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Infant modulates stress responsiveness in lactating female rats

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CONTRIBUTION OF CO-AUTHORS

Dr. Walker is my thesis supervisor and was involved in all aspects of this work.

Pups presence eliminates the stress hyporesponsiveness of early lactating females to a psychological stress representing a threat to the pups.

Author participation

I did the stress testing with the help of Dr. Walker and I performed the hormone assays as well as the *in situ* hybridization for CRF. Dr. Walker helped me with data analysis, the writing and editing of the manuscript. Dr. Woodside participated in the design of experimental protocols and also helped in clarifying and conceptualizing these studies.

LIST OF ABBREVIATIONS

ACTH: adrenocorticotropin hormone
AOB: accessory olfactory bulb
B: corticosterone
BLA: basolateral nucleus of the amygdala
BNST: bed nucleus of stria terminalis
cAMP: cyclic adenosine mono-phosphate
CeA: central nucleus of the amygdala
CRF: corticotrophin releasing factor
GABA: gamma-aminobutyric acid
HPA: hypothalamic-pituitary-adrenal
MeA: medial nucleus of the amygdala
MPOA: medial preoptic area
mRNA: messenger RNA
OT: oxytocin
PPD: post partum day
PVN: paraventricular nucleus of the hypothalamus
SON: supra-optic nucleus
vBNST: ventral bed nucleus of stria terminalis
VMH: ventro-medial nucleus of the hypothalamus
VTA: ventral tegmental area

ABSTRACT

In these studies, we first compared the neuroendocrine responses between early (EL, PPD3-5), late (LL, PPD 15) lactating and virgin (V) females to a male intruder in the home cage. We next investigated whether the presence of the pups at the time of exposure to stress could modify the magnitude of the hormonal response to a male intruder in the home cage or to a predator odor (fox urine) in a novel environment. In the male intruder paradigm, levels of CRF mRNA expression in the PVN and CeA were lower in LL compared to EL or V females and plasma ACTH and B secretion was also reduced in LL compared to EL females. In EL females, the presence of the pups with their mothers at the time of stress significantly increased plasma ACTH and B responses to either male intruder or predator odor compared to EL females without their pups for 2.5hrs or 48 hrs. These studies point out to the critical role of the pups in modulating the maternal response to stressors that represent a threat for the litter.

RÉSUMÉ

Dans ces études, nous avons comparé la réponse neuroendocrinienne à l'intrusion d'un male dans la cage entre des femelles vierges (V) et des femelles à différentes périodes de la lactation (jours de lactation 3-4, et jour 15 de lactation). De plus, nous avons cherché à déterminer si la présence des bébés avec la mère au moment d'un stress psychologique pouvait modifier la réponse hormonale chez les mères. Nous avons utilisé comme stimulus l'intrusion d'un male dans la cage ou l'exposition à une odeur de prédateur dans un nouvel environnement. Suite à l'intrusion du male, les niveaux d'expression de l'ARNm dans le noyau paraventriculaire de l'hypothalamus et dans le noyau central de l'amygdale étaient moins élevés chez les femelles au jour 15 de lactation comparé aux femelles aux jours de lactation 3-4 et aux V. La réponse hormonale (ACTH et corticostérone) était également moins élevée chez les femelles aux jours 15 de lactation comparé aux deux autres groupes.

Les femelles aux jours 3-4 de lactation soumises à l'intrusion du male ou à l'odeur de prédateur en présence de leurs bébés ont démontré une nette augmentation d'ACTH et de corticostérone dans le sang par rapport aux femelles qui ont été testées 2.5 heures ou 48 heures après avoir été séparées de leurs bébés. Ces études démontrent que la présence des bébés joue un rôle important dans la modulation de la réponse à un stress représentant une menace pour l'enfant.

I. INTRODUCTION AND REVIEW OF THE LITERATURE

1. Introduction

Lactation is a unique period characterized by numerous physiological changes designed to fulfill the demands of the offspring. The peripartum period is characterized by a large energy demand due to milk production (1) and variations in energy balance are observed including increase in food intake (2), elevated basal temperature (3) and an increase in insulin and insulin receptors (4). Lactation is also characterized by a chronic hypercorticalism (5), which is particularly important since glucocorticoids increase milk production by interacting directly with the glucocorticoid receptor on the mammary gland (6) and are thus important to initiate and maintain lactation (3). In addition to these metabolic changes, lactation is associated with variations in several neuroendocrine functions including the reproductive and stress axes. The neuroendocrine variations occurring during lactation are accompanied by changes in emotional and aggressive behavior and together, these alterations are designed to ensure a proper physiological and behavioral adaptation of the mother to the different metabolic and psychological demands placed upon her during lactation.

2. The hypothalamo-pituitary-adrenal axis

The primary role of the hypothalamo-pituitary-adrenal (HPA) axis is to orchestrate the adaptive response to stress. In addition, it plays an essential role in controlling the activity of various homeostatic processes and participates in the control of certain immunologic reactions. Following exposure to a stressful stimulus, numerous neuronal structures converging to the Paraventricular nucleus (PVN) of the hypothalamus are activated. The PVN synthesizes Corticotropin Releasing Factor (CRF), a 41 amino acid peptide which is secreted at the level of the median eminence into the hypothalamo-hypophysial portal system and acts on the anterior pituitary to promote the release of adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal gland to

secrete glucocorticoids (corticosterone in the rat and cortisol in humans) in the general circulation. Glucocorticoids are able to dampen the activity of the HPA axis by an inhibitory feedback mechanism at several levels in the axis, including the pituitary, the hypothalamus (PVN) and extrahypothalamic structures such as the hippocampus (7). Peripherally, glucocorticoids maintain the efficiency of food absorption and lipid metabolism by increasing the stimulatory effects of insulin on lipid metabolism and lipolysis in adipocytes (8).

Numerous structures including the brainstem, the hippocampus, the lateral septum, the medial preoptic nucleus (MPOA), the amygdala and the Bed Nucleus of Stria Terminalis (BNST) send afferent inputs to the PVN and modulate the activity of the HPA axis (9). For the purpose of our studies, we will focus more particularly on the BNST and the amygdala because these two structures have important modulatory roles in relation to emotional processing of stressors (150).

2.1 The Paraventricular Nucleus of the hypothalamus

The stress axis is controlled primarily by neurons of the hypothalamic PVN. The PVN contains neurons that can be divided in three different categories, depending on their morphology, function and projection pathways (9, 10). The large magnocellular neurons project to the posterior pituitary where they release vasopressin and oxytocin (OT) into the general circulation (10). These neurons are activated in lactation since there is an increase in OT and vasopressin secretion due to the suckling stimulus and the fluid demand imposed by milk secretion. The two other groups of neurons are smaller cells and constitute the parvocellular component of the PVN. The parvicellular neurons containing CRF and other neuropeptides project to the external zone of the median eminence (9-11). In the rat, approximately 50% of parvocellular neurons projecting to the median eminence show a colocalization of vasopressin with CRF (12). However, during lactation, the degree of colocalization of vasopressin with CRF in these parvocellular PVN neurons, hypothalamic processes and median eminence is increased compared to virgin females (13). A second group of parvocellular neurons sends projections to the brainstem, spinal cord and to other parts of the

hypothalamus (9, 10). These parvocellular neurons show colocalization of OT with CRF (14). CRF is the primary neuropeptide regulating the HPA axis and its expression in the PVN increases following different types of physical and psychological stressors. (15).

The PVN receives several afferents from various brain regions. In particular, noradrenergic and adrenergic afferents from the ventrolateral and dorsomedial medulla oblongata constitute the major excitatory input to the parvocellular neurons of the PVN (16). In general, the majority of studies indicate a stimulatory action of brainstem catecholamine systems on the activity of the HPA axis (17). Administration of norepinephrine appears to activate PVN neurons and promote the secretion of CRF into the portal circulation through an a-adrenergic mechanism (18, 19) Indeed, injection of the a-1 receptor antagonist prazosin, attenuates stress-induced depletion of median eminence CRF (20).

2.2 The Bed Nucleus of Stria Terminalis

The BNST is a structure which relays projections from several inputs to the PVN such as the amygdala and the hippocampus (151). Projections from the BNST to the PVN are organized in a topographic fashion and different regions of the BNST send either excitatory or inhibitory projections to the PVN (21). Lesions of the anterior BNST cause a decrease in CRF mRNA in the medial parvocellular PVN whereas lesions of the posterior BNST lead to an increase in CRF mRNA expression across the whole extent of the PVN (21). This suggests that the anterior BNST sends excitatory glutamatergic fibers to the PVN while the posterior BNST is responsible for inhibitory (GABA) actions on the PVN. Moreover, the lateral divisions of the BNST are functionally related to the central nucleus of the amygdala and may relay excitatory inputs from this nucleus to the CRFergic neurons of the PVN (22). Bilateral lesions of the BNST completely block the adrenocortical response following olfactory stress stimuli (23), suggesting that the BNST has an overall excitatory effect on the activity of the HPA axis. Further implication of the BNST in the modulation of the stress response comes from retrograde tract tracing studies demonstrating that a small

group of CRFergic neurons in the BNST projects to the PVN (24). During lactation, basal CRF mRNA expression is increased only in the dorsolateral portion of the BNST and not in the ventral portion of this nucleus (25).

The BNST presents high-affinity binding sites for OT (26), the density of which is increased during lactation (27). In addition, neurons of the BNST have been shown to be stimulated by OT *in vitro* (28), suggesting the implication of this structure in the milk ejection process. Important variations take place in the oxytocinergic system of the BNST during lactation. They will be discussed in section 4.5.2.

2.3 The amygdala

The amygdala is a heterogeneous nuclear complex of the limbic system which is mainly responsible for the control of the autonomic and endocrine responses to stress (151) as well as for the integration of the various stimuli with affective properties (150). The amygdaloid complex is composed mainly of the central amygdaloid nucleus (CeA), the medial amygdaloid nucleus (MeA) and the basolateral amygdaloid nucleus (BLA), and has direct anatomic connections with other limbic regions and brain structures such as the thalamus, the hypothalamus, and with autonomic regions of the brainstem (150).

The amygdala is thought to participate in the regulation of the stress response since ablation of the amygdala inhibits adrenocortical responses to stress-inducing somatosensory and olfactory stimuli (29). Also, restraint stress causes an increase in CRF mRNA in the rostral CeA, supporting the notion that these neurons are implicated in the stress response (30).

The CeA is of particular interest for us since it plays an important role in the stress response due of the projections it sends towards the PVN and the majority of CRF-containing cell bodies are found in this subnucleus (31). Changes in the expression of CRF mRNA in the CeA have been shown to occur in lactation. They will be discussed in section 3.

