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Alterations in neurogenesis following the transition from virgin to maternal/lactating female

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Abstract

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The hippocampus displays hormone induced plasticity during estrus. Pregnancy, which exposes a female to a significantly longer duration of elevated estrogen and progesterone, results in even greater changes in neurons in the CA1 region of the hippocampus, with late-pregnant and lactating females displaying a significantly higher concentration of apical dendritic spines. Hippocampal astrocytes were increased in number and showed more and thicker processes in late-pregnant and lactating females. Such anatomical changes may also enhance spatial learning and memory. Changes in reproductive capacity may influence the rate at which new neurons are born, so called neurogenesis. In two experiments, neurogenesis in female rats was examined by quantifying the number of new neurons using the DNA marker, bromodeoxyuridine (BrdU), in the region of the DG of virgin, late-pregnant, lactating females, and foster mother virgins. Experiment 1 focused on examining the role of reproductive experience on neurogenesis, and lactating animals showed an increase in the level of neurogenesis with longer duration of pup exposure compared to late-pregnant animals. Experiment 2 examined the role of pups on neurogenesis and showed that animals with longer exposure

to pups had a decreased level of neurogenesis compared to foster mothers and primiparous mothers without pup exposure. These data suggest that reproductive experience modifies neurogenesis. The events surrounding pregnancy may focus both resources and mechanisms of neuronal programmed cell death on the production of the maternally efficient brain. Pups may represent a kind of enriched environment, which, has been shown to enhance neurogenesis. Females thus equipped may better respond to the new learning demands and challenges characteristic of motherhood.

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

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ALTERATIONS IN NEUROGENESIS FOLLOWING THE TRANSITION FROM VIRGIN TO MATERNAL/LACTATING FEMALE

By

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Reproduction is necessary. In order for genes to be passed from one generation to the next, the female (primarily) faces the daunting task of overcoming many natural and unnatural obstacles. Any advantage given to her, therefore, is likely to translate into increased reproductive fitness. In mammals, the focus of the current work, mating, pregnancy, and lactation change the behavior and physiology that are geared toward achieving this evolutionary goal. Research that examines maternal behavior, hormones, changes in the morphology of the brain, and learning, has begun to address basic questions pertaining to reproduction and how it changes the life of an organism, effecting successful reproduction and subsequent genetic representation.

Maternal Behavior

Parental care is a complex and crucial behavior required for the survival of the offspring of many species, particularly mammals. The physical development of the offspring at time of delivery determines the degree to which parental care is needed from both the mother and the father. For most mammals, it is the mother who provides primary care to the offspring due to lactation (Numan, 1994). In the rat, offspring are born altricial, immobile, and helpless, thus requiring constant maternal care. Among

other physical limitations, newly parturient pups are incapable of sight, temperature regulation, and waste elimination (Numan). Thus, pup survival is totally dependant on the abilities of the mother rat to properly care for and meet the needs of her young. Evolutionarily, it is the mother who is most adept at parenting that will ensure offspring survival and the representation of her genes in future populations. So, what does the mother rat do to care for her offspring successfully? Is she born with the necessary maternal behavior "knowledge" or is it learned? In other words, is maternal behavior the result of changes in the brain or exposure to pups? The answers are both intricate and intriguing.

In most rodents, maternal behavior (MB) generally consists of nest building, crouching, nursing, pup retrieval, anogenital licking, and defense of the young. Each of these behaviors has a particular purpose for the survival of the mother and her pups. Nest building, which begins during the last few days of pregnancy, provides an area that promotes warmth for the mother and her pups (Kinder, 1927; Leblond, 1940), as well as, protection from predators. Crouching over the pups provides additional warmth and presents the mammary glands of the mother for nursing by the pups. While at the nest site, the mother grooms and licks the anogenital region of the young pups to stimulate urination and defecation and to re-ingest water lost through lactation (Numan, 1994). If

perchance a pup wanders out of the nest, the mother will retrieve it and will be aggressive (i.e. defend, attack, attempt to drive off) towards intruders of the nest, so called postpartum aggression. These seemingly simple behaviors exhibited by the mother can be clearly divided into two types of behavior: pup-directed behavior (licking, nursing) and non-pup-directed behavior (nest building, maternal aggression; Numan). Both types of behavior are paramount to the survival of the young and are stimulated as a result of reproductive experience, hormones, and/or pup exposure.

Nulliparous/virgin female rats (Bridges, 1990), gerbils (Clark et al., 1986) and hamsters (Swanson & Campbell, 1979), for example, display low levels of interest towards pups (when initially introduced), often ignoring them, whereas animals with reproductive experience immediately exhibit strong MB towards pups at parturition. When the above mentioned nulliparous animals are continually exposed to pups they will begin to exhibit MB (consisting of nest building, pup retrieval, grooming, and crouching) after 5-7 days (Rosenblatt, 1967; Fleming & Rosenblatt, 1974; Bridges, 1990; Clark et al.; & Swanson & Campbell.). Additionally, studies have shown that maternal responsiveness develops gradually during pregnancy (with an early onset of MB during late-pregnancy) as opposed to just appearing suddenly at parturition in rats (Numan, 1994), hamsters (Buntin et al., 1984) and sheep (Poindron & Le Neindre, 1980). These

observations indicate that the state of pregnancy, parturition, and the associated endocrinological changes facilitate a more rapid onset of maternal responsiveness.

Hormones and Maternal Behavior

Female animals exhibit different mating behavior during different phases of the estrus cycle as a function of changes in hormonal levels (Nelson, 1995). When estrogen and progesterone levels peak during the proestrus phase (see Figure 1, from Ganong,1993), the female animal becomes sexually receptive and exhibits copulatory behaviors toward a male. These behaviors consist of ear wiggling, hopping and darting, and lordosis, the female mating posture (Nelson). Without the transient stimulation of these behaviors via specific endocrinological changes, the female fails to become sexually receptive and produce offspring. Once an animal becomes pregnant, however, there are a significant number of changes taking place (both hormonally and behaviorally) which leads to the development of maternal behavior.

During pregnancy and parturition, steroid hormones such as estrogen and progesterone (Bridges, 1984), protein hormones such as placental lactogens and prolactin (Bridges, 1994), and the neuropeptide, oxytocin, (Insel, 1990) combine with additional hormones to contribute to the onset of MB. Estrogen levels in the pregnant rat remain low during the beginning of pregnancy and increase substantially during late-pregnancy

(day 18-21; Bridges, 1984). Progesterone levels, however, peak around day 6 of pregnancy, decline during mid pregnancy, and then peak again around gestation day 12 (Bridges). Prolactin (PRL) levels remain low during gestation and increase just before parturition (see Figure 2; Svare, 1990). During lactation, estrogen and progesterone levels remain low. Prolactin peaks around the fourth postpartum day, begins to subside by the twelfth postpartum day and returns to low levels by the time the pups are weaned (see Figure 3; Svare).

Bridges (1994) demonstrated that in a nulliparous rat primed with estradiol (E_2; synthetic form of estrogen) and progesterone (P), PRL itself could stimulate MB. Animals that have undergone hypophysectomies (removal of the pituitary gland) and lack PRL fail to exhibit MB when given E_2 and P (Bridges). When these same animals are then given ovine PRL or an anterior pituitary graft (which produces PRL), MB latencies are decreased (Bridges). It was determined that prolactin levels were significantly higher in animals that responded faster, therefore, creating a correlation between elevated levels of PRL and decreased latencies of onset of MB (Bridges). Bridges offered further evidence of the role of prolactin in MB by suppressing natural prolactin secretion using bromocriptine, a dopamine agonist. Administration of bromocriptine subsequently

prevented MB; this effect was reversed by concurrent administration of ovine prolactin, thereby overriding the behavioral effects of bromocriptine.

Placental lactogens, PRL-like molecules secreted by the placenta, are present in the blood in high levels throughout the pregnancy of the rat (Voogt et al., 1982; Tonkowicz & Voogt, 1983). They may be directly involved with the onset of MB (Bridges, 1994). When injected into hypophysectomized, steroid treated (E_2 and P) nulliparous animals, placental lactogens stimulate maternal care (Bridges). Additionally, oxytocin (OT), released from the pituitary gland, has been identified as an important hormone involved in MB. It has been established that OT released immediately after parturition is associated with the onset of maternal responsiveness. Further evidence suggests that this release of OT stimulates PRL release as well (Ben-Jonathan et al., 1989). Once the MB is established however, the brain no longer requires brain OT arousal for continued maternal care (Panksepp, 1998). Thus, whereas OT may be paramount to the initial trigger of MB, social learning, other hormones (i.e. prolactin), and sustained changes in brain morphology (via hormonal influences) are important for sustaining MB once it is triggered and established (Panksepp).

