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LOOKING INTO THE MIND OF THE MOTHER:

PUP EXPOSURE AND REACTIVATION OF MATERNAL CIRCUITS

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The female rat, among other species, undergoes a fundamental brain re-modeling as a consequence of experiencing the normal and natural events of pregnancy and offspring stimulation. Compelling data show that maternal experiences produce neurobiological modifications in the female leading to specific maternal behaviors, affective states, and the basic underlying female neurobiology necessary to raise viable offspring. This study aims to evaluate the number, quality and selective activation of neurons that develop during the maternal experience. The study showed a trend toward supporting the hypothesis that a “maternal-circuit” is formed through the proliferation of neurons during late-motherhood and lactation, and is selectively reactivated by mothers exposed to foster pups.

Looking into the Mind of the Mother:

Pup Exposure and Reactivation of Maternal Circuits

The successful propagation of genes makes reproduction an outstandingly motivated behavior amongst species, particularly mammals. Researchers over the past several decades have recognized that the importance of reproduction is vital from an evolutionary standpoint, and invested their time examining mating behaviors. Because reproduction causes a multitude of changes, the directions research has taken are innumerable. From behavioral to hormonal and neurological changes, much has been discovered in regards to the impact of reproduction on both maternal and paternal caregivers. However, the majority of changes usually occur within the mother, thus maternal behavior is a notably researched area, as well as the focus of the current study.

Maternal Behavior - Learning and Memory

A range of behavioral changes occurs with the transition from virginity to motherhood in female mammals. In order to protect and provide for her offspring, a mother must adapt quickly and consistently to their demands. The shift in mainly self-survival behaviors to those behaviors benefiting her offspring is primarily driven by hormonal changes, leading to the production of new neurons, which then leads to the activation of maternal behavior circuits and inhibition of inhibitory pathways for these behaviors and production of new neurons. These new behaviors include retrieval, grooming, crouching licking, nursing, foraging and nest building.

Referred to by Leckman & Herman (2002) as “a highly conserved set of behavioral capacities that are crucial for reproductive success,” these behaviors become the focus of a new mother rat. Newborn rat pups are completely dependent on maternal behaviors for survival because they are essentially immobile and unable to thermoregulate. Maternal behaviors are natural, being caused by many factors including environment and hormones. These behaviors necessary to a mother, which increase the likelihood of pup survival, create a female with abilities beyond that of a non-mother.

Mothers have been found to perform better on a number of tasks when compared to virgin rats, one being predatory behavior, which is critical for pup survival. Kinsley and Bardi, et al. (2006) found the latencies to catch a cricket in an open field for food-deprived nulliparous females (NP or non-mothers) were significantly larger than those for lactating females (mothers). In a similar study, Lambert et al. (2005) exposed female rats of different reproductive experience to pups for 21 days. They were then required to find a piece of food in one of many wells in an open maze. Primiparous females (PP or one reproductive experience) outperformed both nulliparous (NP) groups, being those exposed to pups and not exposed to pups. However, nulliparous females who were exposed to pups outperformed the nulliparous females who were not exposed to pups for 21 days. Lambert et al. (2005) suggests that both reproductive experience and pup-exposure improve foraging ability. Both studies provide support for enhanced foraging and predatory behavior with motherhood and exposure to pups.

In addition to an increase in foraging and predatory behavior, some research has indicated that reproductive experience and/or pup stimulation is beneficial to learning and memory. Pawluski et at. (2006) tested multiparous (more than one reproductive experience), primiparous and nulliparous females in a radial arm maze task in which they searched for food in four out of eight baited arms. Three different types of memory errors were assessed depending on which arms the rats entered. Reference memory errors, working memory errors and working/reference memory errors were all assessed. Primiparous females made the fewest errors, regardless of type, out of the three groups. There was also a trend of multiparous females outperforming or having fewer errors than nulliparous rats. This demonstrates that while one-time mothers were the most successful with the task, both groups with reproductive experience made fewer errors than the group without reproductive experience. A similar study (Gatewood et al., 2005) used a dry land version of the Morris water maze and also a reversal task, in which the location of the baited well was changed, to determine the long-term spatial memory affects of motherhood. They tested multiparous, primiparous and nulliparous females at 6, 12, 18 and 24 months. Primiparous and multiparous females learned both spatial tasks significantly better than virgin females and also exhibited attenuated memory decline up to 24 months of age. These studies display enhancement in both learning and memory as a result of reproductive experience, as well as showing support for long-term memory enhancement also due to motherhood.

Gatewood and colleagues demonstrate that motherhood changes memory and

learning processes not just during pregnancy or until weaning occurs, but for a lifetime. So what exactly is occurring during pregnancy and the postpartum period to create these drastic changes between females with reproductive experience and those without? One possibility is the effects of hormonal changes. Changes in hormone levels lead to neurogenesis, which may then alter specific brain structures. Research in these areas and links between them will be examined, followed by the application of past findings to the current study.

Neurogenesis

The behavioral changes of motherhood are only the physically observable traits that occur during this time. Because behavior is represented by a change in the brain, in order to find out why the behavioral changes are occurring in mothers, the neural circuitry must be examined. As explained by Kolb, Gibb and Robinson (2003), the brain is not fixed as we once thought but instead has the ability to adjust according to developmental and environmental variables. The field of neuroscience assumed for over 100 years that new neurons were not added to the adult brain. Recently, belief in this dogma declined with the understanding that the adult brain is structurally modifiable through many different types of experiences (Gross, 2000). A few techniques developed around the 1990s that confirmed the reality of neurogenesis in the dentate gyrus of adult rats. A method used to label and examine new cells is the administration of the synthetic thymidine analogue BrdU or 5-bromo-3'deoxyuridine. Proliferating cells take up BrdU during the s-phase of

mitosis and continue to label their progeny as well (Gross, 2000). This method was also used in the current study.