Most projections from the amygdala to the PVN are indirect: the amygdala sends fibers to the BNST which in turn projects to CRF neurons in the PVN (21). To a lesser extent, the amygdala also sends some direct fibers to the CRF-containing

cells of the PVN (32, 33). The indirect projections from the amygdala to the PVN are generally stimulatory, as illustrated by studies showing the ACTH response to immobilization stress is significantly reduced following lesion of the CeA, although the ACTH baseline remains unaffected (34). The CeA sends excitatory projections to the anterior and lateral BNST (32), which in turn sends excitatory projections to the lateral parvocellular neurons of the PVN. Although the nature of these projections from the CeA to the BNST or PVN is still debated, it is likely that they include CRF and glutamatergic fibers. The projections from the amygdala to the PVN are organized in a topographic fashion (32). Anterograde tracing methods demonstrated that the CeA projects to the caudal medial and lateral parvocellular PVN where some of the CRFergic neurons are located (32).

Similar tracing methods show that the MeA sends direct excitatory projections to the anterior parvocellular PVN (32, 35). Lesion of the MeA appears to blunt the ACTH and glucocorticoid responses to acoustic and photic stimulation, also supporting the notion that the MeA, like the CeA, stimulates the HPA axis (36). Similarly to the CeA, the MeA also sends indirect projections to the PVN through the BNST. The BNST plays an integrative role in HPA stress regulation and modulation of PVN CRF neurons since it integrates various inputs from different amygdaloid nuclei as well as from the hippocampus. These studies suggest that the CeA and the MeA could affect pituitary ACTH release through direct actions upon the CRF-releasing cells in the PVN.

The BLA does not send projections to extra-amygdaloid structures but it sends afferent fibers to the CeA (151). The BLA receives afferent inputs from the thalamus (suprageniculate nucleus and medial geniculate body) and is involved in emotional conditioning (37). In addition, the BLA receives afferent projections from the olfactory system, and in particular predator odors are known to activate preferentially this nuclei compared to the CeA (38). This is important for our studies since we will use a predator odor as an emotional stressor and we will measure the expression of CRF mRNA in the CeA, which might be affected by projections from the BLA. We did not determine CRF mRNA expression in the BLA because this nucleus is not well recognized for it's implication in emotional processing, compared to the CeA.

3. The HPA axis in lactation

Lactation is characterized by a blunted neuroendocrine response to stress, which is illustrated by a reduction in stress-induced plasma ACTH, corticosterone (B) (39, 40), prolactin and OT (41, 42) secretion.

Lactation is associated with variations in basal and stress-induced activity of the HPA axis. In cycling females, the HPA axis exhibits two main characteristics: pulsatile secretory episodes and a diurnal rhythm. Studies in rats have shown that lactation is associated with a chronic hypercorticalism (5) which involves a flattening of the diurnal rhythm of secretion illustrated by elevated nadir levels of B and a decrease in peak evening levels (40, 43, 44). This blunting of diurnal rhythmicity does not appear to be due to a change in adrenal sensitivity to ACTH (43). Earlier studies have reported that suckling stimulates the adrenocortical system and thus, might account for the elevated trough of ACTH and B concentration (3, 43). Since the sensitivity to glucocorticoid feedback remains unaltered during lactation (43, 45), the reduced CRF mRNA expression observed during this period is likely to be a direct consequence of raised nadir levels of B (46).

Despite the increased basal activity of the HPA axis, basal CRF mRNA expression is reduced in the PVN of females in middle lactation (PPD 10-14) (45, 47). Moreover, Walker et al (2001) demonstrated that CRF mRNA levels in the CeA were lower in mid-lactating females (14 days after delivery) compared to virgin females. In opposition to these data, a recent study reports an increased basal CRF mRNA expression in the PVN and CeA of early lactating females (PPD 3) compared to virgin females (48). These results do not correlate with the stress hyporesponsiveness characteristic of this state.

While CRF mRNA expression is reduced during middle and late lactation, the expression of vasopressin mRNA is greatly enhanced in the parvocellular neurons of the PVN in lactating females compared to virgins (47). Vasopressin is a weak ACTH secretagogue but it potentiates the effect of CRF on pituitary corticotrophs (49) and both neuropeptides are required to elicit a full ACTH response to an acute stimulus (50). This is relevant to our project since studies

done in our laboratory have demonstrated that lactating females exhibit a reduction in pituitary sensitivity to CRF stimulation, resulting in a diminished secretion of ACTH in response to CRF stimulation (51). This diminished pituitary responsiveness to CRF appears to be linked to the suckling stimulus since removal of the pups allowed partial recovery of the ACTH response to CRF (52). In contrast to being less sensitive to CRF stimulation, the pituitary seems to be more responsive to vasopressin during lactation. This shift in pituitary sensitivity (away from CRF and towards vasopressin) might represent a counterregulatory mechanism to ensure a proper ACTH secretion in response to robust stressors during lactation even in the absence of proper CRF stimulations (51).

The blunted stress responsiveness of the HPA axis can be partly explained by a reduced activation of neuronal structures implicated in the stress response. The phosphoprotein Fos, a product of the immediate early gene c-*fos* is a well known marker of neuronal activation (152). Upon stressful stimulation, c-*fos* expression rises in several structures implicated in the stress response including the PVN and the amygdala (53). C-*fos* response to immobilization stress in the PVN, MeA and BNST of early lactating (PPD 3-4) and late lactating females (PPD 10-14) is reduced compared to virgin females (54). Furthermore, CRF-induced c-*fos* mRNA expression in the BNST is lower in lactating females (PPD 7-8) than in virgins (55). This reduction in stress-induced levels of c-*fos* mRNA in lactating females is consistent with the reduced stress-induced activation of CRF mRNA expression and HPA axis at this time (45). Other studies using urethane treatment have also shown that c-*fos* expression in the PVN was attenuated in lactating rats compared to nonlactating animals (56).

Although lactation is associated with a hyporesponsiveness to stress, there is considerable variability in the magnitude of the endocrine response. This is due to the type of stressor used and to the stage of lactation. Our lab demonstrated in earlier studies that the hormonal response to stress was greater in early lactating females (PPD 8-10) compared to late lactating females (PPD 17-19) (57).

4. General hyporesponsiveness to stress during lactation

Many stressors have been used to document the hyporesponsiveness of lactation. These include acute physical and psychological stressors such as ether stress, hypertonic saline injection, swim stress, immobilization, noise or social stress. As mentioned earlier, the stressor type is one of the important variables modulating the magnitude of the neuroendocrine response.

4.1 Psychological stressors

Lactating females on postpartum days 7-10 submitted to a noise stress display reduced ACTH and B responses compared to virgin females (5). However, the blunted hormonal response can be restored to normal after 72 hours of litter separation (5), suggesting that the suckling stimulus modulates the stress responsiveness in mothers. Lactating females also exhibit reduced ACTH response to the forced swim test compared to virgin females (57). Despite the elevated basal B levels in lactating females, these rats did not show an increase in retention of immobility compared to virgin females (57). This is surprising, knowing that retention of an acquired response in the forced swim test is dependent on the secretion of glucocorticoids (58).

4.2 Physical stressors

Similarly, lactating females display a blunted neuroendocrine response to some physical stressors. For example, lactating females show an attenuated B response and reduced prolactin and OT secretion in response to immobilization stress compared to virgin females (41, 42, 54). Lactation is also associated with reduced ACTH and B response to ether stress (43) and to lipopolysaccharide injections (59). Finally, lactating females on PPD 7 exhibit a reduced CRF mRNA and B responses to intraperitoneal hypertonic saline injection compared to cycling females (45).

4.3 Stress hyporesponsiveness in women

The hyporesponsiveness to stress which characterizes lactation is not a unique feature of rodents since a similar phenomenon is observed in nursing women. For instance, plasma ACTH and cortisol responses to 20 minutes of graded treadmill exercise were significantly attenuated in breast-feeding compared to bottle-feeding women (60). Breast-feeding women also display a reduced autonomic response to psychological stressors compared to bottle-feeding women. In response to videotapes of their child, nursing women exhibited lower increase in heart rate and also a reduced baseline galvanic skin conductance (a measure of sympathetic nervous system activity) compared to bottle-feeding women (61).

Similarly, lactating women who were administered the Trier Social Test 15 minutes after feeding their infant had a reduced ACTH and cortisol response compared to lactating women who did not feed their infant prior to the stress interview (153). This study refers to the acute effects of the suckling stimulus, and it might explain why these findings seem to contradict those of Altemus et al (2001) who report no differences in the ACTH and cortisol response to the Trier Social Test between nonlactating and lactating women tested at least 40 minutes after their last infant feeding (62).

4.4 Mechanisms underlying the blunted response of the HPA axis during lactation

Several central and peripheral mechanisms could underlie the stress hyporesponsiveness during lactation. These include changes in afferent inputs to the PVN (either excitatory or inhibitory) (63), reduced sensitivity of anterior pituitary corticotrophs to the main ACTH secretagogues (64), and enhanced inhibition of the HPA axis responses to stress by glucocorticoids, prolactin (65) and OT.

4.4.1 Noradrenergic and adrenergic afferents to the PVN

We have previously demonstrated that noradrenergic inputs from the brainstem were facilitatory on ACTH and B secretion in virgin females but not in lactating females (63). This decrease in excitatory inputs innervating the PVN could explain, at least in part, the general stress hyporesponsiveness observed during lactation (63).

A lower noradrenergic activation of PVN neurons is further supported by studies using methoxamine. Administration of this a-1 receptor agonist has a differential effect depending on the reproductive status of female rats. Methoxamine-induced CRF mRNA accumulation and B secretion are significantly reduced in lactating female rats compared to non-lactating animals (47). These results suggest a selective downregulation of a-1-mediated activation of the HPA axis during lactation (47) leading to a reduction in the sensitivity of PVN neurons to a-1 receptor activation in lactating females compared to cycling females. This diminished sensitivity to a-1 receptor activation might result from a decrease in a-1 adrenoreceptor mRNA expression observed in the PVN of middle lactating females (PPD 10-12) (66). These results support the fact that the attenuation of stress-induced activation of the HPA axis in lactation might be due to down-regulation of the afferent pathways (47).

4.4.2 Sensitivity of Anterior pituitary corticotrophs

In addition to being linked to variations in noradrenergic inputs, the stress-hyporesponsiveness has been linked to alterations at the level of corticotrophs in the anterior pituitary (64). ACTH and B secretions following the elevated plus-maze combined to forced swim stressors were significantly reduced in pregnant and lactating females (PPD 9-12) compared to virgin females. As mentioned earlier, the attenuated response of the HPA axis is likely to be partly due to a reduced pituitary sensitivity to CRF stimulations as demonstrated *in vivo* by a reduced CRF-induced ACTH secretion from the anterior pituitary in the blood and *in vitro* by lower stimulation of cAMP production in corticotrophs by CRF (64). Moreover, receptor autoradiography revealed a significant decrease in CRF

receptor binding density in the anterior pituitary following these same stressors in late pregnant and lactating females compared to cycling females (64).

