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Reproductive experience and stress responsiveness

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Reproductive Experience and Stress Responsiveness

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Abstract

Hormonal fluctuations and maternal behavior associated with pregnancy and post-partum care of pups induce many changes in the female rat. Circulating hormonal surges during pregnancy modify the female brain in preparation for motherhood. Past studies identify the medial preoptic area, the hypothalamus and the basal forebrain as structures dense in hormonal receptors involved in controlling reproductive behavior. The hippocampus and amygdala possess many hormonal receptors. Neurons exposed to pregnancy hormones develop new synapses and increased spine density, changes reflected in behavioral preparations, such as nest building and increased foraging, for the new pups. Following the experience of birth, pup stimulation interacts with hormonal alterations to continue to change the brain and resultant behavior of the mother rat. Recently, behaviors not traditionally associated with maternal behavior, specifically learning and memory, have been found to be enriched following reproductive experience. Attenuation in stress responsiveness may be related to this observation.

This study examined stress responsiveness in primigravid, multigravid, primiparous, multiparous and nulliparous female rats exposed to a stressor (an open field) for thirty minutes, then processed for c-fos immunoreactivity of the amygdala and hippocampus (structures associated with stress perception and response). The nulliparous (control) group displayed the highest stress responsiveness on both behavioral and biocellular (c-fos expression) measures. Gravid animals exhibited the greatest stress attenuation on these measures. Parous groups displayed less stress attenuation than the gravid groups but more than nulliparous groups indicating these changes may persist beyond the reproductive experience.

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Reproductive Experience and Stress Responsiveness

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Reproductive Experience and Stress Responsiveness

Many human females report that the experiences leading up to the production of second and third children are vastly different from those associated with the first child; further, those females' behavioral and physiological responses to first versus subsequent children is similarly altered (Kojima, 1999; Gottlieb & Mendelson, 1995). In animals, modifications in brain and behavior change significantly as the females undergo from one to two and more reproductive experiences. The hormonal fluctuations characteristic of pregnancy and lactation, coupled to the many sensory cues provided by the offspring, play a major role in the parous female's transition from nulliparous to parous female. The current work examines one set of responses in the female rat, stress responsiveness, across different reproductive conditions. We will document to what extent both behavior and brain change following reproductive experience.

Hormones and Pregnancy

Many changes occur in the female rat following the onset of pregnancy. Specifically, ovum fertilization and resulting blastocyst implantation affect a variety of hormone levels circulating in the female rat. Following the onset of pregnancy (day 1), estrogen levels begin to increase on day 5 and continue a steady and gradual rise until the day of pup birth (day 21). Estrogen levels surge to the highest level on day 21 then return to baseline. While progesterone levels mirror this continuous increase, they peak on day 15 then drop to trace levels by parturition (Rosenblatt, 1979). Oxytocin levels, important for muscle contractions, also increase around the time of birth (Numan, 1990). Prolactin levels accompany these changes on day 21 with a sudden dramatic increase and maintain a consistently high concentration for lactation following pup delivery (Rosenblatt, 1979).

These hormonal changes are important for sustaining the pregnancy and preparing the female rat's body for pup delivery. These changes also prepare the female rat for pup care (Bridges, 1990).

Hormones associated with pregnancy modify the female brain in preparation for the demands of pup care. Studies of terminated pregnancies at varying stages reveal the precise onset of maternal behavior in rats. In one study, female rats whose pregnancies were terminated before day 16 failed to exhibit maternal behaviors when exposed to pups from another litter. Female rats that maintained their pregnancy to day 16 exhibited maternal behaviors even without any prior pup handling experience (Bridges et al., 1978). In another study, ovariectomized rabbits were given estradiol injections for 18 days and progesterone injections for days 2 through 15 (the first day of estradiol injections designated day 1). These rabbits displayed nest building behavior on the 18th day (Zarrow et al., 1963). Likewise, female rats treated with estradiol for 11 days, progesterone for days 6 through 9, and prolactin on days 9 through 11 displayed full maternal behavior within 3 days of pup exposure in comparison with untreated controls (which usually take twice as long; Moltz et al., 1970).

Some studies have attempted to further identify the unique role individual pregnancy hormones play in eliciting maternal behavior. Work by Fuchs and Dawood (1980) demonstrated oxytocin, which causes contractions, may induce other physiological changes that initiate maternal behavior. When oxytocin is directly injected into the ventricles of an estrogen-primed ovariectomized rat, maternal behavior can be observed within one hour (Pedersen et al., 1982). Further, McEwen et al. (1994) noted maternal behavior in rats following purely estrogen treatment. These behaviors included

constructing a more elaborate nest, licking, retrieving, crouching-over and feeding pups (Gubernick & Klopfer, 1981; Bridges, 1984). Examination of the effect of estrogen on specific brain regions has revealed a direct influence on the brain.

Increasing hormonal levels associated with pregnancy, specifically estrogen, appear to facilitate these maternal behaviors. Daniels et al. (1997) reported that neuronal changes following estrogen implantation in ovariectomized female rats persisted throughout behavioral training. Studies suggest changes are occurring following exposure to estrogen in the brain of the rat. The hormonal influence of estradiol on brain anatomy appears to involve structures directly related to maternal behavior, such as the medial preoptic area, the hypothalamus and the basal forebrain (McEwen, 1994). These structures display dense areas of estrogen receptors and are involved in controlling reproductive behavior (McEwen et al., 1997). McEwen et al. (1997) also reported the formation of new excitatory synapses with estradiol treatment in the hippocampus.

Recent work has focused on neuronal changes following estrogen administration in other brain areas not traditionally associated with maternal behavior: the hippocampus and amygdala (McEwen et al., 1997). Such work demonstrates the complex and diffuse effect of estrogen on brain processes. Koch and Ehret (1989) demonstrated brain plasticity following estrogen exposure in late-pregnant mice (a time when estrogen levels are particularly high). In this study, the highest number of estrogen receptor labeled cells was found in the late pregnant group. These changes were particularly conspicuous in the anterior amygdala and bed nucleus of the stria terminalis. McEwen et al. (1997) noted these structures respond well to estrogen and progesterone treatment during the natural estrous cycle. In related studies, estrogen treatment induced the extension of

dendritic spines and the development of new synapses in the ventromedial hypothalamus and increased the density of spines on pyramidal neurons in the hippocampus (McEwen & Wooley, 1994). These changes have been associated with enhanced learning and memory capabilities (Kinsley et al., 1999). Such physiological and behavioral alternations in the pregnant female rat brain appear to persist even after parturition.

Hormones and the Post-Partum Period

Hormonal levels continue to fluctuate even after pup delivery. High prolactin levels continue to rise during days 1-14 of lactation, then lessen as pups begin to grow and are weaned. This hormone is also associated with nesting time and increased appetite in the female rat (Leon et al., 1990). As pups age, more mutually initiated contact is observed. Whereas the mother continues to initiate pup care, often pups will approach her for warmth and suckling, which in turn stimulates milk production (Stern et al., 1992). An autocrine mechanism is likely responsible for this relationship given that sustained pup suckling results in increased galanin expression which amplifies lactotroph stimulation (Ren et al., 1999). Endogenous opioid peptides also are believed to play a role in supporting this process (Callahan et al., 2000). Also remarkable for milk-ejection is an enhanced oxytocin receptor expression, binding and release in the hypothalamus and other limbic structures (Kendrick & Keverne, 1992). When the nipple is stimulated by the feeding pup, oxytocin is released. Circulating oxytocin then causes cells in the mammary glands to contract and milk is secreted (Stern et al., 1992).

A number of factors appear to initiate the decline of lactation. Increasing thermoregulation in growing rat pups causes the mother rat to decrease maternal contact. (Leon et al., 1990; Fleming, 1986). In addition, glucocorticoids are believed to play a

role in declining nest time and weaning. ACTH increases have been noted during the first two weeks of lactation. As this increase raises levels of corticosterone in the blood, the mother rat begins to spend less time in the nest and the pups begin to forage for food and water resources on their own (Toufexis, et al, 1999).

