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Differential Effects of Reproductive and Hormonal State on bFGF and GFAP Immunoreactivity in the Hypothalamus and Cingulate Cortex of the Female Rat.

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A Thesis in the Department of Psychology

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ABSTRACT

Differential Effects of Reproductive and Hormonal State on bFGF and GFAP Immunoreactivity in the Hypothalamus and Cingulate Cortex of the Female Rat.

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In experiment 1, we compared the effect of hormonal and reproductive state on astrocytic basic fibroblast growth factor (bFGF) and glial fibrillary acidic protein (GFAP) immunoreactivity in various hypothalamic nucleii and the cingulate cortex (CC), area 2. The results of this study showed differential patterns of bFGF and GFAP immunoreactivity across regions examined. Furthermore, a dramatic increase in both bFGF and GFAP within CC in lactating rats on Day 16 postpartum compared to cycling late pregnant and ovariectomized (OVX) rats. In experiment 2a, the dependence of the changes in expression of these proteins in CC on the lactational stage was examined. Six groups of females were included in the study; OVX, cycling and lactating females on Days 4, 10, 16, and 24 postpartum. Brains were processed for bFGF and glial fibrillary acidic protein (GFAP) immunoreactivity. The results of this study showed that all groups of lactating rats showed higher levels of both bFGF and GFAP immunoreactivity than cycling females. Consistent with our earlier study females on Day 16 and 24 postpartum also had higher levels of these proteins than OVX females. Furthermore, Experiment 2b showed that pup removal either on Day 1 or Day 16 postpartum had no effect on GFAP expression in females examined on Day 4 or Day 24, respectively. Together, these data suggest that bFGF and GFAP within the CC is elevated across all stages of lactation. Furthermore, these changes do not correlate simply with hormonal state or the presence of suckling young.

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GENERAL INTRODUCTION

Pregnancy, parturition and lactation are periods of dramatic physiological, and behavioral change. In the rat, gestation lasts from 22-23 days with parturition usually producing approximately 12-16 pups. Pregnancy is followed by a period of lactation that may last four weeks. For the first 14 days of this period dams are the sole source of nutrition for the young. Pregnancy and lactation place unique demands on the mother and it is assumed that the changes observed in females at this time whether an increase in synapses on oxytocinergic cells in the PVN and SON (Theodosis& Poulain, 2001) or an increase in the absorptive capacity of the gut (Cripps & Williams, 1975) represent adaptive changes that allow the female to maximize the survival of her young and hence her own reproductive success. It is interesting that whereas some of the adaptive changes observed in pregnant or lactating rats revert to the cycling state once pups are removed e.g. connectivity in the SON (Majdoubi et al, 2000), others, such as the readiness to engage in maternal behavior itself are permanent (Bridges, 1975, 1977).

Although a considerable amount of research has been devoted to understanding the neural substrates for these changes, thus far the role of neurotrophic factors has not been investigated. The studies described in this thesis are an initial step in this process. In order to better understand pregnancy, parturition, and lactation in the rat, I will introduce my experiments by giving a resumé of the behavioral, hormonal and neurobiological profiles typical of these periods in the rat.

Behavior

Perhaps the most dramatic behavioral change seen during pregnancy and lactation is the onset of maternal behavior itself. Maternal behaviors are typically defined as any behavior that serves to increase the chance of survival of the young (Roseblatt, 1989). These can include nest building, crouching over pups, pup retrieval, and anogenital licking, as well as aggression towards an intruder (Numan, 1994). A virgin rat does not show any of these behaviors, in fact, a virgin rat will typically avoid or bury pups, and may in fact be aggressive towards them (Fleming & Rosenblatt, 1974). However, if a virgin rat is housed continuously with pups, she will begin to show maternal behavior in 2-6 days, depending on the size of the cage in which they are housed (Terkel & Rosenblatt, 1971). It is believed that this process is particularly lengthy because a virgin not only needs to learn about the pups, but more importantly, has to reduce an innate avoidance response to the pups, which has been shown to be olfactory mediated (Fleming& Luebke, 1981).

An integral part of pregnancy and lactation is the passage of nutrients from mother to young. Typically, at the time of weaning, the rat dam has produced a litter whose aggregate weight is approximately equal to her own. This phenomenal growth is completely supported by the dam's own energy output to the embryos throughout pregnancy and by milk production during lactation. Therefore, female's ingestive behavior undergoes immense change, from a 3-fold increase in food intake to changes in nutrient absorption in order to maximize efficiency of caloric utilization throughout pregnancy and lactation (Cripps & Williams, 1975).

Hormones

The hormones of pregnancy in the rat are typically characterized by increasing levels of progesterone and estrogen, with a final surge of estrogen, and a drastic drop in progesterone just prior to parturition (Bridges, 1984). It is believed that this drop in progesterone is necessary for parturition to occur. It is the negative feedback from the high levels of progesterone during the earlier parts of pregnancy that may contribute to the acyclicity of females during pregnancy. Lactogenic hormone levels either from pituitary (prolactin) or placental sources (rat placental lactogen) also fluctuate across pregnancy. Until day 10 of pregnancy there are twice-daily surges of pituitary prolactin, after which placental lactogens rise, first placental lactogen 1 and then placental lactogen 2 until pituitary prolactin peaks again just prior to parturition (Bridges, 1984; Pihoker et al, 1993).

Estrogen and progesterone have been shown to be sufficient to induce maternal behavior in the rat, more specifically; a regime of hormones that simulate the hormonal profile of pregnancy, chronic estrogen, progesterone, followed by the removal of progesterone, will induce maternal behavior in a way similar to pregnancy (Bridges,1984). This pattern of ovarian hormone replacement is also sufficient to induce the surge in pituitary prolactin release typical of late pregnancy (Bridges et al, 1997).

Shortly after parturition, at the onset of lactation, prolactin release from the pituitary peaks and declines gradually thereafter. This corresponds to the amount of suckling stimulation received from the pups, as it is around Day 15 that pups begin to eat solid food, although weaning itself is not completed until approximately Day 25 postpartum (Lee, et al, 1989; Olivier-Bousquet et al, 1993). Similarly, progesterone rises and falls slowly throughout the period of lactation, peaking at around Day 10-12, and

coming back down to levels of a normal cycling female around Day 16 (Smith & Neill, 1977). Circulating levels of estrogen remain quite low early in lactation and only begin to rise slowly beginning at around Day 15 postpartum (Smith & Neill, 1977). Finally, the last day of pregnancy is marked with an increase in basal corticosterone that remains elevated throughout lactation (Atkinson & Waddell, 1995).

Neurochemistry and Neurophysiology

Pregnancy and lactation are especially interesting because they are a robust model of neural plasticity: one way in which a female copes with pregnancy and lactation is to remodel her brain and body. In the brain, this is marked by morphological and electrophysiological changes, as well as changes in neurochemistry. The period of lactation, for example, has been shown to be marked by a decrease in CRH mRNA in the parvocellular region of the PVN, and an increase in vasopressin mRNA in this same region (Shanks et al, 1999; Walker et al, 2001). Moreover, there appear to be changes in the responsivity of the hypothalamic-pituitary-adrenal axis; for example, the effectiveness of CRH and vasopressin to activate the HPA axis is diminished during pregnancy and lactation (Neumann et al, 1998; Toufexis et al, 1999). There are also changes in dopaminergic activity throughout lactation, seemingly related to the presence of the pups as appetitive social stimuli (Hansen et al, 1993; Gingrich et al, 2000), and to the ability of prolactin to modulate the dopaminergic system (Chen & Ramirez, 1988; Chen & Ramirez, 1989). An increase in neuropeptide Y (NPY) mRNA expression, coinciding with a decrease in pro-opiomelanocortin (POMC) mRNA in the hypothalamus has also been shown during lactation. Johnstone & Higuchi (2001) hypothesized that these changes may in fact occur in response to decreased circulating levels of leptin, which

may, in turn, play a role in the hyperphagia necessary for adequate milk production to occur. Indeed, Woodside et al (2000) showed that exogenous administration of leptin did decrease food intake during lactation. Changes in dopaminergic activity, hormone release, hypothalamus-pituitary responsiveness, NPY, POMC, and leptin are but a few examples of the transformations that the brain must undergo during pregnancy and lactation.

One significant change in electrophysiology and neurochemistry important to the questions addressed in this thesis, and which is at least in part mediated by morphological changes, is in the oxytocin system in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) during parturition and lactation. It has been shown that oxytocin release is crucial for stimulating the uterine contractions that are an integral part of parturition, and is also essential for milk let down during lactation (Wakerly & Lincoln, 1973). The release of oxytocin is characterized by a bolus release of the hormone, triggered by an atypical synchronous firing of the oxytocin neurons (Higuchi et al, 1986). It has been postulated by Theodosis & Poulin (2001) that this phenomenon may be at least in part mediated by excitatory glutamate stimulation to these neurons (and possibly indirectly via noradrenalin excitation of glutamate cells), and through inhibitory actions of GABA via the $GABA_A$ receptor. Furthermore, it seems that this synchronous firing may be facilitated by morphological changes in glial cells, whereby glia withdraw their processes which typically extend between oxytocin neurons, exposing new, now interactive boutons which form synaptic contacts between cells, and thus facilitate the synchronous firing of the oxytocin neurons (Theodosis & Poulin, 2001). Changes in hypothalamic glial cells of the lactating rat have also been demonstrated in response to

pup exposure. More specifically, Featherstone et al. (2000) found that multiparous rats exposed to pups for 24 hours had more astrocytes (as labelled by GFAP immunoreactivity) in the medial preoptic area of the hypothalamus (MPOA) than primiparous rats exposed to pups for 24 hours, virgin and multiparous cycling animals. These data are especially interesting because they show a long-term change in the responsivity of astrocytes in the MPOA that might provide an explanation for the increased sensitivity of the multiparous rat to rat pups (Featherstone et al, 2000).

