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Fos expression in the prefrontal cortex and mesencephalic dorsal raphe nucleus in lactating rats after social instigation

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Abstract

Females are often less aggressive than males, but they exhibit high levels of agonistic behavior against an intruder in the area of the nest during lactation. This behavior is referred to as maternal aggression. In rats, maternal aggressive behavior occurs more often from postpartum day 3 (PPD 3) to PPD 12. Social instigation is an experimental protocol used to increase the levels of aggression that are typical of the species. In the present study we used social instigation to analyze the expression of a marker of neuronal activity, *c-fos*. Lactating rats on PPD 5, in the presence of their pups, were divided into four groups: (1) no social instigation and no aggressive behavior, (2) social instigation and no aggressive behavior, (3) no social instigation and aggressive behavior, and (4) social instigation and aggressive behavior. Sixty minutes after the aggression test we used immunohistochemistry to detect Fos in two brain regions, the ventral-orbital region of the prefrontal cortex (VO PFC) and dorsal raphe nucleus (DRN). Our results showed that rats with aggressive behavior that were provoked exhibited an increase in Fos expression in the VO PFC compared with the control group (i.e., no social instigation and no aggressive behavior). No change in Fos expression was found in the DRN. These results complement previous findings with microinjection of serotonin 5-hydroxytryptamine-1B receptor agonists into the same region, demonstrating that the VO PFC is an important region in the modulation of maternal aggressive behavior.

Keywords: social instigation, maternal aggression, Fos, prefrontal cortex, dorsal raphe nucleus.

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Introduction

The behavioral repertoire of females during maternity changes drastically compared with other periods of the reproductive cycle (Ferreira, Pereira, Agrati, Uriarte, & Fernández-Guasti, 2002). Motherhood involves changes in the perception of mothers toward offspring (Kinsley & Bridges, 1990; Rosenblatt, 1975), providing a link between mothers and offspring and causing significant changes in the response of mothers to environmental stimuli (Agrati, Zuluaga,

Fernández-Guasti, Meikle, & Ferreira, 2008). Females are often less aggressive than males, but they exhibit high levels of agonistic behavior against an intruder in the area of the nest during lactation (Bridges, 1996; Erskine, Barfield, & Goldman, 1980; Giovenardi, Consiglio, Barros, & Lucion, 2000; Olivier & Young, 2002; Lonstein & Gammie, 2002). In rats, maternal aggressive behavior occurs more frequently from postpartum day 3 (PPD 3) to PPD 12. During this period, females direct intense care toward their pups

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(Consiglio & Bridges, 2009; Erskine, Barfield, & Goldman, 1978).

Social instigation is a social experimental protocol used to increase aggressive behavior, in which excessive levels of aggressive behavior can be induced in laboratory animals. Attacks by mice, rats, and hamsters begin with a very low latency and high frequency when tested with an intruder in the resident's box or an unfamiliar place where the animal was previously provoked by an opponent (De Almeida & Miczek, 2002; Fish, Faccidomo, & Miczek, 1999; Potegal, 1991). Our research group recently showed that lactating rats exhibit a significant increase in the levels of aggression when subjected to social instigation (Veiga, Miczek, Lucion, & De Almeida, 2011).

Analyses of the expression of immediate early genes such as *c-fos* have been used to understand the neural basis of maternal behavior (Gammie & Nelson, 2001). In rats, aggressive confrontations activate Fos protein in the prefrontal cortex (PFC; Halász, Tóth, Kalló, Liposits, & Haller, 2006; Haller, Tóth, Halász, & De Boer, 2006). The ventro-orbital PFC (VO PFC) has been identified as an important area for the inhibitory control of subcortical circuits that mediate aggressive and impulsive behavior (Blair, 2001, 2004; Cardinal, Winstanley, Robbins, & Everitt, 2004; Kheramin et al., 2005; Séguin, 2004; Veiga, Miczek, Lucion, & De Almeida, 2007). Another region that has been implicated in the modulation of maternal aggression is the dorsal raphe nucleus (DRN). Our group recently showed that microinjection of the serotonin 5-hydroxytryptamine-1A (5-HT_{1A}) receptor agonist 8-OH-DPAT into the DRN increased aggressive behavior (Veiga et al., 2011).

