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CIRCULATING PROLACTIN, MPOA PROLACTIN RECEPTOR EXPRESSION AND MATERNAL AGGRESSION IN LACTATING RATS

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Abstract

Maternal aggression is most intense in lactating rats from the 3rd to the 12th day postpartum. The purpose of this study was to determine if plasma prolactin (PRL) and prolactin receptor (PRL-R_L) mRNA expression in the medial preoptic area (MPOA) of lactating rats are altered in association with maternal aggression. Lactating Sprague Dawley rats were divided into 5 groups and exposed for 10 minutes to an intruder male or to an object on postpartum day 8. Trunk blood and the brain of the dams were collected 30 or 240 minutes after exposure and from a non-exposed group. Lower levels of prolactin were found 30 minutes after the aggression test. No change was detected in the number of cells expressing PRL-R_L mRNA by *in situ* hybridization histochemistry (ISHH) as a function of testing. However, the correlation between plasma PRL and PRL-R_L mRNA expression in the mothers changed from positive in control females to negative in intruder exposed animals. These data support the concept that a maternal aggressive experience, while acutely altering PRL secretion, fails to affect PRL-R_L mRNA expression.

Keywords

Maternal aggression; prolactin; prolactin receptor; *in situ* hybridization; intruder male; MPOA

INTRODUCTION

Maternal aggression is vigorously expressed by lactating female rats towards male or female intruders that approach the nest area. In rats, maternal aggression occurs most frequently between the 3rd to 12th day postpartum during which time the mother's caregiving behaviors directed towards the pups are intensive [12]. Previous studies in rodents have established that maternal aggression is linked to both hormonal changes at the end of pregnancy and the presence of the pups [12,20]. Moreover, the number of pups in the litter increases the likelihood that the mother will attack an intruder [28]. In virgin female mice, suckling is also able to

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induce aggressive behavior [36], whereas in rats suckling alone does not appear to elicit maternal aggression [14].

The interactions between the endocrine and neural systems in the regulation of maternal aggression are not well characterized. One hormone that plays an important role in bringing about the induction of maternal behavior at parturition is the anterior pituitary hormone prolactin [3], a molecule that also is crucial for milk production and in most mammals is secreted in large amounts in response to suckling during lactation [39]. PRL has been shown to stimulate the onset of maternal behavior when infused into the medial preoptic area (MPOA) of steroid-primed inexperienced, nulliparous rats [6]. The MPOA of female rats also exhibits increased cFOS immunoreactivity (cFOS-IR) following contact with pups, a condition necessary for the continued expression of maternal aggression [15]. Likewise, in mice cFOS expression increases significantly in the MPOA as well as in the claustrum, bed nucleus of stria terminalis, paraventricular nucleus, medial amygdala and cortical amygdala in association with maternal aggression [19]. The involvement of the MPOA in maternal aggression has been reviewed recently by Gammie [18].

PRL's action in the regulation of pup-directed maternal behaviors appears to be mediated through its action on the PRL receptor system. In PRL "knock out" mice deficient in PRL receptors, both null mutant homozygotes and heterozygotes females exhibit defects in pup retrieval [25]. These changes are likely mediated through PRL receptors in the MPOA, since in rats infusions of a PRL antagonist into the MPOA delays the onset of maternal behavior in steroid-primed, virgin rats [7]. Physiologically, over the course of pregnancy and lactation PRL-R_L mRNA expression in the MPOA changes with significant increases observed just prior to parturition [26]. Moreover, exposure of ovariectomized rats to a pregnancy-like regimen of the steroid hormones, progesterone and estradiol, increases PRL-R_L expression in the MPOA [5]. Based upon the findings of behavioral and physiological studies, the possible involvement of the neural MPOA PRL system in maternal aggression seems tenable and worth examination.

Therefore, the goal of the present study is to assess the possible acute effects of postpartum maternal aggressive experience on a specific part of the PRL neurochemical system of the mother to determine whether the PRL receptor system within the MPOA plays a role in this response. In order to assess a possible role of the PRL system in maternal aggression, both central PRL-R_L and circulating PRL levels were measured as a function of maternal aggression. In addition, the PRL response of the intruder male was evaluated. We hypothesize that a slower drop in PRL-R_L mRNA expression in the MPOA would follow a faster decline in PRL plasma after maternal aggression.