Following pup weaning, the maternal brain is believed to return to pre-pregnancy levels. Recent data, however, indicate this may not be the case. In addition to transient hormonal alterations during the acute phase of pregnancy and lactation, Trainer et al. (1997) reported that neuronal changes in the hippocampus emerged and persisted even when reproductive experience terminated. In this study, Trainer et al. (1997) noted increases in dendritic spine concentrations following reproductive experience. Gifford et al. (1999) further described enduring cellular augmentation (i.e., increased glia cell number and glia cell morphology) in the parous female. Clearly these fundamental changes are paramount to the virgin rat's transition to the demands of motherhood and endure more extensively (and possibly permanently) in the female brain than once believed.

Reproductive Experience

Reproductive experience and maternal behavior modify the female rat. For example, age-matched females with reproductive experience (parity) exhibit improved learning ability compared to age-matched female rats without reproductive experience (nulliparity) (Kinsley et al., 1999). This improved learning/memory ability has been proven by the parous female rat's ability to negotiate the eight-arm radial and dry land mazes more quickly and more efficiently (Kinsley et al., 1999). Preliminary data from the University of Richmond neuroscience laboratory also suggests that stress responsiveness

may decrease with reproductive experience and these changes may persist even when reproductive experience is terminated (Wartella et al., preliminary observations). It is believed this change in stress responsiveness is due in part to the experience of pregnancy and pup stimulation (Kinsley et al., 1998).

Daniels et al. (1997) further noted behaviors not specifically associated with reproductive experience, mainly learning and memory, were enhanced by parous hormones and maternal parent/offspring interactions. In addition to these maternal behaviors directly involved in pup care, other indirect brain processes are refined as well. For example, greater nutritional demands may direct the mother to find and remember the location of additional food and water resources. Whereas hormones of pregnancy have been noted to play a role in these responses, the presence of pups also appears to stimulate plasticity in the female rat brain independent of hormonal influences. Structural changes in the brain may also arise from repeated pup exposure and maternal behavior itself (Kinsley et al., unpublished data). Frequent sensory input related to pup exposure has been found to alter the plasticity of the nulliparous female rat's brain particularly with regard to her perception of pups (Leon et al., 1992).

Whereas nulliparous female rats appear stressed and avoid pups, repeated exposure improves the likelihood that virgins will make contact (Fleming, 1986). In addition, virgin rats display maternal behaviors similar to parous animals following pup exposure, including modified crouching and pup retrieval (Lonstein et al., 1999). Sugiyama et al. (1996) further demonstrated how reproductive experience and hormones interact with experiential factors. In this study, pup contact induced the expression of long form prolactin receptor mRNAs in the choroid plexus of the brain in both

ovariectomized and hypophysectomized virgin rats. As such, it appears not only do hormones mediate maternal behavior, but also experiential factors influence hormonal expression and brain plasticity. Hormonal levels and pup stimulation associated with pregnancy appear to interact to enhance brain chemistry and brain morphology known to be important for enhanced cognitive functioning.

Reproductive Experience and Stress

Post-partum female display attenuated stress responsiveness following pup delivery (for the purpose of this study, stress responsiveness is defined as a biological and behavioral reaction to an aversive stimulus that interferes with the organism's homeostasis). Whereby most rats experience an aroused hypothalamo-pituitary-adrenal (HPA) axis and hypothalamo-neurohypophysial system (HNS) as a result of a stressor, this network appears blunted in female rats as early as day 15 of pregnancy and continues throughout lactation. Alterations in levels of corticotrophs in the adenohyophysis are due to reduced ACTH secretion (Neumann et al., 1997). Corticotrophin-releasing hormone is likely responsible for this reduction through pup delivery and early lactation. Stress induced rises in plasma hormone concentrations were found to attenuate on day 15 and progress through late pregnancy and lactation (Douglas et al., 1998). In a related study, Komesaroff et al. (1998) reported reduced glucocorticoid responses to stress in female sheep treated with estradiol. Furthermore, lactating rats have been known to show reduced prolactin and oxytocin responses to restraint stress and abated HPA responses to restraint, ether stress and forced swimming (Carter et al., 1987; Higuchi et al., 1988; Higuchi & Negoro, 1989; da Costa et al., 1996). In human studies,

estrogen lowers systemic blood pressure and to lessen the incidence of cardiovascular disease in women (symptoms traditionally attributed to stress; Huang et al., 1998).

The presence of oxytocin as early as day 15 has been identified as a potential mediator of the attenuated stress responsiveness of the female rat. Basal levels of oxytocin differ significantly among virgin and pregnant rats on days 18 and 21 (with drastic increases found in the late pregnant groups; Neumann et al., 1997). Oxytocin continues to circulate in the plasma of the female rat in response to nipple stimulation. In addition, the number of oxytocin receptors increases in the limbic and hypothalamic regions of the brain during pregnancy and remain throughout lactation (Freund-Mercier et al., 1994; Insel, 1990). Whereas stress will increase oxytocin levels in virgin rats, these levels remain stable in pregnant or lactating rats exposed to a stressor (Douglas et al., 2000). Pregnant and lactating rats already possess high levels of oxytocin and perhaps increasing these levels due to stress may cause harm to the unborn or newborn pups. The concurrent presence of endogenous opioids during late pregnancy and lactation appears to interact with the HPA/HPN axis to further inhibit oxytocin expression to stress as a protective mechanism against excessive circulating stress hormones (Neumann et al., 2000). As such, da Costa et al. (1996) suggested increased oxytocin and opioids resulting from pregnancy and lactation may reduce stressor perception in limbic structures. Such structures have been noted to be associated with reduced stress-induced c-fos mRNA (an immunocytochemical marker of stress) expression in late pregnant and lactating rats as well (da Costa et al., 1996).

Interestingly, glucocorticoid receptors have been identified in hippocampus, as well as amygdala, areas of the brain known for advanced cognition and emotional

reactivity (McEwen et al, 1968). Glucocorticoids are involved in the animal's reaction to stress (Davis et al., 1994). Recently, alterations in stress responsiveness in these areas has been reflected at the behavioral level. Kinsley et al. (1999) noted parous rats were able to learn an eight arm radial maze faster than virgin controls. These reproductively experienced rats exhibited less freezing behaviors and explored the maze in less time than the virgin group. Such behaviors have been associated with stress in rats. Neumann et al. (2000) recently observed a mitigated stress reaction in late pregnant and lactating rats placed in the elevated plus maze as well.

Preliminary data suggest that age-matched animals with reproductive experience exhibit an attenuated stress response in comparison with nulliparous animals when exposed to varying types of stressors (Wartella et al, preliminary data). Recent pilot data from a stress study considering c-fos expression (in the CA3 region of the hippocampus and amygdala) resulting from a half an hour of restraint (via restraint tube) suggested parous animals display less c-fos expression than nulliparous animals. Parous groups in this study had been weaned from their pups for ten days at the time of testing. These measurements were taken on day 52 (pups weaned 10 days), a time when the now parous animal's body returns to its pre-pregnant state. Whereas these data and other studies (Neuman et al., 1998; Komesaroff et al., 1998; Smith et al., 1997) suggested permanent changes at the biochemical level, no study to date has considered how these changes may manifest themselves in brain anatomy related to advanced cognitive functioning. Further, additional behavioral data are needed to further describe and understand the impact of parity on stress responsiveness in rats.

Parity, Stress and The Current Project

Recent studies have indicated that repeated reproductive experience causes long-term, possibly permanent, physiological and behavioral changes in the female rat. As the experience of pregnancy, lactation and pup exposure interact to modify the female rat, it follows logically that these effects may become multiplicative with additional parturitions. Mann et al. noted this possibility in finding differential hypothalamic proopiomelanocortin (POMC) gene expression among female rats with varying degrees of reproductive experience. In this study, multiparous animals revealed an increase in the number of POMC cells in comparison to the primiparous and virgin groups (Mann et al., 1997). Plasma prolactin, dopaminergic and opioid levels also vary as number of parturitions increases (Bridges et al., 1993; Felicio et al., 1996; Mann et al., 1988). Mann and Bridges (1992) even demonstrated reductions in behavioral and neural sensitivity to morphine as a function of number of pregnancies and/or lactations in the female rat. Given the connection between these endogenous systems and maternal behavior, it seems likely that long term alterations of parity may affect stress responsiveness as well (Kinsley & Bridges, 1988).