The majority of studies on aggression have focused on physiological mechanisms (Davis & Marler, 2004), but little is known about the neural mechanisms of maternal aggressive behavior (Consiglio, Borsoi, Pereira, & Lucion, 2005; De Almeida, & Lucion, 1997; Factor, Mayer, & Rosenblatt, 1993; Ferreira, Dahlof, & Hansen, 1987; Giovenardi, Padoin, Cadore, & Lucion, 1998; Insel, 1986; Lonstein & Gammie, 2002; Nelson & Trainor, 2007; Russel & Leng, 1998; Svare, 1990). The aim of the present study was to evaluate VO PFC and DRN activation reflected by Fos expression in lactating rats that were subjected to social instigation and aggressive behavior.

Materials and Methods

Animals

We used primiparous female Wistar rats ~90 days of age from the Universidade Federal do Rio Grande do Sul. Each rat was placed and kept individually in transparent acrylic boxes (46 cm × 31 cm × 17 cm) with free access to water and food. The date of birth of the pups was controlled and established as PPD 0. On PPD 1, the pups were standardized to eight per litter, regardless of the sex of the pups. The animals were

kept under controlled temperature ($21 \pm 1^\circ\text{C}$) and a 12 h/12 h light/dark cycle (lights off at 6:00 PM). To test aggressive behavior in lactating females, we used ~150 g male rat intruders. Instigators were also used, which were protected by an acrylic tube and did not have direct contact with the residents. Intruders and instigators were kept in groups of five animals per cage. The instigators were never previously used as intruders. The experiments were performed in accordance with the standards of the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethics Committee of the institution.

Resident intruder confrontation

On PPD 3, females were selected for aggressive behavior as in Veiga et al. (2011). The behavioral test was conducted in the female resident's box in the presence of the pups at the beginning of the dark period.

Social instigation

Social instigation was performed on PPD 5 (Figure 1). Social instigation consisted of placing an acrylic tube with holes (28 cm length, 10 cm diameter) that contained an opponent (stimulus) male or instigator (Figure 1A) for 5 min in the resident female's box (Veiga et al., 2011). Residents typically threaten the protected stimulus male and attack the perforated cylindrical tube. The pups remained in the box with their mothers during social instigation.

Maternal aggressive behavior

Five minutes after the social challenge on PPD 5, maternal aggressive behavior against a male intruder was assessed for 10 min. The behavioral repertoire that we analyzed was described previously (De Almeida & Lucion, 1997). Social instigation and aggressive behavior were videotaped and later analyzed by the experimenter using Observer software (version 3.0, Noldus, The Netherlands). We determined the frequency of aggressive behaviors including lateral attacks, bites on the body, and an intrusive and aggressive stance.

Lactating rats were divided into the following groups: (1) no social instigation and no aggressive behavior (NI + NA; the acrylic tube was placed empty, without the stimulus rat, into the resident's box, and the rats were not subjected to maternal aggressive behavior), (2) social instigation and no aggressive behavior (I + NA; the acrylic tube was placed into the resident's box with the stimulus rat, and the rats were not subjected to maternal aggressive behavior), (3) no social instigation and aggressive behavior (NI + A; the acrylic tube was placed empty, without the stimulus rat, into the resident's box, and the rats were subjected to maternal aggressive behavior), and (4) social instigation and aggressive behavior (I + A; the acrylic tube was placed into the resident's box with the stimulus rat, and the rats were subjected to maternal aggressive behavior).