As can be seen by this brief review, pregnancy and lactation are associated with multiple changes within the central nervous system. The focus of this thesis is on the potential role of basic fibroblast growth factor, bFGF or FGF-2 in the morphological changes in glial cells that take place throughout pregnancy and lactation. In addition, the contribution of hormones to these effects was examined.

The trophic effects of bFGF during the development of the central nervous system have been examined extensively in the rat. bFGF has been shown to induce neuronal proliferation, and differentiation, as well as the phenotypic expression of certain types of neurons (Marchetti, 1997). For example, compared to other neurotrophic factors, bFGF created the most efficient medium for LHRH neurons to differentiate and proliferate (Gallo et al, 2000). Furthermore, there is an increasing amount of evidence that points to a role for bFGF in the adult brain. bFGF is induced following a variety of insults and has effects both in neurons and in glial cells. For example, Rowntree & Kolb (1997) showed that blocking central bFGF by means of a neutralizing antibody retards functional recovery from injury to the motor cortex. Injured animals treated with the bFGF antiserum showed a reduction in spine density and arborization compared to lesioned,

untreated rats. bFGF has also been shown to protect neurons from glutamate toxicity (Mattson et al, 1989).

A wealth of evidence obtained from both in vivo and in vitro studies has implicated bFGF in the processes underlying morphological change in glial cells (for a review, see Unsicker et al, 1993). For example, intra-cerebral injections of bFGF made following electrolytic lesions activate glial cells and further accelerate gliosis (Eclancher et al, 1996). bFGF is elevated along with GFAP in response to focal brain ischemia and is believed to play a role in neuroprotection (Matsushima et al, 1998).

In addition to its induction following insult, bFGF expression in many areas of the brain changes in response to fluctuations in steroid hormone level. Exogenous administration of corticosterone in the adult rat, for example, increases expression of bFGF in various brain regions, including the hippocampus, prefrontal cortex, hypothalamus and nucleus accumbens (Mocchetti et al, 1996). Consistent with these data, adrenalectomy has been shown to significantly reduce bFGF expression in the hippocampus, prefrontal cortex and the striatum (Riva et al, 1996).

Gonadal hormones also modulate bFGF expression in the brain. Galbiati et al (2001) have shown that bFGF mRNA in the hypothalamus fluctuates across the estrous cycle, peaking on the evening of proestrus. These changes seem to reflect, at least in part, fluctuations in circulating estrogen because exogenous estradiol administration significantly increased levels of bFGF mRNA in ovariectomized animals (Galbiati et al, 2001).

Changes in bFGF in response to manipulations of ovarian hormones have also been reported in regions outside of the hypothalamus. Ovariectomized female rats were

shown to have a greater number of bFGF- immunoreactive cells in cingulate cortex area 2, the ventral tegmental area (VTA), and the entorhinal cortex (EC) than cycling controls (Flores et al, 1999). Interestingly, in a second experiment it was shown that estradiol replacement to ovariectomized rats decreased bFGF expression in the VTA and EC whereas those changes previously seen in the cingulate cortex were only attenuated.

The ability of ovarian steroids to modulate bFGF levels in the brain together with the role attributed to bFGF in morphological plasticity within the CNS led us to investigate whether bFGF might also play a role in the neural and glial plasticity that occurs within the hypothalamus during late pregnancy and lactation. As described earlier, there are profound changes in the connectivity of magnocellular cells of the PVN and SON in late pregnancy that persist throughout lactation. A major element in this remodeling is that glial cells change shape, withdrawing their processes, which typically separate neuronal membranes and thereby allow increased synaptic contacts. Although extensive research has documented roles for both steroid and peptide hormones in these effects, a role for trophic factors has not yet been examined. The experiments in this thesis will investigate whether bFGF and GFAP change in the hypothalamus and the cingulate cortex as a function of reproductive and/or hormonal state. The studies described in Chapter 2, focus on changes observed during lactation, in an effort to define their time course as well as the stimuli inducing them.

EXPERIMENT 1- Effects of reproductive and hormonal state on bFGF and GFAP immunoreactivity in the hypothalamus and CC

The first experiment described in this thesis was carried out to examine changes in both bFGF and the astrocytic protein, GFAP, within the SON and magnocellular PVN as a function of reproductive and hormonal state. As noted earlier, bFGF is a growth factor that is a known mitogen in astrocytes. GFAP has been well documented as a marker of astrocytic cytoskeleton, more specifically; the antibody used in this experiment, labels intermediate fibrillary proteins (Sigma).

To determine the specificity of these changes to magnocellular neurons other hypothalamic areas including the parvocellular PVN and arcuate nucleus were also examined. We also chose to examine two other areas: the medial preoptic area (MPOA) and cingulate cortex 2 (CC). Both of these areas have been reported to show changes in either bFGF or GFAP staining as a function of reproductive (MPOA) or hormonal (CC) state. To examine the effects of reproductive state on bFGF and GFAP immunoreactivity in these areas, cycling, late pregnant and lactating rats were included in the study. To evaluate the contribution of ovarian steroids to any effects observed, a group of ovariectomized, cholesterol treated rats was included together with two groups of rats in which the hormonal profile of late pregnancy was simulated by sequential treatment with estrogen and progesterone followed by progesterone withdrawal. The latter hormonal treatment has been shown previously to induce many of the physiological and behavioral changes associated with late pregnancy including stimulating increases in the peptide hormones prolactin and oxytocin, and the onset of maternal behavior.

METHODS

Subjects

Virgin female Wistar rats weighing 220-240 grams when obtained from Charles River Breeding Farms (St. Constant, Quebec) were subjects in this experiment. Rats were housed in groups of five, in stainless steel cages (50 cm x 20 cm x 15 cm). All animals had ad libitum access to food and water and were kept on a 12-hour light/ dark cycle (lights on 08:00h/lights off 20:00h) at a room temperature of 20 ± 2°C throughout the experiment. All procedures used were approved by the Concordia University Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Procedures

A total of six groups were used in this study. To examine the effects of reproductive state on bFGF and GFAP expression, brains were obtained from three groups of rats: Cycling (metestrus phase), Late Pregnant (Day 21 post conception), and lactating (Day 16 postpartum). Three additional groups of rats were included to assess the contributions of hormonal state to any changes observed: two groups of rats ovariectomized and administered a hormonal replacement regimen designed to mimic late pregnancy and consisting of estrogen and progesterone treatment followed by progesterone withdrawal. Females in one of these groups were sacrificed 48hr after removal of the progesterone implants (E+P-P48hr) and those in the other group 72hr after progesterone withdrawal (E+P-P 72hr). A third group of rats was ovariectomized and given cholesterol implants (OVX+ Cholesterol),

Surgery

Ovariectomy: Bilateral ovariectomies were carried out under ketamine-xylazine anaesthesia (5.7 mg ketamine and.86 mg xylazine/100 g of body weight). Bilateral dorsal incisions were made in both skin and body wall. After removal of the ovaries, incisions in the body wall were closed with silk sutures and the skin incisions were closed with stainless steel wound clips. Both incisions were sprinkled with antibiotic powder (Cicatrin ^{TM}).

Hormone Implantation: All hormones were implanted subcutaneously between the scapulae under isoflurane anaesthesia. Incisions were closed with stainless steel wound clips after application of antibiotic powder (Cicatrin ^{TM}).

Hormone Replacement

One week after ovariectomy, hormone replacement began. On Day 1 of hormone treatment, rats in the ovariectomized +cholesterol group received one cholesterol implant and those in 48hr and 72hr groups received 1x2mm E implant. On Day 3 of treatment rats in the OVX+Cholesterol group received 3x30-mm cholesterol implants, and rats in the 48hr and 72hr groups received 3x30-mm P implants. On Day 14 of treatment, P and cholesterol implants were removed. Brains were obtained from animals in the E+P-P48hr group on Day 16 of treatment and those in the E+P-P72hr group were sacrificed on Day 17 of treatment. Half of the rats in the OVX+Cholesterol group were sacrificed at the same time as the 48hr group and half were sacrificed at the same time as the 72hr group.

Mating Procedures

Vaginal smears were obtained daily from females assigned to the cycling and late pregnant groups. Only animals that showed at least two consecutive four day cycles were

included in the Cycling group. These rats were sacrificed on the day of metestrus. Rats in the late pregnant group were housed overnight with a male on the evening of the day of proestrus. If sperm were observed in a vaginal smear taken on the morning after housing with a male, rats were individually housed in plastic cages (45 cm x 25 cm x 20 cm) until Day 21 of pregnancy.

Females assigned to the lactating group remained in group housing and were mated by adding one male to each cage. Eighteen days after the introduction of the male, late pregnant females were individually housed in plastic cages (45 cm x 25 cm x 20 cm). On the day after birth (Day 1 PP), all litters were culled to eight pups. Mothers and litters were housed together until Day 16 PP.

Immunocytochemistry

Between 1100 and 1300h, on the appropriate day, rats were given an overdose of sodium pentobarbital and, after clamping of the descending aorta, transcardially perfused with 150 ml of .9% saline and 150 ml of 4% paraformaldehyde in .1M Phosphate Buffer (PB, Sigma). Brains were removed and post-fixed in 4% paraformaldehyde in .1M PB containing 30% sucrose for 48h., then stored at -80°C until sectioning. Forty micron sections were obtained from approximately Plate10 to Plate 42 of Paxino and Watson's Atlas of the Rat Brain, and stored at -20°C in a Watson's cryoprotectant () until processing for GFAP and bFGF immunocytochemisty. All tissue was processed in two assays and sections from rats in each of the experimental groups were included in each assay.