Multiparous rats stressed in a restraint tube for 30 minutes displayed less c-fos immunoreactive cells in the amygdala and hippocampus than their age matched primiparous rats (Wartella et al., 1999). While primiparous rats displayed more c-fos immunoreactive cells than multiparous rats, they displayed less immunoreactivity than virgin rats. These findings suggest that multiple experiences of pregnancy, lactation and pups may inure a female to stress and render her less susceptible to the behavioral or other disruptions that stress sensitivity can produce. Such additive changes in the female

brain may be related to the neural alterations that accompany successive experiences of pregnancy, lactation and pup stimulation.

Whereas previous studies have attempted to better understand this mechanism on a biochemical level, no study to date has considered how parity may relate to stress responsiveness during the acute and post-partum stages of pregnancy. This project seeks to confirm whether or not an attenuated stress response is related to reproductive experiences. Further, it asks whether there will be a cumulative difference between the parous female groups (i.e., the multiparous (MP) group is expected to display an additive effect related to the experience of two pregnancies versus only one in the primiparous (PP) group). Given the connection between the experience of stress (amygdala and hippocampus) and cognition (hippocampus), these predicted differences in stress responsiveness will be measured using an open-field apparatus to elicit behavioral differences exploring a novel and stressful open environment.

In addition to the aforementioned groups, this study also seeks to better understand changes in stress responsiveness during acute reproductive time periods. As differences are found in neuron proliferation rates at different stages of pregnancy (Amory et al., 1999), so too might differences in stress responsiveness account for these changes and provide another example of the potential neuroprotective effects attributable to high levels of hormones during pregnancy and increased pup stimulation. The current project will consider the stress responsiveness of late pregnant (gravid) animals exposed to one (primigravid (PG)), two (multigravid (MG)), and zero (nulliparous group (N)) pregnancies. Consistent with pilot study trends, additional stress mitigation (defined as crossing more grids, more rearing, less defecation) is expected in gravid groups compared

to parous and nulliparous groups. Further, multigravid animals should display the least amount of stress thereby demonstrating the additive effect of additional reproductive experiences and acute hormonal exposure.

In the final phase of this experiment, immunocytochemical techniques will be used to measure biocellular indicators of stress responsiveness. If changes are found at the behavioral level between the aforementioned groups (N, PG, MG, PP and MP), differences in c-fos expression (an immunocytochemcial marker of stress in the brain) are expected to confirm such changes as well.

Open Field Testing & c-fos Quantification

An open field apparatus was used to assess stress responsiveness (a wooden square box (3 feet by 3 feet) with the top of the box left open for observational purposes). The enclosing walls are approximately 2 feet high and the plexiglass floor is sectioned into 36 6-in x 6-in grids to mark the location of the rat as she explores the area. Munn was the first to examine this design and develop testing procedures for evaluating stress behavior (1950). Gershenfeld and Paul (1997) used a similar design to measure the effect of anxiolytic drugs. They measured "wall-seeking" tendency as indicator of stress, as rats fear open spaces and usually seek the wall for protection and comfort (Gershenfeld and Paul, 1997). Ramos et al. (1997) extended this measurement to include exploratory measurements (average grids crossed) and avoidance of stressful stimuli (the center of the box). Defecation and urination were also measured in this design (Ramos et al., 1997). Poltyrev et al. added the measurement of rearings (standing on hind legs) to further describe exploratory behavior in open fields (Poltyrev et al., 1996). Preliminary data included recording the number of startle responses (rat visibly jumps) as a measure of

acute stress (Kinsley, unpublished observations). Open field behavioral testing provides observable measurements of stress and exploratory behaviors, which can be confirmed by a cellular measure of brain activation. The amount of anatomical c-fos expression was quantified to establish and compared to behavioral responses.

C-fos is a proto-oncogene that is activated under a variety of conditions, including encounters with a stressful, threatening or novel stimulus. A proto-oncogene is a gene that regulates protein synthesis. The product of c-fos plays a role in cell proliferation, differentiation and signal transmission as the 'third messenger' which then regulates the transcription of other genes. As such, c-fos is the key that turns on the animal's cellular response to a stimulus including stress (Curran et al., 1984). When, for example, the animal perceives stress, c-fos becomes activated and sets off a chain of biochemical events. Active c-fos initiates the protein synthesis of molecules (fos) that stimulate glucocorticoids to initiate the hypothalamic-pituitary-adrenal stress arc and prepare the animal for a fight or flight response. As noted earlier, previous studies reported that estrogen reduced cortisol levels in ovariectomized female sheep (Komesaroff et al, 1998). Further, a relationship between stage of estrous in intact female sheep also indicated differences in cortisol secretions (Komesaroff et al., 1998). Komesaroff et al., however, neither analyzed brain morphology nor recorded behavioral responses of the female sheep to the experimental stressors (dog barking). Thus, the current work seeks to better understand the relationship between these changes in the female rat at different stages and with varying amounts of reproductive experience.

Methods

Animals

Forty (N=40) adult nulliparous female Sprague-Dawley rats were assigned to one of five age matched (50-56 days old) groups: Nulliparous (Virgin/No maternal experience)(N=8), Primigravid (one mating/first pregnancy)(N=8), Primiparous (one-mating/one pregnancy)(N=8), Multigravid (two matings/two pregnancies) (N=8), and Multiparous (two matings/two pregnancies)(N=8). Animals assigned to the nulliparous group were single-housed in a plastic, opaque rat cage and left undisturbed for 114 days following confirmed mating of the multiparous group (see proposed study timeline in appendix A).

Multiparous animals were mated according to the schedule indicated in the appendix (see appendix for mating time line). Pregnancies (and time line) were tracked from the first day of confirmed mating (confirmed mating was designated Day 1). Upon delivery at day 21, pup litters were culled to six pups to provide equal stimulation to the mother. The pups were then weaned from their mother at day 42 (21 days following birth). Ten days after weaning, the now primiparous rats were re-mated at day 62. Following confirmed mating, 21 days of pregnancy was again tracked. Litters were culled to 6 pups. Following delivery and 21 days of lactation, pups were weaned from their mother. On day 114 (10 days post second pregnancy weaning), the now multiparous female rat's behaviors were tested in the open field during the morning.

Animals assigned to the multigravid group were mated at day 43. Following 21 days of gestation, 21 days of lactation and 10 days of pup weaning, the group was remated (day 95). Following confirmation of mating, the day count resumed (with

confirmed second mating = day 95). Following another 21 days of gestation (day 114), multigravid animals began behavioral testing the morning before giving birth.

The primiparous group was mated at day 62. Following 21 days of pregnancy, 21 days of lactation and 10 days of weaning, primiparous animals began behavioral testing on the morning of the 114th day.

Finally, animals assigned to the primigravid group were housed likewise and were mated on day 83. The offspring from these groups were culled to 6 pups. Following 21 days of gestation, this group began behavioral testing on the morning of the 114th day.

All protocols were performed in accordance with the University of Richmond guidelines for use of animals in research.

Behavioral Testing

On day 104, animals were individually placed in a 3-ft x 3-ft open apparatus. Each animal was placed initially in the center block at the start of the experiment and all relevant behaviors (number of startle responses, rearings, freezings, number of blocks crossed and number of center four blocks crossed (as a measure of how often the animal moved away from the wall) and number of fecal boli) were recorded for thirty minutes. Startle responses are indicated when the rat visibly jumps. Rearings were defined by the rat standing on her hind legs (with both front legs completely raised off the floor). Freezing occurred when the animal ceased all movement. Number of blocks crossed was defined as the total number of blocks the animal crossed within thirty minutes. All four paws had to be located in the block in order for that block to be counted. Number of center blocks crossed is a measure of how often the animal explored the center-most four

blocks. All four paws had to cross a center block in order for it to be included. These measures were adapted from Munn's (1950) review of psychological research on the rat.

All behavioral testing was coded blindly by observers. The open field was cleaned with alcohol between trials to control for olfactory influences remaining from previous trials. Consistent with standard procedure reported in the literature, animals were returned to their own cages for a half an hour following behavioral testing to maximize specific c-fos expression.