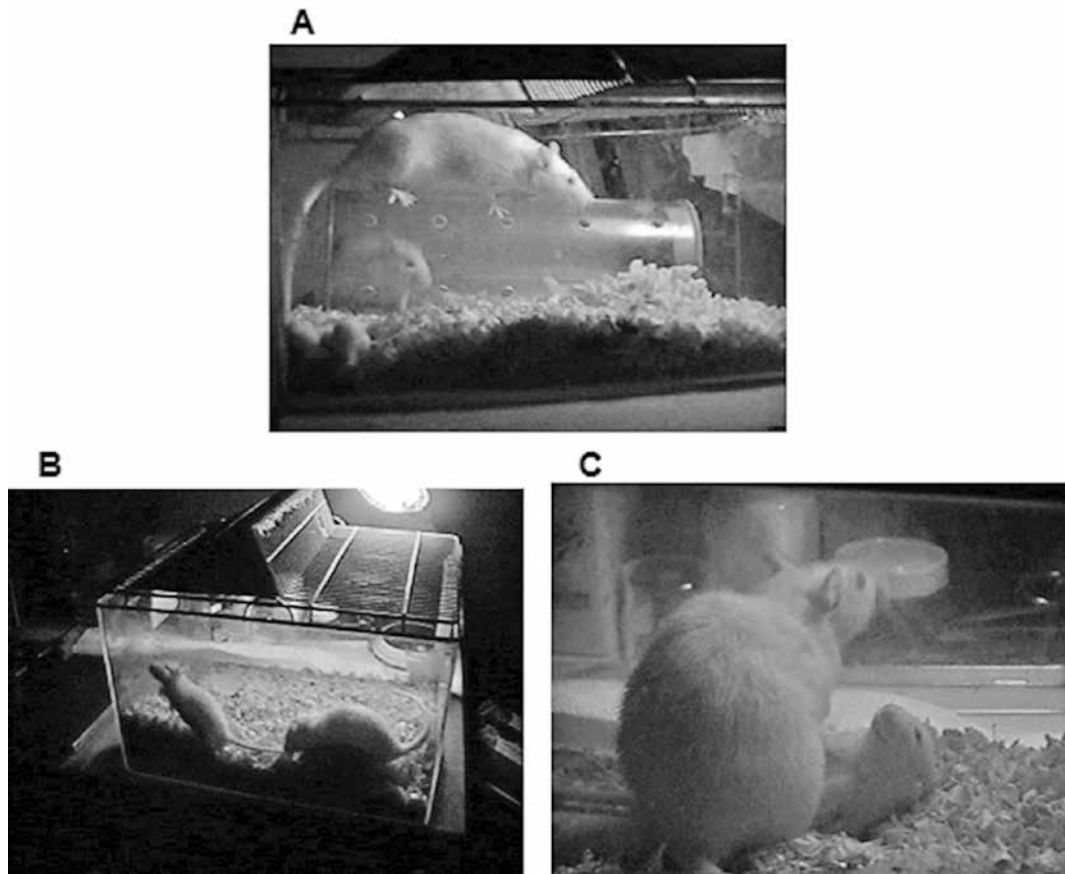


Figure 1. (A) Resident lactating rat and instigator rat in the acrylic tube during social instigation in the resident's box on postpartum day 5 in the presence of pups. (B) Investigative behavior of the female rat during resident intruder confrontation in the box for 10 min. (C) Resident dominating the intruder.

Immunohistochemical detection of Fos protein immunoreactivity

On PPD 5, 80 min after the beginning of social instigation, the four experimental groups of rats were analyzed for Fos protein expression in the DRN and VO PFC. The control group was not subjected to social instigation or aggressive behavior before the beginning of this experimental phase, with only an empty tube placed inside the resident's cage. Simultaneously, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and transcardiac perfusion was performed by injecting 150 ml saline followed by 4% paraformaldehyde diluted in 150 mg of 0.1 M sodium phosphate buffer, pH 7.4. The brains were then removed and kept in a 4% paraformaldehyde fixative solution for 8 h at room temperature. Subsequently, the brains were cryoprotected in a 30% sucrose solution at 4°C. The brains were then cut into 50- μ m-thick coronal sections using a cryostat and collected free-floating. The slices were kept immersed in an antifreeze solution (phosphate-buffered saline [PBS] that contained distilled water, sucrose, and propylene glycol; Vetec, São Paulo, Brazil) and stored at -70°C until the day of immunohistochemical Fos detection. All of the brains were processed together to detect Fos protein using avidin-biotin-peroxidase as in Cezario, Ribeiro-Barbosa, Baldo, & Canteras (2008).

The brain sections were washed three times for 10 min each in PBS and incubated in buffer solution with Triton X-100 (Sigma, St. Louis, MO, USA), normal goat serum, and rabbit anti-Fos antibody (Ab-5; Calbiochem, San Diego, CA, USA) at a 1:20,000 dilution for 48 h at 4°C with continuous stirring. After this period, the sections were incubated for 90 min at room temperature in a 1:250 solution of biotinylated goat immunoglobulin G anti-rabbit (Vector Laboratories, Burlingame, CA, USA), mixed, and then incubated for 90 min in a solution that contained avidin-biotin peroxidase at a dilution of 1:250 (ABC Elite Kit; Vector Laboratories). The visualization of the peroxidase complex occurred after exposure for 10 min in a chromogen solution that contained 0.02% 3,3-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) with 0.3% nickel sulfate in 0.05 M Tris buffer (pH 7.6), followed by 5 min incubation in a 0.01% solution of glucose oxidase (glucose oxidase Type VII from *Aspergillus niger*; Sigma) and 10% Pd-glucose (Sigma) to produce a dark blue color. For negative control of enzyme activity some sections were randomly processed by omitting the primary antibody. All sections were then washed with buffer, collected on previously gelatinized slides, dehydrated in ethanol, cleared with xylene, and mounted with coverslips using distyrene/plasticizer/xylene.