Neuronal marking was also a technique that helped to phase out the belief of the static adult brain. Cell-type specific markers such as NSE for labeling neuron specific enolase and NeuN for neuronal nuclei (used in the current study) expressed in adult-generated cells are solid evidence for the now accepted notion that new neurons are born in the adult brain (Gross, 2000). Gould and colleagues (1999) were one of many researchers to demonstrate that not only are new neurons born in the hippocampus, but that this brain region is specifically effected by and involved in associative memory formation. In this study, BrdU was used to label new cells following specific behavioral tasks, using the Morris water maze. Gould and colleagues found the number of neurons in the granule cell layer to increase in adult rats following a spatial learning task using the Morris water maze. They were compared to a control group with no spatial learning task. A significant number of the BrdU labeled cells found in the dentate gyrus of the hippocampus were also immunoreactive for TOAD-64, the marker of immature neurons, illustrating further the generation of neurons in this area as a result of spatial learning. The result of Gould and colleagues study demonstrates the direct connection between learning dependent on the hippocampus and neurons generated in this area.

Although Gross (2000) and Gould et al. (1999) among many others provide much evidence of new neuron birth, the actual incorporation of the newly generated neurons to previously established neuronal circuitry is less clear. Recently,

however, Kee and colleagues (2007) examined more closely the integration of new neurons into already established memory (specifically spatial) network circuitry in the dentate gyrus of the hippocampus. They found that as the new granule cells began to mature, they became increasingly more likely to be incorporated into established circuits. Through BrdU and Fos expression, Kee et al. found neurons around 6-8 weeks of age were the most likely to be recruited into established memory networks. This demonstrates that the birth of new neurons, specifically in the dentate gyrus of the hippocampus, as found by many researchers (Gould et al., 1999 & Gross, 2000) are not without significance, but once mature, make preferential contributions to memory processing. As mentioned previously, research has documented that learning and memory is enhanced through the duration of pregnancy and following parturition (Kinsley et al., 1999, Pawluski & Galea, 2006). The current proposed study will therefore then use the BrdU labeling method combined with neuronal marking techniques in order to trace the development of new neurons in the dentate gyrus of the hippocampus during motherhood.

Hormones and Brain Plasticity

Why is it that such changes are occurring in the brain circuitry? It is possible that the environment or genetic changes lead to newly formed cells and neuronal pathways. It is also likely that a demand for new circuitry arrives with pregnancy, in addition to parturition and lactation. According to Kolb, Gibb & Robinson (2003), brain plasticity in general can be influenced by a number of

factors. They include growth factors, diet, genetic factors, disease, stress, brain injury, psychoactive drugs, and anti-inflammatory agents among others. However, those most applicable to the behaviors and changes that occur during pregnancy are pre- and postnatal experiences and hormones (Kolb, Gibb & Robinson, 2003). Kinsley et al. examined these factors (1999 & 2006), demonstrating that hormones induce morphological modifications to the hippocampus. The first study (Kinsley et al., 1999) found that improvement in certain cognitive tasks, such as navigating efficiently through open land mazes to find baited wells (Lambert et al., 2005) or radial arm mazes (Pawluski et al., 2006), may be due to an increase in hippocampal dendritic spine concentration, which in turn may enhance cognitive abilities. This dendritic spine increase is a result of the spike in progesterone and particularly estradiol when the female rat becomes pregnant. The total synapse surface area is larger, because of the increase in dendritic spines, which may be the reason for improvement in learning and memory and enhanced cognitive skills in female rats with reproductive experience (Kinsley et al., 1999).

Kinsley et al. established this further in 2006 by examining the concentration of dendritic spines in the CA1 region of the hippocampus during three stages of the estrus cycle (virgin females), late-pregnancy rats and lactating rats for a total of five groups. Spine density was increased in both the late-pregnancy and lactating females when compared to all three virgin groups. These results show that although during certain stages of the estrus cycle there is an increase in estrogen and progesterone (particularly in proestrus), only pregnancy was shown to alter

hippocampal neurons (increased dendritic spine concentration), which may in turn help to regulate certain aspects of maternal behavior, such as nest building, or foraging in order for the mother to better provide for her offspring (Kinsley et al., 2006).

Providing further hormone-induced neurogenesis support, Shingo et al. (2003) examined the forebrain subventricular area in female mice during pregnancy. This brain region is related to the motherhood experience through its connections and effects on the olfactory system. An increase of the hormone prolactin occurs during pregnancy and also during mating. Prolactin is secreted in surges in the afternoon and night during the early stages of pregnancy (Bridges et al., 1993). It is suppressed mid-pregnancy when placental lactogen secretion occurs and surges again the night before parturition (Brunton & Russell, 2008). This surge of prolactin directly before delivery acts in the brain in order to elicit maternal behavior to ensure a successful transition from pregnancy to motherhood (Mann & Bridges, 2001). More specifically, the prolactin surge stimulates the production of neuronal progenitors in the forebrain subventricular area, which then migrate to the olfactory system, creating new interneurons in this area (Shingo et al., 2003). This neurogenesis is likely occurring because olfactory discrimination is important in mating, offspring recognition and rearing, which are all crucial aspects of reproduction.

Oxytocin is another hormone that triggers a succession of changes leading to brain plasticity of the parous female. Labor and lactation are controlled by the

release of oxytocin, activating hippocampal plasticity, there by improving learning and memory in mothers (Monks, Lonstein & Breedlove, 2003). This was further demonstrated in virgin mice, which received intracerebroventricular injections of oxytocin (mimicking the release that occurs in pregnant females), and demonstrated improved long-term spatial learning. In addition, an oxytocin antagonist administered to multiparous mice significantly inhibited the improved spatial memory gained from the impact of oxytocin release during motherhood (Tomizawa et al., 2003). These studies are excellent representations of the importance of hormones and neurogenesis and their significance in the brain changes linked to pregnancy and motherhood.