For each assay, sections were first rinsed 3 x 10 minutes in Tris Buffered Saline (TBS, Sigma and then incubated for twenty four hours at 4°C in primary antibody (1:500, anti- FGF-2, Upstate Biotechnology), diluted in .3% Triton X (Sigma), 3% Normal Horse Serum (NHS, Vector Laboratories), and TBS. Sections were then washed in TBS, (3 x 5 minutes), and incubated in secondary antibody (1: 200 anti-mouse IgG H+L, Vector Laboratories) for one hour at room temperature. The secondary antibody was diluted in 1.5% NHS and TBS. After incubation in the secondary antibody, sections were again washed in TBS (3 x 5 minutes) and processed with avidin-biotin complex (ABC Elite kit, Vector Laboratories) for half an hour at room temperature. After a further wash in TBS, (3 x 5 minutes) staining was visualized by immersing sections in Diaminobenzidine-2, 3 (DAB) using a DAB-peroxidase kit from Vector Laboratories. For the processing of bFGF, the nickel chloride step included in the DAB kit was used, yielding a black nuclear stain. Once processing for bFGF immunocytochemistry was complete, sections were rinsed in TBS (3 x 5 min) and incubated in primary antibody (1: 400, anti-glial fibrillary acid protein (GFAP), monoclonal clone no. G-A-5, IgG1 Sigma), diluted in .3% Triton X (Sigma), 3% Normal Horse Serum (Vector Laboratories) and TBS, for a period of forty-eight hours at 4°C. After a further rinse in TBS (3 x 5 min) sections were transferred to the secondary antibody (1: 200 anti-mouse IgG H+L, Vector Laboratories) and incubated for one hour at room temperature. The secondary antibody was diluted in 1.5% NHS and TBS. After one hour, sections were again washed in TBS (3 x 5 minutes) and processed with an ABC kit (Vector Laboratories) for half an hour at room temperature, after which, sections were again washed in TBS (3 x 5 minutes). Staining for GFAP was visualized by immersing sections in a DAB-peroxidase kit

(Vector Laboratories), and, in order to better differentiate double-labeled cells, nickel-chloride enhancement was omitted, yielding a light-brown stain for GFAP stained cells Sections were then rinsed in TBS (3 x 5 minutes) and stored at 4°C until sections were mounted onto gelatin-coated slides. Slides were then dehydrated in graded ethanol bathes and rinsed in Xylene (Fisher) and coverslipped using Permount (Fisher).

Image Analysis

Sections were visualized using a Leica DMR- HC microscope, mounted with a Hitachi 3CCD camera (model # HV-C20) and images captured to a Macintosh G4 computer using Scion Image Software 1.66. Perimeters of the brain areas examined; SON, magnocellular and parvocellular compartments of the paraventricular nucleus, arcuate nucleus, medial preoptic area and cingulate cortex area 2 were established based on Paxinos and Watson's Atlas of the Rat Brain. Images of at least 3 sections were taken through each area for each animal at 40x magnification. Images were then coded and cells counted by an observer blind to group membership. Counts of the number of bFGF-positive cells and GFAP-positive cells were taken in all areas and the average number of stained cells was calculated across all images/ per area/ per animal. GFAP surface cell density was calculated in the supraoptic nucleus, medial preoptic area, and the cingulate cortex by drawing a grid of 14 by 11 lines, 50 pixels apart on each image and counting every time a GFAP positive process crossed a line. This means that the same area (in $mm^{2)}$ of tissue was examined in each brain region. Using this method, a higher number could reflect either a greater number of processes or longer processes, or more cells per area. Again, the total counts for each section per area/ per animal were averaged.

Data Analysis

The effects of hormonal and reproductive state on levels of bFGF and GFAP-positive cells were assessed by using a one way between groups analysis of variance with condition as the independent variable and number of positive cells or processes crossing lines as the dependent variable. Separate analyses were conducted for each brain area, and for bFGF and GFAP. The level of significance was set at $p < \text{or} = .05$. When significant effects were found post-hoc pairwise analyses were carried out using Fisher's LSD. All analyses were conducted using STATVIEW *TM statistical software on an IMAC G4 Macintosh computer.

RESULTS

HYPOTHALAMIC NUCLEII

Magnocellular PVN

Overall, there was no significant difference between groups in the number of bFGF- positive cells in this area ($F_{(5,30)}$= .639, p>.05) , see Figure 1. (OVX+CHOL n=7, Late Pregnant n=7, Lactating n=4, E+P-P 48hr n=6, E+P-P 72hr n=5, Cycling n=7)

Parvocellular PVN

The number of bFGF- positive cells/section varied significantly across treatment groups ($F_{(5,30)}$= .639, p≤.05, see Figure 2). Post-hoc analysis revealed that the late pregnant group had significantly more bFGF-positive cells than the E+P-P48hr, E+P-P72hr, and cycling groups. Furthermore, the OVX+Cholesterol group had significantly more bFGF- positive cells than the cycling group. (OVX+CHOL n=7, Late Pregnant n=7, Lactating n=4, E+P-P 48hr n=6, E+P-P 72hr n=5, Cycling n=7)

SON

As Figure 3 shows, the mean number of bFGF- positive cells in the SON varied significantly across the six groups ($F_{(5,31)}$= 2.931, p≤.05). Post-hoc analysis revealed that the OVX+Cholesterol group had significantly fewer bFGF- positive cells than the late pregnant, lactating, and cycling groups. Furthermore, the late pregnant group had significantly more bFGF- positive cells than the E+P-P72hr group. (OVX+CHOL n=7, Late Pregnant n=8, Lactating n=3, E+P-P 48hr n=5, E+P-P 72hr n=8, Cycling n=6)

Figure 4 shows the mean estimated GFAP surface density in the SON. Overall, there was a significant difference among treatment groups ($F_{(5,32)}$= 2.710, p≤.05). Post-hoc pairwise comparisons showed that GFAP surface density was lower in the

OVX+Cholesterol than the E+P-P72hr group and Cycling. In addition, there was a lower density of GFAP staining in the E+P-P48hr then Cycling groups. Finally, there was a trend for the late pregnant group to have less GFAP surface density then the Cycling group. (OVX+CHOL n=7, Late Pregnant n=8, Lactating n=3, E+P-P 48hr n=5, E+P-P 72hr n=8, Cycling n=6)

We were unable to count the number of GFAP-positive cells in the SON because this area was so dense, it was hard to reliably delineate one cell from another.

MPOA

Figure 5 shows the mean number of bFGF- positive cells in the MPOA as a function of group membership. There was an overall significant difference in the number of bFGF- positive cells among treatment groups ($F_{(5,30)}= 2.838$, $p \leq .05$). Post-hoc analysis revealed that the lactating group had significantly more bFGF-positive cells than the OVX+Cholesterol, E+P-P48 hr, E+P-P72 hr, and cycling groups, but did not differ from the late pregnant group. In addition, the late pregnant group had significantly more bFGF- positive cells than the E+P-P72 hr group. (OVX+CHOL n=6, Late Pregnant n=7, Lactating n=4, E+P-P 48hr n=5, E+P-P 72hr n=8, Cycling n=6)

Figure 6 shows the mean estimated GFAP surface density in the MPOA. The overall effect of treatment was not significant in this area ($F_{(5,28)}= 1.599$, $p > .05$). (OVX+CHOL n=6, Late Pregnant n=7, Lactating n=4, E+P-P 48hr n=5, E+P-P 72hr n=8, Cycling n=6)

We were unable to count the number of GFAP-positive cells the MPOA because like the SON, this area was so dense, it was hard to reliably delineate one cell from another.

Arcuate Nucleus

Figure 7 shows the mean number of bFGF- positive cells in the region of median eminence of the arcuate nucleus as a function of group membership. There was no overall significant difference in the number of bFGF- positive cells between treatment groups ($F_{(5,31)}$= 1.627, p>.05). (OVX+CHOL n=6, Late Pregnant n=8, Lactating n=3, E+P-P 48hr n=6, E+P-P 72hr n=8, Cycling n=6)

We did not assess GFAP surface density in this area as there was no significant difference in bFGF among treatment groups.

CINGULATE CORTEX

As shown in Figure 8, overall, the number of bFGF-positive cells differed significantly across treatment groups ($F_{(5,31)}$= 3.679, p≤.05, see Figure 4) in the CC. Post-hoc analysis showed that the lactating group had significantly more bFGF-positive cells than any other group. Furthermore, the late pregnant group had significantly more bFGF-positive cells than the cycling group; there was also a tendency for a higher number of bFGF-ir cells in the OVX+Cholesterol group than in the cycling group. (OVX+CHOL n=7, Late Pregnant n=8, Lactating n=4, E+P-P 48hr n=6, E+P-P 72hr n=7, Cycling n=5)

Figure 9 shows the mean estimated GFAP surface density in the CC. There was an overall significant effect of treatment ($F_{(5,31)}$= 2.620, p≤.05) on this parameter. Post-hoc analysis revealed that the lactating group was significantly higher than the E+P-P48hr, E+P-P72hr, late pregnant, and cycling groups. There was also a trend for the lactating group to have greater surface density than the OVX+Cholesterol group.