All behavioral data between groups were compared using ANOVA SPSS statistical software.

Tissue Preparation

Animals were euthanized by overdose of sodium pentobarbitol. Brain tissue was fixed using the following standard immunocytochemical procedures: The animals were perfused transcardially by 100 ml of phophate buffered saline solution for 2-3 minutes and fixed with a 200 ml solution of 2% paraformaldehyde for 20 minutes. The brain was then be removed from the skull and placed in a container of paraformaldehyde for two hours. Paraformaldehyde was then drained from the container and then replaced by a 20% sucrose solution in phosphate buffered saline. The brain was submerged in this solution for approximately 12 hours (or until the brain sank to the bottom of the container indicating absorption). Brains were then blocked for the area of interest (hippocampus CA3 region and the basolateral amygdala) and frozen to 21 C. Upon freezing, every sixth section (to control for overlap) was taken at 40 microns using a cryostat apparatus and placed two to a well. After washing the tissues with phosphate buffered saline, the primary antibody was added (1:1,000 concentration) and incubated overnight (12 hours).

The tissues were then rewashed and incubated one hour in a solution containing the secondary antibody and biotinylated mixture. Thereafter, a final series of washes was completed and di-amino benzene (DAB) added to stain areas of c-fos activation. Stained tissues were mounted on subbed slides and left to dry overnight. The following morning, slides were cleared in 70, 90, and 100% alcohol, then xylene. and coverslipped using permount bonding solution and a cover slip. Covered slides were left to dry one week before quantifying on a microscope.

C-fos Quantification

Dried slides were quantified using a Neurolucida Image Analysis software package which displays microscope images on a computer screen. At 40 x magnification, c-fos stained cells were counted bilaterally for each area of interest (CA3 hippocampus and basolateral amygdala), and the total number of c-fos stained cells for each slide summed. Hippocampus sections were taken once all three regions of the hippocampus (CA1, CA2 and CA3) had emerged at low (10X) magnification. Eight sections were taken total. The CA3 region of the hippocampus was determined by moving approximately 333 micrometers to the left (in the left hippocampus, or right in the right hippocampus) and approximately 171 micrometers down and centering the crossbar on that location, then increasing the magnification. The basolateral amygdala was determined by locating the inferior external capsule and centering the crossbar immediately inward approximately 281 micrometers. These measures varied slightly with larger hippocampus and amygdala structures. Numbered slides were assigned another letter by another experimenter to ensure blind coding and eliminate prior knowledge of group assignment bias. Four slides were quantified for each brain (with

two sections to slide). All totals for each structure were averaged together for each reproductive group to get the average total for that structure. Total c-fos for each group was compared using an ANOVA SPSS statistical package.

Results

Behavioral Data

A one way between subjects analysis of variance was performed using SPSS on six dependent variables (DV) by pregnancy group: Rearing, freezes, number of blocks crossed, number of center blocks crossed, startles, and fecal boli. Post hoc analysis using Tukey HSD were performed on all significant findings.

Significant differences were found in every behavioral measure except the number of startles, $F(4, 36)=1.071$, $p>.05$ and number of fecal boli, $F(4, 36)=1.242$, $p>.05$. For rearing behaviors, significant differences were found among reproductive groups, $F(4, 36)=39.98$, $p \leq .001$. Whereas post-hoc analysis revealed all three stages of pregnancy (i.e., both parous groups were significantly different from virgins and gravid groups; Gravid animals were significantly different from virgins) displayed significantly different number of rearings, number of pregnancies (i.e., whether the animal had experienced more than one pregnancy) did not appear to influence this relationship except whereby the animal had no experience (i.e., the nulliparous group) (i.e., there were no significant differences between the multiparous and primiparous animals. Further, there were no differences between multigravid and primigravid animals). Nulliparous animals demonstrated the least number of rearings and this was significantly different from all other pregnant groups.

Significant differences in the amount of freezing behaviors were also found among reproductive groups, $F(4, 36)=16.68$, $p \leq .001$ (see Figure 2). Post-hoc tests revealed the number of freezes was only significantly different between the nulliparous (no reproductive experience) and reproductive experienced groups (i.e., Primiparous, multiparous, primigravid and multigravid). No other differences were noted among reproductive groups.

A similar pattern of significant differences was found in crossing individual grid blocks with Total number of blocks crossed, $F(4, 36)=10.18$, $p \leq .01$ (see Figure 3) and Total number of center blocks crossed, $F(4, 36)=6.384$, $p \leq .001$. There were no significant differences in Total number of blocks crossed, nor the Total number of center blocks crossed among animals with reproductive experience on post-hoc analysis. Nulliparous animals, however, crossed a significantly fewer blocks and a significantly fewer center blocks than all other reproductive experienced groups.

No significant differences were found in the number of fecal boli, $F(4, 36)=1.242$, $p=.31$ (see figure 5), nor startle behaviors, $F(4, 36)=1.071$, $p=.24$ (see figure 6).

c-fos Data

A one way between subjects analysis of variance was performed using SPSS to measure differences among reproductive groups in c-fos expression in two stress influenced anatomical areas: the CA3 hippocampus and the basolateral amygdala. Post hoc analysis using Tukey HSD were performed on all significant findings.

Significant differences in the amount of c-fos expression were noted in the hippocampus, $F(4, 36)=59.37$, $p \leq .001$ (see Figure 7). Post-hoc analysis revealed significant differences in c-fos expression among stages of pregnancy, but not number of

pregnancies (i.e., both the primigravid and multigravid groups exhibited a significant difference in c-fos expression in comparison to primiparous and multiparous groups, but these groups did not show differences among c-fos expression as a result of number of pregnancies). The nulliparous group displayed a significantly higher number of c-fos immunoreactive cells than any of the reproductive groups.

This pattern of significant differences was consistent in the amygdala as well, $F(4, 36)=26.26$, $p \leq .001$ (see Figure 8). The nulliparous group had significantly higher c-fos expression than the reproductive experienced groups. Both the multiparous and primiparous groups displayed higher c-fos expression than both primigravid and multigravid groups. Multiparous and primiparous animals did not show any significant differences in c-fos expression among each other, nor did multigravid and primigravid animals show any significant difference.

Relationships between Measures

Pearson correlations (see Table 1) were conducted on all behavioral and c-fos measures. Moderate to strong associations were noted among behavioral and c-fos measures of stress. Freezing, in particular, was highly correlated with c-fos expression in the amygdala ($r=.763$, $p<.001$) and hippocampus ($r=.784$, $p<.001$). Number of blocks crossed and number of center blocks crossed were also highly inversely related with c-fos expression (Hippocampus: $r=-.937$, $p<.001$; $r=-.623$, $p<.001$; respectively; Amygdala: $r=-.928$, $p<.001$; $r=-.600$, $p<.001$). Moderate correlations included the number of fecal boli and c-fos expression in the amygdala, $r=.414$, $p<.05$, but not hippocampus, $r=.299$, $p=.072$. Startles were only minimally associated with c-fos expression in the amygdala,

r=.373, p<.05, but not hippocampus, r=-.321, p =.053. Rearing did not correlate with any behavioral or c-fos measures.

Discussion

Overview of Results

Female rats with reproductive experience exhibited an attenuated stress response in behavior and in the brain. These findings are consistent with several studies that demonstrated modifications in behavior (Neumann et al., 2000; Madonia et al., 1999; Mann et al., 1997) and biochemistry (McEwen et al., 1997; Trainer et al., 1997; Costa et al., 1997; Higuchi et al., 1988; Felicio et al., 1996; Bridges et al., 1993; Mann et al., 1988) following the onset of pregnancy in rats. While greater effects were observed during the acute phase of pregnancy on most behavioral and biochemical measures, these changes were maintained (though less prominent) even after lactation ceased. Whereas changes in behavioral and biochemical stress reactivity were observed at different stages of the maternal experience, additional pregnancies did not appear to further modify stress responsiveness.