Hormonally Induced Changes in the Brain

Hormones elicit MB via binding to specific receptor sites in the areas of the brain particularly associated with MB (Numan, 1994). The medial preoptic area (mPOA), for example, is directly related to MB and the hippocampus (HI) is directly associated with learning and memory. These areas of the brain are of special interest to researchers because the new demands placed on the mother rat include not only the appropriate MB but also require efficient learning and memory to successfully overcome the challenges of motherhood. These changes in MB led researchers to investigate the relationship of reproductive cycles, reproductive experience, the associated hormones of the two, and potential changes in the morphology of the brain.

Estrogen and progesterone, which modulate many behaviors in the female rat, can affect morphology of neurons in adult females (Gould, Woolley, Frankfurt, & McEwen, 1990). During the estrus cycle, the HI displays hormone induced plasticity (Woolley & McEwen, 1992; Woolley & McEwen, 1993; & McEwen and Woolley, 1994). More specifically, during proestrus when estrogen is at the highest level, the apical dendritic spines in the CA-1 region of the HI were found to have a higher density than those during phases with less estrogen. Horner (1998) has proposed that these differences in dendritic spine morphology may contribute to the neural basis of differences in behaviors such as learning and memory. Earlier work done by Woolley et al. (1996) suggested such an

effect as well. The frequency of multiple synapse boutons in CA-1 region of the hippocampus as a function of E_2 treatment in the female rat was examined (Woolley et al.). Quantitative analysis revealed that in ovariectomized estradiol treated animals, there was a 25.5% increase in the number of synapses formed compared to control animals.

Woolley and McEwen (1992) suggest that circulating levels of E_2 could also regulate activity in the CA- 1 region of the HI. Observed changes in asymmetric (excitatory) synapses support the possibility that changes in density of dendrites during elevated levels of E_2 may effect CA-1 pyramidal cell excitability (Woolley and McEwen, 1992). Furthermore, this E_2 effect suggests that cognitive function (learning ability), which is dependent on the synaptic communication of neurons, may also be affected by these hormonally induced changes in synaptic density. Gould et al. (1990) demonstrated that gonadal steroids are necessary for the maintenance of adult CA-1 hippocampal pyramidal cell structure. Whereas removal of gonadal steroids via ovariectomy resulted in a dramatic decrease in CA-1 dendritic spine density, estradiol replacement prevented it, therefore suggesting a neuroprotective effect of E_2. Progesterone enhanced the effect of E_2 within a short time period of approximately five hours (Gould et al., 1990).

Trainer et al.(1997) asked what would happen to these same spines during pregnancy, when gonadal steroids are significantly higher and for a significantly longer

duration. It was shown that the brain of a nulliparous animal is very different than that of a late pregnant or lactating female, particularly in the HI. The CA- 1 HI neurons in the late-pregnant (LP) female produced more dendritic spines than their nulliparous counterparts (Trainer et al., 1997). In addition, HI CA-1 neurons from primiparous (one litter of pups) animals showed a greater concentration of dendritic spines compared to the nulliparous animals. In addition to these changes in neurons, changes in glial cells have been found. Gifford (1999) found that reproductive experience affects the concentration and complexity of glial cells (measured by glial fibrillary acidic protein immunoreactive [GFAP-ir] staining) in both the HI and the mPOA. It was demonstrated that glial cells were increased in complexity and concentration in late-pregnant and lactating females compared to nulliparous animals. These data on glia are significant because glia act as support structure for neurons, providing functional and physical support for newly born neurons in order to survive and migrate to their destination in the brain (Pinel, 1997). Such neural changes can be looked at as responses to the increased behavioral demands of the mother. Therefore, the brain of a late pregnant female appears to have an enormous capacity for change in both structure and, consequently, function.

The effects of E_2 and P on the structure and function of the brain are considerable and memory and other cognitive functions show concomitant fluctuations. The cyclicity

in the quality and quantity of hippocampal synapses (increased dendritic spine numbers accompanied by increased numbers of synapses on spines) may determine spatial learning and memory (McEwen and Woolley, 1994). Further, the role of E_2 in performance on a radial arm maze which requires spatial working memory was examined (Daniel et al., 1997). Three separate experiments demonstrated that E_2 treatment of ovariectomized rats resulted in significantly improved working memory performance on the radial arm maze (Daniel et al.). This finding supports the idea that neural changes in the brain (resulting from hormonal changes similar to those mentioned above) may underlie and/or promote learning and (spatial) memory.

Kinsley et al. (1999) have shown that female rats with maternal experience performed better on radial arm and dry land mazes, learning faster with fewer errors compared to nulliparous animals. These changes in learning are significant because learning and memory is essential for new mothers; it is crucial in the caring of pups to remember where food is stored, water can be found, nest sites are located and so forth. These findings support that learning and memory may be sensitive to hormonal fluctuations and/or changes in mother's environment. Was it the exposure to pups, the hormones, or both that contributed to the enhanced learning taking place by the reproductively experienced rats?

Regarding the environment, past research has demonstrated that environmental enrichment has a critical influence on brain morphology and function. Researchers have shown that animals living in an enriched environment make fewer errors in a maze compared to those in an isolated environment (Cummins et al., 1973). Additionally, the same animals living in an enriched environment have significantly heavier brain weights (Cummins et al, 1973; Cummins et al., 1977), dendritic branching (Volkmar & Greenough, 1972), and number of glial cells (Altman & Das, 1964). Recent research has revealed that changes in the environment, again, by way of enrichment, not only affect learning and memory but cell proliferation in the brain of adult female rats (Nilsson et al., 1999). Adult rats housed in an enriched environment showed improved performance on a water maze spatial learning task (Nilsson et al.). Additionally, enhanced neurogenesis (birth of new neurons) in the dentate gyrus (DG) of the HI was observed as a function of enriching environmental cues (Nilsson et al., 1999; Kemperman et al, 1997). These findings are interesting as we learn that the brain has the potential for remarkable plasticity and change due to a simple change in the environment.

Neurogenesis and Hippocampus

When one examines the numerous significant changes occurring in the brain of the pregnant female, they resemble another significant time in the life of the female -

That of early development which also includes changes in neurons, dendritic spines, glia, and the production of new neurons, so called neurogenesis. For example, during development, estrogen treatment has affected CA-1 and CA-3 HI volume as well as spatial navigation behavior (Isgor & Sengelaub, 1998). The female brain during pregnancy undergoes changes that may influence neurogenesis. For decades, it was believed that adult brains could not make new neurons and that through programmed cell death, stress, disease, etc., they lose cells for the remainder of life.

Thirty five years ago it was first determined that granule neurons in the adult rat were being produced in the DG of the HI (Altman & Das,. 1965). Although relatively unexplored for many years after that (according to Gould et al., 1999), Goldman and Nottebohm (1983) found neurogenesis in the song system (vocal control nucleus) of canaries. This was of particular interest because the production of new neurons correlated with the learning of bird song (Goldman & Nottebohm).

Recently, researchers have discovered that neurogenesis occurs throughout the life span into adulthood of some animals and, most recently, in humans (Kempermann & Gage, 1998). Cameron, Woolley, McEwen, and Gould (1993) reported that in the DG of rats, neurogenesis occurs postnatally and continues into adulthood. Additionally, Gould et al. (1998) discovered that in the DG, neurogenesis occurs throughout the adult life of

primates (marmosets) and can be negated by stressful experiences. Further, Tanapat et al. (1999) report that stereological analysis revealed a larger but transient increase in number of new neurons in female rats than males in the dentate gyrus of the HI. The production of these hippocampal neurons appears to be affected by ovarian hormone levels as demonstrated by a diminished number of neurons in ovariectomized females and a reversed effect for animals that received E_2 replacement (Tanapat et al., 1999). It was also noted that neurogenesis was altered as a function of different stages in the estrus cycle. Tanapat et al. (1999) showed that females in the proestrus stage produced more cells compared to those in estrus and diestrus. After examination of pyknotic (dying) cells during these phases of estrus, it was determined that E_2 not only stimulates cell proliferation, but enhances survivability of the neurons as well (Tanapat et al., 1999).

Whereas researchers have detected neurogenesis, no research has been done on the changes in reproductive capacity and the rate at which neurons are born. If the above-mentioned changes were taking place in the female brain as a result of pregnancy, (via hormonal changes, enriched environment etc.) why would neurogenesis not be affected? The changes in behavior coupled with the physiological changes in glia and existing neurons leads us to believe that neurogenesis may be increased and/or altered as a result of pregnancy and motherhood.

In two experiments, neurogenesis in female rats was examined by quantifying the number of new neurons using the DNA marker, bromodeoxyuridine (BrdU), in the region of the DG of virgin, late-pregnant, lactating females, and foster mother virgins. Experiment 1 focused on examining the role of reproductive experience on neurogenesis, whereas, Experiment 2 examined the role of pups on neurogenesis. Essentially, our hypothesis was that neurogenesis will be significantly enhanced by the reproductively experienced animals. Further, we hypothesized that pups will significantly affect neurogenesis in mother (including foster group) rats due to the stimulation pups provide the mother. It has been strongly documented that an enriched environment increases neurogenesis in the dentate gyrus of adult rodent (van Praag, et al., 1999; Kempermann et al., 1998a; Kempermann et al., 1997; Kempermann et al., 1998b).