4.4.3 Presence of pups

It is well known that the suckling stimulus determines basal levels of ACTH and B in lactating rats (43) and also modulates the neuroendocrine response to stress (5, 43, 45, 67), the pituitary sensitivity to CRF stimulation (51) and CRF mRNA expression (45). All these changes are restored after various durations of pup removal. For instance, full normalization of morning basal levels of ACTH and B occurs 24 hours after litter removal (43). The accumulation of CRF mRNA in the PVN following intraperitoneal saline injection in early lactating females (PPD 7) becomes only fully restored 48-72 hours after separation from the litter (45). Finally, lactating females submitted to a noise stress in the presence of their pups do not show a significant increase in plasma ACTH and B concentrations. However, administration of the same stressor 72 hours after lactating females were weaned from their litter produces a significantly greater B response compared to females previously tested in the presence of their pups (5).

Despite exhibiting a reduced neuroendocrine response to stress, lactating females still displayed a behavioral response to the noise stress that included increased exploratory behaviors, rearing and grooming and increased pup directed activities (5). Thus, a full behavioral response was seen and it was clearly dissociated from the neuroendocrine response.

Dissociation between behavioral and neuroendocrine response is further supported by a study in which lactating and virgin females were submitted to the Porsolt forced swim stress. Even though lactating females displayed reduced ACTH secretion in response to the stressor compared to virgin females, they did not exhibit increased immobility scores compared to the cycling females (57). Increased retention of immobility was expected in hypercortisolemic lactating females since glucocorticoid secretion has been shown to facilitate the retention of immobility in the forced swim test (58). The lack of increased immobility

scores in lactating females despite their ACTH response to the stressor suggest a dissociation between the hormonal and behavioral responses to stress.

Together, these studies show that the reduced neuroendocrine response of the mother is mainly a consequence of changes induced by the suckling stimulus provided by the pups. Another psychological component of the pups' presence could affect the neuronal response to stress. Indeed, it is important to determine if, by their presence only, pups can modulate the neuroendocrine response of the mother at the time of stress.

Few studies measuring the HPA axis activity in lactating females have taken into account the way the mother perceives events in her close environment. In an earlier study, it was shown that the state of the pups has an impact on the stress response of the mother (68). Mothers reunited with their previously shocked pups showed a greater elevation of plasma B compared to females reunited with pups which were only handled (68). Therefore, it appears that the magnitude of the adrenal stress response of mothers can be modulated not only by the presence of pups, but also by their state. Shocked pups are likely to produce more ultrasonic vocalizations which would increase the arousal level in mother rats.

This earlier study provides a key element for our experiments, because it demonstrates that the response of the mother can be selective to stimuli produced by her pups. Similarly, we want to determine that the response of the mother to psychological stressors representing a threat to the infant is contingent upon the presence of the pups. More precisely, we would expect that lactating females who are submitted to a pup-relevant psychological stressor would show a greater neuroendocrine response, only if they are in the presence of their litter. We hypothesize that by their presence, pups can enhance the emotional salience of a psychological and emotional stimulus and therefore modulate the neuroendocrine response of the mother to the stressor.

4.5 Other major neuroendocrine changes in lactation

Lactation has to be considered as a dynamic state because of the constant variations in hormonal levels and differences in the magnitude of the stress

response across the various stages of lactation. Several hormones, including primarily the ovarian steroid hormones (estrogen and progesterone) and prolactin, change drastically during pregnancy and in the peripartum period. In addition, the brain oxytocin (OT) system is activated during lactation, as indicated by an increased OT and OT receptor synthesis in the hypothalamic magnocellular neurons and an enhanced sensitivity of OT neurons to stimulations (69). In the rat, progesterone levels are high during pregnancy but decline rapidly from day 19 of gestation onwards. Progesterone levels remain low during the postpartum period in human (154) while in the rat, there is a steady rise of progesterone until about day 12 of lactation, after which progesterone levels begin to decrease (70). Estrogen, OT and prolactin are low during pregnancy and peak at parturition. The onset of maternal behavior at parturition is primarily caused by OT, and less importantly by estrogen and prolactin (71, 72).

4.5.1 Prolactin

Lactating females exhibit hyperprolactinemia, triggered by the suckling stimulation (73). It is well documented that increase in prolactin production in lactation is due to a decrease in dopaminergic inhibitory tone on pituitary cells and to an increased sensitivity to thyrotropin-releasing hormone and to other stimulators of prolactin (155). Besides its well known peripheral effects on the mammary gland to regulate milk production and composition, prolactin also plays a significant role in the initiation and regulation of maternal behavior (74, 75). The behavioral effects of prolactin are thought to be mediated via central and peripheral prolactin release and also through the actions of placental lactogens (76). Pituitary prolactin gains access to the brain by crossing the blood brain barrier through a receptor mediated process (76). In addition to pituitary production, prolactin is also produced independently in the brain. Prolactin mRNA expression has been found in the hypothalamus, the thalamus, the brainstem and in the amygdala (77). Prolactin immunoreactivity has also been found in areas involved in maternal behavior, such as the medial preoptic area (MPOA) and the amygdala (78). Administration of prolactin in the MPOA triggers the onset of

maternal behavior in rodents (75) and prolactin-receptor knock-out mice show an impaired or absent maternal behavior, supporting a role for prolactin in maternal behavior (79). Despite these evidences, the importance of central prolactin is still debated.

Recently, Torner et al (2002) demonstrated that activation of the brain prolactin system during lactation modulates anxiety-related behavior and neuroendocrine responses to stress (65). Indeed, intracerebroventricular injection of antisense oligonucleotides against prolactin increased the anxiety-related behavior on the elevated plus maze, impaired maternal behavior and caused an increase in stress-induced ACTH release, pointing to an inhibitory role of prolactin on the HPA axis response to stress during lactation (65). In the rat, prolactin levels decrease as lactation proceeds, but suckling stimulates prolactin release from the pituitary which promotes continued milk production (80). In women, blood levels of prolactin are about twenty times greater than normal at parturition, but the level of the hormone remains elevated for only about one week in the absence of suckling. In breast-feeding women, blood concentrations of prolactin remain high for the duration of lactation (156)

4.5.2 Oxytocin

OT is a nine amino acid hormone synthesized mainly in the magnocellular neurons of the supraoptic nucleus (SON) and PVN. These neurons send their axons to the posterior pituitary where OT is released in the peripheral circulation following appropriate stimuli (81). In the lactating rat, OT mRNA expression in the SON is increased several folds (69) and OT pituitary levels reach maximal levels just before parturition (157). This increase correlates with a rise in OT receptors in the myometrium and mammary gland (81).

OT is primarily involved in female reproductive functions such as uterine contraction during labor and milk ejection during lactation (158). OT also enhances social contact, as demonstrated in studies in which social interactions

with an opposite-sexed partner is enhanced in male rats receiving centrally administered OT (82).

In addition, there is a striking reorganization of the oxytocinergic synapses in the SON occurring in the peripartum period. Whereas in virgin females most magnocellular neurons are separated one from the other by glial cells and neuropile elements, in lactating females the membranes of cell bodies and dendrites are in direct apposition, with retraction of glial interposition (83). Furthermore, many of these neurons are contacted by the same presynaptic terminal (double synapses) (84). This restructuring of oxytocinergic neurons during lactation, in addition to the increased sensitivity of OT neurons to autostimulation by the released OT (85), allow a synchronization of the firing of OT neurons which ensures proper secretion of OT upon the suckling stimulus and results in milk ejection (86). The maintenance of these anatomical rearrangements in the SON in lactating females is closely linked to the suckling stimulus (87). Neuronal remodelling in lactation also occurs in other hypothalamic areas including the preoptic and mediobasal hypothalamic areas, the arcuate nucleus and in the ventromedial hypothalamic nucleus (83). Changes observed include variations in the density, structure, organization and size of synapses and in the glial coverage of neurons (83).

4.5.3 Estrogen and progesterone

Within 24 hours following parturition, levels of estrogen and progesterone in the blood are typical of the estrus stage, with the usual rise and fall of estrogen followed by a peak and decline in progesterone secretion (159). Upon parturition, estrogen and progesterone trigger the onset of maternal behavior which is notably characterized by an increased alertness and attraction to pup-related stimuli (88, 89).

During the first two weeks of lactation, females exhibit low diestrous concentrations of estrogen in the plasma (around 45 pg/ml). Plasma levels of

progesterone rise steadily until day 10-12 of lactation, after which point progesterone levels start to decrease (70)

5. Emotional behaviors during lactation

Lactating females react differently to emotionally challenging and threatening situations and exhibit different levels of anxiety compared to cycling females. Behavioral studies have shown that lactating females exhibit reduced fearfulness to the novel environment of an open field as shown by increased exploratory behavior and a reduced latency to enter the open field, compared to virgin females (52, 90). Moreover, the ACTH and B response to a novel cage stressor are lower in lactating females compared their cycling counterparts (91), suggesting that lactating females are less anxious than cycling rats in a novel environment. However, in another test of anxiety, the Elevated Plus-Maze, lactating females showed a decrease in the time spent in the open arms compared to the closed portions of the maze in comparison to virgin females (92).

Other studies also indicate that the mother rat is less fearful than a virgin female rat (67). As an index of fear, the duration of freezing behavior in response to a sudden auditory stimulus was compared between lactating and virgin females. Lactating females froze markedly less than cycling females and interestingly, the reduction in freezing was observed only when lactating females were interacting with their pups (67). This experiment is interesting for our paradigm because it shows that the presence of pups is able to modulate the behavioral response of the mother to a psychological stimulus.

When studying the emotional response to a psychological stressor, the amygdala is a critical structure as outlined above. More specifically, the CeA plays a critical role in the expression of fear because of its direct projections to areas involved in the autonomic expression of fear such as the lateral hypothalamus and dorsal motor nucleus of the vagus (93, 94). The CeA sends

many projections to the central gray and both of these structures are thought to play a role in fear-related freezing (94).

The CRFergic neurons in the CeA are activated during stressful situations such as social defeat situations or in the elevated plus-maze. CRF in the CeA appears to play an important role for mediation of anxiety. Administration of a CRF antagonist in the CeA considerably reduces the heightened emotionality experienced by an intruder exposed to an aggressive resident. Similarly, injection of a CRF antagonist in a male rat significantly decreases the anxiety associated with the elevated plus-maze, as indicated by an increased time spent in the open arms (95).