METHODS

Animals

Nulliparous female (225–250 g) and male (175–200 g) Sprague-Dawley rats (Crl:CD[SD]BR) were purchased from Charles River Laboratories (Kingston, NY). Subjects were housed in polypropylene cages (45 × 25 × 20 cm) in a 14:10 light:dark cycle (lights on 0500 h) with food and water available *ad libitum* throughout the study. The animals used in this study were maintained in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals, National Academy of Sciences, 1996). The research protocol was approved by the Institutional Animal Care and Use Committee at Tufts Cummings School of Veterinary Medicine.

Treatments

Lactating Dams—One set of female rats were bred to males from our rat colony and then the females were individually housed. Pregnancy and parturition were monitored daily. Each litter was culled to 10 pups (5 females/5 males) on postpartum day 1. On postpartum day 8 between 0900 and 1200 h, mothers with pups were tested for maternal aggression during a 10 minute session following the introduction of either an adult male intruder (used only once) or a 12 × 3 cm piece of a previously cleaned plastic object. Five groups of lactating dams were studied. The main control group (G1, n=8), served as a common control group for the lactating plus intruder (G2= at 30 min, n=8; G3=at 240 min, n=8) and lactating plus object (G4=at 30 min, n=8; G5=at 240 min, n=8) groups. It is noted that all maternal aggression testing was performed in the presence of pups.

During the aggression test session the following behaviors were recorded: frontal and lateral attacks, biting, boxing, lateral threats, sniffing, locomotion, nest building and pup interaction [8]. At least 30 minutes after the aggression testing, the females were observed in their home cages to assess hovering over pups and crouching behavior (both were included as "crouching behavior") in the test room.

Control groups included mothers and offspring under undisturbed conditions representing the situation before the maternal aggressive test. They were not exposed to an intruder or to a novel plastic object. Brains were removed following sacrifice and subsequently processed for mRNA for the long form of the rat prolactin receptor (PRL-R_L) by *in situ* hybridization histochemistry (ISHH) as described below. ISHH quantification was performed blind to the treatment of the animals. Trunk blood samples from mothers were collected and assayed for PRL content by radioimmunoassay.

Intruder Samplings—As with mothers, intruder males were decapitated under undisturbed conditions representing the situation before the maternal aggressive test and at 30 and 240 min after the end of the ten minute aggression test (either to a lactating female or to a cycling female in diestrus). Five intruder groups were sampled (n=8 males/group). The G_I control group intruders were removed from their home cages which they shared with another male and then sacrificed. For the G_{II} and G_{III} groups, an intruder was exposed to a lactating female rat with her pups for 10 min in her home cage (and sampled at 30 and 240 min). For the G_{IV} and G_V groups, an intruder was exposed to a regular cycling, diestrous, nulliparous female rat for 10 min in the diestrous female's home cage (and sampled at 30 and 240 min). Trunk blood samples from the intruders were collected and assayed for PRL content by radioimmunoassay.

Prolactin Measurements

Plasma concentrations of PRL were measured using the NIDDK rat PRL kit which was supplied by the National Hormone Pituitary Program and Dr. A. F. Parlow. This kit included reference preparation NIDDK-rPRL-RP-3 and anti-rat PRL S-9. All plasma samples were assayed in duplicate at volumes ranging from 1 to 75 μl. Assay sensitivity was 1 ng/ml of plasma. Inter- and intra-assay coefficients of variability were 11 and 9%, respectively.

In Situ Hybridization Histochemistry

Probe Preparation—The ISHH protocol employed was similar to that previously reported [5]. The template used to prepare the riboprobe specific for the long form of the prolactin receptor (PRL-R_L) supplied by Dr. Paul Kelly (INSERM 344, Paris, France) was a 269-bp *Hin*dIII-*Xba*I cDNA fragment of the full length clone complementary to the cytoplasmic domain of the receptor. The plasmid was linearized with *Hin*dIII and transcribed using SP6 (antisense) and T7 (sense) in the presence of ^{33}P-UTP (ICN; Irvine, CA) and transcription

buffer. This probe that recognizes the long form (PRL-R_L), but not short form (PRL-R_S) of the prolactin receptor was extracted and purified as previously reported [5].