(OVX+CHOL n=7, Late Pregnant n=8, Lactating n=4, E+P-P 48hr n=6, E+P-P 72hr n=7, Cycling n=5)

Figure 10 shows the mean number of GFAP positive cells in the CC. There was an overall significant effect of treatment ($F_{(5,31)}$= 3.679, $p \leq .05$). Post-hoc pairwise comparisons revealed that the lactating group was significantly higher than the late pregnant E+P-P48hr, E+P-P72hr and Cycling groups. There was a trend for the lactating group to be higher than the OVX+Cholesterol group, and for the OVX+Cholesterol to be higher than the cycling group. (OVX+CHOL n=7, Late Pregnant n=8, Lactating n=4, E+P-P 48hr n=6, E+P-P 72hr n=7, Cycling n=5)

DISCUSSION

In this experiment, we investigated whether the morphological changes in glial cells of the SON (reviewed in Hatton, 1997, Theodosis & Poulin, 2001) typically seen at the end of pregnancy were associated with changes in bFGF expression. To determine how general such changes might be, bFGF-ir in other hypothalamic areas including the medial preoptic area, the arcuate nucleus and the paraventricular nucleus was assessed. In addition, bFGF immunoreactive cells in the cingulate cortex area 2, where changes in bFGF immunoreactivity in response to hormonal state have previously been reported (Flores et al, 1999), were also counted. GFAP immunoreactivity in each of these areas was also examined. Both GFAP surface density and, where possible, the number of GFAP immunoreactive cells were estimated.

bFGF

In the SON, cycling, late pregnant and lactating animals all had significantly higher numbers of bFGF immunoreactive cells than ovariectomized animals, thus, levels of bFGF do indeed change as a function of reproductive state; however, there is no marked effect of late pregnancy itself nor of the hormonal milieu associated with late pregnancy. Interestingly, the pattern of results obtained in the SON was dissimilar from that seen in the magnocellular PVN where no significant variation in bFGF was observed across experimental groups. This is particularly surprising because both of these areas contain magnocellular oxytocin and vasopressin neurons and both areas act in a coordinated fashion to control OT and AVP release. These areas have also been previously described as being similar in both their morphological and electrophysiological changes at the time of parturition and lactation (Hatton, 1997). It

might be premature to conclude, however, that bFGF has no role in the synaptic remodeling of magnocellular neurons in these areas. It is perhaps, more likely that the time point chosen to represent late pregnancy was simply not appropriate for observing changes in bFGF. If bFGF expression precedes changes in GFAP expression, for example, the time point chosen in the current study may have been to close too parturition itself. Finally, it is noteworthy that the levels of bFGF-ir in the cycling, late pregnant and lactating groups were high. Thus, the lack of difference between these groups might reflect a ceiling effect, and a different technique for measuring bFGF might be required to detect differences among these groups.

The lack of effect of the hormonal replacement schedule designed to mimic late pregnancy with respect to bFGF-ir was also surprising. This hormonal profile has been previously shown to induce many of the physiological and behavioral changes seen around the time of parturition including increasing oxytocin mRNA and NOS staining in the PVN and SON (Popeski et al, 1999) and stimulating the onset of maternal behavior and has also been shown to induce morphological changes in magnocellular oxytocin neurons (Montagnese et al, 1990). Again it is possible that the failure to observe the expected effects on bFGF in this study may reflect an inappropriate choice of time of sacrifice.

In the arcuate nucleus as in the magnocellular PVN bFGF-ir did not change as a function of hormonal or reproductive state. In contrast, in the parvocellular region of the PVN, late pregnant and OVX animals had significantly more bFGF immunoreactive cells than cycling animals. Further, in two brain areas, the MPOA, and CC2, lactating animals had significantly more bFGF immunoreactive cells than any other group. Together, these

data show not only that reproductive and hormonal states are related to changes in bFGF in many areas of the brain, but also that the direction of these changes can vary extensively from nuclei to nuclei. They also suggest that no one aspect of hormonal state e.g. estrogen level can easily explain these changes.

A simple way to account for the differential response of these areas to changes in hormonal and reproductive states are differences in distribution of hormone receptors, such as estrogen receptor α and estrogen receptor β. However, a simple analysis of the regional distribution of these receptors alone does not seem to give a clear relationship between the pattern of results and the pattern of the receptor distribution. For example, the cingulate cortex and the MPOA have both been shown to contain estrogen receptor β, as do the magnocellular neurons of the PVN, furthermore these magnocellular neurons contain few estrogen receptor α. (Shughrue 2001, Kritzer, 2002,) However, the results obtained in this study show disparate and varying patterns between the MPOA, CC, and the magnocellular neurons of the PVN. An analysis of this type is complicated by the fact that hormone receptor density typically varies with circulating hormone level and with reproductive state and most of the data available on the distribution of hormone receptors has been obtained from cycling rats.

Perhaps the most surprising aspect of these results is the changes observed in CC2. As in previous studies (Flores et al, 1999), we found that ovariectomized rats had higher levels of bFGF staining than cycling rats, however, we found that lactating rats had ten times the levels seen in cycling rats. These results were unexpected as the cingulate cortex is not an area usually associated with the behavioral and physiological

changes that accompany pregnancy and lactation. These data raise two issues: the stimuli that induce these changes and their functional significance.

Flores et al showed that removal of estrogen increased bFGF-ir in CC2 and it is well known that lactation is associated with low circulating estrogen. This is unlikely to be the sole contributing factor, however, because lactating rats had higher levels of bFGF than OVX rats, which had been without E for a similar amount of time. Lactation is also characterized by high progesterone and prolactin levels and it is possible that these hormones play a role in inducing bFGF in CC2. Lactating dams also experience a significant increase in tactile, auditory, and olfactory sensory inputs through their interaction with their litter. As well as being a source of increased sensory input, the young also exert an energetic drain on their mother. Recent data has implicated the cingulate cortex in ingestive behavior and thus this too might contribute to the changes observed in the current study.

Finally, both areas in which very high levels of bFGF-ir were seen in the lactating group - CC2 and the MPOA are part of the mesocorticostriatal dopaminergic loop. (Domesick,1988). Moreover, previous research has suggested a role for dopamine in maternal behavior. For example, a microdialysis study by Hansen et al (1993) showed increased dopamine release in the ventral striatum of maternal rats when mother and pups were reunited following an overnight separation. Even further release of dopamine was shown when mothers engaged in various maternal behaviors such as pup retrieval and licking. Byrnes et al (2002) have shown that administration of dopamine antagonists during parturition disrupts maternal care (pup retrieval and crouching) as well as retention of maternal behavior. The notion that increases in bFGF of lactating animals in

both the MPOA and the cingulate cortex is mediated by underlying changes in the dopaminergic system is an intriguing one. Further studies could investigate this possibility; perhaps a first step would be to look at whether dopamine antagonists could block changes in bFGF in the cingulate cortex and the MPOA.

Flores et al (2000) showed that repeated amphetamine administration increased bFGF expression within the VTA and have argued that this increase reflects a response to hyperactivation of the dopamine system. If lactation is associated with similar increases in dopaminergic activity albeit in different systems then the changes we observe here might reflect a similar process. Nevertheless, the question of how, or whether, the changes that we have observed in bFGF and GFAP in CC2 are reflected in the behavior of the animal remains.

The cingulate cortex is usually an area associated with emotionality, impulsivity and decision-making (Yamasaki, 2002). There is an extensive body of research on the psychological consequences of lesions (either post-trauma, or from a surgical procedure, often in a last resort treatment to epilepsy) to the prefrontal cortex. Some of this work has shown that people who have lesions in areas including the CC often have noticeable changes in their personality pre and post incident (Hornak, 2003). Clinically, these changes are often characterized by impulsivity, lack of control over emotionality and attention, and poor decision-making skills (Hornak, 2003). In the laboratory, activation of the CC has been shown to occur in response to attentional and emotional stimuli (Yamasaki et al, 2002). These data have also been supported by rodent research, suggesting that the prefrontal cortex is an important structure for attention and behavioral sequencing (Delatour & Gisquet-Verrier, 2001). Interestingly, among the many

behavioral and physiological adaptations that lactating rats show is hyporesponsiveness to both physical and psychogenic stressors and behaviorally what has been described as a reduction in emotionality (Fleming, A. S., & Luebke, C., 1981). It is possible, therefore that the changes in bFGF that we observe in CC2 is related to this state.

GFAP

Almost identical patterns of bFGF and GFAP immunoreactivity were seen in the CC; ovariectomized rats had more GFAP-ir than cycling animals, with lactating rats showing a dramatic increase in GFAP compared to cycling animals. Therefore, many of our aforementioned hypotheses with respect to changes in bFGF in the CC (hyperdopaminergic activity, attention, focus and hyporesponsiveness to stress) may also apply to the changes observed in GFAP.

The pattern of GFAP-ir among treatment groups in the SON was similar to that of bFGF with the exception of the E+P-P72hour group. This group showed higher GFAP-ir than the E+P-P48hr group, although these groups did not differ in bFGF staining. being having increased GFAP immunoreactivity as compared to the E+P-P48hr group. Whether this dissociation reflects an underlying difference in response to progesterone withdrawal between bFGF and GFAP awaits replication and a more refined time course analysis. The pattern of GFAP-ir in the MPOA was different from that of bFGF, as there was no significant effect of treatment on GFAP-ir in the MPOA. However, it should be noted that the MPOA had an extremely large number of GFAP-positive cells, indeed, counts were almost three times greater than in other areas, given the same measured area, so these results may very well represent a ceiling effect. It may also be that our method of evaluating surface density is not sensitive enough to evaluating such large amounts.