The amygdala plays an important role in the acoustic startle response (ASR) and in the fear-potentiated startle (96), a model of conditioned fear. The ASR is a short-latency reflex that occurs in response to a sudden loud acoustic stimulus (97). Lactating females in early, middle or late lactation show a decrease in the ASR compared to cycling females (52). However, the behavioral hyporesponsiveness to the auditory stimulus appears to be reversible upon fear-conditioning (52), suggesting that activation of neural pathways underlying the fear responses can rapidly restore the behavioral response of lactating females. In view of these results, we hypothesize that lactating rats are more responsive to environmental stimuli with a fear component representing a danger for their pups. This should ultimately allow them to protect adequately their litter.

6. Maternal Behavior

After parturition, the mother rat develops a whole range of behaviors directed towards the pups, such as retrieval of the litter in the nest, grooming and nursing (allowing the pups to suckle). These maternal activities could increase the arousal level of the lactating female rat, and might account for the increased endocrine response of a lactating female placed in a novel environment with her pups. The hormonal events of late pregnancy, especially the rise in prolactin, OT and estrogen levels and the rapid drop in progesterone levels, are required for

the onset of maternal behavior at parturition (160). OT plays an important role in the generation of maternal behavior, as demonstrated in a study in which 42% of estrogen-primed virgin female rats developed maternal behaviors following intracerebroventricular (ICV) administration of OT (98). Estrogen appears to be obligatory for the onset of maternal behavior (160) and is thought to enhance maternal behavior partly through its effects on central oxytocinergic systems (99). Furthermore, OT administration facilitates the approach towards pups in virgin female rats, who are otherwise neophobic and either avoid or attack pups (160). These hormones act on brain mechanisms to decrease the fear or aversion of infant stimuli or to increase the approach towards the infant (100).

Also, nonpregnant rats that have been exposed to hormonal fluctuations mimicking late pregnancy show a strong attraction to olfactory stimuli associated with pups (88), supporting the important role of hormones in the induction of maternal behavior. Maternal behavior can be induced in nulliparous females by exposing these females to pups for a few days (160) and the latency for the emergence of maternal behavior can be reduced by making these females anosmic (161). These findings suggest that nulliparous females find the olfactory stimuli emitted by pups aversive and that maternal behavior occurs after habituation, or when hormones have acted on certain brain regions to decrease the aversion to such stimuli.

6.1 Brain regions involved in maternal behavior

Several brain regions are implicated in maternal behavior in the rat, but the most important ones are the MPOA, the ventral BNST (vBNST) and the MeA (160). It is proposed that these regions act to promote the approach towards pup-related stimuli and to trigger the motor pathways associated with maternal behavior (101).

Destruction of the MPOA and the vBNST with excitotoxic lesions prevents maternal behavior (102, 103).On the other hand, there is evidence that prolactin and estrogen act on these brain regions to stimulate maternal behavior (75). The role of the MPOA and vBNST in maternal behavior is further supported by a

study in which Fos immunoreactivity in these regions was greater in lactating females which interacted freely with their pups, compared to lactating females that were exposed to their pups enclosed in a mesh bag, where females could see, hear and smell them, but not physically interact with them (104). Furthermore, it has been shown that the expression of Fos increases in the MPOA and vBNST of maternal females, even if they had their olfactory bulbs and nipples removed, suggesting that the induction of Fos expression is not only the result of olfactory and suckling stimulation, but is closely related to the performance of maternal behavior (104).

Interestingly, the MPOA and vBNST can remain activated or "sensitized" to stimulations provided by pups for long periods of time following litter removal. Lactating females separated from their pups on postpartum day 5 and exposed to either their pups or to a candy (control) 3 days later displayed greater Fos expression in the MPOA and the vBNST compared to the control females (105).

In summary, full interaction with the litter and performance of maternal activities such as grooming and nursing, along with stimulations emitted by pups are important stimulators of Fos expression in the MPOA and vBNST.

Anterograde tract tracing studies revealed that MPOA neurons project most importantly to the lateral septum, to the ventromedial nucleus of the hypothalamus (VMH), to the periaqueductal gray and to the ventral tegmental area (VTA). On the other hand, neurons of the vBNST project mainly to the VTA, to the retrorubral field and to the VMH (100). Several of these target areas are also involved in the facilitation of maternal behavior. For example, the lateral septum is thought to dampen aggressive behaviors and to suppress aversive reactions towards pup-related stimuli (106), whereas stimulation of the VMH induces aggressive behavior in several species (107). Numan and Sheehan (1997) hypothesize that in lactating females, MPOA projections serve to activate the lateral septum and to dampen the activity of the VMH in order to decrease the neophobia towards the pups (100). Alternatively, projections from the vBNST to the retrorubral field and the VTA are thought to stimulate limbic and prefrontal areas involved in reward (100).

The fact that nulliparous females tend to avoid pup-related stimuli and that this aversion is reversed upon parturition led to the idea that a neural system might inhibit maternal behavior in virgin females (162,163). It was proposed that exposure of cycling females to pups activates brain regions that are normally activated by threatening or stressful stimuli, therefore causing an avoidance to pups and an inhibition of maternal behavior (162,100). It is suggested that the MeA might inhibit maternal behavior in cycling females since lesions of this structure promote maternal behavior and also decrease the pup active avoidance in virgin females (89, 108). The maintenance of this inhibition in virgin females is believed to be due to chemosensory cues that reach the MeA through inputs from the olfactory bulb (volatile odors) and the vomeronasal organ (non-volatile odors) (161).

Since lactation is associated with a blunted HPA axis response to stress, it is conceivable that the neuroendocrine response to threatening stimuli is also involved in decreasing the aversion to pups through similar pathways or mechanisms.

6.2 The olfactory system

Olfactory stimuli provided by pups are critical for the generation of maternal behavior since disruption of the olfactory system induces the expression of maternal behavior in virgin females within one day of pup exposure (109).

The olfactory system in the rat is closely linked to specific hypothalamic and limbic areas. Anatomical studies have demonstrated that inputs from the olfactory bulb and the vomeronasal organ are capable of reaching directly the MPOA, the MeA and the BNST (110, 111). In turn, the MeA projects strongly to the BNST (112) and to the MPOA, which is known for its role in maternal behavior (104). Target neurons of the accessory olfactory bulb (AOB) express gonadal steroid receptors and consequently might be modulated directly by circulating hormones (164).

In the context of inhibition of maternal responding in virgin females, it has been suggested that the MeA is part of a neural pathway through which olfactory inputs

from pups induce aversion (89, 108). This is supported by olfactory bulbectomy studies which have resulted in a reduction in Fos immunoreactivity in the MeA and in the BNST during maternal behavior (104).

Sheehan et al (2000) propose a circuitry by which olfactory inputs from the pups inhibit maternal behavior in virgin female rats and how hormones counteract this effect to promote maternal behavior. On one side, the olfactory bulb projects to the MeA which in turn sends inhibitory projections to the MPOA/vBNST, thereby inhibiting maternal behavior. On the other side, olfactory inputs from pups reaching the MPOA/vBNST would promote maternal behavior. These two circuits have opposing functions; maternal behavior is promoted by olfactory inputs to the MPOA/vBNST and is inhibited by olfactory inputs reaching the MeA.

During lactation, estrogen stimulates maternal behavior by interfering with these circuits. Estrogen receptors in the MeA, BNST and MPOA become upregulated prior to parturition (88). It is hypothesized that estrogen suppresses the effects of olfactory inputs to the MeA (therefore preventing the amygdala from inhibiting the MPOA and BSNT) and enhances the activity of the MPOA/BNST in response to olfactory stimuli related to pups. Therefore, estrogen has an important role to play in the disinhibition of maternal behavior (113).

The MeA is also known to be sensitive to other olfactory stimulations than those related to pups. For example, predator odors such as cat odor or fox urine strongly activate the MeA (114). This is relevant to our studies since we will measure the responsiveness of lactating female rats to a predator odor.

6.3 Maternal aggression

Although lactating females display reduced aversive reactions towards their pups, they demonstrate a high level of aggression towards con-specific intruders (115). The aggressive behavior exhibited by females against an intruder is directly related to the protection and defence of their litter and is part of the complex activities of maternal behavior (165). Lactating females display high levels of aggressive behaviors toward intruders during the first 10 days of lactation, after which, levels of aggression decline considerably (166). In a recent study by

Neumann et al (2001), the level of aggressive behavior displayed by lactating residents on PPD 6-7 was positively correlated with their ACTH stress response to an intruder and also to the reproductive stage of the intruder (91).

Maternal aggression cannot be induced in nulliparous females, suggesting that the onset of aggressive behavior depends on pregnancy hormones and parturition (115).

Volatile olfactory stimuli are important in aggression since bilateral olfactory bulbectomy reduced the expression of aggression in lactating rats, but removal of the vomeronasal organs did not alter such behavior (116). There is a dual olfactory system in rats: the main olfactory system processes volatile odors while the accessory olfactory system, which comprises the vomeronasal organs, detects pheromones of low volatility (164). Similarly, mother rats with lesioned olfactory epithelium displayed less aggressive behavior towards an intruder, further suggesting that the sense of smell is important for maternal aggression in rodents (117).

When mothers can smell their pups without engaging in contact (pups enclosed in a nylon mesh bag), they remained aggressive towards an intruder whereas mothers with pups placed in a glass container did not. This suggests that olfactory cues from the offspring are important in maintaining the aggressive behavior in lactating rats (118). The maintenance of aggressive behavior is dependent on the presence of pups since upon removal of the litter mother rats do not display aggressive behaviors towards intruders (118, 119).

Among the numerous structures implicated in maternal aggression, the PVN appears to be an important site. Electrolytic lesions of the PVN of lactating females with pups significantly decreased the frequency and duration of attacks against a male intruder compared to control sham-lesioned animals (120). It is believed that the consequence of these lesions on magnocellular neurons from the PVN is to decrease the secretion of OT in the plasma, which may cause the diminished aggressiveness observed in lactating females (120). In hamsters, administration of OT in the amygdala causes an increase in maternal aggression, suggesting that this peptide is involved in the modulation of aggressive behavior (121). In addition, it has been shown that the decline in anxiety during lactation

might facilitate the increase in aggression and nest defence exhibited by mother rats (119, 122). It is possible therefore that the reduced HPA axis responsiveness to stress during lactation paired with an increased aggression would allow a better defence of the nest against an intruder.

7. Conclusion

The blunted neuroendocrine responsiveness to various stressors promotes maternal behavior in lactating females and allows them to take care adequately of their pups. In addition, the suppression of the HPA axis response to stress may also have various adaptive functions, such as conservation of energy for milk synthesis and diminished psychological arousal or anxiety associated with the demands of pup care.