BrdU is a thymidine analog that is incorporated into the S-phase of dividing DNA. During cell proliferation, the DNA must replicate before the cell is divided into 2 daughter cells. This close association between DNA synthesis and cell doubling makes the measurement of DNA synthesis very attractive for assessing cell proliferation. If labeled DNA precursors are added to cell culture, cells that are about to divide incorporate the labeled nucleotide into their DNA. The BrdU is then easily identified through single label immunohistochemical technique (Sigma, unknown).

Methods

Experiment 1

Animals:

Adult female Sprague-Dawley rats (N= 19; age 70-90 days) were non-systematically assigned to one of the four experimental groups. The groups consisted of a nulliparous animals (V), late-pregnant (LP) animals, and two different lactating groups which were differentiated by length of lactation before the animals are sacrificed (LA & LB). Animals were individually housed in standard cages with food and water *ad lib.* All animals were housed in a light:dark (14:10) and temperature (21°C) controlled room. Animals assigned to LP, LA, or LB were mated with stock males in our laboratory and day one of pregnancy was recorded on the first day that sperm was observed in the vaginal lavage.

Procedure:

Animals in the V group were weighed everyday and given injections intraperitoneally of 50mg/kg body weight of the DNA marker bromodeoxyuridine (BrdU) twice a day (~0900h & ~1900h) for three days (injection amount and procedure adapted from Tanapat et al., 1999). The LP group followed the same procedure but

received injections on days 18, 19, and 20 of their pregnancy. The Lactating A group received the same procedure but was given the injections on day 21 of their pregnancy and postpartum day 1 (p1) and p2, respectively (litters will be culled to six pups per animal for control over amount of stimulation). Finally, animals assigned to LB group received the above procedure but received injections on p1, p2, and p3. All animals were sacrificed on the fourth day, anesthetized, and perfused transcardially with 4% paraformaldehyde. Brains were removed and postfixed overnight in 4% paraformaldehyde and placed the next morning in 1.OM phosphate buffer and kept at 4°C until immunohistochemical processing.

Experiment 2:

Animals:

Adult female Sprague-Dawley (N=17) rats were non-systematically assigned to one of three experimental groups. The groups consisted of primiparous lactating animals p-14 (P+), an age matched primiparous group minus the pups (P-) (pups were removed from mothers at time of birth), and a foster group, which consisted of V animals exposed to pups daily. Foster pups were removed and replaced every morning to assure proper feeding and postnatal care. All animals were individually housed in standard cages with food and water *ad lib* with a light:dark (14:10) and temperature (21°C) controlled room.

Animals assigned to P+ and P- were mated with stock males in our laboratory and day one of pregnancy was recorded on the first day that sperm was observed in the vaginal lavage.

Procedure:

Animals in P+ and P- were weighed daily and given injections following the protocol above. P- and P+ animals received injections on p-11, p-12, and p-13 and were sacrificed on p-14. Foster moms received injections at the same times as P- and P+.

Immunohistochemistry

On the first day of processing, 40μm Vibratome sections were collected into cold 0.05M phosphate buffered saline (PBS) and processed using BrdU immunohistochemistry. Sections were washed in cold PBS (2x5min.) and incubated in glycine (0.05M PBS/0.1M glycine) for at least 30 minutes. Next, sections were washed again in PBS (3x5min.) and incubated in a .05% sodium borohydride solution for 15 minutes. The sections again were washed in PBS (4x5min.) and incubated in a 1%DMSO, 1%H202 PBS mix for 15 minutes. Sections were washed in PBS (3 x 5min.) and incubated in a 2N HCL solution at room temperature. Next, sections were washed in PBS again (3x5min.) and incubated in 20% normal goat serum (NGS)/ 0.3% Triton-X

solution for more than fifteen minutes. After this step was complete the last solution was removed from the wells and incubated overnight at 4°C in primary antisera (BrdU mouse monoclonal; 1:250 dilution; Becton-Dickinson).

The next day, all steps were executed at room temperature. Sections were rinsed in a PBS wash solution containing 1.0% NGS/ 00.02% Triton-X solution. Following the two-hour incubation in the secondary antibody, sections were rinsed in a PBS wash. Finally the sections were incubated in Avidin-Biotin Complex for an hour and then stained using DAB/Ni staining procedure. Once the sections were stained, they were mounted on gelatinized slides, cleared, and cover-slipped.

Quantitation

Slides were coded prior to quantitative analysis to ensure an unbiased cell count using BIOQUANT image analysis computer software. Every sixth section (for a total of 10 sections) of the DG (as defined by Gould and Cameron, 1996) was quantified to represent the entire structure (procedure adapted from Kempermann, Kuhn & Gage, 1998).

Results

All statistics were calculated using the SPSS statistical package. Figures 4 & 5 represent medians for all treatment conditions (additionally, means and standard errors for each treatment condition are represented in Tables 1 & 2). Data were analyzed with a Kruskal-Wallis non-parametric one-way analysis of variance (ANOVA; two tailed with an alpha level of 0.05) for each experiment to determine statistical significance. A Mann-Whitney U test (one tailed, alpha level of 0.05) was used to examine significant differences between all possible pairs of treatment conditions within both experiments and between experiments. Non- parametric tests were used because the data was unable to meet the assumptions for parametric tests due to a small N. It should be noted that the results of non-parametric test are less generalizable to the population (Gravetter & Wallnau, 1996).

The animals included in this experiment were run over the course of several different replicates of the protocol over eighteen months to insure that there were no seasonal/temporal confounds. The independent variable was the stage of reproduction and the seven levels were: late pregnancy, foster virgins, control animals (virgin), three stages of lactation, and a pup minus group. The dependant variable is quantity of BrdU-immunoreactive neurons in the dentate gyrus of the hippocampus.

Experiment 1:

Means of BrdU-immunoreactive (ir) neurons in the dentate gyrus of the hippocampus and standard errors were calculated for each IV (see Table 1) Medians for each treatment condition are represented in Figure 4. The Kruskal Wallace non-parametric one-way ANOVA revealed that a significant difference existed between the experimental groups of Experiment 1 ($p = .0476$). The Mann-Whitney U test revealed significant differences between the Virgin and Late-pregnant group ($p=.0340$) with the Late-pregnant group having a lower amount of BrdU-ir neurons. Late-pregnant and Lactating B group ($p=.0053$) were significantly different with the Lactating B group having more BrdU-ir neurons and Lactating A and Lactating B group ($p=.0417$)were significantly different with the Lactating B group having more BrdU-ir neurons.

Experiment 2:

Means of BrdU-immunoreactive (ir) neurons in the dentate gyrus of the hippocampus and standard errors were calculated for each IV (see Table 2). Medians for each treatment condition are represented in Figure 5. The Kruskal Wallace non-parametric one-way ANOVA revealed that a significant difference existed between the experimental groups of Experiment 2 ($p = .0240$). The Mann-Whitney U test revealed significant differences between the Primiparous plus pups (P+) group and the Primiparous minus pups (P-) group ($p=.0053$) with the P+ group having significantly

fewer BrdU-ir neurons and a significant difference between the P- group and Foster group (p=0340) was revealed with significantly fewer BrdU-ir neurons in the Foster group.

Other relevant comparisons:

The Mann-Whitney U test also revealed a significant difference between the Lactating B and Lactating C+ treatment conditions across both experiments (LB had more BrdU-ir neurons; p=.005). A trend was revealed between the Virgin and Lactating C- treatment group (p= .0873) with more BrdU-ir neurons found in the Virgin group.

Discussion

Experiment 1

The current results suggest that neurogenesis is modified by reproductive experience and pup exposure. Specifically, the results in Experiment 1 indicate that whereas neurogenesis is high in the nulliparous/virgin (V) treatment group, it is decreased significantly in the late-pregnant (LP) group and begins to rise again during the two phases of early lactation, lactating group A (LA) and lactating group B (LB). What factors might cause such a dramatic decrease in neurogenesis during LP yet stimulate neurogenesis during early lactation?

Several possibilities exist for the significant decrease in neurogenesis during the transition from the V to LP treatment conditions. During late-pregnancy in rats (specifically the last three days) stress hormones (glucocorticoid concentrations) increase markedly, reaching a maximum at parturition (Dupouy, Coffigny, & Magre 1975; Voogt, Sar, & Meites, 1969; Challis, Kendall, Robinson, & Thornburn, 1977). Furthermore, it has been well documented that stress, which releases high levels of glucocorticoids that bind to receptors in hippocampus (HI), significantly inhibit neurogenesis in adult tree shrews (Gould et al., 1997), marmoset monkeys (Gould et al,. 1998), and rats (Cameron & Gould, 1994; Gould et al., 1992). Whereas these glucocorticoids decrease neurogenesis and can be a determining factor in the late-pregnant female neurogenesis levels, it should be noted that there are other factors working to enhance neurogenesis during the same period of late-pregnancy.