Environment and Brain Plasticity

The neurogenesis or brain plasticity causing the behavioral modifications in mothers are not just the result of pregnancy and hormones associated with it, but also the environmental changes that occur. These environmental changes, according to Kolb, Gibb & Robinson (2003) are one of several factors leading to brain plasticity. Mothers are immersed in a variety of new sensory stimuli when their pups are born, creating an enriched environment and need for adaptations on the part of the mother. Lambert et al. (2005) reported that nulliparous females who were exposed to pups for 21+ days demonstrated enhanced performance on both plus maze and open maze foraging tasks as compared to isolated nulliparous females.

Similarly, Tashiro, Makino, & Gage (2007) suggest that there is a critical period during the first three weeks of the development of new cells during which experiences can determine the survival of the new neurons. In the current study, the female rats will be housed with their offspring for the first 21 days following birth and the BrdU injection, which will mark the cells born during this critical time period. Tashiro, Makino & Gage (2007) suggests that this 3-week exposure period could be a mechanism by which the dentate gyrus is altered permanently.

Pawluski & Galea (2007) also focused on the postpartum period of the female rat, specifically hippocampal alterations. The four groups included consisted of multiparous, primiparous and nulliparous females, as well as one group of nulliparous females exposed to pups. All groups were injected with BrdU within 24 hours of birth (pup-exposure in the case of the nulliparous females) and perfused either 24 hours or 21 days following the injection. This split was necessary to examine both early and later postpartum effects. Interestingly, results showed primiparous and multiparous rats had significantly decreased cell proliferation in the dentate gyrus during the early postpartum period (one day following BrdU injection). During the later postpartum period (21 days following BrdU injection) primiparous rats expressed a decrease in cell survival in the dentate gyrus, as compared to all other groups, including pup-exposed. However, both nulliparous females with brief pup-exposure (about 24 hours) and 22 days of pup exposure resulted in increased cell proliferation and cell death in the dentate gyrus. It is important to keep in mind that with cell proliferation must come cell death, in order

to maintain a sort of homeostasis. The older neurons can be "replaced" so to speak because of the generation occurring along side the death.

To reiterate the findings of Pawluski & Galea (2007) both cell proliferation and cell death are more prominent in nulliparous females exposed to pups as compared to those not exposed to pups A decrease in cell survival was found in multiparous and primiparous females, showing a longer lasting trend among first time mothers. This neurogenesis found in the hippocampus of the nulliparous females is likely to be initiated by external stimuli from pups. Is it possible then, for the cell survival decrease in multiparous or primiparous females to be hindered by pup re-exposure? Would there be more cell survival if mothers were re-exposed to pups, as the case was for nulliparous females?

Current Study

The current research examines the possible re-activation of the cells generated during early motherhood of primiparous females, by means of foster pup exposure. The study aims to evaluate the number of cells that develop during the maternal experience of first time mothers as well as the selective activity of those cells. It is hypothesized that the states of late-pregnancy and early lactation will stimulate the proliferation of a set of cells that will be integrated into a form of "maternal circuitry" within the mother's brain, specifically focusing on the neurogenesis occurring in the dentate gyrus of the hippocampus. With re-exposure to pups, it is proposed that the maternal circuit formed during the early postpartum

period will be reactivated. This finding would demonstrate that once a female rat becomes a mother, the mechanisms by which her behaviors and motivations have adapted to fill the maternal, caretaker role are forever changed. In other words, once a mother, always a mother.

Previous studies suggest that motherhood influences aspects of cognition, emotionality, neural plasticity, and neuronal health not just during pregnancy, but for a lifetime. Finding that even one of these effects generalizes from rats to humans opens the door to the investigation of variables that may provide therapeutic benefits for existing neurobiological threats to the female brain. These threats include depression, anxiety disorders, maternal-offspring interactions and neural degeneration, among others. This study is only a small step, but may provide a foundation for further research dealing with these threats to the female brain, specifically to mothers. Although generalizations from rats to humans should be made with appropriate caution, the natural maternal rat serves as a valuable model for investigation. It is essential to generate competent biomedical models of the complex reproductive-related neuroendocrine and behavioral modifications experienced by the female in order to further the knowledge of neurobiological phenomena and their effects on the mother.

Study Design & Methods

Subjects

Eighteen Sprague-Dawley virgin rat females weighing approximately 300g were used for this study (n=9 for both group). Ten male rats were also used in this study for mating purposes only, in addition to six host female rats, which were not used in the study themselves, but provided the foster pups.

The females were age matched and placed randomly in either the experimental or control group. Rats in both groups were kept in 20 x 45 x 25 cm clear polypropylene cages and exposed to human contact only for the purposes of feeding and cage cleaning. The cages were lined with corncob bedding and changed once a week. A wire top covered the cage and provided Purina Rat Chow and water to the rats *ad libitum.* All mothers were housed with their pups until weaning at 21 days (+ or – 1 day) after delivery. The animal housing rooms were controlled for both temperature and light for the duration of the study. All animal maintenance and the procedures used in this study were strictly conducted according to the standards set forth by the University of Richmond Institutional Animal Care and Use Committee and the National Institutes of Health (for which approval was received: #06-05-6).

Materials

Behavioral Assessments

Other than mating, no behaviors or assessments were evaluated in this study. Each subject was exposed to either foster pups or a control object (hand or novel object) after weaning from their genetic litter but the interaction was minimally assessed. Basic information was recorded regarding the nature of the interaction the female had with either the exposure to foster pups (non-genetic re-exposure) or the control object. Subjects exposed to foster pups were recorded as either displaying motherly behaviors or not. The subjects exposed to the novel object were recorded as either noticing and investing the object or ignoring it. There were minimal behavioral investigations because the focus of this study was not on behavior or interactions but on the neurobiological changes that occur during late-pregnancy and early lactation.