Reproductive Experience, Stress and Behavioral Outcomes

Female rats with reproductive experience displayed less behavioral stress responsiveness than virgin female rats. Female rats in the acute stages (i.e., primigravid and multigravid groups) of pregnancy (day 21) exhibited fewer freezes during a half an hour of open field stress. In addition, these animals demonstrated more exploratory behavior than any other group. They reared up on their hind legs to look around the new environment and actively explored even the center four blocks, an area typically regarded as stressful in the open field.

This finding is consistent with the previous work of Kinsley et al. (1999) who noted less exploratory behavior in virgin rats placed in an eight arm radial maze to explore and remember the location of food and water caches. Reproductive experienced animals in the present study also demonstrated less stress responsiveness by more actively moving about the open field (as reflected in the total number of blocks crossed) than any other group. These changes have great ecological and adaptive value to the pregnant rat that is faced with the demands of pregnancy and raising pups. In addition to having to forage for additional food and water sources to compensate for the greater nutritional demands associated with pregnancy, it is also important that this additional demand does not cause an increase in circulating stress hormones which could adversively affect developing pups. In addition, stress mitigation might also ensure that additional stress hormones (such as oxytocin or glucucorticoids) will not harm the developing young in late pregnant females. (Fleming, 1986).

This observed change in stress mitigation in parous females is likely due to dampened perception of stress whereby stimuli that would normally evoke unnecessary stress is now evaluated as less threatening in the absence of an acute stressor. Contrary to prediction, however, no further attenuation of the stress response was seen in female rats with more than one pregnancy. As such, the initial experience of motherhood might alter the stress reactivity of the maternal brain as a primary, rather than cumulative, modification. Small sample sizes might also explain the lack of significant statistical differentiation in stress responsiveness between the two reproductively experienced groups.

These changes appear to be maintained, although less so, in the primiparous and multiparous groups of female rats. These groups both displayed significantly more exploratory behavior and less stress responsiveness than the virgin group, yet higher stress responsiveness and lower exploratory behavior than both the primigravid and multigravid groups. The primigravid and multigravid females also crossed a higher number of center blocks and higher number of total blocks than virgins, yet less than either primiparous or multiparous group. By contrast, the primiparous and multiparous groups displayed the greatest number of rearings. Despite the likelihood that such high numbers of rearings may have been difficult for the primigravid and multigravid groups due to their awkward and more weighted abdomens, these animals still reared more often than nulliparous animals.

Virgins had the greatest number of freezings. In addition, freezes were higher in the primiparous and multiparous groups in comparison to primigravid and multigravid groups. As such, it appears some of the exploratory behaviors associated with an acute pregnancy stage (day 21) decline after the pups are weaned from the mother. Whereas these findings are consistent with past studies that indicate a decline in stress mitigation following pregnancy (Neumann, 2000), past work failed to delineate precisely how long these effects remained. Although the exact mechanism by which these two reproductive groups differ is not known, the presence of pregnancy hormones appears to influence this outcome in combination with pup exposure (Neuman et al., 1998; Komesaroff et al., 1998; Smith et al., 1997). Regretfully, this study was unable to determine at what point this change may occur.

Since the primigravid and multigravid groups displayed more stress attenuation than primiparous and multiparous groups with pup exposure, acute hormonal fluctuations associated with pregnancy are likely responsible for changes in the female brain. As these changes were noted post pup weaning, however, alterations in the parous animal's behavior likely endure indefinitely beyond the reproductive experience. This finding has great adaptive value to the reproductively experienced female that will likely deliver additional litters of pups throughout her lifespan. As such, less energy would be expended in reinitiating the mechanism for stress mitigation associated with the demands of motherhood. Additional behavioral and biochemical testing at longer intervals post-weaning may help substantiate this possibility.

Nulliparous female rats displayed the most stress responsiveness (freezing) of all groups. Nulliparous females spent more time along the wall than both gravid or parous groups, as was evident in the fewer number of center blocks crossed. In addition, their exploratory behavior was less than other groups as reflected in their low number of total grids crossed. This finding was consistent with past findings of nulliparous groups in novel situations (Neumann, 2000; Madonia et. Al., 1999; Komesaroff et al., 1998). Remarkably, nulliparous animals only have to forage for their own survival, not that of pups. As such, a mitigated stress responsiveness is not as necessary as it is for gravid and parous groups.

Finally, although startle behaviors and number of fecal boli did not differ significantly between groups, nulliparous rats were startled more and produced more fecal boli than any other group. It appears acute stress might have been more often expressed in freezing behavior than in startle jumping. It seems logical that a rat placed

in a perceived threatening situation might choose to cease movement rather than call greater attention to itself. Of special note, extraneous noise was kept to an absolute minimum, which eliminated additional unpredictable stress to the testing animals. Furthermore, a small sample size may have prevented statistical significance from being achieved. These possibilities could explain the lack of significant differences found between the groups.

Reproductive Experience, Stress and Biochemical Outcomes

In addition to less behavioral stress, female rats with reproductive experience exhibited less c-fos expression than virgin female rats. Female rats in the acute stages (primigravid and multigravid groups) of pregnancy (day 21) revealed the least amount c-fos immunoreactive cells in both the amygalar and hippocampal regions of the brain. In this way, both brain structures associated with anxiety and learning (respectively) were altered by the acute experience of pregnancy. Both primiparous and multiparous groups displayed significantly more c-fos immunoreactive cells in the amygdala and hippocampus than either gravid group, yet significantly less c-fos immunoreactive cells than nulliparous rats. Nulliparous rats displayed the highest number of c-fos immunoreactive cells in both the amygdala and hippocampus than any other group. This finding was consistent with previous work by Costa et al. (1997) who also noted decreased immunoreactivity of c-fos in cognition sensitive areas of the brain (hippocampus and amygdala, in particular) during lactation. Concordant with the behavioral results in this study, no statistically significant differences were found as a function of number of pregnancies (i.e., no differences were found in animals with more than one pregnancy within each stage (late pregnant or post-weaned) pregnancy. .

Remarkably, however, groups with prior reproductive experience (more than one pregnancy) at each respective stage of pregnancy revealed less c-fos activation than those that were in their first (or had only had) one pregnancy at that precise stage. As such, the number of reproductive experiences does appear to have some additive affect although significant changes are not being reflected in this small sample.

These findings have important implications for the animal transitioning from a nulliparous state to the responsibilities of motherhood. For a female rat confronted with a novel stimulus, the hippocampus actively perceives the degree of realistic threat this new stimulus presents (McEwen et al., 1997). For a female hyper-reactive to stress, more care would be taken to protect oneself than accurately interpret the presenting situation. Learning would be clearly compromised in such a situation. However, a hippocampus more adept at accurately making this determination with the use of refined cognitive abilities would be a great advantage to the animal preparing to deliver and care for pups. The amygdala and hippocampus of such an animal likely interact and delay and/or inhibit overly reactionary responses based on present and prior learned information. Rather than just reacting, perhaps the mother (or pregnant) rat can more efficiently interpret whether or not a realistic threat exists and thereby engages in fight or flight behavior. If the threat is determined to be invalid, further exploration and resistance to the disruption from the task at hand provides the mother rat not only with greater efficiency but also improves the likelihood she will be able to care for her pups.

Behavioral and Biochemical Measures of Stress

Behavioral and biochemical measures of stress were clearly related in this study. Pearson correlations suggest moderate to high associations between behavioral and

biochemical measures of stress responsiveness. As such, a female rat with high c-fos immunoreactivity in the hippocampus and amydala displayed increased amounts of stress reactivity (i.e., more freezes, more defecation) and lower amounts of exploratory behaviors (i.e., less rearing, less blocks crossed, lower number of center blocks crossed). Animals with lower c-fos activation displayed opposite trends (less freezing, less defecation/more rearing, more blocks crossed, higher number of center blocks crossed). Whereas previous studies have considered behavioral and biochemical stress reactivity independently in parous rats, this study demonstrates the dynamic interaction of parity between the brain and behavior in a female rat's proclivity for reacting to potential stressors.

Although the mechanism by which group differences occurred was not tested in this study, these correlations suggest reproductive experience altered the brain which, then, affects the animals' responses behaviorally. The c-fos data in the present study can, however, help to explain where these differences in behavior may originate.