Quantification of Fos-positive cells

The VO PFC and DRN regions were identified according to the Paxinos & Watson (1998) rat brain atlas. To quantify cells that were immunohistochemically stained for Fos protein, we used a Zeiss Axioscop2 microscope with a 20× objective and a charge-coupled device video camera coupled to an Apple Macintosh 8600-300 computer and NIH Image 1.62f analysis system. Neuronal quantification was performed using both hemispheres (Davis & Marler, 2004; Madruga, Xavier, Achaval, Sanvitto, & Lucion, 2006), and neurons were counted inside a 13,887 μm^2 frame (i.e., test area) according to previous studies of Fos expression in rodents (Davis & Marler, 2004; Gammie & Nelson, 2001). Neurons outside the outer forbidden lines were not counted (Camozzato et al., 2009; Wilkelmann-Duarte et al., 2007). Slices were made from each region along its entire length with a 200- μm gap. In the VO PFC, slices were made 3.70–5.20 mm anterior to bregma. In the DRN, slices were made 7.04–8.0 mm posterior to bregma (Paxinos & Watson, 1998). For the DRN, the cell counts reflected the sum of the proximal and distal parts with the aqueduct as a reference. Data are expressed as the average number of neurons in each region within the test area. All quantifications of labeled cells were performed by two experimenters unrelated to the study.

Statistical analysis

The results are expressed as mean \pm SEM. Fos immunoreactivity results in the VO PFC and DRN in the four experimental groups (NI + NA, I + NA, NI + A, and I + A) were analyzed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls *post hoc* test. Values of $p < .05$ were considered statistically significant. Aggressive behavior in females in the two groups tested for aggressiveness (NI + A and I + A) were analyzed using Student's *t*-test.

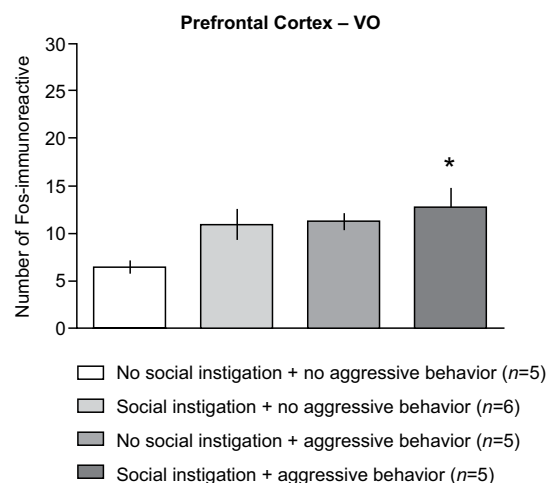
Results

Microscopic analysis of Fos protein expression in the VO PFC showed that lactating rats subjected to social instigation and aggressive behavior exhibited a statistically significant increase in the number Fos-positive cells ($F_{3,17} = 3.30$, $p < .05$; Figures 2 and 3) compared with the control group (i.e., no social instigation and no aggressive behavior). No significant difference was found between groups in Fos expression in the DRN ($F_{3,16} = .64$, $p = .5$; Figures 4 and 5).

Socially instigated lactating rats subjected to aggressive confrontation exhibited a significant increase in the frequency of lateral attacks ($t_9 = 2.43$, $p < .04$; Figure 6) compared with the control group (i.e., no social instigation and no aggressive behavior).

Discussion

The present study showed that lactating rats subjected to social instigation and aggressive behavior



*: $p < 0.05$ compared to NI + NA group

Figure 2. Mean (\pm SEM) number of Fos-immunoreactive cells in the ventral-orbital (VO) prefrontal cortex in rats subjected to social instigation and aggressive behavior on postpartum day 5 in the presence of pups. * $p < .05$, compared with control group (no social instigation and no aggressive behavior).