During late-pregnancy estradiol (E_2) levels are elevated (Rosenblatt, 1990). So when examining the decrease in neurogenesis between V and LP it is important to consider that E_2 has been shown to stimulate neurogenesis (Tanapat et al., 1999), as well as, exert neuroprotective effects over neurons by preventing neuronal death (experimentally induced by kainic acid; Azcoitia, Sierra & Garcia-Segura, 1998). What

could be taking place during this time of late pregnancy and equally importantly, why does neurogenesis appear to be at the highest level in the V treatment group?

First, we do not know anything about the role of apoptosis (programmed cell death) in any of the animals for this project. It could be that in the V group, a high rate of apoptosis occurs, therefore increasing the "need" for new neurons. In the LP group, perhaps high levels of estrogen are protecting existing neurons from the detrimental effects of glucocorticoids, therefore resulting in less of a "need" for new neurons. Or, as mentioned earlier, E_2 has been shown to be stimulatory of existing neuronal structure in the HI such as dendritic branches (Woolley & McEwen, 1992; Woolley & McEwen, 1993; & McEwen and Woolley, 1994) and number of synapses (Woolley et al., 1996), so perhaps E_2 is stimulatory to existing neurons in structure and function and decreases the above mentioned "need" for new neurons. Secondly, it could be as simple as resources being diverted during fetal development from the mother to the pups. Products such as tissue, placenta, fetuses and amniotic fluid formed via glucose and amino acid absorption from the mother during pregnancy, are energetically and metabolically expensive for the mother (Numan, 1994). After all, survival depends on not only parental care, as mentioned earlier in this paper, but proper development of the pups as well.

Significant differences between the LB group and LP and LA group indicates that neurogenesis is possibly affected by early pup exposure. In the first several days of lactation, the mother rat receives large amounts of stimulation (olfactory, auditory, tactile etc.) from the pups (Numan,1994). These stimuli may serve as an environmental enrichment model for the mother rat. As discussed earlier in this paper, it has been demonstrated that environmental enrichment affects brain morphology. Additional research confirms that an enriched environment stimulates neurogenesis, as well as, inhibits apoptosis in the HI of adolescent rats (Young, Lawlor, Leone, Dragunow & During, 1999).

Other factors significant to the period of early lactation of the mother rat include hormonal changes and acquisition of MB. Recall that oxytocin (OT) and prolactin (PRL) surge at parturition and levels remain high during the LA and LB treatment groups. It is possible that in addition to hormones stimulating MB, they stimulate neurogenesis too. In other words, the increase in neurogenesis could be due to the direct effects of the hormones, or to the learning, memory, or physical activity associated with MB and the increased demands of motherhood (Kinsley, 1999).

Learning stimulates neurogenesis in the DG of the HI of adult male rats (Gould, Beylin, Tanapat, Reeves & Shors, 1999). Gould et al. (1999) showed that adult-

generated hippocampal neurons are specifically affected by and possibly involved in associative memory formation (including trace eye-blink conditioning and spatial water-maze training). More specifically, Gould et al. suggest that the learning about space (via water maze training) and time (via trace eye-blink conditioning) under specific conditions has a trophic effect on adult generated HI neurons, thus suggesting further a function for these new neurons in different kinds of learning. If there is a temporal dimension of memory, could the neurons being produced in the lactating maternal female be considered "maternal neurons", those that exist for maternal learning only? Gould et al.'s learning and neurogenesis model parallels what is happening with the new mother again, she must remember where the food and water caches are, where the nest site is located etc. During this time in the mother's life, activity levels increase as she retrieves pups, crouches over them and forages for food.

Van Praag, Kempermann and Gage (1999) examined activity level and neurogenesis and demonstrated that voluntary running increases cell proliferation in the DG of adult mice compared to yoked swim controls. Van Praag et al. report that the level of neurogenesis that was detected in the "runners" was similar to that level detected in mice living in an enriched environment. More recent research by Van Praag, Christie, Sejnowski and Gage (1999) revealed that running not only enhanced neurogenesis, but

learning and long term potentiation (LTP) as well. Again, these findings are relevant to changes taking place in the maternal female because LTP is associated with the learning and memory regulated by the HI. We have already examined evidence that suggests that reproductive experience effects learning and memory (Kinsley et al., 1999), so this finding about LTP is significant in establishing further the changes that can potentially take place in the brain of a pregnant or lactating female. Although our current data does not allow us to examine and separate all the possible factors affecting neurogenesis in the reproductively experienced female they do pave the path for future projects.

Experiment 2

The results in Experiment 2 suggest that pup exposure significantly affects neurogenesis in the mother rat but not in the direction that we had originally hypothesized. As the data depict in Figure 5, the P+ treatment group had the lowest median number of BrdU-ir cells with the Foster group second to lowest and the P- group with the highest median number of BrdU-ir cells. This was surprising because there was an increase (although not significant) from LA to LB in Experiment 1 with the only difference between the two groups being longer duration of pup exposure in the LB treatment group. We expected to see an increase in neurogenesis as duration of pup exposure increased based on the previously mentioned environmental stimulation studies

and the increase that was noted from the LA to LB group in Experiment 1. There are several factors that may explain our results.

When we first formulated our hypotheses we speculated that longer exposure to pups would be analogous to a longer duration in an enriching environment. What was not considered was that by the time the mothers in both the P+ and Foster groups were given injections of BrdU, the pups were becoming more independent and moving about the cage more freely than they ever had. This suggests that the mothers may not be receiving the same stimulation, as they have less and less contact with the pups as each day passes. Furthermore, during the end of the second week of lactation, glucocorticoids begin to rise, which consequently raise maternal body temperature, and the mother begins to spend less time in the nest to avoid over-heating (Numan, 1994). This rise in glucocorticoids is believed to be stimulated by release of suckling stimulated ACTH (Numan). As mentioned in the discussion for Experiment 1, the effect of glucocorticoids on neurogenesis is inhibitory.

The level of neurogenesis in the Foster mother group may provide an explanation that there are effects of pup stimulation but that without going through pregnancy and associated hormonal changes, the brain may not be "primed" for more significant changes to take place. The higher level of neurogenesis found in the P- group could be

due to those animals beginning to cycle regularly, essentially returning to physiological state of the nulliparous or virgin animal.

Other relevant comparisons

The significant difference found between the LB and P+ groups could indicate that long term exposure to pups suppresses neurogenesis, via lack of stimulation or habituation (also considered a form of learning). Habituation to pup odors, tactile stimulation, auditory stimulation etc. occurs in the mother rat thus suggesting that the "novelty" of the pups has decreased since the early periods of lactation. Additionally, MB is firmly established during the P+ group thus possibly suggesting that acquisition of MB plays a role in neurogenesis as well. In other words, perhaps the learning taking place by the new mother (LB) is stimulating neurogenesis and once it is firmly established, higher levels of neurogenesis are no longer required (P+).

It is of some interest that informal observations of maternal behavior and levels of neurogenesis were performed and a casual correlation revealed a trend for a negative correlation. Simply, this may suggest that higher levels of BrdU are associated with earlier onset of MB. Additionally, a trend was revealed between V and P- suggesting that there may be a parity effect. Perhaps reproductive experience reduces baseline levels of neurogenesis.

Whereas this study answers only a couple questions, it sets the groundwork for many interesting studies to follow. We do not know anything about the neurons long term, for example. What happens to these new neurons? Do they die as quickly as they are born? Kempermann et al. (1997) revealed that an enriching environment has a "survival-promoting effect" for neurons proliferating in the dentate gyrus (DG) of the HI in adult mice. Additionally, Kempermann, Kuhn, & Gage (1998) demonstrated that neurogenesis in aging mice can be stimulated by living in an enriched environment. This finding in conjunction with the findings that suggest neuroprotective effects of estrogen have major implications for the aging brain. This could be a potential direction for the future research of neurodegenerative diseases, such as Parkinson's.

Examining levels of apoptosis in our same experimental groups would add insight into the fate of the neurons as well. Also, what happens when an animal goes through multiple pregnancies? Does the level of neurogenesis plateau or keep fluctuating? In other words would the fluctuations that we observed in primiparous animals occur again in the same degree for multiparous animals (multiple pregnancies), or does the first pregnancy establish a baseline level of neurogenesis so that the female doesn't have to "reinvent the wheel," so to speak, for each subsequent pregnancy? Further, if these new neurons or "maternal neurons", as mentioned earlier, are serving a specific role in MB

perhaps once they exist they do not have to be "reborn" for future reproductive experience.