The full mechanisms underlying the stress hyporesponsiveness of lactation have not been completely elucidated. We intended to study whether hyporesponsiveness to stress is specific to particular stressful situations. Since lactating females display a reduced emotionality in stressful situations and react differently depending on the state of their pups, we postulate that a component of the hyporesponse to stress observed in lactation might be dependent on the emotional content of the stressor. Therefore, the relevance of the stressor to the infant would determine the magnitude of the neuroendocrine stress response of lactating rats. We focused our attention on the amygdala because of its implication in emotional processing (150), in the processing of olfactory stimuli and in the modulation of the stress response. Increased emotional salience of a stressor could activate neural systems that are stimulatory to the HPA axis and supersede the central systems normally downregulated during lactation.

II. RATIONALE AND OBJECTIVES OF THE RESEARCH

It is well established that lactating female rats exhibit modifications in the function of their HPA axis and in particular a dampened response to various physical, metabolic and psychological stressors compared to virgin females (5, 43, 45, 54, 57). Central adaptive mechanisms are thought to underlie this stress hyporesponsiveness but the importance of external psychological factors in modulating the magnitude of the stress response has not been thoroughly investigated. For instance, both the presence and the state of pups might critically modulate the reactivity of the HPA axis to stress in nursing females (68, 123). The studies described in this thesis were designed to explore 1) whether the hyporesponsiveness to stress during lactation it could be eliminated when the mother is exposed to specific stressful situations that are threatening for her litter and 2) whether specific changes in the HPA activity could be associated with differential neuronal activation of specific brain nuclei involved in the processing of fear and emotions. The central aim of our experiments was:

To determine if the hyporesponse to stress observed during lactation is dependent upon the relevance of the stressor to the infant.

In addition to exhibiting reduced neuroendocrine responsiveness, lactating females also show altered emotional behaviors such as reduced fear (67). In previous experiments done in our laboratory, we demonstrated that lactating females were less emotional in the open field and displayed reduced acoustic startle response compared to virgin females (52). However, this reduced acoustic startle response can be rapidly reversed upon fear potentiation, suggesting that neural pathways normally activated during fear responses critically modulate the behavior of lactating females (52). Previous reports have demonstrated that the presence of pups is essential for the enhanced freezing response to a loud noise (67) and for maternal aggression (119). Associated with changes in behavior is the reduced neuroendocrine response to stressors observed in lactating females.

These results suggest that the hyporesponsiveness of the HPA axis seen during lactation is reversible upon weaning.

The question that arises is whether or not the emotional salience of the stressor in terms of relevance to the infant is critical to differentially activate neuronal structures implicated in the stress response such as the amygdala and the PVN. In these studies, we compared virgin and lactating female rats in their response to two emotionally relevant stressors such as intrusion of a male in the home cage and exposure to a predator odor. Lactating females were tested at different stages of lactation and in the presence or absence of their pups. We wanted to verify if the neuroendocrine response to stress varied according to the stage of lactation and whether or not the presence of pups modulated the neuroendocrine response to psychological and threatening stresses during lactation. We determined plasma ACTH and corticosterone levels at different time points following exposure to the stressor and measured the level of CRF mRNA expression in the PVN and central nucleus of the amygdala.

III. ARTICLE

Pups presence eliminates the stress hyporesponsiveness of early lactating females to a psychological stress representing a threat to the pups.

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Abstract

Blunted neuroendocrine responses to stress are reported in lactating females after exposure to various stressors. However, many of the stimuli used in these studies have little ethological relevance for maternal protection of the litter in a threatening environment. The question that arises then is whether the relevance of the stressor to the infant is critical in the "gating" of the neuroendocrine response. We hypothesize that the presence of pups with their mothers at the time of exposure to an intruder or a predator odor is an effective way to increase the emotional salience of the psychological stressor, thus eliminating the stress hyporesponsiveness in lactating females. We first compared neuroendocrine responses (CRF mRNA in the PVN and central amygdala, CeA, plasma ACTH and corticosterone) between early (EL, PPD3-5), late (LL, PPD 15) lactating and virgin (V) females to a male intruder in the home cage. We next investigated the effect of pups' presence at the time of stressor exposure on the magnitude of the hormonal response to a male intruder in the home cage or to a predator odor (fox urine) in a novel environment. In the male intruder paradigm, levels of CRF mRNA expression in the PVN and CeA were lower in LL compared to EL or V females and plasma ACTH and corticosterone secretion was not as elevated in LL compared to EL females. Aggression towards the intruder was high in EL females in the presence of their pups and a positive correlation was found with the integrated ACTH response. Aggression rapidly declined after pup separation (2.5 or 48 hrs) or in LL nursing females. In EL females, the presence of the pups with their mothers (EL+pups) at the time of stress significantly increased plasma ACTH and corticosterone responses to either male intruder or predator odor compared to EL females without their pups for 2.5hrs or 48 hrs (EL-pups). Plasma ACTH response to fox urine in EL+pups females was comparable to that of virgin females, suggesting that increasing the salience of emotionally-relevant stimuli by keeping the pups present in the cage could eliminate the hyporesponsiveness detected for EL females without their pups. These studies point out to the critical role of the pups in modulating the maternal response to stressors that represent a threat for the litter. We

hypothesize that the amygdala, because of its ability to process olfactory stimuli and stimuli with affective properties, might play an essential role in "gating" the neuroendocrine response to stress during lactation.

Introduction

Lactating females exhibit reduced hormonal responses to a variety of physical and metabolic stressors including hypertonic saline injection, swim stress, ether stress, LPS injections, (1-4) as well as psychological stressors including noise, immobilization or social stress (5-8). The hyporesponsive state is thought to result from adaptive mechanisms occurring at various sites and spanning the late pregnancy (9) and lactation periods. For instance, reduced activity of afferent pathways to the paraventricular nucleus of the hypothalamus (PVN) (10-12) and differential modulation of receptor-mediated activation of PVN neurons (13,14) have been demonstrated. Modifications in the neuronal phenotype of PVN neurons (15) and/or pituitary sensitivity to ACTH secretagogues (14,16) might also participate in blunted stress responses during lactation.

Altered emotional behaviors are also observed during this period as lactating females exhibit reduced fear and neophobia (6,17,18) reduced acoustic startle responses (19) and they appear to be less emotional in the openfield (8,19). Interestingly, however, the diminished acoustic startle response could be quickly restored to normal by fear potentiation, suggesting that the behavioral hyporesponsivity to stress of lactating females might be situation specific. Although we did not measure the neuroendocrine responses to stress in the fear conditioning paradigm previous studies showed that the hormonal response to a stressor in lactating rats can also be modulated by situational variables such as the state of the pups (20). Mothers reunited with their previously shocked pups showed a greater activation of the HPA axis and consequently a greater elevation of plasma corticosterone compared to females reunited with pups that were only handled (20). Therefore, it appears that the magnitude of the maternal stress

response can be modulated not only by the presence of her pups, but also by their state.

The question arises then whether the relevance of the stressor to the infant is also critical in the "gating" of the neuroendocrine response. Threat to the infant could provide a sufficient fear component to eliminate stress hyporesponsiveness and induce potent neuroendocrine and behavioral responses in the lactating mother. We hypothesize that the presence of the pups with their mothers at the time of exposure to an intruder or a predator is an effective way to increase the emotional salience of the psychological stressor. These studies were designed to test this hypothesis using the male intruder paradigm and exposure to fox urine as psychological stressors.

It is well known that lactation is a dynamic period and that the stage of lactation influences the behavioral and neuroendocrine stress response. Mothers at earlier stages of lactation (during the first week) are behaviorally more responsive to stimuli emitted by pups (21), display greater hormonal response to stress compared to later in lactation (3) and do not yet display reduction in CRF mRNA expression in the hypothalamic PVN and amygdala (12). Thus in the present study, we focused our attention on the early lactational period (PPD3-5) and measured the hormonal response and expression of CRF mRNA in the hypothalamic PVN and amygdala following male intrusion or predator odor. Our results demonstrate that the presence of the pups at the time of exposure to predator odor eliminates the blunted hormonal responses in early lactating females.

Materials and methods

Animals

Sprague-Dawley virgin or pregnant females on day 17-18 of gestation (Charles River, St Constant, Quebec, Canada) were received in our animal

facility and housed individually in plastic cages under controlled conditions of light (12h light: 12h dark, lights on at 08.00h), temperature (24-26°C) and humidity (70-80%). Rats were fed a normal rat chow diet and water *ad libitum*. The day of birth was designated as day 0 of lactation, and on day 2, litters were culled to 10 pups per mother. Mothers were singly housed with their litters throughout the experimental procedure. Lactating females were tested on postpartum day (PPD) 3-5 (early lactating, EL), and 15 (late lactating, LL). All procedures were approved by the Committee on Animal Care at McGill University and followed ethical guidelines edited by the CCAC.

Blood sampling and brain collection

Two days before testing, jugular catheters (Silastic, Dow Corning Corp., Midland, MI, USA) were implanted in virgin (248 ± 5.8 g) or lactating (298 ± 4.3 g) females under ketamine (8.5 mg/100g BW, ip)-xylazine (1.5 mg/100g BW, ip) anesthesia. On the day of testing, the jugular catheter was extended using 50-60 cm of PE50 tubing (Clay Adams, Parsippany, NJ, USA), and rats were left undisturbed for 120 minutes in their home cage before the onset of blood sampling. All testing occurred between 10 AM and 2:30 PM. When females were tested only once, testing occurred between 10-11AM. For some of the experiments (2.5hr separation paradigm), females were tested twice within a day, with or without their pups with a 2.5 hr interval between sampling episodes and in a counterbalanced order design. The first and second sampling episodes occurred between 10-11AM and 1:30-2:30PM respectively. For the male intruder paradigm, not all the animals could be bled repeatedly for the 2 sampling episodes, and thus we did not reach a pure within-subject design. Therefore, we were not able to use statistical analyses to compare the AM vs PM response in our experimental groups. However, a dissociation of AM and PM responses is shown in Figures 3 and 6. For females sampled a second time after 48hrs of pup separation, all blood samples were collected in the AM between 10-11AM. For the exposure to fox odor, we were able to use a within-subject design with a

counterbalanced order design. Blood samples (350 µl) were taken before stress and at various times after the onset of stress (i.e. 5, 10, 15, 30, 60 min) and blood volume was replaced after each sample with 300 µl of a 0.9% sterile saline solution. Blood samples were kept on ice, centrifuged at 12 000 r.p.m. for 2 min. and plasma was stored at -20 °C prior to hormone assays. At the end of the sampling protocol, animals were killed by decapitation and trunk blood was collected in tubes containing 150 µl EDTA (60 mg/ml). Brains were rapidly removed, frozen in isopentane and kept at -80 °C for *in situ* hybridization. When EL females were tested in the absence of their pups, the entire litter was removed and either sacrificed (48hrs separation group) or placed on a warming pad until being returned to their mother after testing.