Neural Assessments

To prepare the BrdU (bromodeoxyuridine) for intraperitoneal injection, BrdU was dissolved in 0.1 M phosphate buffered saline (PBS), and heated to 50-60 °C. Following testing, sodium pentobarbital was used to overdose the animals. For the perfusion (discussed in detail later) phosphate buffered saline (PBS) and 4% paraformaldehyde (PF) were used.

Analysis of the collected brain tissue was done using fluorescent immunohistochemistry in order to selectively stain neurons in the dentate gyrus of the hippocampus. This process required the use of the following substances: sodium

pentobarbital, phosphate buffered saline (PBS), paraformaldehyde (PF), sucrose, antibody, fluorescing secondary antibody, and clearing solutions (alcohols and xylenes). A cryostat was used to cut the desired hippocampal sections from the brain. Microplate wells, slides, cover slips, and microscopes were also used. The uses for each of these substances and materials are described below.

Procedures

Once obtained, the eighteen female virgin rats were mated accordingly. After mating, each female was placed alone in a cage (described earlier) until they completed pregnancy and delivered pups. If the female did not become pregnant she was mated again. Within twenty-four hours of cessation of delivery, each mother was administered an injection of 200mg/kg bromodeoxyuridine (BrdU) intraperitoneally. This dosage is comparable to that used in Shors 2004; Shors et al., 2001 and Van Praag et al. 2002) and Van Praag et al. (2002). BrdU is used as a marker of proliferating cells and their progeny (Holmes & Galea, 2002). The females were housed with their pups until weaning at 21 days after delivery ± 1 day.

One week post weaning, nine randomly selected subjects (now primiparous females) were each exposed to ten newborn foster pups, which were no more than ten days old. The primiparous female and ten foster pups were left in a 25 x 45 x 25 cm polypropylene cage for sixty-minutes. The remaining nine subjects were divided into two control groups, predicted and demonstrated to have no difference between them. One group (containing four subjects) was exposed to a novel object (small

beaker) for sixty minutes. The second control group, containing five subjects, had a hand quickly placed into and withdrawn from their cage at the beginning of the sixty minutes. This control group was to ensure consistency among all three groups, having the only variance between them be what is actually placed into the cage. The two control groups were predicted to have no difference, and were therefore merged together into one control group. If, however, a difference is found between the two control groups, the study will be separated into three groups. The number of subjects would stay the same (9 experimental subjects, 5 control exposed to a novel object and 4 control exposed to a hand) but the analysis of the data would be modified.

Following the single sixty-minute exposure to foster pups, or the novel object or sixty minutes after the brief hand exposure, all animals were overdosed using sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. Following the perfusion, brains were blocked for the area containing the dentate gyrus, and the entire span of this region was sliced at 50 –microns using a cryostat. This tissue was processed for BrdU-immunoreactivity and c-Fos expression in addition to the labeling of mature neurons through the use and expression of NeuN. BrdU is a thymidine analogue that is used to identify proliferating cells. C-fos is an indicator of recently activated cells. Using this component allows visibility of cells specifically activated in response to the presence of the foster pups or novel object. Finally, the NeuN antibody, marks cells of mature neurons, as it specifically recognizes the DNA-binding neuron-specific protein NeuN present in most neuronal

cells in the central and peripheral nervous systems.

Following the immunohistochemical procedures (described in detail below), the number of triple-labeled BrdU/c-Fos/NeuN cells in the dentate gyri of foster pup-exposed mothers was compared to the mothers in the control group using a Carl Zeiss Axioimager fluorescent microscope. NeuN labeled cells expressing both BrdU and c-Fos are predicted to be higher in number in mothers exposed to foster pups versus those subjects with no foster pup interaction.

Immunohistochemistry

The animals were first administered an overdose of sodium pentobarbital. They were then transcardially perfused, pumping the vascular system first with PBS (phosphate buffered saline) followed by chilled 4% PF (paraformaldehyde) to begin the preservation process. The brain was post-fixed in 4% paraformaldehyde overnight, followed by immersion and storage in 20% sucrose 80% PBS solution until the tissue could be assessed. Each brain was blocked for the hippocampus at the optic chiasm and cerebellum. Slices measuring 50 –microns were taken from the blocked area using the cryostat set at -16^{o}C. Sections of tissue were saved starting with the first sighting of the dentate gyrus. The entire span of the dentate gyrus was taken, but only ten slices from each brain were collected and placed into microplate wells (2 slices per well) for the immunohistochemical process (the remaining slices were saved in the event that further processes are desired).

Basic fluorescent immunohistochemical techniques were used to stain the obtained brain tissue (Kee, Teixeira, Wang, & Frankland, 2007). Primary and secondary antibodies with fluorescent tags were used to locate and bind to BrdU, c-Fos and NeuN triple labeled cells.

To begin the staining process, the tissue first went though 3 five-minute washes at room temperature of TBS or tris buffer, followed by a 2 hour incubation of 50% Formamide at 65 ^{o}C. The tissue was then rinsed in 2xSSC at room temperature for 15 minutes. Sections were then exposed to 2 N HCL for 30 minutes at 37^{o}C followed by a 10-minute rinse at room temperature in 0.1M Borate Buffer and 6 fifteen-minute washes in TBS (tris buffer). In preparation for the primary antibody cocktail the tissue was blocked for one hour in TBS++. The primary antibody cocktail of BrdU (sheep anti-BrdU polyclonal – GeneTex, 1:500,), c-Fos (rabbit anti c-fos polyclonal – GeneTex, 1:1000, and NeuN (mouse anti NeuN – Chemicon, 1:1000) was then applied. The tissue was incubated in the cocktail for 24 hours at 4^{o}C.