Whereas we examined only the HI, neurogenesis has been detected in other areas of the brain as well. Gould, Reeves, Graziano, & Gross (1999) have found neurogenesis in the neocortex of adult primates. The neocortex is important for cognitive function, so it would be interesting examine the cortex (homologous structure in the rat) in the same treatment groups of our experiment. Would acquisition of MB, pup stimulation, or hormones change neurogenesis in an area of the rat brain associated with cognition? If estrogen boosts memory and neurogenesis in the HI of rats (Daniel et al., 1997) could it boost cognition and neurogenesis in the cortex as well? Additionally, the role of additional hormones (PRL and OT) could be examined in the same fashion due to their association with the onset of MB.

We have identified several possible factors that might influence neurogenesis but it is surprising that very little is known about the mechanisms underlying the regulation of neurogenesis. Specifically, what are the pathways between environmental enrichment and changes in neurogenesis? Kempermann et al. (1997) suggest that trophic factors, such as fibroblast growth factor 2 and epidermal growth factor, can influence the fate of neuronal progenitor cells. Cameron, McEwen, & Gould (1997) revealed that NMDA

receptors effect neurogenesis. Specifically, it was demonstrated that stimulation of NMDA receptors decreased the rate of adult hippocampal neurogenesis. Closely related to this is the finding that running enhances LTP (Van Praag et al., 1999). NMDA receptor activity induces LTP (Pinel, 1997). Using the aforementioned findings, a question for future research could be to examine the "upstream" signals for neurogenesis.

And last but not least, what about males? It has been demonstrated that males are capable of exhibiting parental behavior towards pups following prolonged exposure to the pups (Rosenblatt, 1967). As we saw differences in the foster mother brains pertaining to levels of neurogenesis perhaps the levels in males following pup exposure might change as well. Brain equals behavior, so changes in behavior must be reflective of changes in the brain. What about bi-parental males? What are the mechanisms that regulate paternal behavior of both parents and would that effect neurogenesis to a different degree in one or both parents?

In conclusion, our data reveal that reproductive experience and pup exposure effect the birth of new neurons in the adult female rat. These data are important both as applied to basic neuroscience and clinical neuroscience. Basic neuroscience benefits from research such as this by getting a better understanding of what changes our brains and under what conditions they change. Clinically, determining what influences cell

proliferation in the brain may contribute the development of new drugs used for the treatment of brain damage whether it be by disease or trauma. As most studies, these experiments present more questions than they answer, but that is the beauty of scientific research.

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Table 1

Mean number of BrdU-ir neurons (±SEM) of different treatment conditions for Experiment 1

Treatment Condition	Means (±SEM)
Virgin	777.48 (144.96)
Late-Pregnant	437.53 (38.98)
Lactating-A	521.23 (75.24)
Lactating-B	780.88 (87.16)

Table 2

Mean number of BrdU-ir neurons (±SEM) of different treatment conditions for Experiment 2

Treatment Condition	Means (±SEM)
Primiparous +	262.10 (40.83)
Primiparous -	583.48 (73.46)
Foster	378.42 (63.88)

Figure Captions

Figure 1. Levels of circulating estrogen and progesterone during the ovarian cycle in rats (adapted from Ganong, 1993).

Figure 2. Circulating levels of progesterone, estradiol, and prolactin during pregnancy in the rat (adapted from Rosenblatt,1990).

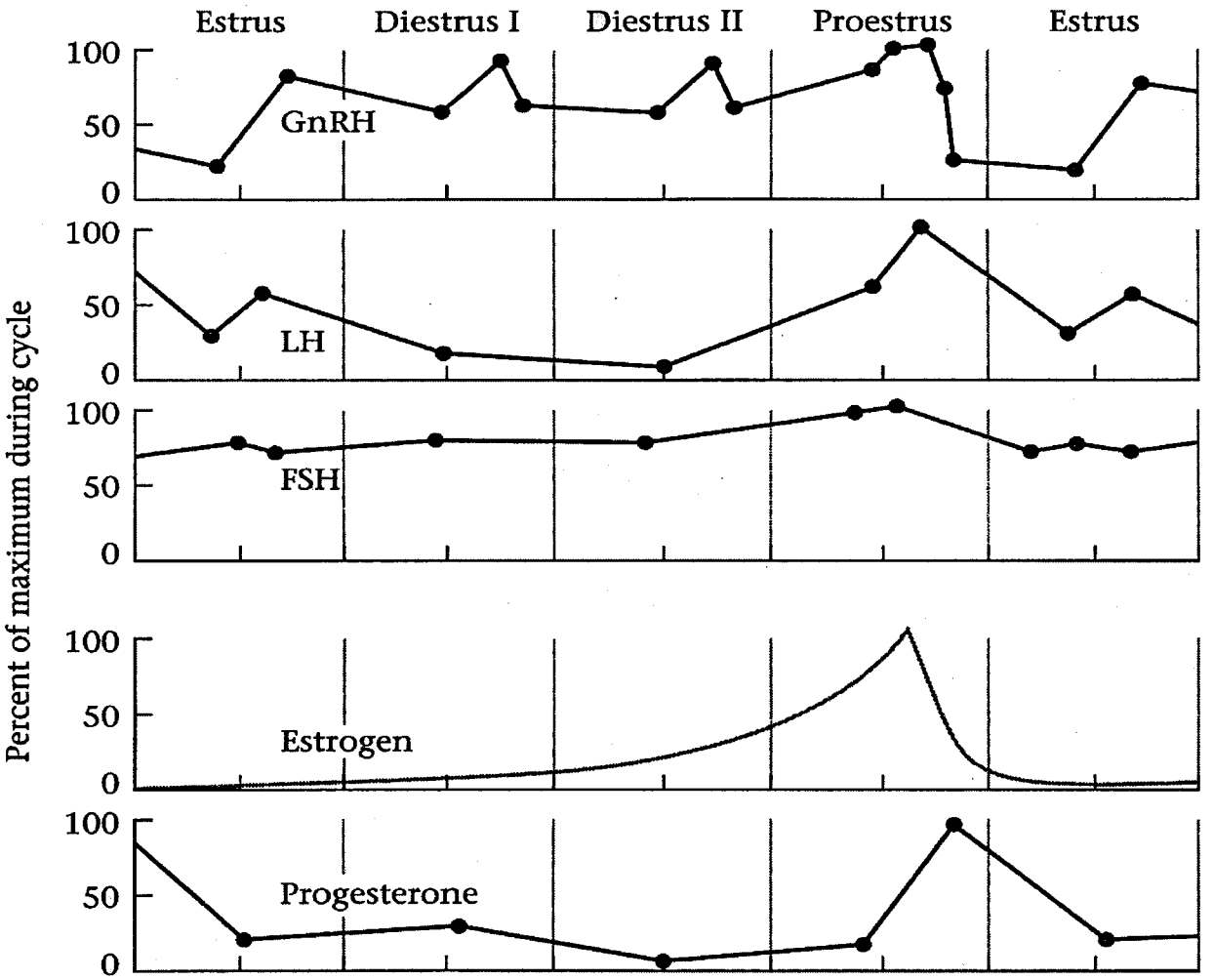
Figure 3. Serum hormone levels of Rockland-Swiss albino mice during gestation and lactation (adapted from Svare, 1990).

Figure4. Median number of BrdU-ir neurons in the dentate gyrus of the hippocampus in Virgin (range: 394.1 – 1222; N=5), Late-pregnant (range:337.1 – 578.5; N= 6), Lactating A (range: 320 – 639; N=4) and Lactating B (range: 627 – 994; N=4) groups of Experiment 1. (* Significantly different from Late-pregnant; ** Significantly different from Lactating A).

Figure5. Median number of BrdU-ir neurons in the dentate gyrus of the hippocampus in Primiparous+ (range: 160 – 396; N=6), Primiparous- (range: 386.2 – 760; N=5), and Foster (range: 182.2 – 606.3; N=6) groups of Experiment 2. (* Significantly different from Primiparous+; ** Significantly different from Foster group).

Figure 6. BrdU-ir nuclei in the dentate gyrus of the hippocampus (total mag x10; scale bar = ~ 150μm). A) Nulliparous group; B) Late-pregnant group (LP d21); C) Lactating A group (PP d1); D) Lactating B group (PP d3).

Figure 7. BrdU-ir nuclei in the dentate gyrus of the hippocampus (total mag x10; scale bar = ~ 150μm). A) Primiparous + group (PP d14); B) Primiparous – group (PP d14); C) Foster mother group.