Stress testing

Resident-intruder paradigm: Litters from nursing females were either kept with their mothers or removed for 48 hrs or 2.5 hrs prior to testing. All females (body weight virgins: 248±5.8 g, lactating: 298±4.3 g) were kept in their home cage (with or without their litters) and a male rat (300-400g) was introduced in the cage for a total duration of 5 min. The latency to display the first aggression was recorded for each female and blood samples (350 µl) were taken at time 0, just prior to the male intrusion, and at 5, 15, 30 and 60 min. following the onset of male intrusion. None of the pups in the litters were seriously injured by the male during the 5 min intrusion period. For the EL group, some females in the 2.5hr separation condition were sampled twice per day (once with or once without their litters in counterbalanced order) and an interval of 2.5 hrs was left between the two sampling periods. In this case, females were exposed to a different male in each of the sampling periods.

Predator (fox urine) odor: All females were kept in their opaque home cage for the basal sampling (t=-2 min). Females were then transferred to a larger clear cage with or without their litters and a blood sample was taken immediately after the transfer to the novel cage (t=0 min). A plastic cup containing a piece of cotton

absorbed with 0.8 ml of concentrated fox urine (Buck Expert, Quebec) or control 0.9% saline was placed in the cage for 10 min. Testing with fox urine and control odor was never done at the same time or in the same room to ensure that results from control animals were not biased by the fox odor. After 10 min., the cup was removed from the cage and taken outside the experimental room. Blood samples (350 µl) were taken at time –2 min in the home cage, just prior to exposure to the odor in the new cage (t=0), and at 10, 30 and 60 minutes following exposure to the predator odor. To determine the specific response to fox odor, virgin females were exposed to both experimental conditions (fox or control odor) but lactating females were only tested with fox odor.

Hormone assays

Plasma ACTH and corticosterone (B) concentrations were measured by specific radioimmunoassays as previously described (11). For the ACTH assay, Plasma was incubated with a specific anti-ACTH antiserum at a final dilution of 1:15,000. The ^{125}I-ACTH tracer (1 µCi/vial), Incstar, Stillwater, IN, USA) was added the next day and after 24h incubation at 4°C, the bound fraction was separated using goat antirabbit IgG (Antibodies INC, Davis, CA, USA). Typical total binding ranged between 32-36% and non-specific binding averaged 1.48%. The limit of detection was 15.6 pg/ml and the intra and interassay variability was 8% and 26%, respectively. Plasma corticosterone concentrations were assayed by RIA using a kit from ICN (Medicorp, Montreal, Canada) and ^{125}I-corticosterone as the tracer. The limit of detection was 0.2 µg/dl, and the intra and interassay variability was 3% and 12%, respectively. All samples were run in duplicates.

In situ hybridization for CRF

Brain sections (20 µm) were collected onto slides coated with poly-L-lysine and stored at -80°C until hybridization. The CRF probe was a 45-base pair oligomer directed against bases 523 to 667 of the second exon of the CRF gene (Sheldon Biotechnology Center, Montreal). The probe was 3' -end labeled with

^{35}S and terminal deoxynucleotydltransferase using a kit from Boerhinger Manheim (Laval, Quebec) and purified on Sephadex G50 columns (Amersham Pharmacia Biotech, NJ, USA). Sections were fixed with 4% paraformaldehyde in 0.1M phosphate buffer for 10 min and dehydrated in graded ethanol prior to being submitted to a series of washes in saline sodium citrate (SSC) 4X containing 1% Denhardt's solution (1 X 1h), SSC 4X (1 X 15 min), 0.2M TEA, NaCl 18% (1 X 5 min), 0.2M TEA, NaCl 18% and 0.25% acetic anhydride (1 X 10 min) and SSC 2X (3 X 5 min). The sections were then dehydrated in graded ethanol, rinsed in chloroform, followed by ethanol 100% and 95%, and air dried. Sections were incubated with 90 µl of hybridization solution containing 1.0-1.5 X 10^6 cpm of the ^{35}S-labeled probe and coverslipped before being incubated overnight at 42°C. The hybridization solution consisted of 0.6 M NaCl, 0.01 M Tris buffer, 500 µl/ml formamide, Denhardt's solution (1X), 0.1 M Phosphate buffer, Sarcosyl (1X), 1mM EDTA, 0.5 mg/ml tRNA and 0.25 mg/ml salmon sperm DNA. The next day, the imperfect hybrids were disrupted by successive washes in SSC 4X (2 X 10 min, ice-cold), SSC 1X (30 min, room temp.), SSC 0.5X (2 x 15 min, 45°C), SSC 2X (2 X 15 min, ice-cold) and the sections were dehydrated again in graded ethanol and air dried before exposure to Beta-Max Hyperfilm (Amersham, Arlington Heights, IL) at -80°C for 10 days to detect signal in the PVN and 25 days to detect signal in the amygdala. Hybridization signal was quantified in the parvocellular PVN and in the central nucleus of the amygdala using computerized densitometry by means of an MCID image analyzer system (Imaging Research Inc., Ste. Catherine, ON). Three rats per group were used and 3-4 sections per animal and per region were analyzed.

Statistical analysis

All hormonal results were analyzed using analysis of variance (ANOVA) with repeated measures across time. In experiments with lactating females exposed to the male intruder (2.5hr pup separation), groups included early lactating females being sampled twice within a day (AM and PM) and females

being only sampled once (either AM or PM). Therefore we were not able to compare AM and PM responses, but performed statistical analysis of the groups over time using an independent subject design. The experimental design was counterbalanced for each condition and we did not observe a sequence effect on the hormonal responses in either test. In experiments with lactating females exposed to the fox odor, we performed a three-way ANOVA with group (pup+AM/pup-PM and pup-AM/pup+/PM), time and cycle (AM and PM) as factors. However, because pup presence and cycle are confounded to some extent in this analysis and the results of the analysis are comparable to a 2 way-ANOVA with time and group (pup+ and pup-) as factors, the latter analysis is presented. In this 2-way ANOVA, individual animals are treated as independent variables and we did not compare AM and PM statistically. Areas under the curve were calculated for some experimental conditions using a trapezoid method (22) with either raw data or normalized data when baseline values were significantly different between groups. Histochemical results were analyzed with one-way ANOVA. Analysis of variance was followed by post-hoc Student's t-test or Newman-Keuls test when appropriate. All values are presented as Means ± SEM and the level of significance was set at $P< 0.05$.

Results

Neuroendocrine and behavioral responses to a male intruder as a function of time of lactation

As shown in figure 1(top, left panel), plasma ACTH concentrations in response to a male intrusion in the home cage tended to vary according to the stage of lactation or reproductive status of the female. Compared to virgin females who did not exhibit significant responses to this psychological stressor, the response of EL females was the greatest, followed by that of the LL females. Repeated measures ANOVA showed that there was a significant difference in the ACTH response in both the early lactating (EL) females (day 4-5 of lactation) and

late lactating (LL) females (day 15 of lactation) compared to the virgin group (overall group effect: P=0.024, time effect: P=0.011). However, because virgin females did not display significant responses, there was no significant group x time effect (P = 0.191). As observed in earlier studies (2), basal concentrations of ACTH were elevated in the virgin group (0min) compared to lactating females ($p<0.01$), possibly reflecting the concomitant effects of increased adrenocortical activity of virgin females when singly-housed after surgery as compared to group-housed females and olfactory disturbances (placing the cage containing the males in the room) introduced in the room shortly prior to basal sampling. The lack of response to the male intruder in virgin females might have been caused by the elevated baseline ACTH values observed in this group. Immediately after the stressor (t=5min) plasma ACTH levels of both the early and late lactating groups differed significantly from the virgin group ($P< 0.05$ or 0.01) while at 30 and 60 min, only LL females showed reduced ACTH levels compared to virgins ($P< 0.05$). Peak ACTH secretion was generally seen at 15 minutes following the onset of stress in both lactating groups.

In order to compare the response of EL and LL females, we calculated the area under the curve (AUC) for the plasma ACTH response to male intruder (figure 1, top, right panel). To account for the differences in basal levels between groups, we normalized individual values to the group baseline and calculated the AUC using these normalized values. The EL females exhibited a significant response compared to virgins ($P<0.05$), while the response of LL females was not significantly greater than the absence of response in virgins. These data are consistent with previous results demonstrating that stress-induced ACTH secretion is greater in early lactating females compared to late lactating females (3). Despite significant changes in ACTH responses between virgin and lactating females, basal or stimulated plasma corticosterone concentrations were not different between experimental groups as depicted in the bottom panel of figure 1. In agreement with ACTH data, basal plasma corticosterone levels were highest in virgin females, but not significantly higher than in the lactating groups. For all three experimental groups, there was a significant time effect, but no group or time x group interaction (overall group effect: P=0.123, time effect: P=0.001,

group x time effect: P=0.872). The peak corticosterone secretion was seen between 15 and 30 min for all groups and the area under the curve integrated over the 60 min period did not show significant group differences (not shown). The significant corticosterone response observed in virgin females after male intrusion further suggests that high basal ACTH levels measured in this group might have obliterated ACTH responses to this stressor.

As reported in earlier studies (23,24), we found that EL females in the presence of their pups displayed elevated levels of aggression and short latencies to first aggression (Table 1). When the pups were removed from the dam for either short (2.5 hrs) or longer (48 hrs) intervals, these EL females rapidly reduced their aggression level. In late lactation, one out of 9 mothers showed signs of aggression and none of the virgin females displayed aggressive behavior towards the male intruder. When latency to the first aggression was expressed as a function of the integrated ACTH secretion (Figure 2), a significant relation was found in EL females (r^2= 0.247, df=17, $p<0.025$), but not in LL females.

The expression of CRF mRNA levels in the PVN and central nucleus of the amygdala (CeA) 90 min after male intruder (5 min test session) is depicted in figure 3. As demonstrated in an earlier report (25) and by others (26), expression of CRF mRNA was reduced in both the PVN and the CeA in LL females (day 15 of lactation) compared to virgins ($P<0.05$). There was no significant difference in CRF expression between virgin and EL females in either structure as reported previously (12).