Study Limitations

Several limitations should be noted when generalizing these data to other areas. Gravid and parous animals were handled more often in the laboratory, particularly with regard to mating, than nulliparous animals. Since nulliparous animals did not experience as much handling, perhaps some of the hyper-reactivity, and even aggression, seen in virgin rats being removed from the open field by the experimenter upon the conclusion of the behavioral test, may have been due to less handling desensitization. Furthermore, while changes in stress responsiveness following reproductive experience appeared to persist (although somewhat abated in comparison to the acute stage of pregnancy), these

observations were noted at ten days post weaning, when hormones of pregnancy and pup stimulation are absent. Additional studies testing animals at longer intervals following the reproductive experience would better confirm the likelihood of permanent changes. In addition, although multiparous animals elicited consistently less stress responsiveness behaviorally and biochemically than primiparous groups, a small sample size may have limited the sensitivity of the statistical analysis in finding significant differences. Finally, this study was unable to discriminate the differences pregnancy hormones and pup exposure could have had on the female rat's behavioral and biochemical stress responsiveness. Additional studies are needed to differentiate these possibly very different influences.

Conclusion

Related to previous studies and consistent with our pilot data, these data suggest definite changes occur both behaviorally and biochemically in the female rat following pregnancy. This finding may help explain other refined cognitive abilities previously observed in learning studies. Less stressed animals will accept the care of pups and forage more efficiently for food and water caches. Such abilities are very advantageous to the female rat adjusting to the new responsibility of caring for large litters of pups. Contrary to expected results, these data do not confirm a greater effect for females exposed to more than one reproductive experience. As such, these changes appear to begin with pregnancy and continue (although to a lessor degree) for some time following the reproductive experience. Additional studies identifying the mechanism by which this modification occurs are needed to further explain these modified brain activities.

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Table 1. Correlations of behavioral and biochemical stress responsiveness measures

	Rearing	Freezing	Number of Blocks Crossed	Number of Ctr Blocks Crossed	Startles	Fecal Boli	Amygdala	Hippoc
Rearing								
Freezing	-.184							
Number of Blocks Crossed	.303	-.750**						
Number of Ctr Blocks Crossed	.317	-.537*	.618**					
Startles	-.120	.309	-.382	.321				
Fecal Boli	-.137	.241	-.350*	-.341*	-.039			
Amygdala	-.307	.763**	-.928**	-.600**	.373*	.414*		
Hippocampus	-.246	.784**	-.937 **	-.623**	.321	.299	.931**	

* $p < .05$
** $p < .001$

Figure Caption

Figure 1. Mean number (+/- standard error) of times rearing was observed in each reproductive group.

Figure 2. Mean number (+/- standard error) of times freezing was observed in each reproductive group.

Figure 3. Mean number (+/- standard error of the mean) of blocks crossed by reproductive group.

Figure 4. Mean number (+/- standard error of the mean) of center blocks crossed by reproductive group.

Figure 5. Mean number (+/- standard error of the mean) of fecal boli by reproductive group.

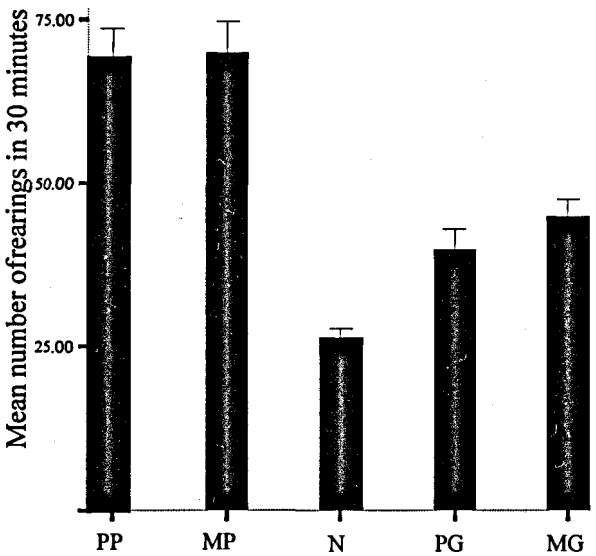
Figure 6. Mean number (+/- standard error of the mean) of startles by reproductive group.

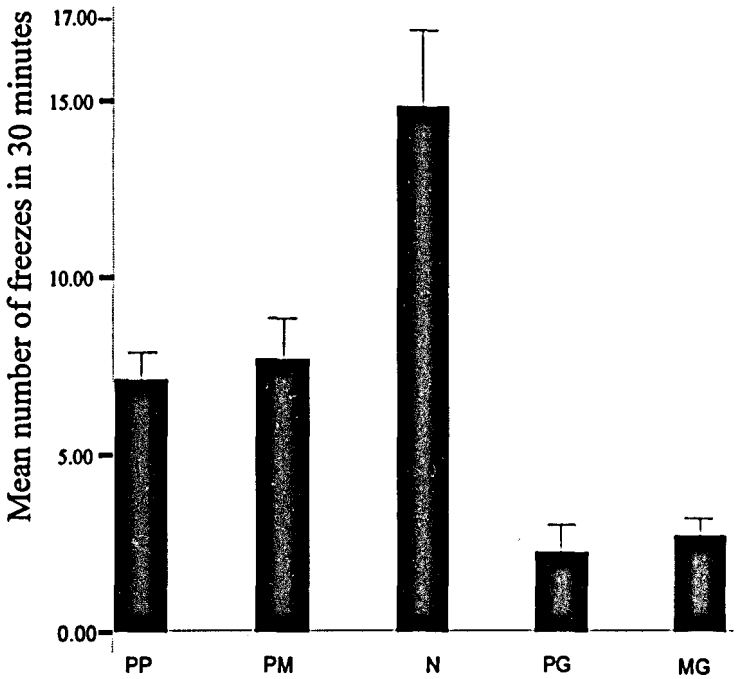
Figure 7. Mean number (+/- standard error of the mean) of c-fos immunoreactive cells by reproductive group in the CA3 hippocampus.

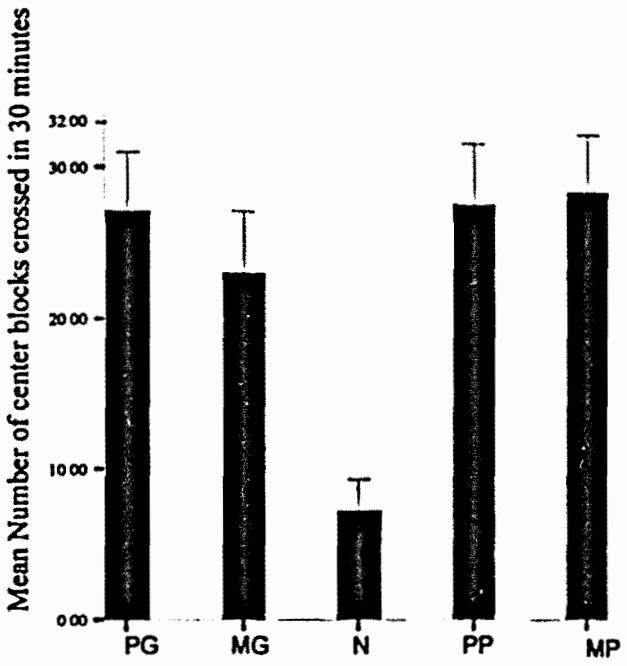
Figure 8. Mean number (+/- standard error of the mean) of c-fos immunoreactive cells by reproductive group in the basolateral amygdala.

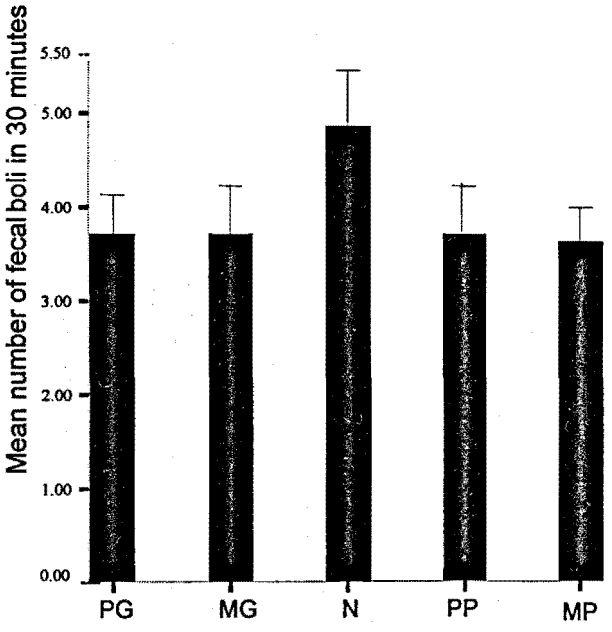
Figure 9. Example of c-fos immunoreactive cells in the CA3 hippocampus by reproductive group (scalebar = 62 *um*).