Estrus
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Percent of maximum during cycle
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50
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GnRH
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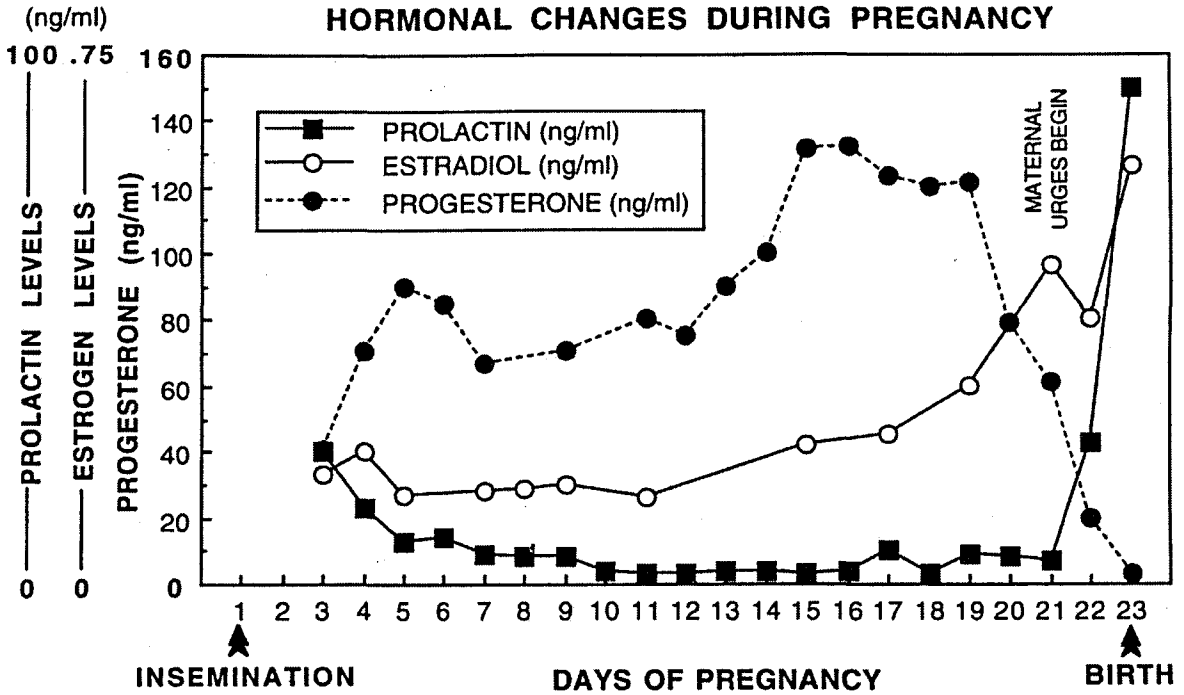
FSH
Estrogen
Progesterone


HORMONAL CHANGES DURING PREGNANCY
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ESTROGEN LEVELS
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160
140
120
100
80
60
40
20
0
PROLACTIN (ng/ml)
ESTRADIOL (ng/ml)
PROGESTERONE (ng/ml)
MATERNAL URGES BEGIN
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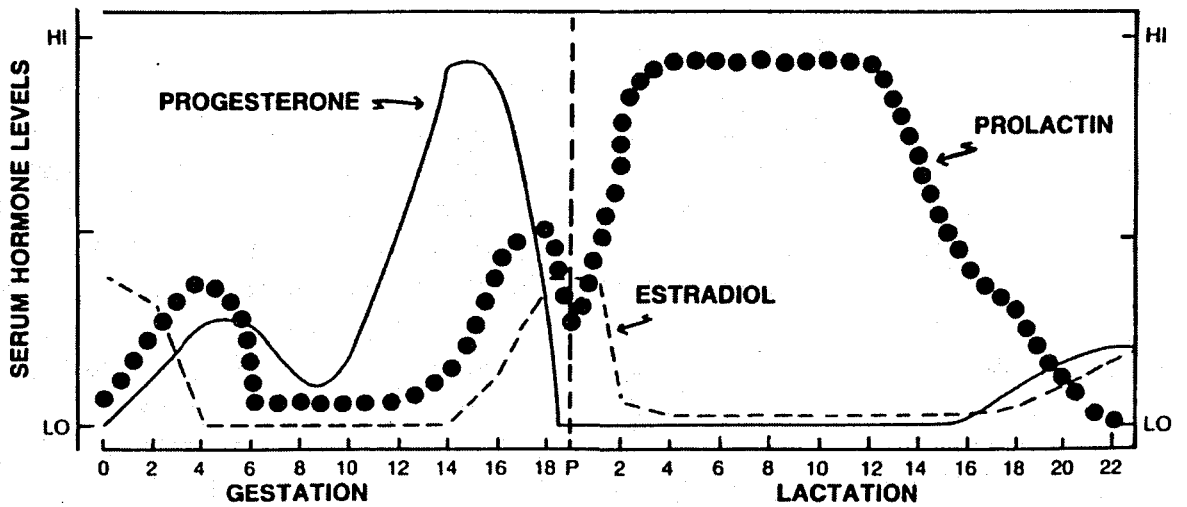
INSEMINATION
DAYS OF PREGNANCY
BIRTH