The second day of staining began with 2 fifteen-minute washes of TBS at room temperature. Again, in preparation for the application of the secondary antibody, the tissue was rinsed for 15 minutes in TBS++. The secondary antibody cocktail was then applied - donkey anti-sheep conjugated with Fluorsecein, donkey anti-mouse conjugated with Aminomethylcoumarin, and donkey anti-rabbit conjugated with rhodamine, Red-X, all Jackson Immuno antibodies with1:500 dilutions. The sections were incubated with these complementary fluorescent

markers for two hours. At this point the tissues became light sensitive, requiring the duration of staining to be conducted in the dark, and storage thereafter. The tissues went through a final series of 7 TBS rinses at room temperature for 15 minutes each.

Following the staining procedure, the sections were mounted with TBS on double-subbed slides and allowed to dry overnight. Once dry, the tissue was cover-slipped using Dabco, an anti-fade fluorescent mounting medium, and sealed on the edges using a clear lacquer.

Microscopic Imaging and Quantification

The sections were examined and quantified using a Carl Zeiss Axioimager fluorescent microscope recently obtained through the National Science Foundation by Dr. Craig Kinsley. A total of 146 images were taken of adequate quality for analysis (100 experimental and 46 control). Tissue and images were excluded from analysis if the dentate gyrus was not apparent in the image or if a quality image could not be taken due to debris or bubbles on the slide obscuring the targeted area. This means that not all animals had tissue adequate for quantification (see expansion in discussion portion). For those images that were deemed adequate for analysis, the number of triple labeled BrdU/c-fos/NeuN cells were quantified by finding the average of the left and right hemispheres for each section. This unilateral score was found for each section of tissue. The number of unilateral scores adequate for analysis was 50 from the experimental subjects and 23 from the control subjects. Images were taken at 20x in order to include the entire “beak” area

of the dentate gyrus. All images were taken, analyzed and quantified by blind researchers.

The Axioimage software program was used to quantify both the number of triple labeled regions and the size of the total area containing triple labeled cells within the dentate gyrus of the hippocampus. The threshold level of areas to be included in the quantification was set at 15%, 25%, or 35% of the overall gray level displayed. This value was dependent on the overall exposure of each individual image. Using these set values allowed the method of quantification to remain reasonably constant from image to image.

Results

Statistical Analysis

The following analyses are a product of unilateral scores, thus the left and right hemisphere of each viable section were combined and averaged. The number of regions of triple labeled staining, regardless of size, was first found in order to then calculate the total area of those regions. The regions were measured in each hemisphere to find the total area in micrometers (uM) of triple labeled BrdU/c-fos/NeuN cells – a more accurate measurement than number of regions alone. Here again, the total area of triple labeled cells was averaged for the left and right hemisphere of each section to establish one unilateral score.

To determine whether there were differential effects of the type of exposure (foster pup or novel object) on the total area of triple labeled cells in the dentate gyrus, a one-way single factor analysis of variance (ANOVA) was calculated. Results revealed that the type of exposure had a marginally significant effect on the total area of triple labeled cells, $F(1,71) = 2.815$, $p < 0.098$ (see Figure 1). This trend suggests that the primiparous females exposed to pups had a larger area (reported in uM) of triple labeled cells in the dentate gyrus (Area sum = 1809067.34, M = 36181.35, SD = 37911.56) than those females exposed to a novel object, (Area sum = 1208266.82, M = 52533.34, SD = 40342.93).

In order to determine the approximate number of triple labeled cells in the areas measured, each unilateral area score was divided by the average area of a single neuron found in the dentate gyrus of the rat hippocampus as discussed by Roy, Seidler, & Slotkin, 2002. This analysis simply allows for easier understanding of the neurobiological interaction being examined.

A one-way single factor ANOVA was also used here to determine the number of triple labeled BrdU/c-fos/NeuN cells in the dentate gyrus of the hippocampus. Results revealed that the type of exposure did not have a significant effect on the number of triple labeled cells in the dentate gyrus, $F(1,71) = 0.035$, $p = 0.852$.

Discussion

The results of the current study demonstrated a trend towards supporting the hypothesis that when a primiparous female is exposed to foster pups, the maternal circuit formed during late pregnancy and the early-lactation period would be reactivated. Represented in Figure 2, is a primiparous female exposed to foster pups. This particular subject also demonstrated maternal behaviors including anogenital licking and attempting to nurse the pups. The staining technique used to target BrdU, c-fos and NeuN are confirmed here and also in Figures 3 and Figure 4. Figure 2 is taken at 20x and Figure 3 at 100x – both experimental subjects. Figure 4 captures the triple label stain of a primiparous female in the control group. This particular female was exposed to a novel object (beaker). During this subject's sixty-minute exposure she investigated the beaker and covered it with the corncob bedding. Both Figure 2 and Figure 4 were taken at 20x capturing the triple labeled green, red and blue staining, marking BrdU, c-fos & NeuN, respectively. The overlapping of the three tags can be seen as a yellowish-green color.

As mentioned previously, the general behavior of the subjects was recorded for both the group exposed to pups and those exposed to the novel object or hand. While these overall behaviors were recorded for each subject they were not coded and therefore not included in the statistical analysis. It is important to note however, that six out of nine of the primiparous females exposed to foster pups displayed maternal behaviors during their sixty-minute interaction, which included grooming, anogential licking, and nursing. One of these six subjects also displayed

aggressive behavior at the end of the sixty-minute exposure when she was being removed from the cage containing the pups. In addition to recording these behaviors, the number of genetic pups in each primiparous females' litter was also noted. This was done to determine whether or not there was a correlation between the number of pups the mothers originally had and their behaviors towards the foster pups. The correlation comparing the number of pups each mother had in their genetic litter to their behavior with the foster pups (6 displaying maternal behaviors and 3 ignoring the pups) showed a positive relationship ($r = .12$). This positive correlation suggests that with an increase in the number of pups, the behavior of the mother with foster pups tends to be more maternal. Finding this positive correlation has interesting implications, as the reasons why this may be occurring are numerous. While this was not the focus of the current study, it would be of value to look deeper into this relationship with future investigations.