In summary, changes in bFGF and GFAP-ir in the SON were decreased in OVX animals, lacking ovarian hormones as compared to lactating, late pregnant and cycling animals. Differential patterns of bFGF and GFAP immunoreactivity were observed in different nuclei throughout the hypothalamus, as well as in functionally different areas of the brain; both the MPOA and the cingulate cortex showed a significant increase in bFGF immunoreactive cells in lactating animals as compared to all other groups. Furthermore, patterns of bFGF and GFAP-ir were similar in the CC.

EXPERIMENT 2 - The effects of day of lactation and pup removal on bFGF and GFAP immunoreactivity in the CC

One particularly interesting finding from Experiment 1 was the high levels of bFGF and GFAP immunoreactivity in cingulate cortex area 2 in late lactation compared to the other groups. The two experiments described in this chapter were carried out to elaborate on these initial findings by investigating the time course of these changes as well as the importance of suckling stimulation from the young for inducing and/or maintaining them.

In experiment 2A, the dependence of the changes in expression of bFGF and GFAP in the CC on the lactational stage of the female was examined. Six groups of females were included in the study; lactating females killed on Days 4, 10, 16, or 24 postpartum (D4, D10, D16, D24, respectively) as well as OVX, and cycling groups. In experiment 2B, the effect of removing the litter, and hence suckling stimulation, on Day 1 postpartum or on Day 16 postpartum on GFAP immunoreactivity on Day 4 and Day 24 postpartum, respectively, was investigated.

METHODS-

The subjects, housing conditions and general procedures for mating and ovariectomy used in these experiments were as described in Experiment 1. Similarly, the methods used for immunocytochemistry, image and data analysis were as previously described.

Experiment 2A

Treatments

To examine the effects of period of lactation on bFGF and GFAP expression, brains were obtained from four groups of rats: lactating animals on Day 4 postpartum (PP) (D4), Day 10 PP (D10), Day 16 PP (D16), and Day 24 PP (D24). Furthermore, brains were taken from a cycling group (Cycling) on the day of metestrus and from ovariectomized animals (OVX).three weeks after surgery.

Experiment 2B

Treatments

To examine the effects of pup removal on bFGF and GFAP expression, brains were obtained from 4 groups of rats: lactating animals on Day 4 Postpartum (D4), lactating animals on Day 4 PP that had their pups removed on Day 1 PP (D4PR), lactating animals on Day 24 PP (D24), and lactating animals on Day 24 PP that had their pups removed on Day 16 PP (D24PR). In addition, brains were taken from a cycling group (Cycling) on the day of metestrus.

RESULTS

Experiment 2A

CC

Figure 11 shows the mean number of bFGF- positive cells in the CC at the four times studied during lactation compared to the number seen in OVX and cycling rats.. There was an overall significant difference in the number of bFGF- positive cells between treatment groups ($F_{(5,21)}$= 2.591, $p \leq .05$). Post-hoc analysis revealed that the D4, D16, and D24 groups all had significantly more bFGF-positive cells than the cycling group. There was also a trend for the D10 group to have significantly more bFGF-ir cells than the cycling group. Planned comparisons showed that D16 animals had significantly more bFGF-ir than OVX animals (F value= -1.974, $p \leq .05$). (OVX n=4, Cycling n-4, D4 n=5, D10 n=6, D16 n= 5, D 24 n=3)

Figure 12 shows the mean surface density of GFAP-positive cells in the CC as a function of group membership. There was an overall significant difference in the surface density ($F_{(5,21)}$= 4.287, $p \leq .05$). Post-hoc analysis revealed that OVX, D4, D10, D16, and D24 animals all had significantly greater surface density than the cycling group. (OVX n=4, Cycling n-4, D4 n=5, D10 n=6, D16 n= 5, D 24 n=3)

Experiment 2B

CC

Figure 13 shows the mean surface density of GFAP-positive cells in the CC as a function of group membership. Although, there were no overall significant effects of

GFAP surface density as a function of group membership in the CC. ($F\ value_{(4,20)} = 2.077$, $p > .05$) Planned comparisons showed that as in Experiment 2 D4 and D24 animals had a significantly greater amount of GFAP surface density than cycling animals. Results from bFGF-ir in experiment 2B remain to be analyzed. (Cycling n=6, D4 n=4, D4PR n=5, D24 n= 6, D 24PR n=5)

DISCUSSION

In these experiments, we elaborated on the finding from Experiment 1: that lactating animals had a significantly greater number of bFGF-ir and GFAP-ir positive cells together with greater GFAP surface density in a region of the cingulate cortex – Cingulate Cortex 2 (Paxinos & Watson, 1986)) than cycling rats. To begin to define the events responsible for these changes we first compared bFGF and GFAP immunoreactivity in females at different stages of lactation – 4, 10, 16 and 24 days postpartum. There was no incremental effect of lactation across the time points examined. Lactating animals appeared to have a greater number of both bFGF and GFAP positive cells than cycling rats, regardless of the stage of lactation.

Hormonal state varies dramatically across the time points chosen. Prolactin levels are very high on Day 4 postpartum and then fall as lactation progresses (Bridges, 1984). Similarly, progesterone levels, which peak at around Day 10 postpartum, have decreased to levels seen in cycling females by Day 16 postpartum (Bridges, 1984). This reduction in circulating prolactin and progesterone levels is accompanied by a slow increase in circulating estrogen, which reaches diestrus levels around Day 15 of lactation (Smith & Neill, 1977). Indeed, given the litter size used in this experiment, 8 pups, one would expect that lactational diestrus had terminated for all the females in the Day 24 group. It seems unlikely, then that the increase in bFGF or GFAP is maintained by a particular hormonal profile. Whether the suckling induced increase in prolactin levels or extremely low estrogen levels seen soon after birth play a role in the initiation of these changes remains to be determined.

Hormonal profile is not the only factor that differentiates lactation from other states. Lactation represents the greatest chronic energetic demand experienced by female mammals. It is also accompanied by a unique pattern of sensory stimulation from the young. Early in lactation, rats spend 80% of their time in contact with their young and for much of this time the pups are attached to the mother's nipples, which have a rich sensory innervation. Mother's also receive more generalized tactile stimulation to the ventrum from contact with the pups and perioral stimulation has been shown to play a critical role in the appropriate performance of maternal behavior (Siegel & Rosenblatt, 1975). In addition to this proximal stimulation, the pups are also a powerful source of olfactory, auditory, and, to a lesser extent, visual cues.

The second of the experiments described in this chapter investigated the importance of proximal stimulation for the induction of bFGF and GFAP in the cingulate cortex. The results of Experiment 2b, showed that separating the mother from her litter for three days, from Day 1 postpartum, or for eight days, from Day 16 postpartum, did not reduce the number of GFAP positive cells and GFAP surface density when compared to females at the same lactational stage. Furthermore, all were significantly increased from cycling animals. These data suggest that tactile stimulation is not important for inducing these morphological changes in CC. However, because the rats in this study were housed in a room with other females kept with their litters they do not rule out the possibility that distal cues such as olfaction may be important in this phenomenon. These results are consistent with the notion that a particular hormonal state is not sufficient to maintain these effects since typically 48h - 72 of pup removal is sufficient to reinitiate estrous cycles in postpartum females.

It appears from the results of Experiment 2b that parturition followed by 24 hours of maternal experience is sufficient to produce the changes in GFAP in the CC that are observed throughout lactation. In this context, it is interesting to note that a single hour of maternal experience is sufficient to change maternal responsivity for at least ten days. In addition, the longer the period of maternal experience, the longer changes in maternal responsivity are retained. After one full maternal episode, parturition to weaning, the female rat's responsivity to pups is changed permanently (Bridges, 1975; 1977).

The fact that removal of the pups for at least a week is not sufficient to return the female to the non-lactating state stands in contrast with the effect of pup removal on morphological changes in other parts of the brain. Some of these changes were described by Theodosis & Poulin (2001), and discussed earlier in this thesis. These authors found that at the termination of lactation, the new synaptic contacts formed between magnocellular neurons disappeared and glial processes extended once more between neuronal cell bodies. If the effects that we observed in CC were simply an adaptation to increased input it would be expected that the animals in our pup-removed lactating groups would have returned to their pre-pregnant state, and yet, the changes that we have observe in the CC remain the same. Perhaps these changes are in fact serving a more permanent function, such as those underlying the maternal responsiveness to pups and those changes seen in the SON are serving direct physiological purposes (the stimulation of uterine contractions and the milk ejection reflex) necessary only during parturition and lactation, after which, the brain assumes it's pre-pregnant state. Further experiments investigating whether these changes are in fact permanent, whether animals who have

ceased lactation for some time still show increases in bFGF and GFAP immunoreactivity in the CC would have to be completed in order to address this issue.

GENERAL DISCUSSION

A number of interesting findings emerged from the results of the first experiment in this thesis: 1) that variation in reproductive state is associated with changes in bFGF and GFAP immunoreactivity in a number of hypothalamic nuclei; 2) that there is no consistent pattern of change even between closely associated nuclei and 3) that there is apparently no simple relationship between circulating hormone levels and bFGF and GFAP expression in any of the hypothalamic nuclei examined. In addition, within brain sites (the CC and the SON) the pattern of change of bFGF and GFAP expression was similar across reproductive state Finally, and perhaps most surprisingly, lactating animals were found to have much higher levels of bFGF and GFAP immunoreactive cells in CC than all other groups in spite of the fact that this area is not typically associated with either reproductive function or maternal behavior.

