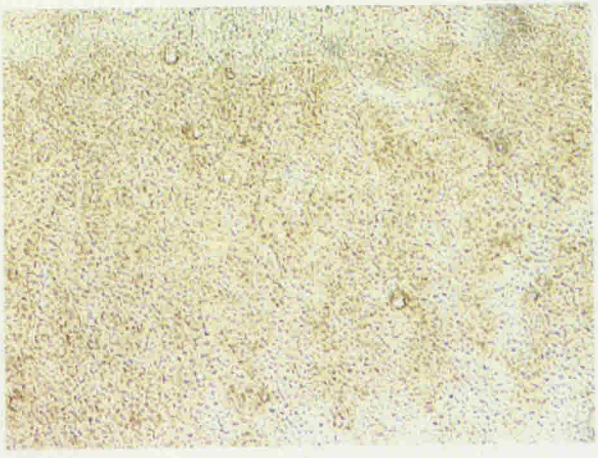
Appendix D

Representative images of SYN-IR in the CA1 hippocampus of each reproductive group

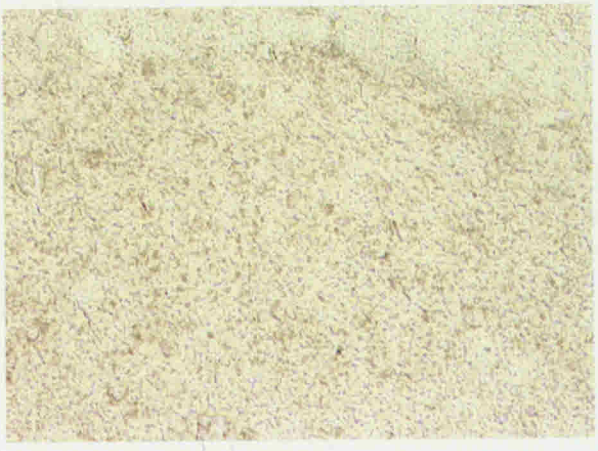


Primiparous

Virgin



Lactating without pups



Lactating