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# DIFFERENTIAL EFFECTS OF REPRODUCTIVE AND HORMONAL STATE ON BASIC FIBROBLAST GROWTH FACTOR AND GLIAL FIBRILLARY ACID PROTEIN IMMUNOREACTIVITY IN THE HYPOTHALAMUS AND CINGULATE CORTEX OF FEMALE RATS

**N. SALMASO, N. POPESKI, L. A. PERONACE AND B. WOODSIDE***

*Center for Studies in Behavioral Neurobiology, Concordia University, 7141 Sherbrooke O. SP-244, Montreal, Quebec, Canada H3G 1M8*

**Abstract—Morphological changes in astrocytes occur in a number of brain regions including the hypothalamus and hippocampal regions as a function of hormonal and reproductive state. Because basic fibroblast growth factor has been shown to play an important role in morphological changes in astrocytes, we investigated whether basic fibroblast growth factor immunoreactivity would also be influenced by reproductive state and circulating gonadal steroids. To do this we compared astrocytic basic fibroblast growth factor and glial fibrillary acid protein immunoreactivity in hypothalamic nuclei and the cingulate cortex, area 2 among groups of cycling, late pregnant and lactating rats as well as in ovariectomized and ovariectomized hormone-replaced females. Significant differences in both basic fibroblast growth factor and glial fibrillary acid protein immunoreactivity were observed across groups in the supraoptic nucleus, parvocellular paraventricular nucleus, medial preoptic area of the hypothalamus and cingulate cortex 2. The pattern of change in basic fibroblast growth factor and glial fibrillary acid protein immunoreactivity varied across regions both in direction and magnitude. For example, although in the supraoptic nucleus ovariectomized rats had lower levels of basic fibroblast growth factor-ir than cycling females, this pattern was reversed within cingulate cortex. Overall the results of this study suggest that reproductive and hormonal states are associated with robust changes in basic fibroblast growth factor and glial fibrillary acid protein immunoreactivity in a number of brain areas but that the changes observed vary in magnitude as well as direction from one brain region to another. © 2005 Published by Elsevier Ltd on behalf of IBRO.**

**Key words: prefrontal cortex, maternal behavior, lactation, hypothalamus, astrocyte, growth factor.**

One of the most well-established examples of morphological remodeling in the uninjured adult rat brain is that observed in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus at the end of pregnancy and throughout lactation (for a review see Theodosis and Poulain, 2001). At these stages, the astrocytic processes of glial cells surrounding oxytocin (OT) neurons are withdrawn which permits close apposition of neuronal membranes and facilitates both the extension of existing synapses and the development of new synapses on oxytocinergic neurons (Theodosis and Poulain, 2001). The result of these changes is increased glutamate, GABA and norepinephrine inputs onto magnocellular OT neurons (for a review see Theodosis and Poulain, 2001). Concomitant with these changes are increases in dendritic bundling and a rise in the number of gap junctions between oxytocinergic neurons (Hatton, 1997, 2002). It has been suggested that this remodeling facilitates the synchronous firing that permits the bolus release of OT necessary to elicit uterine contractions during pregnancy and milk ejection during lactation (Theodosis and Poulain, 2001). Synaptic remodeling of the OT system can be hormonally induced in ovariectomized rats and it appears that intra-nuclear release of OT plays a key role in this process (Theodosis, 2002).

Changes in astrocytic morphology in other brain areas have also been associated with reproductive behavior and physiology. Featherstone et al. (2000), for example, found that multiparous rats exposed to pups for 24 h had more glial fibrillary acid protein (GFAP)-labeled astrocytes in the medial preoptic area of the hypothalamus (MPOA) than either primiparous rats exposed to pups for 24 h, virgin, or multiparous cycling rats. In addition, it is well established that astrocytes within the arcuate nucleus change shape on the afternoon of proestrus, an effect that appears to depend primarily on circulating estrogen levels (Garcia-Segura et al., 1995).

A wealth of evidence obtained from both *in vivo* and *in vitro* studies has implicated basic fibroblast growth factor (bFGF) in the processes underlying morphological change in glial cells (for a review, see Unsicker et al., 1991). For example, intra-cerebral injections of bFGF made following electrolytic lesions activate glial cells and further accelerate gliosis (Eclancher et al., 1996); bFGF and GFAP are also both elevated in response to focal brain ischemia (Matsushima et al., 1998).

Both adrenal and gonadal steroids are potent modulators of bFGF. Exogenous administration of corticosterone to adult rats increases expression of bFGF in the hippocampus, prefrontal cortex, hypothalamus, and nucleus accumbens (Molteni et al., 2001) and adrenalectomy reduces bFGF expression in the hippocampus, prefrontal cortex and the striatum (Riva et al., 1995). In the hypothalamus bFGF mRNA fluctu-

*Corresponding author. Tel: +1-514-848-2424x2226; fax: +1-514-848-4545.
E-mail address: barbara.woodside@concordia.ca (B. Woodside).

*Abbreviations:* AVP, arginine–vasopressin; bFGF, basic fibroblast growth factor; CC, cingulate cortex area 2; DAB, diaminobenzidine-2,3; GFAP, glial fibrillary acid protein; MPOA, medial preoptic area; NHS, normal horse serum; OT, oxytocin; PB, phosphate buffer; pPVN, parvocellular paraventricular nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus; TBS, Tris-buffered saline.