Interestingly, the hypothesized changes in bFGF during late pregnancy and lactation in the SON and PVN were not seen. In fact, there were no differences between late pregnant, lactating and cycling groups in the SON or in the PVN magnocellular and parvocellular regions. Furthermore, patterns of bFGF and GFAP-ir found in the SON were not similar to those seen in the region of magnocellular neurons of the PVN, in fact there were no differences found among treatment groups in the magnocellular region of the PVN. This dissociation is particularly interesting because many of the changes in glial cells described previously by Theodosis & Poulin (2001) are typically seen in both the PVN and the SON. However, it is possible that similar patterns of bFGF and GFAP-ir do exist, but rather that the timing of these changes may be different.

In order to further investigate the changes seen in the CC, in experiment 2a we compared bFGF and GFAP-ir in this area among females at different lactational stage and OVX and cycling animals. We found that bFGF and GFAP was increased early in lactation and remained elevated throughout. In Experiment 2b, we investigated whether proximal pup stimulation was necessary for these changes to occur by removing pups from dams. Results showed that, at least with respect to GFAP, pup removal did not reduce the amount of GFAP-ir to levels of cycling animals. There was in fact no difference between animals not housed with their pups and those who remained housed with their pups.

Among the issues raised by these data is the functional significance of the observed increases in bFGF and GFAP expression within the areas examined both with respect to local neuronal action and more generally with respect to reproductive function and maternal behavior. These questions are particularly intriguing in the context of changes observed in the cingulate cortex of lactating animals because the current findings are the first to associate this part of the brain with lactation. Another interesting question is that of the necessary and sufficient stimulation required to induce these changes. Indeed answering the latter question may help address the former.

A role for bFGF?

As I briefly reviewed in previous sections of this thesis, bFGF is a neurotrophic factor originally believed to be limited to the developing brain, but more recently implicated in plasticity in the adult brain. bFGF has been shown to act as a mitogen in glial cells, inducing morphological changes both in vitro and in vivo (Abe & Saito, 2001). These morphological changes in astrocytes may be important for cell survival

during the gliosis response to injuries such as ischemia and electrolytic lesions (Rowntree & Kolb, 1997; Eclancher et al, 1996). Furthermore, bFGF does not only act on astrocytes, but also produces neuronal differentiation and proliferation, bFGF has also been shown to be a sufficient medium in which several types of neurons can survive. (Gallo, 2000 ; Abe et al., 1990) Abnormalities in bFGF have also been documented in neurodegenerative diseases such as Parkinson's and Alzheimer's disease, although the cause of these abnormalities is not clear (Abe et al., 1990).

Although all of the data on the function of bFGF in the adult brain is new, it points to multiple roles for this neurotrophic factor. Furthermore, bFGF's role does not appear to be limited to an emergency response to a pathological situation. It is also important for normal growth and learning. bFGF has been shown to be necessary for long-term potentiation, a phenomenon suspected to be a cellular substrate for learning and memory. Indeed, bFGF has been shown to improve performance on spatial learning tasks such as the Morris Water Maze and the radial arm maze (Abe et al,1990, Hisajima et al.,1992, Amino et al., 1996).

Given the data that bFGF is an important mitogen for morphological changes in astrocytes, and that bFGF was found to be expressed in a similar pattern to GFAP among treatment groups in the cingulate cortex, a potential causal relationship between bFGF and GFAP must be considered. A first reasonable question to ask is whether bFGF is in fact inducing the astrocytic changes seen during lactation in comparison to cycling animals. One way to test this hypothesis would be to block bFGF expression in lactating animals and see whether GFAP-ir would increase regardless of the absence of bFGF. A

second experiment could attempt to induce changes in GFAP experimentally by administering bFGF in the CC and see whether there are changes in GFAP.

In Experiment 2a, bFGF, like GFAP, was found to remain elevated across all stages of lactation suggesting that bFGF may play a role in the maintenance as well as the induction of GFAP expression; otherwise, we would have expected bFGF to fall once GFAP has peaked. It is, of course, possible that both of these changes (bFGF and GFAP) are occurring independently from one another, and are in fact not causally related to each other, however, the experiments described earlier in this paragraph would help answer this question.

What is stimulating bFGF and GFAP in the CC?

The goal of the experiments in Chapter 2 was to obtain information on factors that might influence bFGF and GFAP expression in CC. The results obtained were somewhat surprising. As described in the general introduction the hormonal profile of lactation varies considerably across time. Regardless of the time of lactation, however, suckling stimulation from the pups makes the primary contribution to maintaining the lactational state. The similarity of bFGF and GFAP expression across lactation, however, argues against an association with any particular hormonal profile. Moreover, because removing proximal pup stimulation did not reverse the changes seen in bFGF and GFAP it is unlikely that they resulted from changes in suckling stimulation.

Another hypothesis as to the nature of the stimuli inducing these changes is simply maternal experience itself. In Experiment 2b litters were removed on Day1 PP, allowing females to have a full 20-24h of maternal experience. Fleming et al, have shown that 1h of interaction with pups immediately after birth is sufficient to induce an increase

in maternal responsivity that persists for several days supporting the notion that the initial phases of mother litter contact can produce long-lasting changes in the maternal brain. To understand the relationship between the induction of maternal behavior shortly after parturition and bFGF and GFAP-ir in the CC, one could compare late pregnant females, rats that gave birth without having the chance to interact with their pups, and rats that have begun to show maternal behaviour. Furthermore, we could see whether parturition alone would be sufficient to induce these changes. Moreover, it would be interesting to see whether inducing maternal behavior in virgin rats would induce similar changes, as this would speak directly to the importance of behavior itself in these effects.

If early maternal experience were critical for inducing the changes in the CC that were reported in this thesis then it would be important to identify the critical element of that situation. In this regard, it is intriguing that the cingulate cortex area 2 has been shown to be important for olfactory learning. For example, Di Pietro et al (2002) have shown a double dissociation between the cingulate cortex area 2 and the cingulate cortex area 1 on olfactory and visual versions of the radial arm maze. When rats are only allowed olfactory cues in order to find a food reward, the cingulate cortex 2 is a necessary structure for this task and the cingulate cortex 1 is not. However, the opposite pattern of results is observed on a visual version of the task using visual cues rather than olfactory ones. This would suggest that the cingulate cortex area 2 is important for olfactory learning and the cingulate cortex 1 is important for visual-spatial learning. Careful qualitative analysis of our sections would suggest that, at least at the level of the CC which we examined, our increases in bFGF and GFAP seem to be contained to the cingulate cortex area 2 (See Figure 14). Given that olfaction plays an important role in

mother-litter interaction and that lactating rats show better olfactory learning than cycling rats it is tempting to suggest both that olfactory input plays a key role in inducing increased bFGF and GFAP expression within CC2 and that these changes might facilitate olfactory discrimination during lactation.

If olfactory input were critical for the induction and maintenance of bFGF and GFAP expression within CC2 then one could ask why litter removal did not reduce GFAP staining. It should be noted, however that in the litter removal experiment although mothers were separated from their litters they were still kept in a testing room with other lactating females and their litters. Thus, although proximal pup stimuli were removed, distal cues such as odors were not.

Even if one were able to isolate immediate postpartum experience as the instigator of these changes and olfactory cues as important in their maintenance, it would still be important to identify the neurochemical mediators. Given that lactation has been associated with an increase in dopaminergic tone, (Byrnes, 2001) and that Flores et al have shown increases in bFGF in the CC in response to repeated amphetamine administration, dopaminergic input to CC2 is an obvious candidate. It would be interesting to determine whether there were any correlation between changes in bFGF and tyrosine hydroxylase staining in CC2 as well as to determine the effects of manipulating dopaminergic input to this area on bFGF and GFAP-ir. Certainly, if dopamine were an important stimulus, then we would have to consider pup sensitization, and motivation for an appetitive social stimulus as possible hypotheses for the functions of changes in bFGF and GFAP in the CC.

What function does the CC serve?

In the preceding section the hypotheses that the increased bFGF and GFAP expression in CC2 might be related to an increased olfactory discrimination and/or a change in dopaminergic input that might ultimately modulate appetitive motivation were discussed. Because of the long-standing notion that the CC is associated with "cognitive" and "emotional" functions, however, another possibility is that the changes that we see in astrocytes in this area are associated with the hyporesponsiveness to stress of lactating rats. Obviously one way to address all of these hypotheses is to lesion this area and to observe the effects on each of the behavioral systems described. Although this would be a rather crude experiment, it might represent a first step in examining the functions that the CC may serve during lactation.

In summary, this thesis describes a set of experiments to investigate a role for neurotrophic factors in the neural adaptations associated with pregnancy and lactation. We observed changes in bFGF and GFAP throughout different nuclei of the hypothalamus and the CC as a function of reproductive state. We found that lactating animals had a significantly higher number of bFGF and GFAP-ir cells in the CC. Furthermore, these changes persisted throughout lactation and were not reversed by removing pups from their dams. Olfactory inputs associated with initial maternal experience, maternal behavior itself, as well as changes in dopaminergic tone may, alone, or in combination, contribute to the initiation of these changes.