Tissue Preparation and ISHH—After rapid decapitation, brains were collected, buried in powdered dry ice for several minutes, and then wrapped in parafilm and stored, desiccated, at −80°C. Frozen cryostat-cut serial sections (14 μm) from the MPOA were cut and mounted on Vectabond-coated glass slides (4 sequential slides with 4 sections per slide) and frozen at −80° C. Sections were collected throughout the entire rostral-caudal extent of the MPOA. Frozen sections were subsequently thawed and fixed in 3.4% formalin at room temperature for 5 min rinsed in 1X PBS for 2 min, washed in 0.1 M triethanolamine, pH 8.0 for 10 min, and then acetylated with 0.25% acetic anhydride for 10 min. Slides were then rinsed in 2X standard saline citrate (SSC) and dehydrated and delipidated in a series of ethanols and chloroform, respectively. The sections were air-dried for one-hour. The slides were hybridized (1×10^7 cpm/ml of hybridization buffer) for 16–20 h at 60°C. Tissues were then rinsed (4x SSC), treated with RNase, and washed in decreasing concentrations of SSC with 10 mM DTT (2X, 1X and 0.5X) for 15 min each at room temperature. The last wash with 0.1 × SSC was for 30 min at 55°C. Slides were then dehydrated, dried, dipped in Kodak NTB 2 nuclear emulsion, and placed in a refrigerator at 5–10° C during the exposure period. Sections were processed (Kodak D19) after 20 days and then counterstained with cresyl violet to visualize cell bodies.

ISHH Data Analysis—Sets of neurons within a 400 × 300 μm of each section were counted under bright field illumination using an Olympus BX50 microscope. Cells with three times the number of grains as background were recorded as containing a cluster of mRNA for the PRL receptor. These cells were counted using Digital images from a Zeiss Axioscope. Data were captured with a Hamamatsu charge coupled device (CCD) video camera together with an 8-bit (256 gray-scale levels) frame grabber board controlled by Scion's version of NIH image. Count data for PRL-R_L mRNA were summated across sections and expressed as the number of clustered cells within the MPOA.

Statistical Analysis

A one-way ANOVA was employed for the dependent variables (plasma PRL, maternal behavior duration) or average number of clusters in MPOA. The PRL values of the lactating female exposed to male animals and control were compared as one set of data and likewise for the lactating female exposed to object and control. The Tukey's multiple comparison test was used to determine significance ($\alpha = 0.05$). The Dunnett's multiple comparison test was used to determine significance for the maternal behavior duration in PPD 8 mothers during exposure to an intruder or object when compared to control. For the intruder male study, since normality test failed, non parametric test was used. Pearson correlation test was used to compare plasma PRL of the mother to the total number of clusters in MPOA after the maternal aggressive test as well as to compare the frequency of aggressive behaviors (total number of the behavior during the 10-minute session) to the duration (total duration in seconds) of crouching behavior after the aggressive test. Correlations of the ratio of PRL to PRL-R_L were calculated to evaluate the relationships between the PRL and PRL-R_L systems and the frequency of frontal attack; in order to exclude the interference of pup interaction on the PRL measurement, only dams with similar pup interaction times during the aggressive test were used in this correlation analysis. This procedure reduces sample size and correlation results should be considered cautiously.

RESULTS

Lactating Rats - Maternal Aggressive Behavior

The lactating mothers displayed characteristic high levels of aggression towards the intruder males during the 10-minute test on PPD 8. These behavioral responses are shown in Table 1. It is noted that aggressive behavior data for the diestrous control animals tested with intruders are not presented, since these latter females failed to display any components of maternal aggression.

Maternal Behavior (pup interaction and nest building)

The lactating female left the nest either after the introduction of an object or an intruder. However, the total time of maternal behavior during the 10 min session is different among those groups. The lowest scores are shown when the intruder is present, whereas similar results are shown for the lactating female exposed to an object or to nothing. So, the presence of the intruder made the mother less interactive with pups (Table 2).

Plasma PRL

The effects of maternal aggression on plasma PRL concentrations are shown in figure 1 (left panel). Plasma PRL levels in PPD 8 mothers were lower [$F(2,20=3,7)$, $p<0.05$] 30 min after the dams were exposed to an intruder, when compared to 240 min after exposure to the intruder ($p<0.05$); plasma PRL levels in PPD 8 mothers were not different [$F(2,21=0,295)$, $p=0.748$] 30 or 240 min after the dams were exposed to an object. *MPOA PRL-R_L mRNA Cells:* Maternal aggression did not affect the average number of MPOA cells that contained PRL-R_L mRNA (figure 2). The number of cells expressing PRL receptor message were similar in animals prior to and after exhibiting maternal aggression. Likewise, the number of MPOA cells expressing mRNA for the PRL-R_L was similar at the 30 and 240 min sampling times in mothers exposed to intruders or plastic objects.