HI
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SERUM HORMONE LEVELS
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GESTATION
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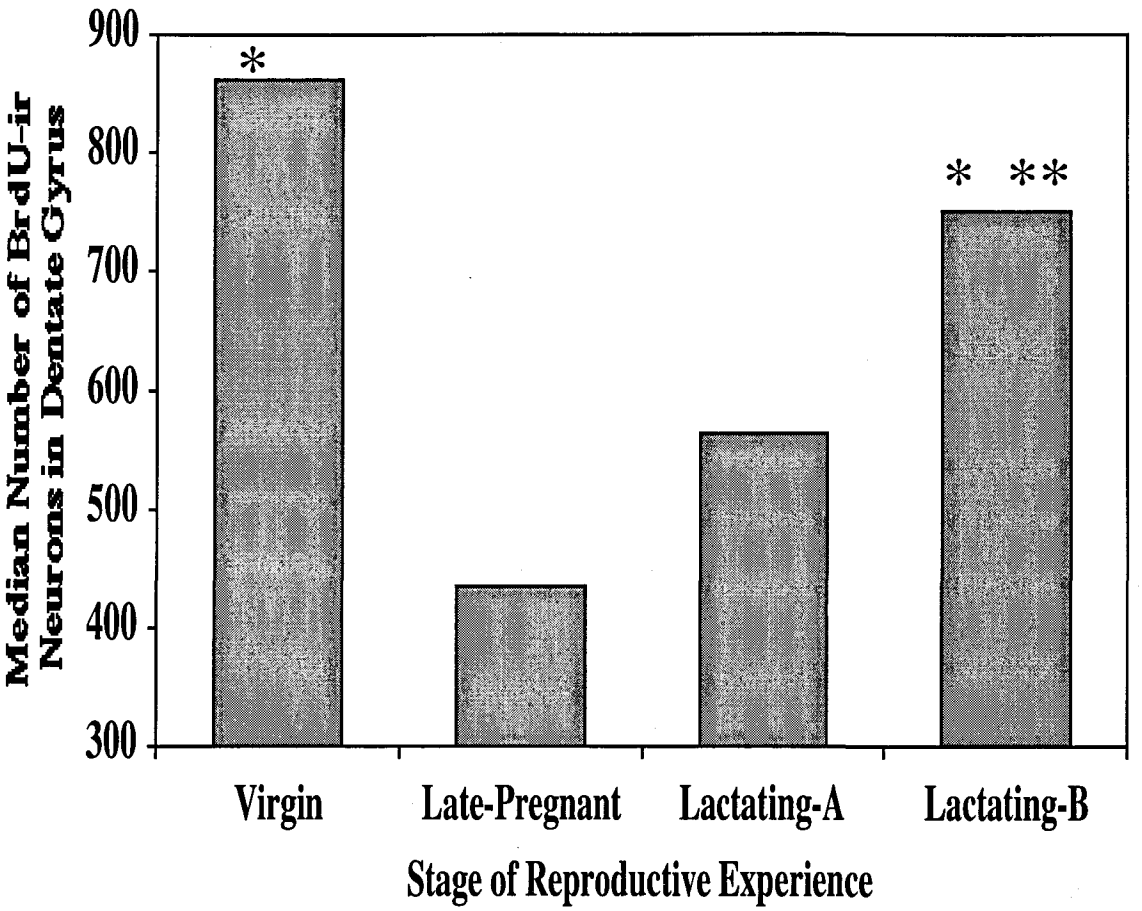
900
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700
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500
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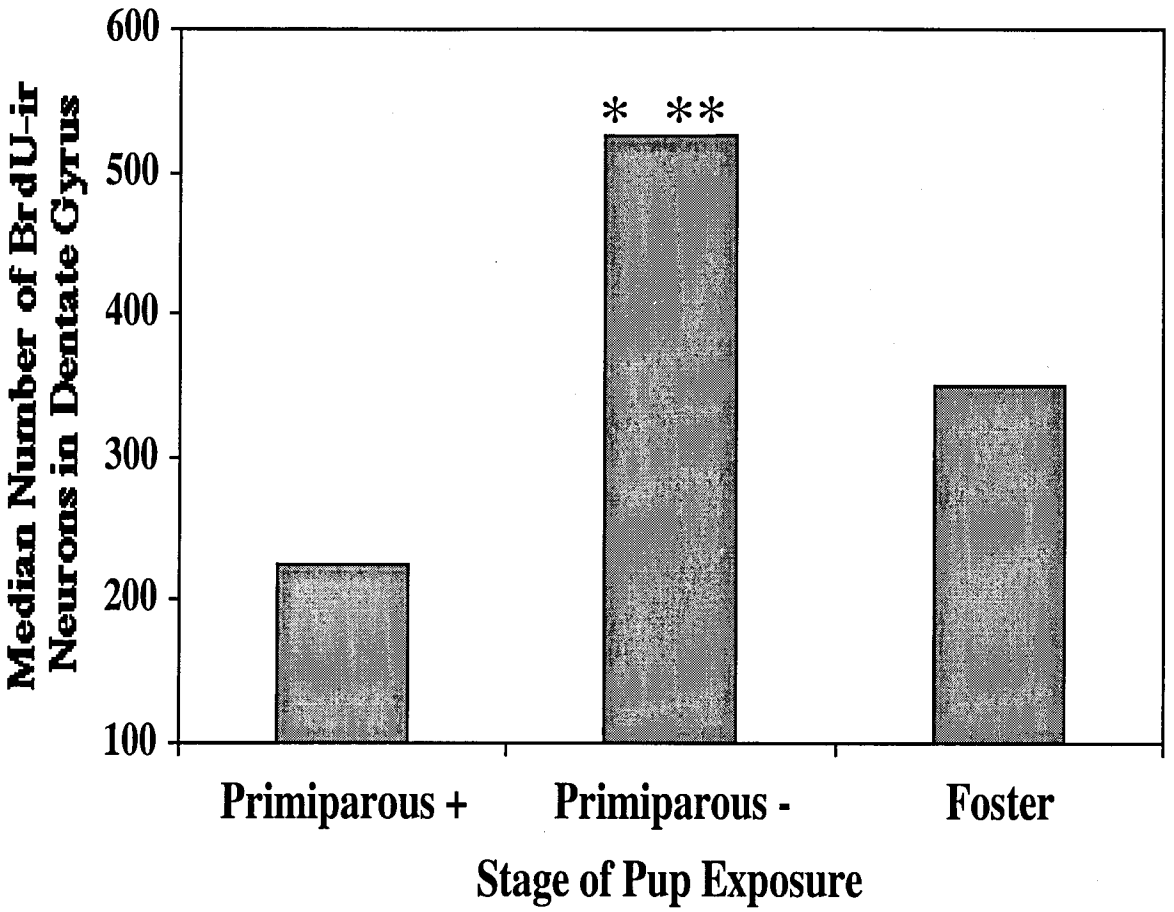
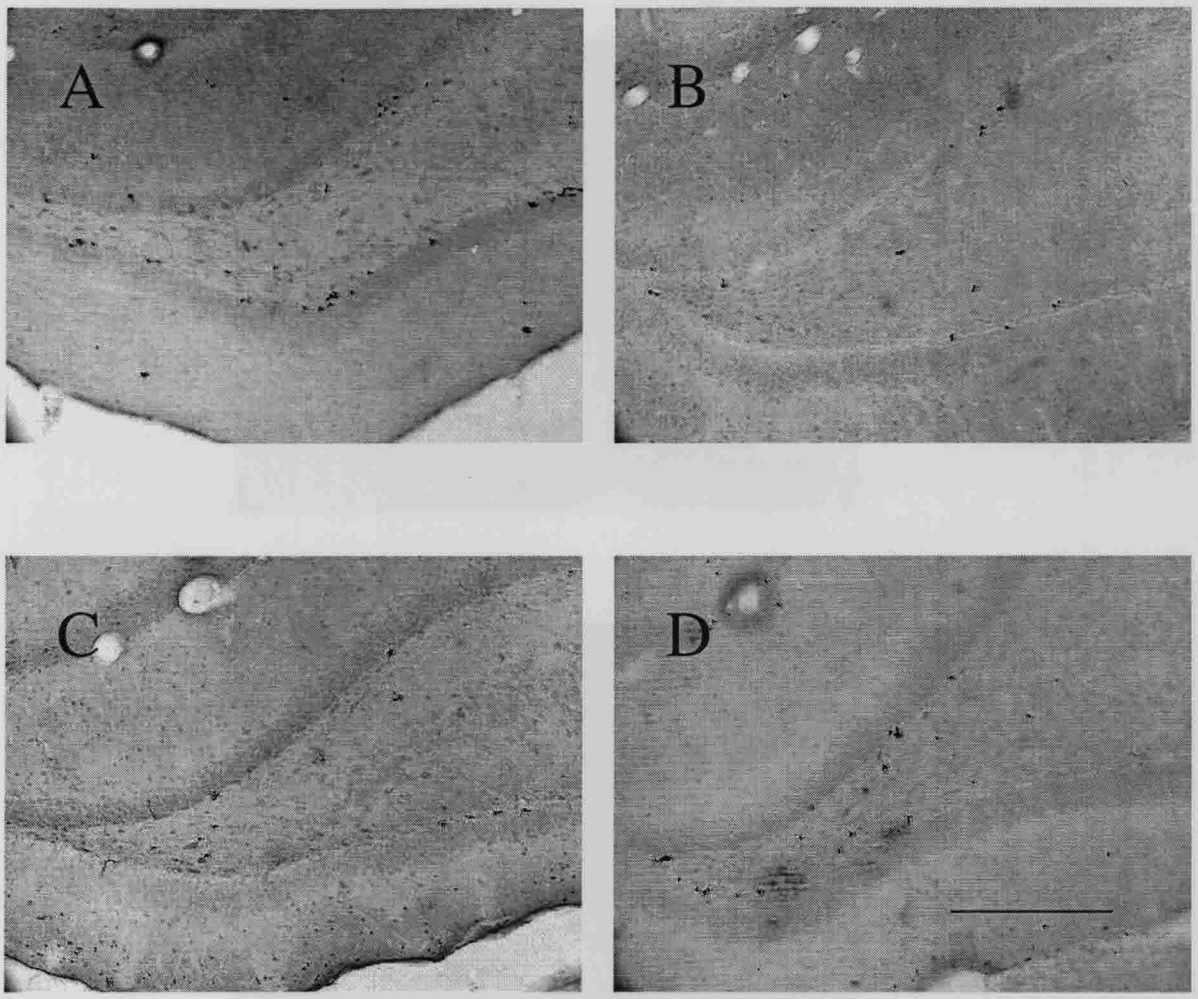
300
Median Number of BrdU-ir Neurons in Dentate Gyrus
*
* **
Virgin
Late-Pregnant
Lactating-A
Lactating-B
Stage of Reproductive Experience


Median Number of BrdU-ir Neurons in Dentate Gyrus
600
500
400
300
200
100
* **
Primiparous +
Primiparous -
Foster
Stage of Pup Exposure


A
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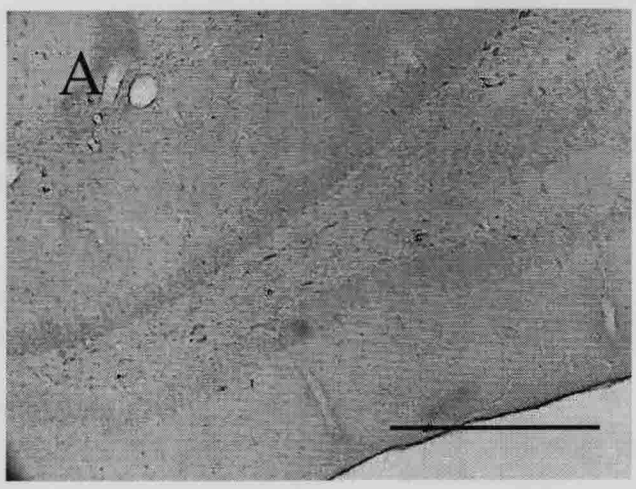
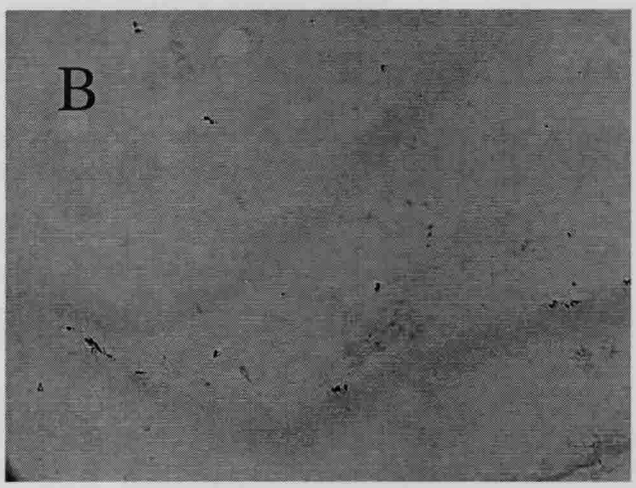
C
D


A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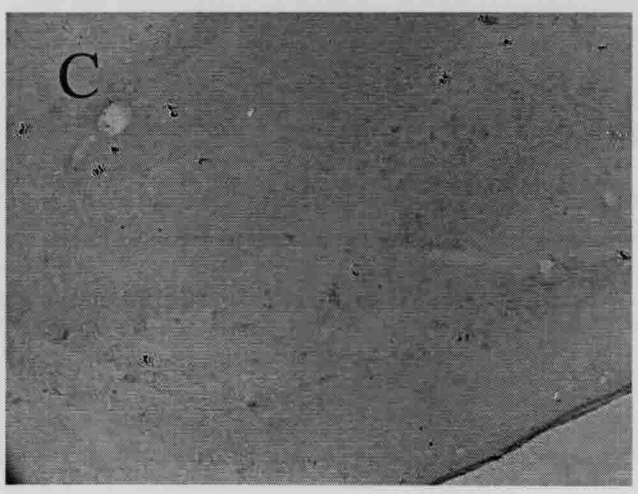
C

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RESEARCH EXPERIENCE

Spring/Fall 1999 — **University of Richmond – Neuroscience Laboratory**
Continuing research on stages of pregnancy and neurogenesis. Assisting fellow graduate students on current projects. Teaching various laboratory techniques to undergraduates and supervising lab projects.