A possibility for the lack of significance found in this study may be due to the differences in the types of mothers included in this study. It is possible that the three subjects, who did not display maternal behaviors towards the foster pups and instead ignored them, were perhaps neglectful mothers. There are no data in this study supporting this inference but it would be of extreme interest and value for future research.

Because varying behavior was found both between and within groups, the lack of more precise behavioral observation and analysis is a limitation on the current study and if included, may have impacted the results significantly. The

implications of the behaviors observed for each subject and their relationship to the number of triple labeled cells in the dentate gyrus would be of significant value and therefore a possibility for future research. This study, as it only includes analyses of neurobiological changes, may not be enough to understand the complex changes occurring during late pregnancy and early-lactation because the behavioral interactions were not considered.

While there is a possibility that some of the subjects included in the current study may have been neglectful or irresponsible mothers, another possible explanation for the lack of a significant difference between the mothers exposed to foster pups or to a novel object, is the amount of time the subjects had with the foster pups. It is possible that in order for the cells born during the subjects' initial maternal experiences to be re-activated by the foster pups, the amount of time may have need to be greater than sixty-minutes, if not in all but perhaps some cases. Some animals may have been more wary of the sudden exposure to young that were not their genetic offspring and thus they may have needed more time to investigate, accept, and begin to care for the foster pups.

Another limitation to mention in the current study involves the histology process and analysis of the tissue, as referenced previously. Not all sections were adequate for analysis for a number of different reasons. Due to poor slicing using the cryostat or poor mounting and cover-slipping, the dentate gyrus could either not be located or was blocked by debris in a large number of sections and therefore were not included in the analysis. The total number of images analyzed was 146.

This accounts for 100 sections from the experimental animals or 50 unilateral scores and 46 sections from control animals or 23 unilateral scores (73 total unilateral scores out of a possible 90). Starting with 10 sections per brain and 18 total brains, the reduction of tissue adequate enough for analysis from 180 to 146 was a considerable decrease. Being that 18% of the sections were not viable and thus not processed for analysis, the implications of this portion of the data on the results of the current study may have been significant.

In conclusion, while the results of the current study demonstrate a trend in supporting the initial hypothesis, they also suggest that simply evaluating the quantity of cell proliferation in the dentate gyrus as an effect of pregnancy and motherhood, and tracing the re-activation of these cells, is not enough to determine that a permanent change is occurring as a result of these experiences. Due to the lack of statistical significance in the current study and the lack of behavioral analysis, it may be inferred that there are differences in the processes and behaviors necessary for the formation and reactivation of the "maternal circuit" and its ability to change the motivations, affective states and overall behaviors of a mother forever. While this may be the case, the methods and procedures of the current study may be a valuable building block for future research.

Past research using animal models suggest that motherhood impacts the emotional, behavioral, cognitive, hormonal and neurobiological aspects of a female. While generalizations from animals to humans must be made with appropriate caution, finding that any one of these effects generalizes from animals to humans

would open the door to investigations that may provide therapeutic benefits to existing neurobiological threats to the female brain. Included in these threats are depression, anxiety disorders, neural degeneration, and maternal-offspring interactions, among others. Maternal behaviors are perhaps the most significant, critical set of behaviors for the propagation and initial survival of mammals, including humans. The importance of this research to gain a greater understanding of these behaviors, maternal motivation and responsiveness and the neurobiological route of them all, would be of great benefit to health providers but more importantly, to mothers and their children.

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Table 1.

Group	*Count*	*Sum*	*M*	*SD*
Experimental	50	1809067.34	36181.35	37911.56
Control	23	1208266.82	52533.34	40342.93

Source of Variation	*df*	*F*	*p- value*	*F crit*
Between Groups	1	2.815	0.097	3.976
Within Groups	71			
Total	72			