Although maternal aggression did not directly affect mRNA expression for the PRL-R_L, a number of interesting correlations were identified between circulating PRL concentrations, PRL receptor expression and the display of maternal aggression. First, as shown in figure 3 (left panel), in lactating rats sampled under basal conditions, there was a positive linear correlation between plasma PRL concentrations and the number of clusters or cells expressing the PRL-R_L ($n=5$, $r = +0.851$, $p = 0.06$). This relationship was reversed 30 min after the mother displayed maternal aggression towards an intruder. Thus, at 30 min, there was a negative correlation between plasma PRL and the total number of clusters with cells expressing mRNA for the PRL-R_L. ($n=4$, $r = -0.994$, $p < 0.01$; figure 3, right panel).

In addition to these correlations between plasma PRL and the number of cells expressing the PRL-R_L in the MPOA, two significant behavioral correlations were found between these variables at 30 min and the frequency of frontal attacks during aggression testing and crouching duration during the 30 minutes after maternal aggressive behavior. As shown in figure 4 (left panel), a positive linear correlation was found between the frequency of frontal attacks during aggression testing and the ratio of plasma PRL to PRL-R_L expression in the MPOA at 30 min ($n=3$, $r = + 0.998$, $p < 0.05$). In addition, a negative correlation was found between the frequency of frontal attacks and biting with the duration of crouching after interaction with the intruder ($n=5$, $r = -0.865$, $p<0.05$ and $r = -0.96$, $p<0.01$ respectively; see figure 4, right panel).

Intruder Males

Prolactin—Plasma PRL levels in the intruder males after exposure either to a lactating mother rat or a cycling, diestrous female rat are shown in figure 1 (right panel). The small increase in plasma PRL levels at 30 min after exposure to a lactating or diestrous female did not reach

significance. Plasma PRL pre-exposure levels (control group) were similar to levels at 240 min. Significant correlations between the maternal endocrine and behavioral variables and intruder plasma PRL were not found.

DISCUSSION

The present study demonstrated that plasma PRL of the mother rat 30 minutes after the aggressive test are lower, but no change in PRL-R_L mRNA expression in the MPOA was detected at this time. The change in the ratio of plasma PRL and the PRL-R_L expression at that time, however, does reflect how the PRL system as a whole is regulated. While in a stable condition (no intruder exposure) the expression of the PRL-R_L at MPOA is positively related to PRL, after the aggressive test it is negatively correlated. That is, PRL levels are lower for those lactating rats which have higher MPOA PRL-R_L expression after displaying aggressive behavior. Different interpretations may arise from this data. It is possible, for example, that higher levels of PRL and PRL-R_L expression may be related to the intensity of the response when the mother is exposed to an intruder.

In order to delineate the relationships between the pup interactions during the maternal aggressive test and PRL system, we also examined the correlations between the aggression scores, crouching duration immediately before sampling, and the PRL system 30 minutes after testing. For example, those rats that displayed frontal attacks, a significant positive correlation was found in mothers between the frequency of frontal attack and plasma PRL and an inverse relationship between frontal attacks and PRL-R_L mRNA expression in the MPOA. This suggests that the frequency of frontal attack is simultaneously related to high PRL levels and inversely to the PRL-R_L mRNA expression in this brain region. Since the ratio of (PRL)/(PRL-R_L) decreased 30 minutes after the aggressive behavior, it is possible that the more aggressive females maintain higher PRL levels than the less aggressive dams. In addition, females that displayed the more defensive responses of frontally attack and biting the intruders, spent less time crouching after these interactions. Thus, even when the physical threat of the male was no longer present, maternal behavior was still affected.

In the present study, lower levels of circulating PRL levels were found shortly after the introduction of the intruder into the test environment. This is in agreement with earlier work [28] in which a decline in plasma PRL was found after an aggressive encounter. It appears that when the mother is engaged in attacking the intruder, she tends to leave her young and the nest site, thereby temporarily interrupting nursing with a concurrent decrease in plasma PRL. This decrease may be attributable to the presence of the intruder, to the mother's voluntary separation from her pups during the aggressive test or to the decreased time of crouching before sampling. It is established that blood PRL levels increase within 1–3 min of nursing initiation, and fall when nursing is terminated [16,22].