Effect of pup separation on the hormonal response to a male intruder

To determine whether the presence of the pups could modulate the neuroendocrine responses to a male intruder in EL females, we examined the effect of pup separation on the ACTH response using two different duration of

litter separation: 48 hours and 2.5 hours (figure 4). The rationale for using these two time points was that by 48 hrs, several of the neuroendocrine modifications of lactation have been found to be returned to normal levels (as seen in virgin females) (2,26) and by 2.5 hrs, the neuroendocrine status of the lactating female still remains comparable to suckled females, except for the rapid decline in prolactin secretion after pup removal (27). With both separation intervals, the response of EL females in the presence of their pups was greater than when they were without their pups. In the 2.5hr separation paradigm (Figure 4, top), females were tested in the AM and/or the PM and separate ANOVA analysis with repeated measures across time was performed for the AM and the PM responses. In the AM (left), there were significant group (EL+pups vs EL-pups, $p=0.05$) and time ($p=0.001$) effects as well as group by time interactions ($p=0.042$). Significant differences between EL+pup and EL-pups were observed at the 5 and 15 min time points ($p<0.05$). The EL+pup group contributed to the time effect significantly (p for the EL+pup=0.007), but not the EL-pup group. In the PM, there were no significant group ($p=0.163$), time ($p=0.354$) or group by time ($p=0.934$) effects. Thus, the ACTH response of females tested with their pups present in the cage was significantly higher than that of females in the absence of their pups in the AM only.

A similar analysis for the 48hr separation time showed a group effect that was close to significance ($P=0.0650$) and no time ($P = 0.162$) or group x time interaction ($P=0.870$). In contrast to virgins who did not display significant responses to a male intruder, EL females separated from their pups for 48hrs still displayed a significant response. The area under the curve (AUC) for the plasma ACTH response after 48hr separation was: EL+pups=530 $\pm$ 91.4 pg/ml x 60 min and EL-pups= 245 $\pm$ 90.5 ($P=0.05$). Basal ACTH secretion in females separated for 48hrs was lower ($P<0.01$) than in EL+pups females. In agreement with previous findings (2), 48hrs separation from the pups significantly ($P<0.05$) reduced basal plasma corticosterone levels in EL females (Table 2). The response to male intruder was significantly different between the two EL groups in the 48hr separation paradigm. Repeated measures ANOVA showed a significant time ($p=0.001$) and group by time interaction ($P = 0.022$), but no

significant group interaction (P=0.173). The corticosterone secretory response of females in the EL+pups group was lower than that of the separated females (AUC on normalized data was: EL+pups= 514 ± 39.8, EL-pups (48h)= 810.5 ± 156.4, p=0.016). In the 2.5hr separation paradigm, there were no group differences or group x time interactions at either the AM or PM sampling time, although the time effect was always significant (p<0.001).

Effect of pup separation on the hormonal response to a predator odor

We first determined that the exposure to fox urine in a novel environment triggered larger responses compared to a control odor in virgin females (figure 5). Repeated measures ANOVA revealed a significant time effect (P=0.045), but no significant group (P= 0.234) or time x group interaction (P=0.0844). The response of virgin females to fox urine stayed elevated after 60 min compared to exposure to control odor (P=0.03). The area under the curve for fox exposure tended to be greater than for control odor (AUC Control = 436.9±72.1 pg/ml x 60min, Fox = 570.3±112.8, P>0.05). Plasma corticosterone concentrations at 60min tended to be higher in virgins exposed to fox compared to control odor (control: 22.8± 3.29 ug/dl, Fox: 27.2 ±2.18).

In the predator odor paradigm, we compared virgins and EL ± pups with the short separation interval (2.5 hrs) and as for the male intruder paradigm, the response was evaluated in the AM or the PM. As shown in figure 6 (top: ACTH, bottom:corticosterone), exposure to fox urine in a novel environment induced a large endocrine ACTH response in EL+pups females, while the response of EL females in the absence of their pups (EL-pups) was significantly diminished compared to EL+pups (or virgin) females. Three way ANOVA using a within subject design showed a significant group x time x cycle interaction for ACTH only (p=0.0006). Although we used a within subject design for the EL females, the confounded group effect (±pups) and cycle effect (AM vs PM) prevented a clear analysis of each variable independently. We next performed a repeated measures ANOVA using independent samples to compare the 2 lactating groups

in the AM and PM. In the AM, presence or absence of the pups in EL females resulted in a significant group effect ($P = 0.05$), time effect ($P < 0.0001$) and group x time interaction ($P = 0.046$) for ACTH secretion. Plasma ACTH levels were significantly different between EL+pups and EL-pups at 10 minutes and 30 minutes ($P<0.05$). In the PM, we found a significant group ($p=0.024$) and time ($p=0.001$) effect for ACTH secretion, but no group x time interaction ($p=0.169$). Group differences were observed at the 10 min time point only ($p<0.05$). As for the male intruder paradigm, ACTH responses to the fox odor appeared lower in the PM than those observed in the AM in both lactating groups. The greater response in EL+pups in the AM is further illustrated with the area under the curve calculated over the 60 min sampling protocol (AM: EL+pups=744.2 ± 124.8 pg/ml x 60 min.; EL-pups=404.4 ± 78.7, $p=0.041$, PM: EL+pups=356.4 ± 33.15 pg/ml x 60 min.; EL-pups=229.8 ± 52.6, $p=0.064$).

Plasma corticosterone responses to fox odor were comparable between the two lactating groups in the AM with only a significant time effect ($p=0.001$), but no group ($p=0.269$) or group x time interaction ($p=0.582$). The lack of group differences in the face of significant variations in ACTH secretion between EL+ and EL-pups suggests a ceiling effect for adrenal corticosterone secretion in the AM. In the PM, however, a significant effect of pup presence was observed (group effect $p=0.018$) on corticosterone secretion, in closer parallel to the corresponding ACTH secretion. There was a significant time effect ($p=0.001$), but no group x time interaction ($p=0.505$) in the PM. The AUC for corticosterone secretion in the PM was greater ($p=0.0318$) in EL+pups (89.98 ± 9.28 µg/dl x 60 min.) than EL-pups (59.5 ± 6.91 µg/dl x 60 min.).

Discussion:

The primary goal of this series of experiments was to determine whether increasing the salience of a threatening stimulus by presenting it to lactating females in the presence of their litters would modify their neuroendocrine response to the stressor. Consistent with our hypothesis we found that on days 3-

5 postpartum, females exposed to either a male intruder or predator odor showed a more robust adrenocortical response to both of these stressors when their pups were present. This effect was seen most dramatically in the predator odor condition, where the presence of the litter completely restored stress responsivity of lactating females to the level of virgin females. By contrast, lactating females separated from their pups for 2.5h prior to exposure to predator odor showed the attenuated stress response typical of lactating females (Figure 6).

We chose male intrusion and predator odor as stimuli in these experiments because they represent ecologically salient stressors for the mother and her litter and because there is a considerable literature to suggest that both stimuli represent potent emotional stressors in rodents. The introduction of a male intruder has been shown previously to induce increases in ACTH and corticosterone levels in resident male rats (28,29). The relevance of this paradigm for lactating rats has been demonstrated recently in a study in which lactating rats served as residents and virgin and lactating rats as intruders (8). Both residents and intruders showed an increase in ACTH and corticosterone in this situation but the response of lactating females was lower than that of virgin females.

Intrusion of a male into the home cage has also been used extensively to study aggression in lactating female rats and it is generally accepted that aggression is part of the complex behavioural patterns included in maternal behavior (30). Interestingly, we found that the magnitude of the hormonal response of lactating females to a male intruder paralleled levels of aggression displayed by the females towards the intruder. The greatest amount of aggression toward the intruder was seen in females tested early in lactation in the presence of their litter and these females also showed the greatest hormonal response to the intrusion. In contrast, females tested late in lactation (day 15 pp.) showed an attenuated hormonal response to the male intruder compared to those tested early in lactation as well as a low level of aggressive behavior. Similarly, after separation from their litters, females tested early in lactation showed both an attenuated hormonal response to stress and low aggression.

Neuman et al (2001) (8) also reported that the level of aggressive behavior displayed by lactating residents was positively correlated with their ACTH response to intrusion. These findings are consistent with the literature on maternal aggression. High levels of aggression are seen during the first 10 days of lactation (31), when, as we show in the current study, endocrine responses to male intrusion are high. In addition, Erskine et al. (23) as well as Ferreira and Hansen (24) also showed that maternal aggression is increased in the presence of the pups. The positive correlation found for the ACTH secretory response and latency to aggression in EL females does not necessarily mean that one process is causal to the other. A number of brain regions activated during maternal aggression overlap with regions implicated in the control of stress responses such as the PVN, amygdala and BNST (32). Aggression and control of ACTH responses thus could be seen as parallel outputs of the same appraisal process rather than causally related. Interestingly, this correlation between ACTH responses and aggression latency is lost in late lactation despite increased suckling intensity. This suggests that along the dynamic process of lactation, there might be a dissociation between the neuroendocrine and behavioral outputs of stress appraisal.

In contrast to studies performed in adult males (28,29), virgin females displayed no increase in ACTH concentrations to male intrusion. The lack of ACTH response might be caused by a lack of activation of the HPA axis in specific stages of the oestrous cycle in virgins (33) or by the fact that the intruder male simply has a very different functional significance for virgin females than for lactating females or resident males. The stage of the oestrous cycle was not monitored in the virgin females in the current study, thus it is not possible to judge to what extent either sexual interest of the female or oestrous stage effects on HPA responsivity might have contributed to this pattern of results. However, since corticosterone responses to male intrusion was significant in virgin females, the lack of ACTH response might also be explained by the abnormally high basal ACTH concentrations that we observed in this group.

It is noteworthy that the presence of pups enhanced the stress responses of early lactating females to a male intruder compared both to females separated from their pups for 2.5 hours (in the AM only) and those separated for 48 hours even though these two conditions would be expected to correspond to very different hormonal and physiological states. Separation from the litter for 2.5 hrs is associated primarily with a decline in prolactin levels (27) and perhaps a reduction in suckling induced ACTH secretion (2) although other aspects of neuroendocrine function would remain similar to suckled females. This short separation period therefore, was expected to provide an adequate baseline from which to examine the influence of pups' presence on neuroendocrine responses without major concomitant hormonal/neuronal changes other than a rapid drop in prolactin secretion. This hormonal change is unlikely to participate in the pup effect on stress responsiveness since a recent study demonstrated that inactivation of central prolactin secretion by antisense infusion increased, rather than reduced, stress-induced ACTH secretion in lactating females (34). Two days (48h) of pup separation, however, is associated with the return to nonlactating levels of many neuroendocrine functions. Previously, for example, we showed that basal plasma ACTH and corticosterone concentrations are normalized by 14 and 24 hours after pup separation, respectively (2). In addition, stress-induced CRF expression in the paraventricular nucleus of the hypothalamus is restored after 48 hours of pup separation (1). This restoration of nonlactating levels of HPA function is paralleled by decreases in progesterone levels and increases in luteinizing hormone pulsatility. In addition, other critical variables such as removal of the metabolic drain of lactation, reduced energy intake, changes in circulating levels of leptin, insulin and fatty acids of separated EL females are likely to contribute to the reduced basal and increased stimulated activity of the HPA axis after forced weaning. As recently suggested in adult male rats (35), glucocorticoid feedback on the HPA axis might be a consequence of the peripheral metabolic actions of this steroid rather than direct actions on the brain. In the dramatic metabolic changes of late pregnancy and lactation, blunted responses to physical stressors might occur as a response to the combined action of metabolic effectors (i.e.leptin, insulin) and glucocorticoids.