Descriptive Statistics and Single Analysis of Variance

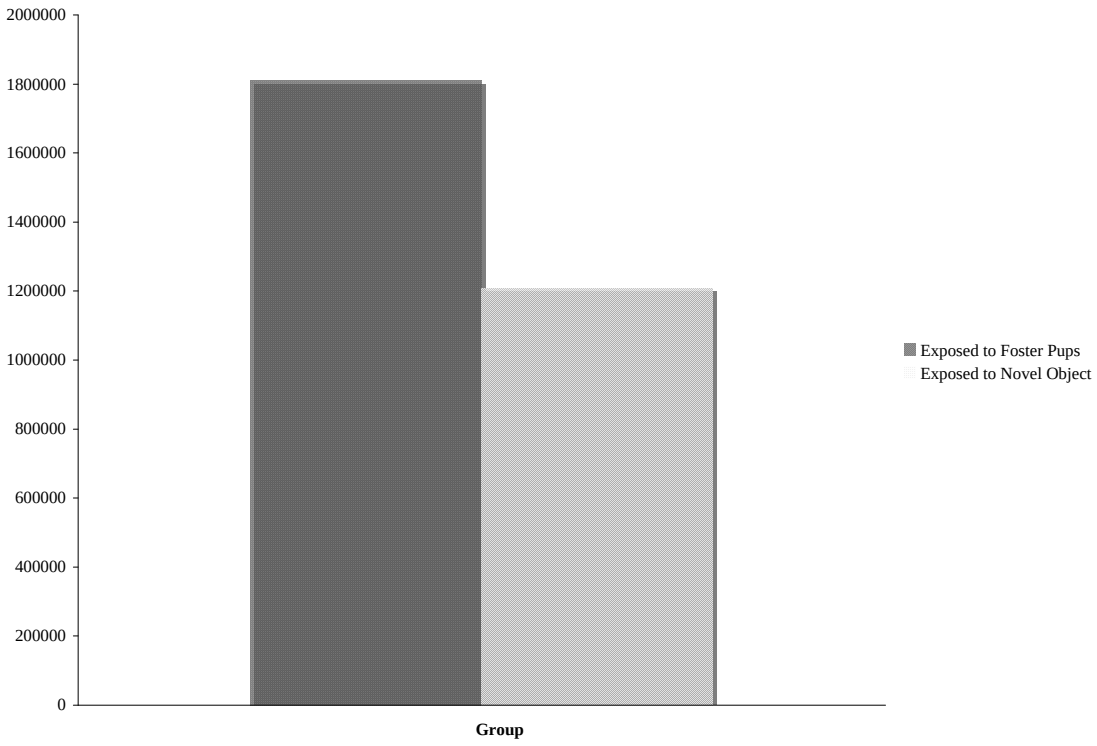

Figure 1. Area of triple labeled BrdU/c-fos/NeuN cells in the dentate gyrus of the hippocampus.

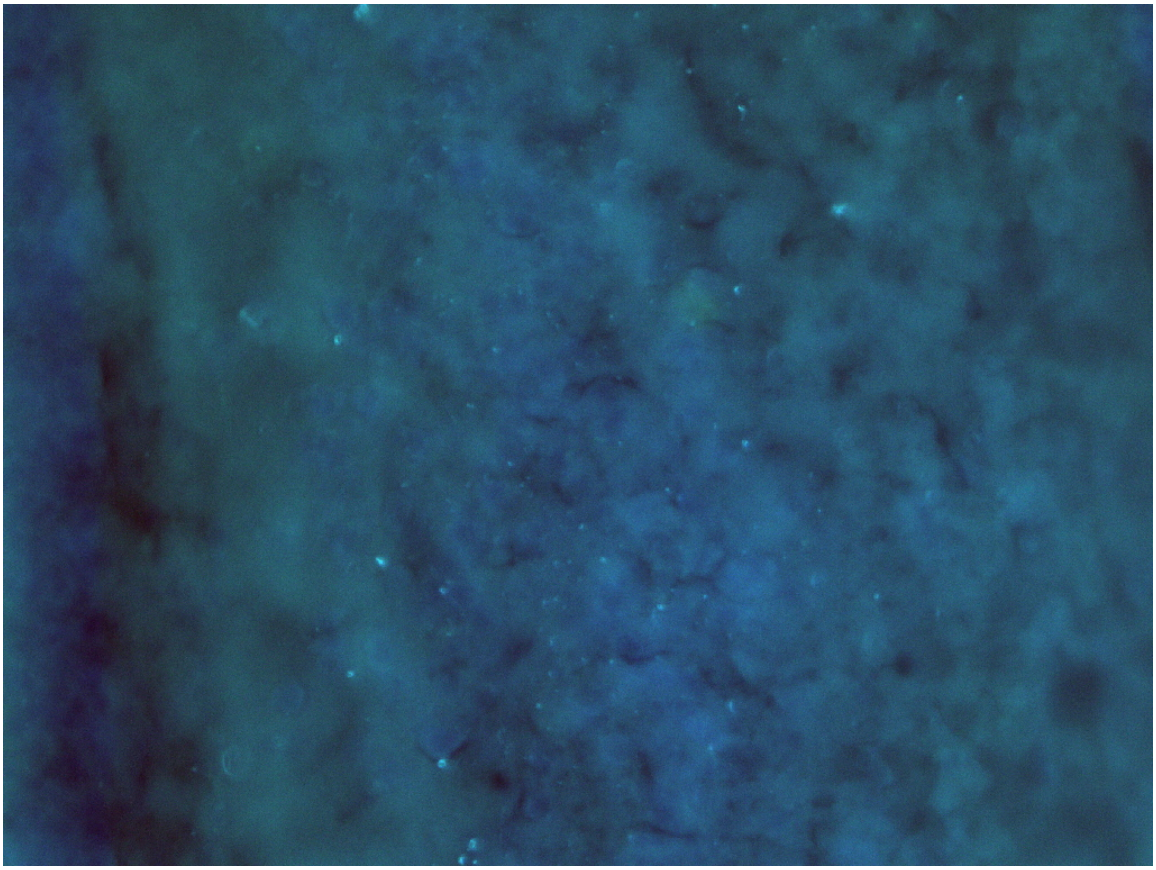

Figure 2. Dentate gyrus triple label BrdU, c-fos, NeuN fluorescent stain. Experimental subject. 20x.