Previous research indicates that ongoing pup-directed maternal behavior as well as maternal aggression is not dependent upon circulating PRL [13,31]. However, the expression of maternal aggression may be associated more with the status of the PRL system. In male rats PRL like immunoreactivity does not decrease after hypophysectomy in a number of extrahypothalamic sites, including the caudate nucleus, brainstem region, cerebral cortex, thalamus [11] despite a significant drop in serum PRL to undetectable levels. Thus, brain PRL is not necessarily regulated by pituitary or circulating PRL levels. The idea that the neural PRL system itself may alter behavior is supported by the work of Torner et al [38] who found that central infusions of PRL-R_L antisense oligodeoxynucleotide were anxiogenic in lactating rats.

Prior studies have found that the long form of PRL-R_L mRNA expression is stimulated by progesterone, PRL or growth hormone (GH) and markedly increased in the brain at mid- and

late gestation as well as during lactation [35]. However, it is still unclear what specific signals differentially regulate PRL receptor expression in distinct neuronal populations and the neurochemical phenotypes of neurons that contain PRL receptors. PRL might be acting to alter the pattern of neuronal responsiveness during lactation [21] which may mediate maternal aggression.

In mice, there is evidence that the MPOA may mediate maternal aggression; a positive trend was noted between total attack time and fos-IR in that area [23]. Likewise, a role for the MPOA is supported by studies that examined the role of GABA in maternal aggression [2]. Other work in rats indicates that the inhibitory actions of cocaine on maternal aggression, possibly involving altered oxytocin levels, are mediated through its action at the level of the MPOA [10].

The results of the present study indicate that the expression of the PRL-R_L in the MPOA does not change acutely in lactating rats that display maternal aggression on PPD 8. Although expression of this receptor as measured with real time RT-PCR has been reported to increase 2 hours after subcutaneous injection of PRL [1], other studies have shown that longer periods of separation from young and the resultant decline in circulating PRL are needed to alter expression of this receptor. Likewise, the ability of treatment with PRL, growth hormone, and progesterone, all of which can stimulate PRL receptor expression [35], is evident after longer periods of exposure. It is possible that had we measured receptor expression by real time RT-PCR that more subtle changes in receptor expression might have been found. It is noted that while real time RT-PCR can be more quantitative than ISHH, RT-PCR generally provides less anatomical specificity.

The mechanisms underlying increased expression of PRL-R_L in hypothalamic nucleus of lactating rats are unknown. Individual hypothalamic nuclei are differentially regulated either by the stimulus of suckling itself and/or PRL during lactation. The expression of PRL-R_L in the ventromedial hypothalamic (VMH), ventrolateral preoptic and ventromedial preoptic nucleus was increased in bromocriptine-treated rats (suppressed PRL) in the presence of suckling stimulus, indicating that PRL-R_L expression may be inhibited by hyperprolactinemia. In the lateroanterior, ventrolateral and paraventricular nuclei PRL-R_L expression was decreased after haloperidol treatment in the absence of pups, suggesting that the PRL-R_L in these areas is most likely regulated by elevations in PRL or the suckling stimulus itself [30]. It is possible that the lower levels of plasma PRL 30 minutes after maternal aggression found in our study may lead to altered PRL-R_L expression sometime later than 4h.

In this study the increase in plasma PRL of the male intruder 30 minutes after being exposed and attacked by a lactating rat and being attacked was not significant. Other researchers have found changes in PRL levels in intruders after aggressive encounters [9,29]. Acute hormonal reactions to agonistic interactions under chronic conditions were found not only to depend on the actual behavioral situation as well as the animals previous behavioral experience. During acute defense and defeat there is a significant increase in plasma PRL in an inexperienced intruder as well as in chronically defeated and stressed subordinates, while there is a weak release of PRL during the encounter in the dominant animal. Therefore, PRL, and to a lesser extent also ACTH, β-endorphin, and corticosterone, secretion is differentially released based upon the social situation and acute offensive and defensive responses [9].

In summary, the results of this study support the idea that this maternal aggressive experience acutely reduces PRL secretion without acutely altering PRL-R_L mRNA in the MPOA of lactating rats. The possible long term effects and mechanisms controlling these events as well as the involvement of other neural areas that mediate maternal aggression are the subjects of future investigations.