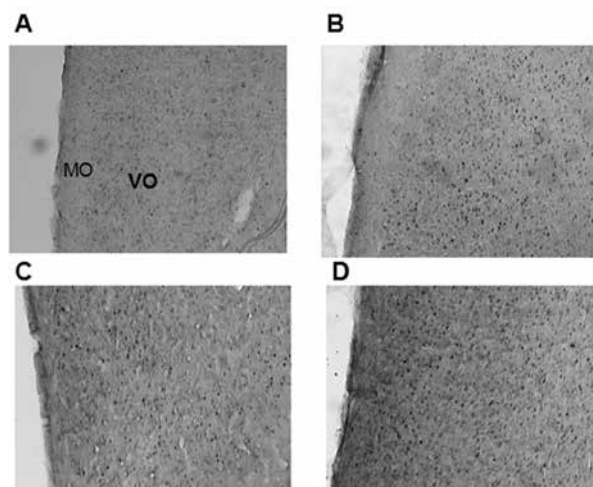


Figure 3. Coronal sections of the rat brain that show the region of Fos-immunoreactive cells in the VO PFC in the (A) control group, (B) social instigation and no aggressive behavior group, (C) no social instigation and aggressive behavior group, and (D) social instigation and aggressive behavior group. A significant increase in the number of Fos-positive cells was found in the VO PFC, concomitant with the expression of maternal aggressive behavior (D).

against a male intruder in the presence of their pups on PPD 5 exhibited an increase in Fos protein expression specifically in the VO PFC. No change was observed in Fos expression in the DRN in lactating rats after exposure to social instigation or aggressive behavior. Analyses of the increased expression of transcription factors such as *c-fos* in association with specific behaviors (e.g., maternal aggression) have provided important indirect evidence of neural activity in a subregion of cells during the execution of a certain behavior (Gammie & Nelson, 2001).

No studies of which we are aware have demonstrated the brain regions that have increased Fos expression in lactating rats subjected to experimental protocols

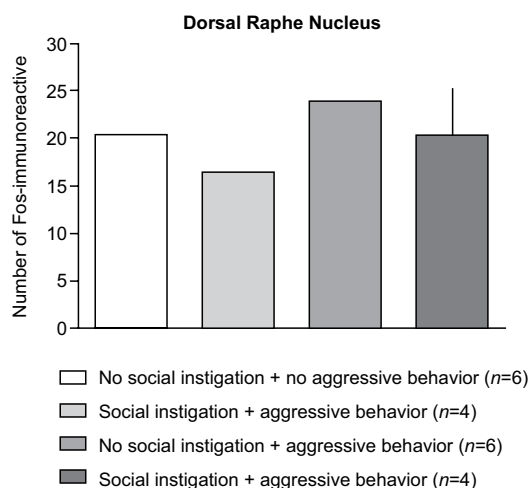


Figure 4. Mean (\pm SEM) number of Fos-immunoreactive cells in the dorsal raphe nucleus (DRN), proximal and distal to the aqueduct, in resident lactating rats subjected to social instigation and aggressive behavior on postpartum day 5 in the presence of pups.

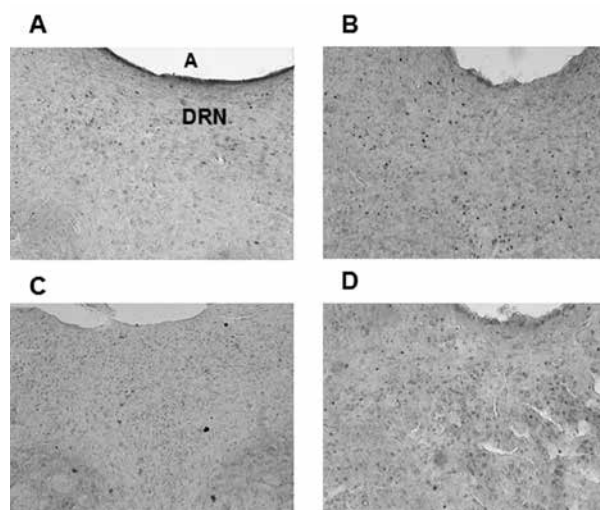


Figure 5. Coronal sections of the rat brain showing the DRN (proximal, relative to the aqueduct [Aq]) where Fos-immunoreactive cells were quantified in the (A) control group (no social instigation and no aggressive behavior), (B) social instigation and aggressive behavior group, (C) no social instigation and aggressive behavior group, and (D) social instigation and aggressive behavior group.