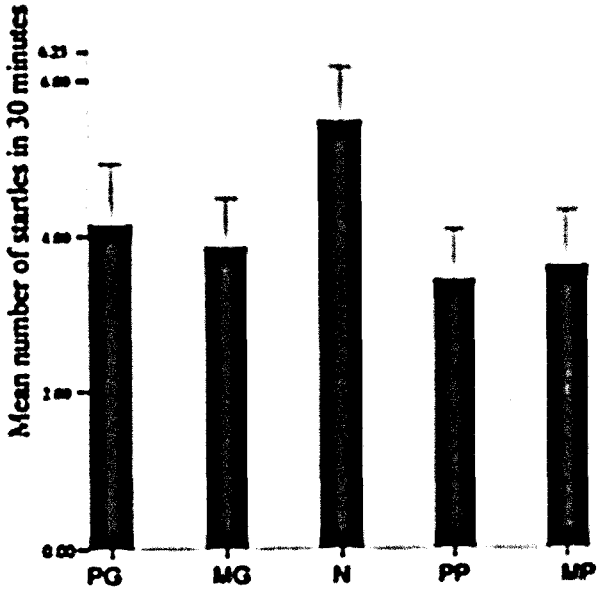
Figure 10. Example of c-fos immunoreactive cells in the basolateral amygdala by reproductive group (scalebar = 62 *um*).

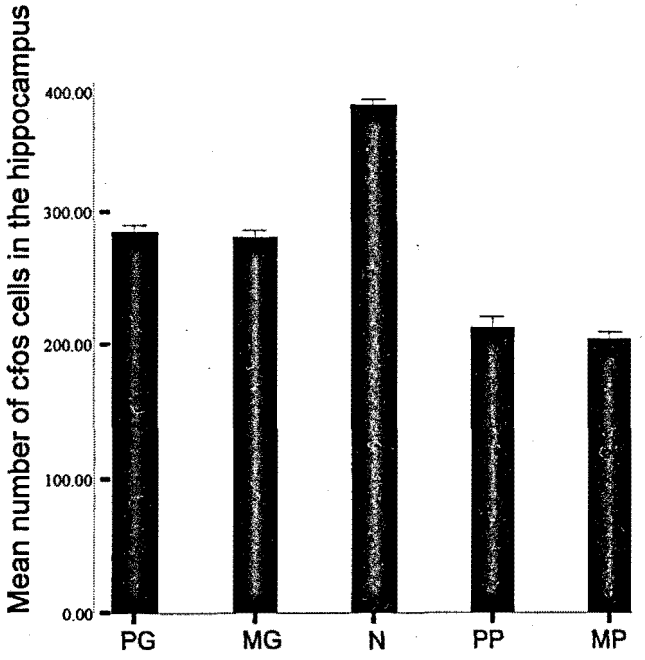

Mean number ofrearings in 30 minutes
75.00
50.00
25.00
PP
MP
N
PG
MG

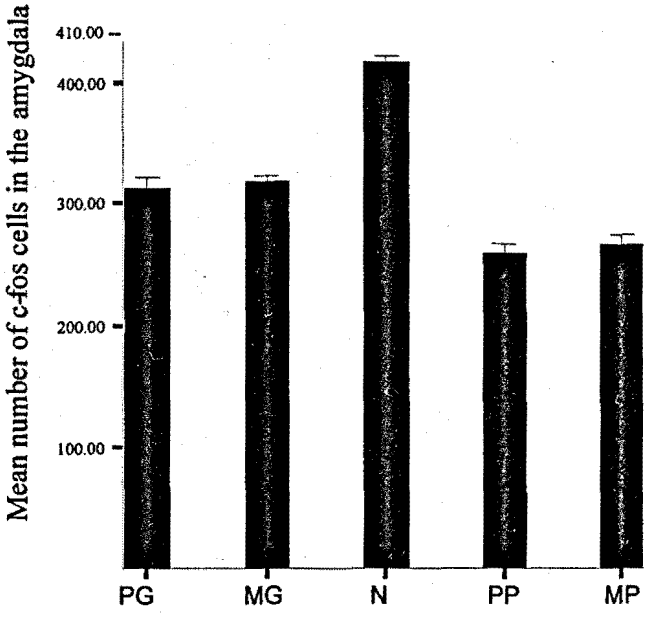

Mean number of freezes in 30 minutes
17.00
15.00
10.00
5.00
0.00
PP
PM
N
PG
MG

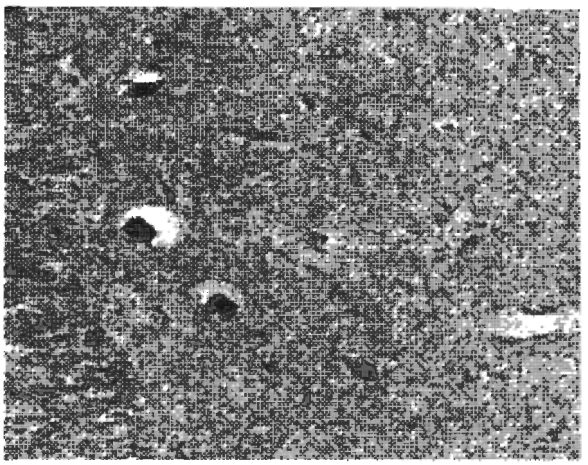

Mean Number of center blocks crossed in 30 minutes
32.00
30.00
20.00
10.00
0.00
PG
MG
N
PP
MP


Mean number of fecal boli in 30 minutes
5.50
5.00
4.00
3.00
2.00
1.00
0.00
PG
MG
N
PP
MP

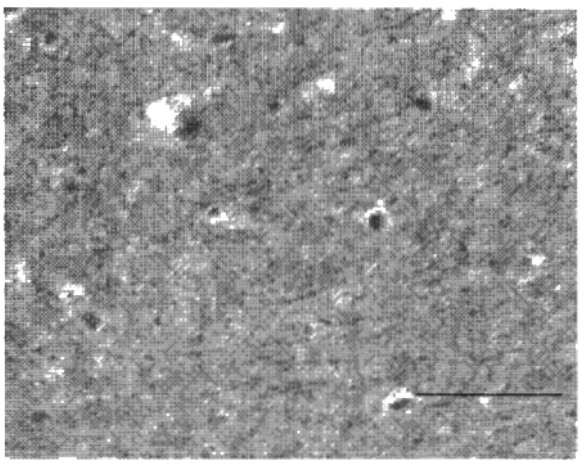

Mean number of startles in 30 minutes
PG
MG
N
PP
MP


Mean number of cfos cells in the hippocampus
400.00
300.00
200.00
100.00
0.00
PG
MG
N
PP
MP

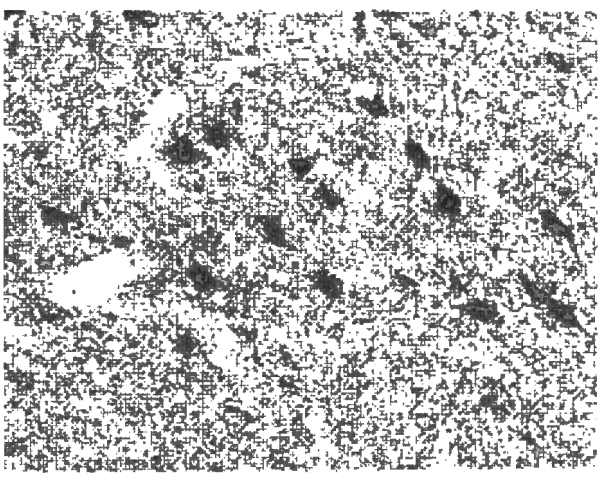

Mean number of c-fos cells in the amygdala
410.00
400.00
300.00
200.00
100.00
PG
MG
N
PP
MP