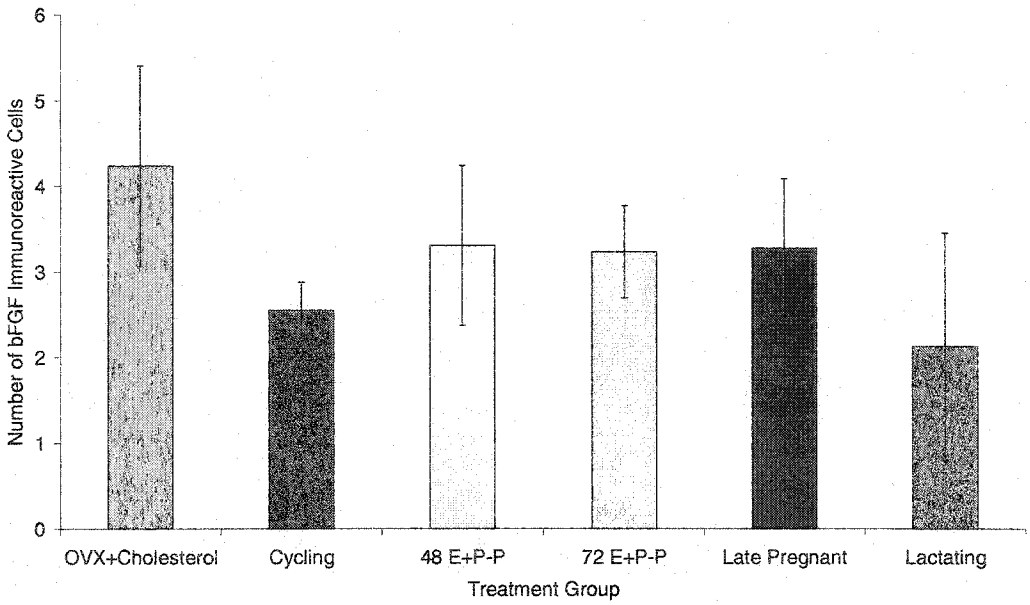

Figure 1. Average number of bFGF-ir cells per section in the magnocellular region of the PVN (means ± standard error). Lines show significant differences between groups.

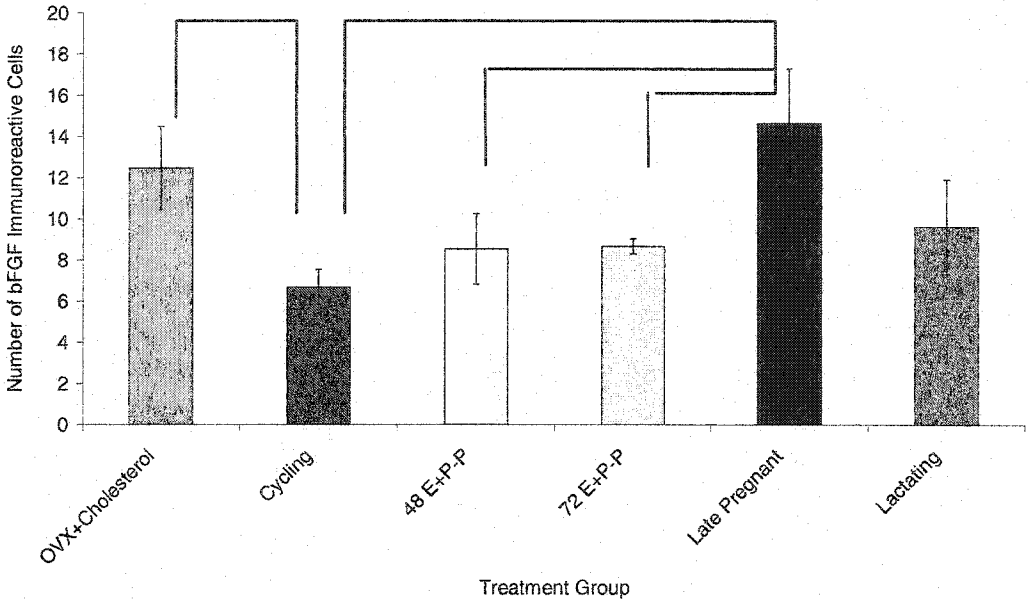

Figure 2. Average number of bFGF-ir cells per section in the parvocellular region of the PVN (means ± standard error). Lines show significant differences between groups.

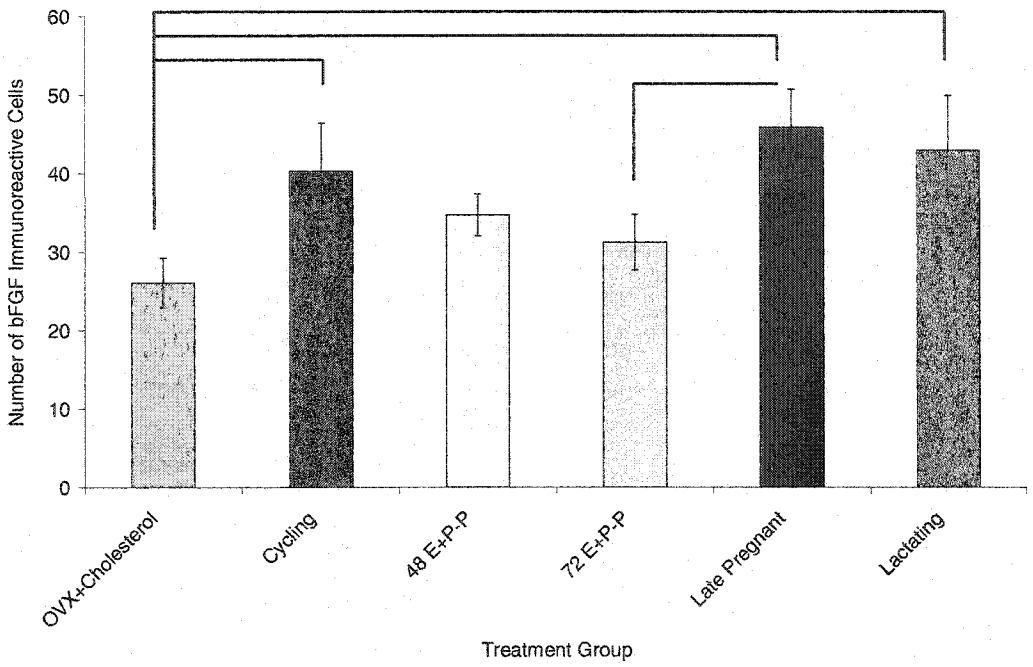

Figure 3. Average number of bFGF-ir cells per section in SON (means ± standard error). Lines show significant differences between groups.

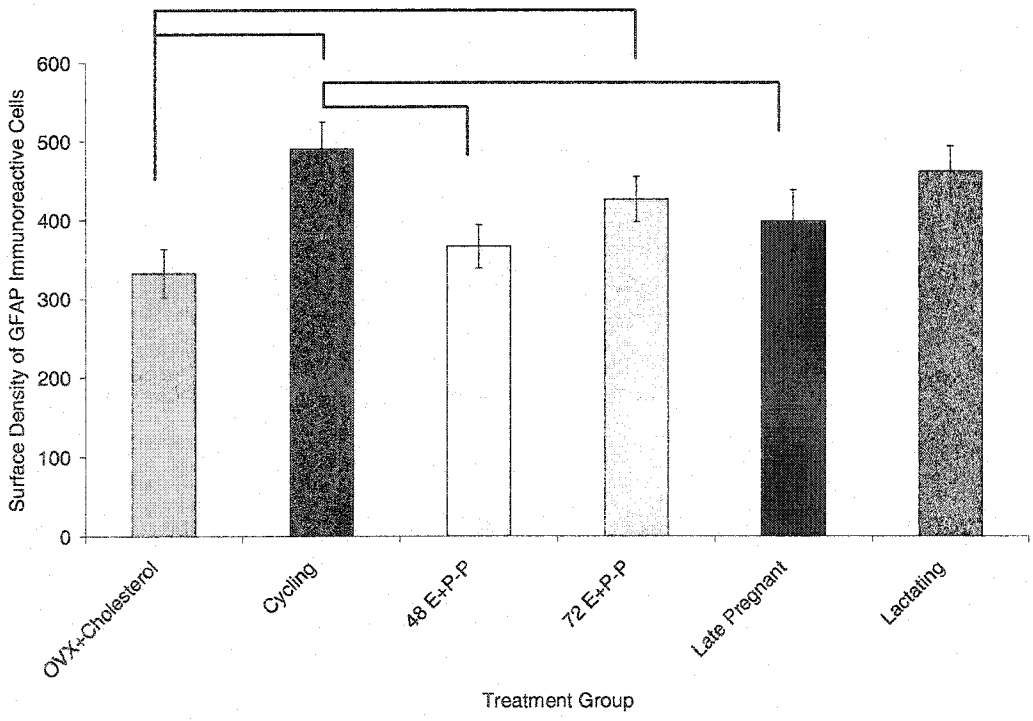

Figure 4. Average surface density of GFAP-ir cells per equal measured area (± standard error) in the SON. Lines show significant differences between groups.