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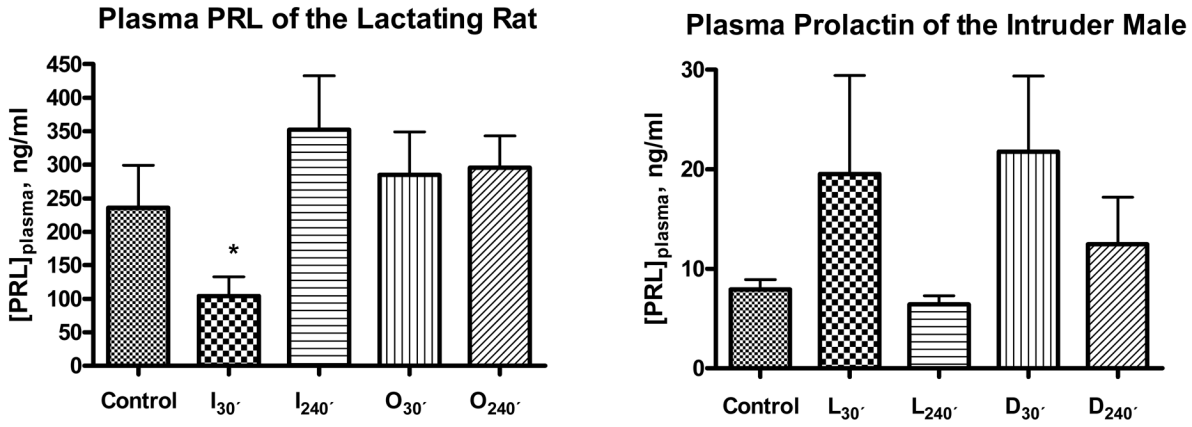

Fig 1.

Plasma PRL concentrations (mean ± SEM) of the lactating rat 30 and 240 min after exposure to an intruder ($I_{30'}$ and $I_{240'}$ respectively) or an object ($O_{30'}$ and $O_{240'}$ respectively) (left); plasma PRL concentrations of the intruder male rat 30 and 240 min after exposure to a lactating ($L_{30'}$ and $L_{240'}$ respectively) or a female in diestrus ($D_{30'}$ and $D_{240'}$ respectively) (right). The main control group represents the undisturbed conditions before the maternal aggressive test. Analysis revealed lower PRL levels in PPD 8 mothers 30 min after exposure to an intruder when compared to 240 min after exposure to the intruder ($^{*}p<0.05$).