MG



PG



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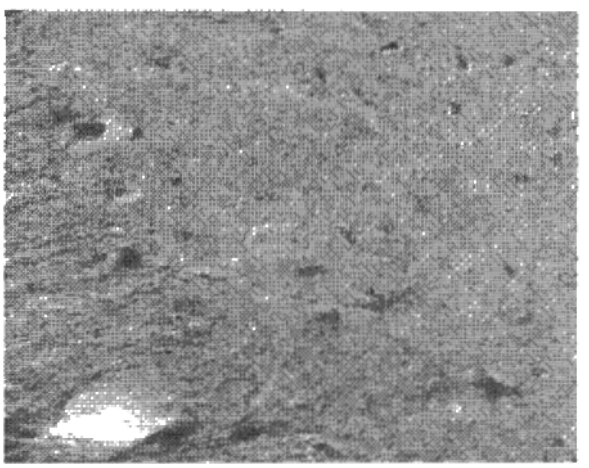

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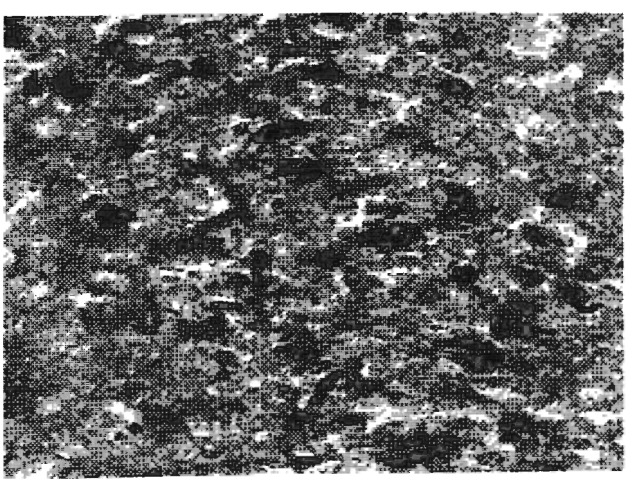

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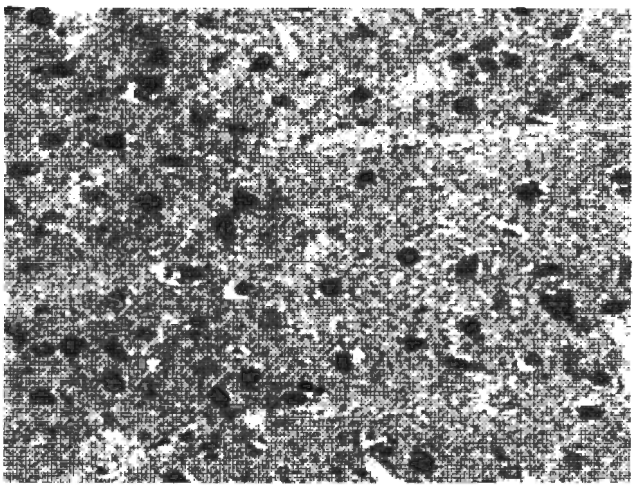

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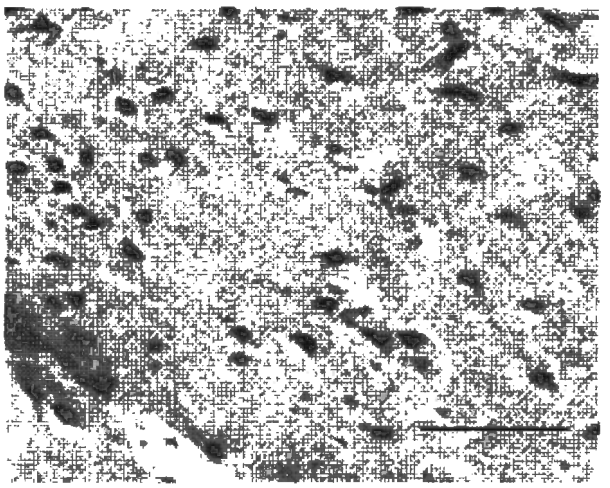

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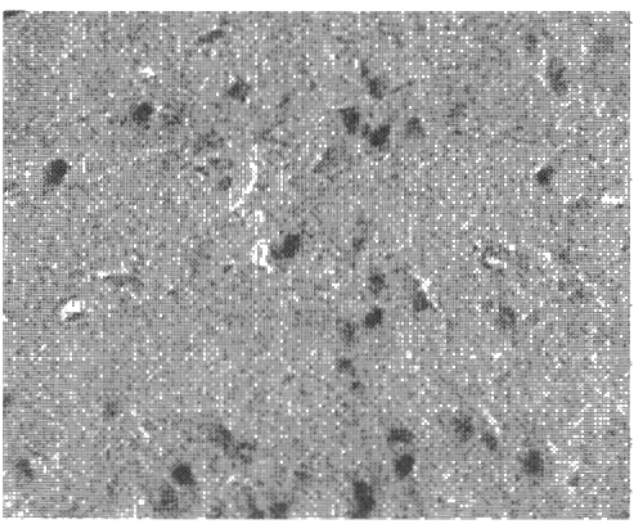

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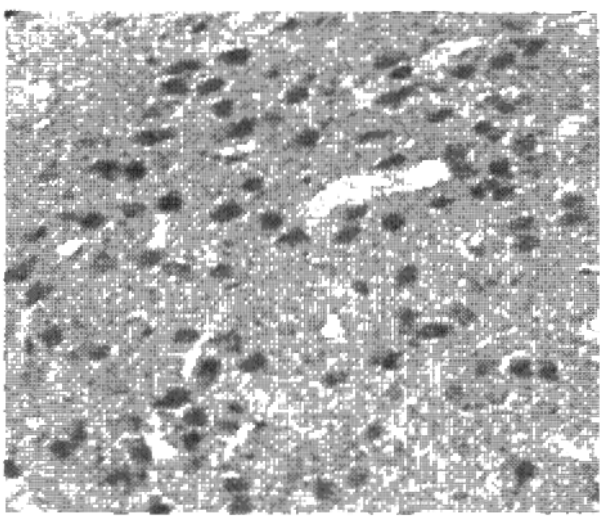

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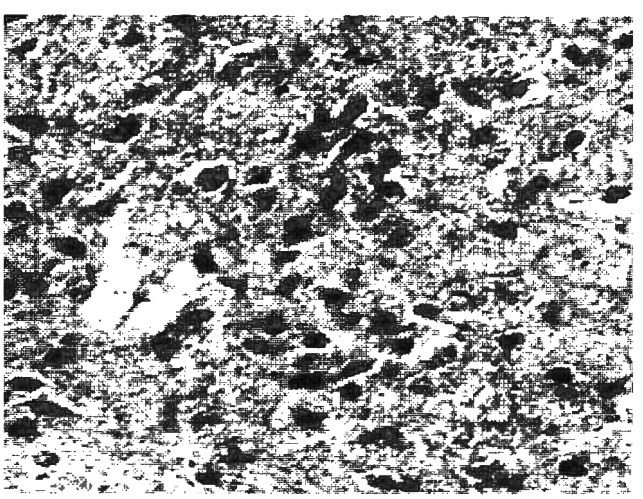

N

Appendix

Reproductive Experience Timeline

Group	D(day)1	D21	D42	D43	D52	D64	D83	D95	D104	D114
PWM	Mate	De	Wean	X	Mate	X	X	De	Wean	B
LPM	X	X	X	Mate	X	De	Wean	Mate	X	B
PWP	X	X	X	X	Mate	X	X	De	Wean	B
LPP	X	X	X	X	X	X	X	Mate	X	B
V	X	X	X	X	X	X	X	X	X	B

PWM = Post-weaned Multiparous Group

LPM = Late-pregnant Multiparous Group

PWP = Post-weaned Primiparous Group

LPP = Late-pregnant Primiparous Group

V = Virgin

X = Nothing done on this day

De = Pups delivered

B = Behavioral Testing