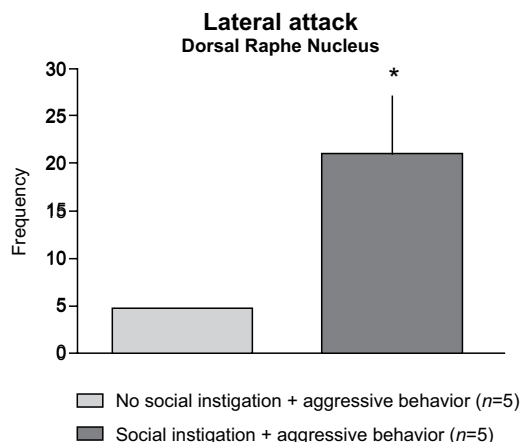


Figure 6. Effect of social instigation on aggressive behavior (i.e., lateral attacks) in lactating rats on postpartum day 5 in the presence of pups.

of aggression such as social instigation. Some studies have shown an increase in Fos expression in the medial amygdala, bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus, and periaqueductal gray after maternal aggression (Gammie & Nelson, 2001). Parental male mice that take care of offspring exhibit an increase in Fos expression in the medial preoptic area, ventromedial hypothalamus, and medial amygdala (Trainor, Finy, & Nelson, 2008), and these same regions also express Fos in the context of maternal aggression (Gammie, 2005). Fos is expressed for only short periods of time (15–90 min) after exposure to a stimulus (Morgan, Cohen, Hempstead, & Curran, 1987; Morgan & Curran, 1991; Gammie & Nelson, 2001; Knyshevski, Connor, Harrison, Ricci, & Melloni, 2005). However, some studies have shown that Fos expression can persist, representing cellular activation in response to chronic physiological (Fenelon, Poulain, & Theodosis, 1993; Miyata, Nakashima, & Kiyohara, 1994) and behavioral (Fenelon et al., 1993; Matsuda, Peng, Yoshimura, Wen, Fukuda, & Sakanaka, 1996) stimuli.

Experiments conducted with male rats found that, compared to LO and VO, regions of the prefrontal cortex cause an increase in neuronal activation after aggressive encounters (Halász et al., 2006), and this activation was particularly higher in male mice previously selected for high rates of aggression (Haller et al., 2006). Thus, the increased expression of Fos in the VO PFC in the present study is similar to previous studies.

The manifestations of abnormal aggressive behavior are related to or caused by decreased prefrontal cortex activity (Damasio, Grabowski, Frank, Galaburda, & Damasio, 1994; Volkow et al., 1995; Golden, Jackson, Peterson-Rhone, & Gontkovsky, 1996; Critchley et al., 2000; Davidson, Putnam, & Larson, 2000; Hawkins & Trobst, 2000; Bassarath, 2001; Best, Williams, & Coccaro, 2002; Veit et al., 2002; Blair, 2004; Halász et al., 2006). In contrast to these results, however, the present study found that social instigation followed by aggressive behavior increased neuronal activation in the VO PFC, and other studies have also shown similar results (Halász et al., 2006; Haller et al., 2006). Halász et al. (2006) argued that this discrepancy with regard to PFC deficiency in humans who exhibit violent behaviors are not found in rodents and can be explained by the differences between the acute and chronic functioning of the PFC. For example, acute activation as observed in the present study and chronic dysfunction in violent persons may affect aggressive behavior through independent mechanisms. Prefrontal cortex deficiencies have been shown to alter moral judgments in humans, and behavioral disturbances have been suggested to occur through this route (Price, Daffner, Stowe, & Mesulam, 1990; Nichelli, Grafman, Pietrini, Clark, Lee, & Miletich, 1995; Greene, Nystrom, Engell, Darley, & Cohen, 2004; Luo, Nakic, Wheatley, Richell, Martin, & Blair, 2006). Thus, prefrontal deficits lead to violence via deficiencies in moral judgments (Halász et al., 2006). In contrast, acute activation of the PFC

may contribute to the expression of aggressive behavior, which is supported by the present results and previous studies in rodents (Halász et al., 2006).

The observation of Fos-positive serotonergic neurons in the DRN is consistent with previous work from our group. Microinjection of the 5-HT_{1A} receptor agonist 8-OH-DPAT into the DRN increased aggressive behavior in lactating rats (Veiga et al., 2011), which possibly exhibit a reduction of serotonin in this area. A more detailed analysis using Fos immunohistochemistry with co-labeling for serotonergic neurons may provide more support for the results obtained in this work.

In summary, the present results demonstrated that lactating rats subjected to social instigation and aggressive behavior on PPD 5 exhibited an increase in Fos expression in the VO PFC in the presence of their pups, but no changes in Fos expression were found in the DRN. Future experiments that analyze Fos immunohistochemistry in other brain regions in lactating rats subjected to social instigation and aggressive behavior may further elucidate the modulation and expression of maternal aggression.

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