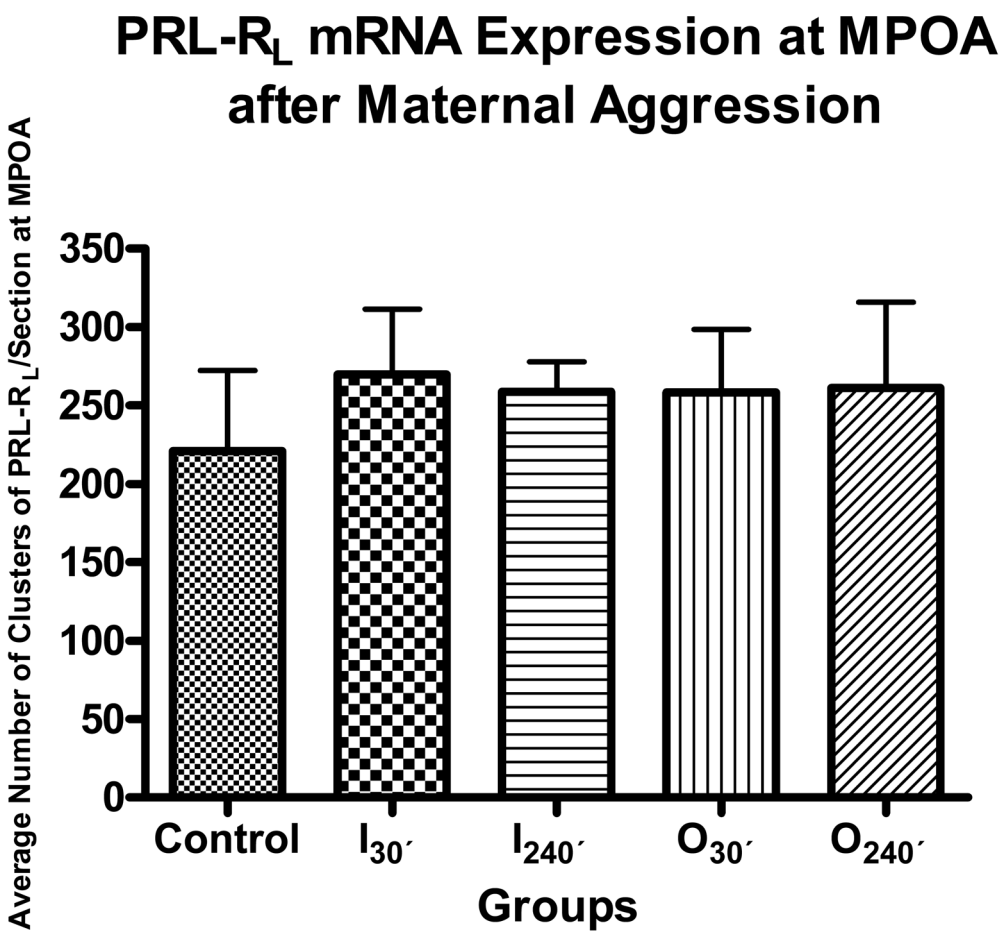

Fig 2.

Average number of clusters/section expressing the PRL-R_L throughout the MPOA of the lactating rat after maternal aggression.

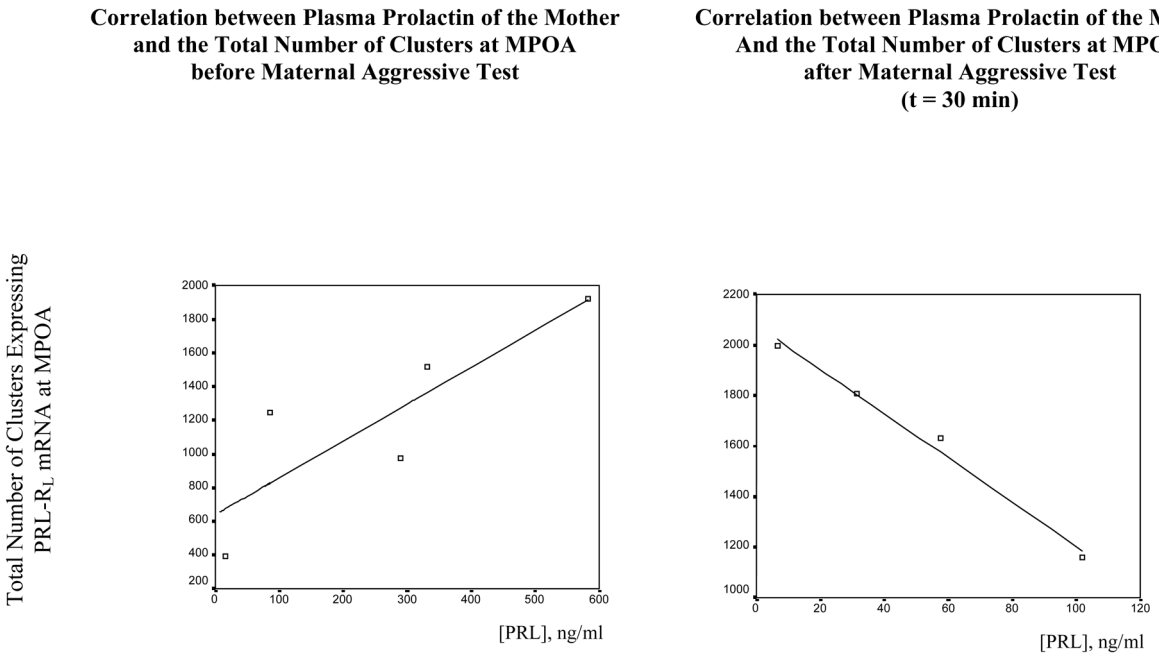

Fig 3.

Correlation between plasma PRL of the mother and the number of clusters expressing the PRL-R_L throughout the MPOA, before (left panel; r = +0.851, p = 0.06) and after (right panel; r = −0.994, p < 0.01) maternal aggression.