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ates across the estrous cycle, peaking on the evening of proestrus (Galbiati et al., 2001). These changes 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). In contrast, ovariectomized female rats have a greater number of bFGF-immunoreactive cells in cingulate cortex area 2 (CC), in the ventral tegmental area (VTA), and in the entorhinal cortex (EC) than cycling controls and these high levels are reduced following estrogen treatment (Flores et al., 1999).

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 increases in bFGF immunoreactivity might accompany the neural and glial plasticity that occurs within the hypothalamus during late pregnancy and lactation. Although extensive research has documented roles for both steroid and peptide hormones in these effects, a possible role for trophic factors has not yet been examined. Thus, this experiment investigates whether increases in bFGF occur in the magnocellular areas of the PVN and in the SON of the hypothalamus of late pregnant and lactating rats. To determine the specificity of these changes other hypothalamic areas including the parvocellular paraventricular nucleus (pPVN) and arcuate nucleus were also studied. GFAP-ir was used as a marker of activated astrocytes.

Inducing the hormonal profile of late pregnancy, by sequential treatment with estrogen and progesterone followed by progesterone withdrawal, stimulates increases in the peptide hormones prolactin and OT, and the onset of maternal behavior (Bridges, 1984; Popeski et al., 1999). To evaluate the contribution of this hormonal profile to any effects on bFGF observed in late pregnancy a group of ovariectomized, cholesterol-treated rats was included in this comparison together with two groups of rats in which the hormonal profile of late pregnancy was simulated.

In addition, because increases in GFAP-ir have been reported in the MPOA of postpartum rats and bFGF-ir in the CC has been shown to decrease in the absence of circulating estrogen, these areas were also examined. Given previous data we were expecting that bFGF-ir would be higher in CC in OVX than in cycling rats and that GFAP-ir in the MPOA would be greater in lactating rats than in any other group.

## EXPERIMENTAL PROCEDURES

### Subjects

A total of 40 virgin female Wistar rats weighing 220–240 g, when obtained from Charles River Breeding Farms (St. Constant, Quebec, Canada), were subjects in this experiment. To examine the effects of reproductive state on bFGF and GFAP expression, brains were obtained from three groups of rats: Cycling (metestrus phase, $N=7$), Late Pregnant (day 21 post-conception, $N=8$), and lactating (day 16 postpartum, $N=4$). Three additional groups of rats were included to assess the contributions of hormonal state to any changes observed: two groups of rats were ovariectomized (ovariectomies were carried out under ketamine–xylazine anesthesia (5.7 mg ketamine and 8.6 mg xylazine/100 g of body weight); incisions were sprinkled with antibiotic powder (Cicatrin™, Glaxo-Smith Kline, UK)) 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 killed 48 h after removal of the progesterone implants (E+P-P48 h, $N=6$) and those in the other group 72 h after progesterone withdrawal (E+P-P 72 h, $N=8$). A third group of rats was ovariectomized and given cholesterol implants (OVX+Cholesterol, $N=7$).

*Hormone replacement.* One week after ovariectomy, hormone replacement began. On day 1 of hormone treatment, rats in the OVX+Cholesterol group received one cholesterol implant and those in 48 h and 72 h groups received 1×2 mm E implant. On day 3 of treatment rats in the OVX+Cholesterol group received 3×30-mm cholesterol implants, and rats in the 48 h and 72 h groups received 3×30-mm P implants. On day 14 of treatment, P and cholesterol implants were removed. Brains were obtained from animals in the E+P-P48 h group on day 16 of treatment and those in the E+P-P72 h group on day 17 of treatment. Half of the rats in the OVX+Cholesterol group were killed at the same time as the 48 h group and half were killed at the same time as the 72 h group in order to control for any effect of time of perfusion. All hormones were implanted s.c. between the scapulae under isoflurane anesthesia. Incisions were closed with stainless steel wound clips after application of antibiotic powder (Cicatrin™, Glaxo-Smith Kline, UK).

### 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 4 day cycles were included in the Cycling group. These rats were killed 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 was observed in a vaginal smear taken on the morning after housing with a male, rats were individually housed in plastic cages (45 cm×25 cm×20 cm) until day 21 of pregnancy.

Females assigned to the lactating group were mated by adding one male to a group housing cage. Eighteen days after the introduction of the male, late pregnant females were individually housed in plastic cages (45 cm×25 cm×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 11:00 h and 13:00 h, on the appropriate day, rats were given an overdose of sodium pentobarbital and, after clamping the descending aorta, transcardially perfused with 150 ml of 0.9% saline and 150 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, Sigma, Canada). Brains were removed and post-fixed in 4% paraformaldehyde in 0.1 M PB containing 30% sucrose for 48 h, then stored at −80 °C until sectioning. Forty micron sections were obtained from approximately Plate 10 to Plate 42 of Paxinos and Watson's (1986) atlas of the rat brain, and stored at −20 °C in a cryoprotectant solution until processing for GFAP and bFGF immunocytochemistry. 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 3×10 min in Tris-buffered saline (TBS, Sigma) and then incubated for 24 hours at 4 °C in primary antibody (1:500, anti- FGF-2, Upstate Biotechnology), diluted in 0.3% Triton X (Sigma), 3% normal horse serum (NHS, Vector Laboratories, USA), and TBS. Sections were then washed in TBS, (3×5 min), and incubated in secondary antibody (1:200 anti-mouse IgG H+L, Vector Laboratories) for 1 hour at room temperature. The secondary antibody was diluted in 1.5% NHS and TBS. After incubation in the secondary antibody, sec-