Summer 1999 — **Paid Laboratory and Research Supervisor**
Laboratory manager for University of Richmond neuroscience lab. Supervised undergraduate projects pertaining to insulin receptors, synaptogenesis, and reproductive experience. Organized and led collaborative grant project examining reproductive experience and learning for University of Richmond and Randolph-Macon College neuroscience labs. Continued research on neurogenesis project.

Fall 1998 — **University of Richmond – Neuroscience Laboratory**
Researched protocol for Immunohistochemical technique using bromodeoxyuridine (BrdU), a DNA marker used for labeling neurogenesis in the brain. Implemented BrdU protocol and began research project on stages of pregnancy and neurogenesis.
Randolph Macon College – Neuroscience Laboratory
Participated in laboratory work of a project pertaining to maternal experience and c-fos expression in the amygdala of male rats.

July/August 1998 — **University of Richmond – Neuroscience Laboratory**
Assisted in laboratory work of graduate student projects pertaining to (1) stage of pregnancy and glial cell production and (2) nos expression in prenatally stressed male rats.

Publications

Kinsley, C.H. & Amory, E.A. Neurogenesis and the reproductive cycle. ***Brazilian Journal of Medicine***, in preparation.

Amory, E.A., Wartella, J.E., Madonia, L.F., Williams, A., Dillon, A., Ploszay, A., Griffin, G., Graber, A., Lambert, K.G., & Kinsley, C.H. Motherhood modifies neurogenesis in hippocampus. ***Media Guide for Society for Neuroscience***, 1999.

Published Abstracts

Amory, E.A., Wartella, J.E., Madonia, L.F., Williams, A., Dillon, A., Ploszay, A., Griffin, G., Graber, A., Lambert, K.G., & Kinsley, C.H. Motherhood modifies neurogenesis in hippocampus. ***Society for Neuroscience Abstracts***, 1999.

Amory E.A., Wartella J.E., Ploszay, A., Williams, A., Dillon, A., Griffin, G., Beresik M., Lambert, K.G. & Kinsley, C.H. Mother knows best: Potential enhancement of neurogenesis in the rat brain during pregnancy and lactation. ***Journal of the Virginia Academy of Science***, 50,154,1999.

Wartella, J.E., Amory, E.A., Ploszay A., Belinsky, E., Lambert, K.G., & Kinsley, C.H. Reproductive experience may modify stress responsiveness in the female rat. ***Journal of the Virginia Academy of Science***, 50,163,1999.

Conference Presentations

Berry, A., Wright, M.E., Yarett, P., Amory, E.A., Zanetto, D.J.C., Devries, A.C., Kinlsey. C.H., Fischer-Stenger, K., & Lambert, K.G. An investigation of the effects of social contact on glucocorticoid and immunological responses to chronic stress in male rats. *International Behavioral Neuroscience Society meeting,* expected 2000.

Amory, E.A., Wartella, J.E., Madonia, L.F., Williams, A., Dillon, A., Ploszay, A., Griffin, G., Graber, A., Lambert, K.G., & Kinsley, C.H. Motherhood modifies neurogenesis in hippocampus. *Society for Neuroscience,* 1999.

Kinsley, C.H., Amory, E.A., Wartella, J., Gifford, G., & Lambert, K.G. Your mother (rat) should know: Pregnancy drives changes in neurogenesis and hippocampal neural and glial morphology and activity. *Maternal Brain Conference*, 1999.

Amory, E.A., Wartella, J.E., Ploszay, A., Williams, A. Dillon, A., Griffin, G., Beresik, M., Lambert, K.G., & Kinsley, C.H. Potential alterations of neurogenesis following the transition from virgin to maternal/lactating female. *Society for Behavioral Neuroendocrinology*, 1999.

Wartella, J.E., Amory, E.A., Ploszay, A., Belinsky, E., Lambert, K.G., & Kinsley, C.H. Differences in reproductive/maternal experience may alter behavioral and neural responsiveness to a stressor in the female rat. *Society for Behavioral Neuroendocrinology*, 1999.

Aurentz, C.A., Felts, P.T., Lowry, C.A., Wartella, J.E., Miller, S.D., Amory, E.A., Gifford, G.W., Kinsley, C.H., & Lambert, K.G. Increased c-fos and tyrosine hydroxylase activity in the extended amygdala of rats displaying compulsive-like running. *International Behavioral Neuroscience meeting*, 1999.

Lowry, C.A., Lambert, K.G., Aurentz, C.A., Felts, P.T., Devries, A.C., Sundstrum, J., Amory, E.A., Wartella, J.E., Gifford, G.W., Miller, S.D. & Kinsley, C.H. An investigation of sex differences and behavioral responses to ecologically relevant stressors. *International Behavioral Neuroscience meeting*, 1999.

Williams, J.J., Aurentz, C.A., Gifford, G.W., Wartella, J.E., Miller, S.D., Amory, E.A., Kinsley, C.H. & Lambert, K.G. Activity stress and alterations in hippocampal astrocyte activity . *International Behavioral Neuroscience meeting*, 1999.

Williams, J.J., Aurentz, C.A., Gifford, G.W., Wartella, J.E., Miller, S.D., Amory, E.A., Kinsley, C.H. & Lambert, K.G. Maternal experience enhances behavioral responsiveness to a predator odor but decreases c-fos activity in the extended amygdala of rats. *International Behavioral Neuroscience meeting*, 1999.

Amory E.A., Wartella J.E., Ploszay, A., Williams, A., Dillon, A., Griffin, G., Beresik M., Lambert, K.G. & Kinsley, C.H. Mother knows best: Potential enhancement of neurogenesis in the rat brain during pregnancy and lactation. *Virginia Academy of Science*, 1999.

Wartella, J.E., Amory, E.A., Ploszay A., Belinsky, E., Lambert, K.G., & Kinsley, C.H. Reproductive experience may modify stress responsiveness in the female rat. *Virginia Academy of Science*, 1999.

Kinsley C.H., Madonia, L., Trainer, R., Gifford, G,. Miller, S., Tureski, K. & Lambert, K.G. Motherhood enhances learning and memory accompanying alterations in neuronal and glial morphology. *Society for Neuroscience*, 1998.

Invited Presentations

Amory, E.A. & Kinsley, C.H. Presentation: *Does pregnancy enhance neurogenesis, the production of new neurons?* University of Richmond Annual Science Symposium, 1999.

Amory, E.A. *Serial Killing: the motivation behind the killer.* Lecture for Motivation and Emotion Psychology course. University of Richmond, 1998.

Amory, E.A. *Dolphin Cognition and Behavior.* Lecture for Motivation and Emotion Psychology course. University of Richmond, 1998.

Amory, E.A. *Dolphin Cognition and Behavior.* Lecture for Behavioral Neuroscience course. Randolph-Macon College, 1998.

Amory, E.A. & Gifford, G.W. Chalk Talk: *Neurogenesis.* University of Richmond Annual Life Sciences Symposium, 1998.

Amory, E.A. & Kinsley, C.H. Presentation: *Neurogenesis and the female reproductive cycle.* University of Richmond Life Sciences Symposium, 1998.

SERVICES

- Participated in three roadshows to local high school and elementary schools to teach students about the field of neuroscience.
- Established and organized neuroscience laboratory duties and supervised 10-15 undergraduates in lab work and participation.
- Served as Chairperson for the Virginia Junior Academy of Science Conference (1999).

ACTIVITIES & INTERESTS

- Associate Member of IMATA (International Marine Animal Training Association 1997,1998
- SCUBA Diving (SSI Open Water Certification, PADI Underwater Photographer Certification & PADI Oceanarium and Zoological Diver)
- VIMS Aquarium (Volunteer)
- YMCA Tutoring Program
- Honor System of Virginia Tech – Case Coordinator and Panel Member
- CPR Certified
- Member of the Society for Marine Mammalogy (1997,1998)

REFERENCES

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