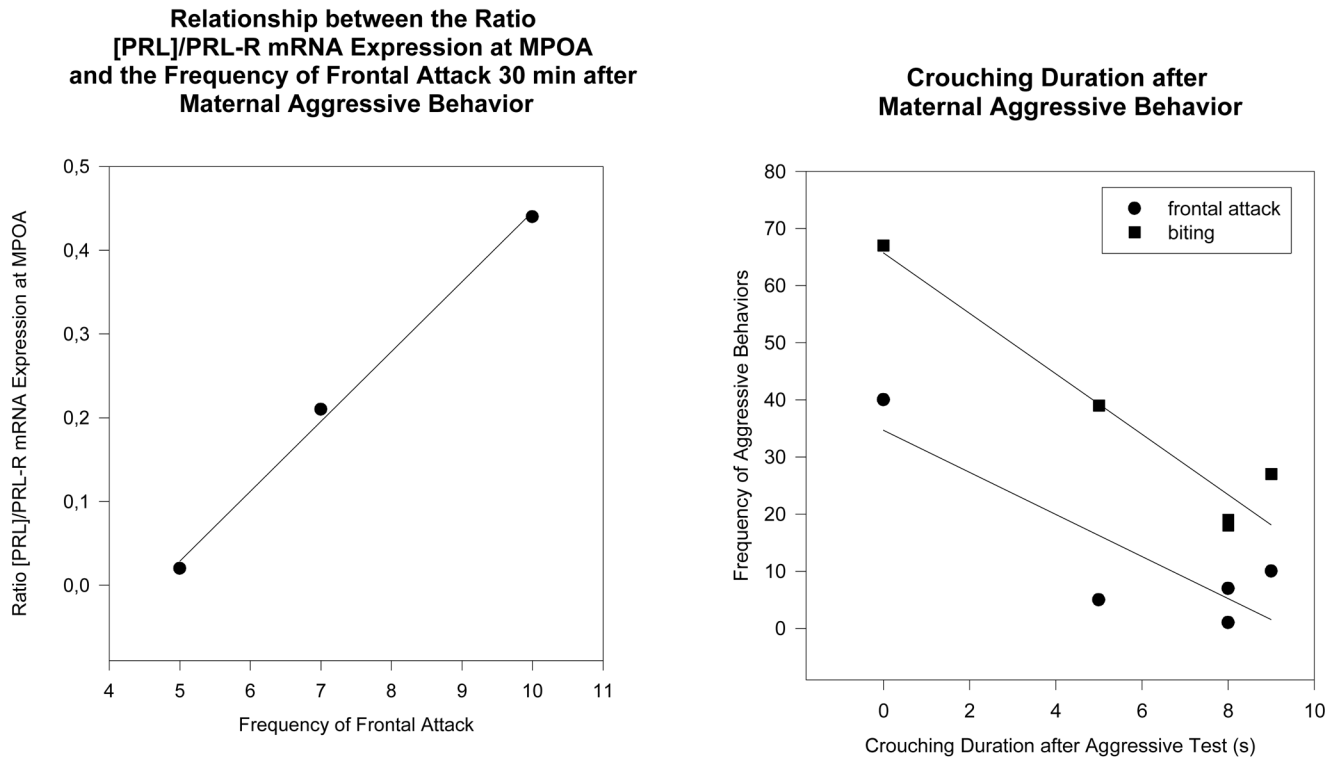

Fig 4.

(Left Panel): Correlation between the ratio [PRL]/PRL-R_L mRNA expression at MPOA of the lactating rat and the frequency of frontal attack 30 min after maternal aggressive behavior ($r = + 0.998$, $p < 0.05$).

(Right Panel): Correlation between the frequency of frontal attack and biting with crouching duration after maternal aggressive behavior ($r = -0.865$, $p<0.05$ and $r=-0.96$, $p<0.01$ respectively).

Table 1

Maternal aggressive behaviors displayed by the lactating mothers towards the intruder males during the 10-min test on PPD 8.

Maternal Aggressive Behavior	Frequency (n)
Frontal attack	6.38 ± 2.34 (16)
Lateral attack	9.06 ± 1.58 (16)
Lateral threat	17.81 ± 2.84 (16)
Biting	19.38 ± 3.99 (16)

Table 2

Maternal behavior duration (s) (mean ± SEM) displayed by the lactating mothers during the 10-min test on PPD 8, while exposed to an intruder ($I_{30'}$ and $I_{240'}$) or an object ($O_{30'}$ and $O_{240'}$). Analysis revealed lower maternal behavior duration in PPD 8 mothers during exposure to an intruder ($I_{30'}$ and $I_{240'}$) when compared to exposure to control (*$p<0.05$).

Group	Maternal Behavior Duration (n)
Control	396.9 ± 86.2 (8)
$I_{30'}$	181.4 ± 30.3 (8)*
$I_{240'}$	179.9 ± 42.3 (8)*
$O_{30'}$	289.2 ± 58.7 (8)
$O_{240'}$	335.6 ± 34.7 (8)