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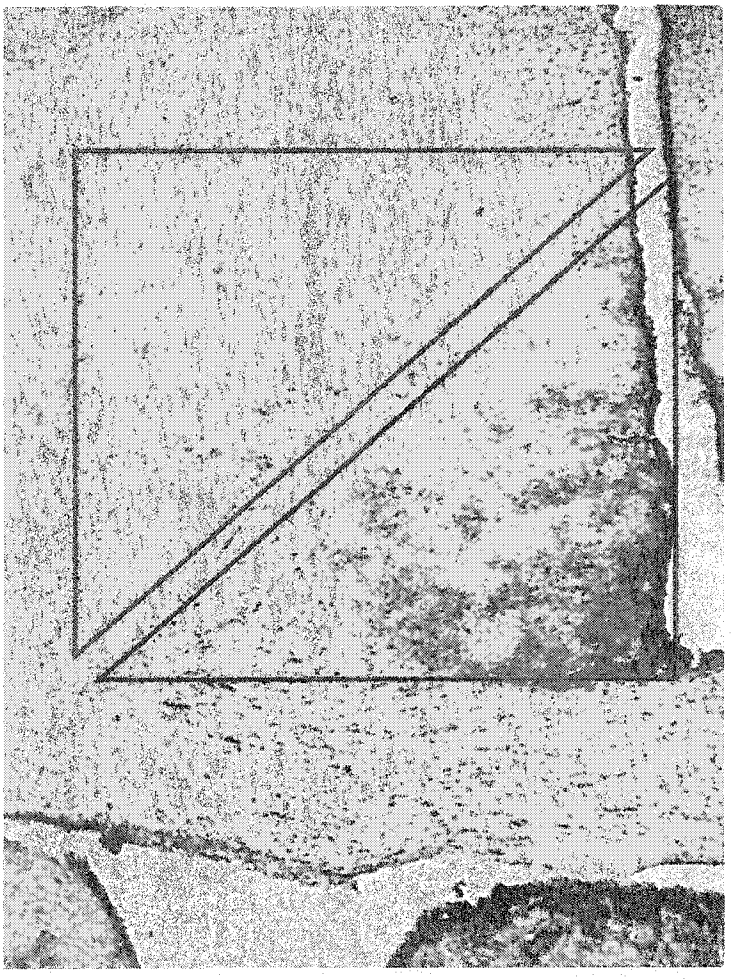

Figure 14. Cingulate Cortex Area 2 (outlined in blue) of a lactating animal on D16 PP. bFGF and GFAP-ir cells are seen predominantly in area 2 of the CC, not in area 1(outlined in red).

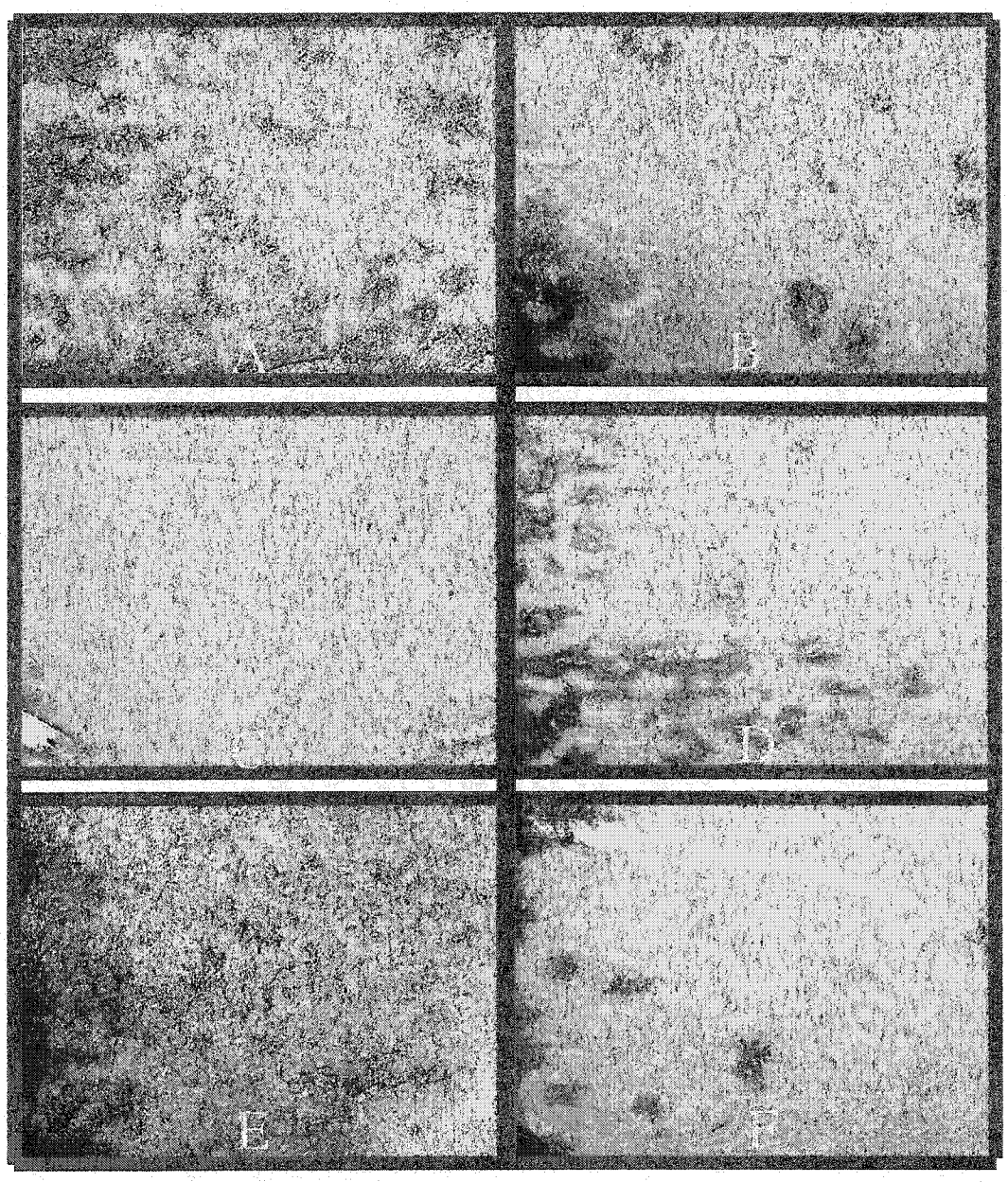

Figure 15. bFGF and GFAP-ir in the CC of OVX+ Cholesterol (A), E+P-P 48hrs (B), Cycling (C), E+P-P 72hrs (D), Lactating D16 (E), and Late Pregnant (F) animals, in Experiment 1.

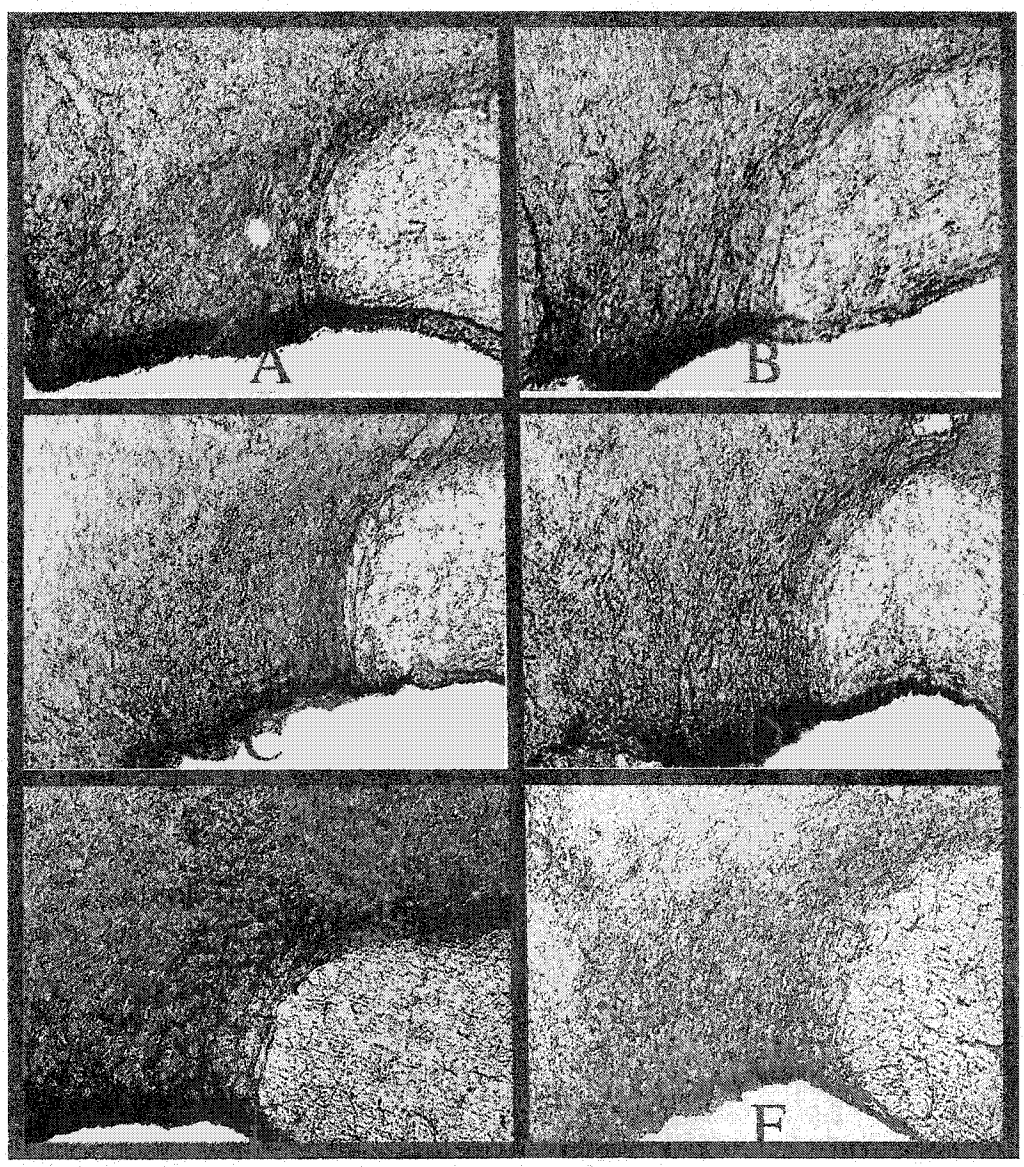

Figure 16. bFGF and GFAP-ir in the SON of OVX+ Cholesterol (A), E+P-P 48hrs (B), Lactating D16 (C), E+P-P 72hrs (D), Cycling (E), and Late Pregnant (F) animals, in Experiment 1.

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