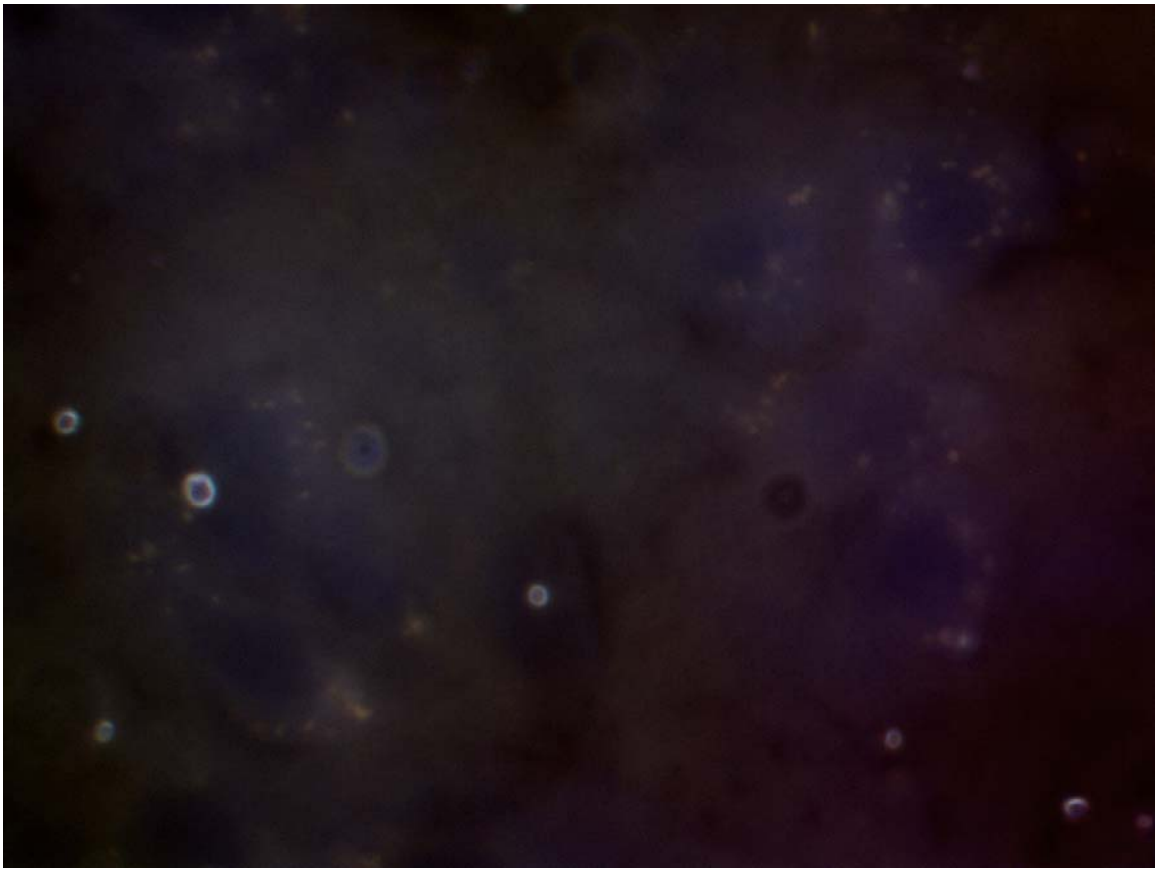

Figure 3. Dentate gyrus triple label BrdU, c-fos, NeuN fluorescent stain. Experimental subject. 100x.

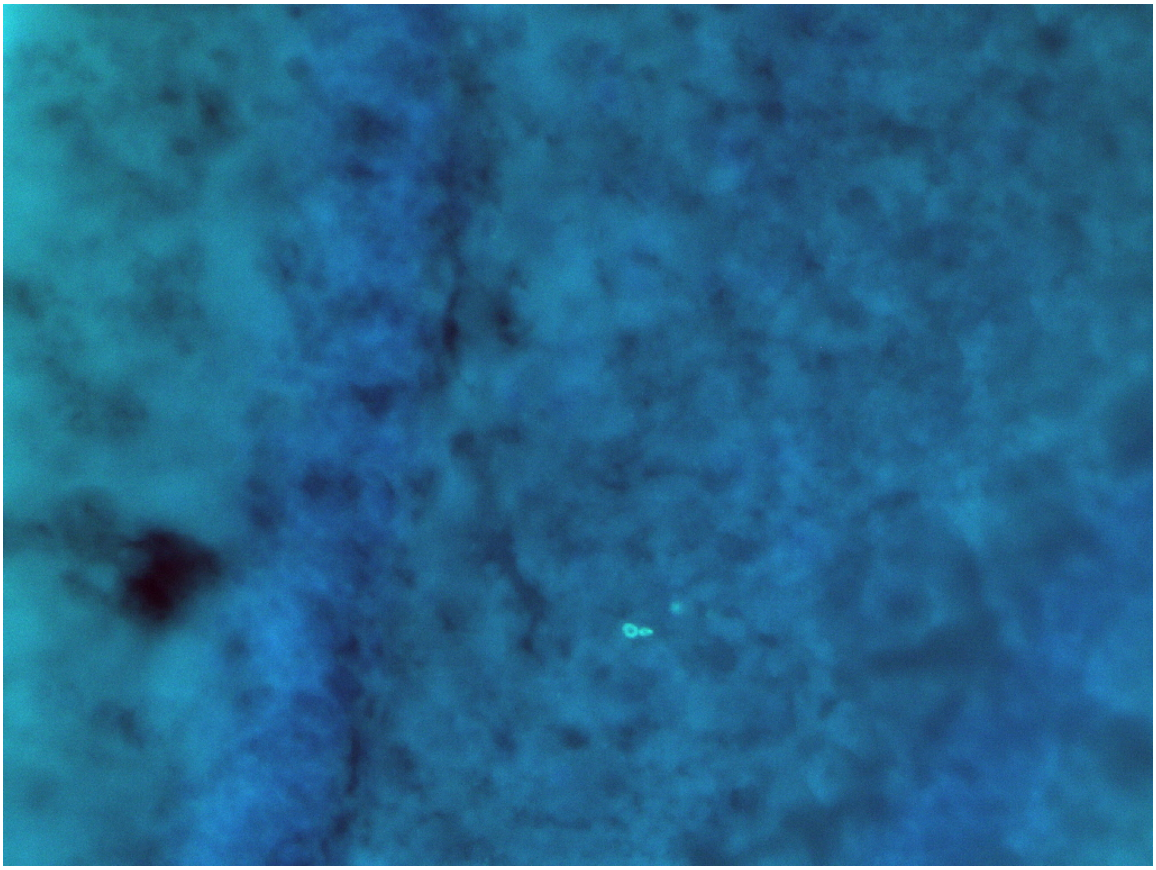

Figure 4. Dentate gyrus triple label BrdU, c-fos, NeuN fluorescent stain. Control subject. 20x.