Exposing early lactating females to a predator odor in a novel environment allowed us to evaluate the effects of the presence of pups on the lactating females' response without the possibly confounding effects of physical contact inherent in the male intruder condition. In addition, predator odor might be expected to be particularly relevant to the mother because olfactory cues during lactation play a critical role in mother-young interactions (36-38). Using the short-separation paradigm, we demonstrated that the presence of the pups with the mother at the time of stress totally eliminated the blunted ACTH response to combined novelty/predator odor in early lactating females. This effect is even more striking when one considers that the responses we observed in the lactating females with their pups in the AM are in the range of responses (2.8-3.0 fold increase) observed in virgins females after exposure to ether stress (2). Interestingly, we found that the ACTH response of EL+pups to either the male intruder or the predator odor appeared larger in the AM compared to the PM and this is consistent with earlier studies showing a greater stress-induced responsiveness of the HPA axis in the AM compared to the PM in male rats (39). When exposed to the predator odor, EL females in the presence of their pups exhibited larger ACTH responses at both times of day, while with the male intruder, the group difference was abolished in the PM. Taken together, these results suggest that the influence of stimulus salience (a threat on pups present with the mother) on HPA responses to stress might be better evidenced at a time when the HPA axis is relatively quiescent and adrenal sensitivity is low. Alternatively, the lower PM responses might represent habituation to a previously encountered stressor although this process generally occurs with more than one exposure to the same stressor.

As in previous studies performed in male rats (40,41), we exposed females to the predator odor in a novel environment to mimic more closely an ecologically valid stimulus and to enhance the stressful aspect of the predator odor. Indeed, in pilot experiments, we found that the response of virgin or lactating females to the fox urine was greater in a novel cage compared to the

home cage (Deschamps, Walker, unpublished), suggesting that the neuroendocrine response might be context-dependent. Indeed, a recent study demonstrated that corticosterone responses to TMT, a potent volatile odor isolated from fox feces was larger when the stimulus was presented in a brightly lit and large openfield eliciting fearful behaviors compared to a more familiar environment (42). In agreement with studies performed in male and virgin female rats (40,41), we found that the fox urine combined with the novel cage stimulus elicited a greater ACTH response in virgin rats than was seen following presentation of a control odor. Because exposure to fox urine was combined with a novel environment in this experiment, we cannot clearly dissociate the contribution of novelty versus predator odor to the adrenocortical responses we observed. However, several reports have demonstrated that lactating females exhibit reduced behavioral, neural and adrenocortical responses to the openfield compared to virgins (8,17,19,43), suggesting that the contribution of novelty to the response might be minimal in lactating females compared to virgins. It remains possible, however that the presence of the pups in the novel environment generated a higher level of arousal in the mother by eliciting pup-oriented behaviors such as retrieval, grooming and nursing attempts. Importantly, the stress response of mothers who did exhibit such maternal behaviors, however, was not different from that of mothers who did not suggesting that increased arousal associated with maternal care was not critically involved in enhancing the stress response.

Compared to virgin females, lactating females display reduced CRF mRNA expression in the PVN and the central amygdala (CeA) (1,5,25) and diminished Fos activation in the hypothalamic PVN (12), brain stem (44) and medial amygdala (Woodside et al, unpublished) following a variety of psychological stressors. However, the current data, together with earlier work by Toufexis et al (1999) (16) showing that the reduced acoustic startle responses of lactating females are rapidly normalized by fear conditioning, suggest that inhibitory mechanisms that are normally in place to maintain these blunted responses can themselves be either inhibited or lifted in some situations.

Whether areas associated with fear processing and potentially modulating inputs to hypothalamic neurons after exposure to a psychological stressor can be strongly activated in lactating females with their pups present at the time of stress is currently unknown. Alternatively, it is possible that, when combined with presentation of a psychological stressor, manipulations such as fear conditioning or the presence of the pups are able, to recruit other mechanisms that drive the HPA axis.

The psychological stressors used in the current experiments activate a number of limbic nuclei as well as the PVN and it is reasonable to suppose that it is at these sites that cues from the pups and the stressor are integrated so as to provide a greater drive onto the HPA itself. Interestingly, the medial amygdala is activated after exposure to a predator odor, a male intruder or to pups themselves (45,46). There is ample evidence to link activation of the medial amygdala to modulation of both neuroendocrine and behavioral responses to stress. For example, Activation of the medial amygdala could influence the activity of the central nucleus of the amygdala which together with the periaqueductal gray is thought to play an important role in mediating fear-related freezing to TMT (47).

In summary, our results demonstrate that the presence of the pups at the time of exposure to a threatening stressor can eliminate blunted HPA axis responses of early lactating females to this stressor. Thus, by increasing the salience of the stimulus, pups presence might modify the level of activation of neural structures implicated in the control of the HPA axis. Because of its ability to process olfactory stimuli and stimuli with affective properties, the integrated amygdala might play an important role in "gating" of the neuroendocrine response during lactation. The rapid normalization of neuroendocrine responses to stressors representing a direct threat to the infant makes perfect ethological sense and is of critical importance to maintain adequate protection of the young.

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Figure legends

Figure 1

Plasma ACTH (top) and corticosterone (B, bottom) responses to a male intruder in virgin females (n=10), early lactating females (EL, n=19; days 3-5 of lactation) and late lactating females (LL, n=5; day 15 of lactation). Females were exposed to the male intruder in the home cage for 5 min. The area under the curve (AUC) for the ACTH response is calculated over the 60 min of sampling (top right panel). All values are represented as means ± SEM. Repeated measures ANOVA for ACTH secretion revealed an overall significant group effect for early and late lactating females compared to virgin females ($P=0.024$), a significant time effect ($P=0.011$), but no group x time effect ($P = 0.19$). In EL and LL females, significant differences were observed at sampling time = 0 (**$P<0.01$), and 5 ($^{*}P<0.05$) minutes compared to virgin females. At 30 and 60 min, only LL females were different from V ($^{*}P<0.05$). Repeated measures ANOVA for corticosterone secretion only showed a significant time effect ($p=0.001$). Horizontal bar indicate presence of the male intruder.

Top right: Area under the curve (AUC) calculated for the ACTH response in V, EL and LL females over 60 min. Values normalized to baseline in each group were used. EL females were significantly different from V ($^{*}P< 0.05$).

**, $p<0.01$; *, $p<0.05$ compared to virgins

#, $p<0.05$ compared to 0 min.

Figure 2

Relation between ACTH secretion over the 60 min after male intruder (area under the curve, AUC) and the latency to display the first aggression in early (EL,) and late (LL,) lactating females. Pearson correlation coefficient for EL females was $r^2= 0.247$ ($p<0.025$).

Figure 3

Top: Coronal brain sections showing CRF messenger RNA expression in the paraventricular nucleus (PVN) of the hypothalamus (left) and in the central

nucleus of the amygdala (CeA, right) in virgins (V), early (EL) or late (LL) lactating females. Expression of CRF mRNA levels was measured by *in situ* hybridization 90 minutes after the onset of the male intruder stressor. Bar represents 2mm. *Bottom*: Mean optical density (nCi/g proteins) of CRF mRNA levels determined in the PVN and CeA of virgin and lactating females. Values represent the mean ± SEM of 3-4 sections/animal and 3 animals/group.

*, $P<0.05$ compared to virgins

#, $P<0.05$ compared to EL females

Figure 4

Effect of pup removal for short (2.5 hrs, top) or long (48 hours, bottom) periods on the plasma ACTH response of early lactating females to a male intruder for 5 minutes. Females were tested either in the presence (EL+pups) or absence (EL-pups) of their pups in the home cage. For the 2.5hrs separation, females were tested in the AM (left) and/or the PM (right). Group, time and group by time effects were significant in the AM only ($p<0.05$). In the 48hrs separation paradigm, repeated measures ANOVA across time revealed a close to significant group effect ($P=0.065$), a significant time effect ($P = 0.039$) but no time x group effect ($P = 0.842$). Horizontal bar indicate presence of the male intruder. All values represent mean ± SEM of 5-14 animals (48 hrs) or 8-10 animals (2.5 hrs)

*, $P<0.05$ compared to EL-pups

Figure 5

Plasma ACTH response of virgin females following exposure to fox urine odor in a novel environment. Females were sampled in their home cage (-2min) just prior to transfer in the novel cage containing the fox or control odor (start at 0 min). The fox or control odor was removed after 10 minutes (black box) and females remained in the novel cage until 60 min. Repeated measures ANOVA revealed a significant time effect ($P = 0.045$), but no significant group ($P = 0.234$) or group x time effect ($P = 0.0844$). Horizontal bar indicates the presence of the fox or control odor. All values are means ± SEM of 11 animals/group.

*, $P<0.05$ compared to control odor

Figure 6

Effect of pup presence on the plasma ACTH (top) and corticosterone (B, bottom) response of early lactating (EL) mothers to exposure to fox urine in a novel environment. Females were tested either in the presence (EL+pups) or absence (EL-pups) of their pups for 2.5 hrs and testing occurred in the AM (left) and the PM (right) in a counterbalanced design. Females were sampled in their home cage (-2min) just prior to transfer in the novel cage containing the fox odor (start at 0 min). The fox odor was removed after 10 minutes and females remained in the novel cage until 60 min. Statistical analysis yielded a significant difference between the EL+pups and EL-pups for ACTH in AM and PM (group effect: AM:$p = 0.05$; PM:$p=0.024$) and a significant time effect in AM and PM ($p < 0.0001$). A group x time effect for ACTH was only observed in the AM. For corticosterone secretion, a group effect was significant in the PM ($p=0.018$) and significant time effects were observed in AM and PM ($p<0.001$). Horizontal bar indicates the presence of the fox odor. All values represent means ± SEM of 5-6 females/group.

*, $P<0.05$ compared to EL-pups

Table 1: Behavioral measures of aggression in virgin and lactating females

Female group	Number of females displaying aggression		Latency to aggression (minutes)
Virgins	1/12	8 %	4.92 ± 0.08
EL + pups	23/29	79%	2.3 ± 0.45 **
EL – pups (48 hours)	2/9	22%	4.3 ± 0.48
EL – pups (2.5 hours)	2/12	16%	4.7 ± 0.24
LL + pups	1/9	11%	4.99 ± 0.01

EL: early lactating females (PPD 3-5), LL: late lactating females (PPD15)

**P < 0.01 compared to all other groups

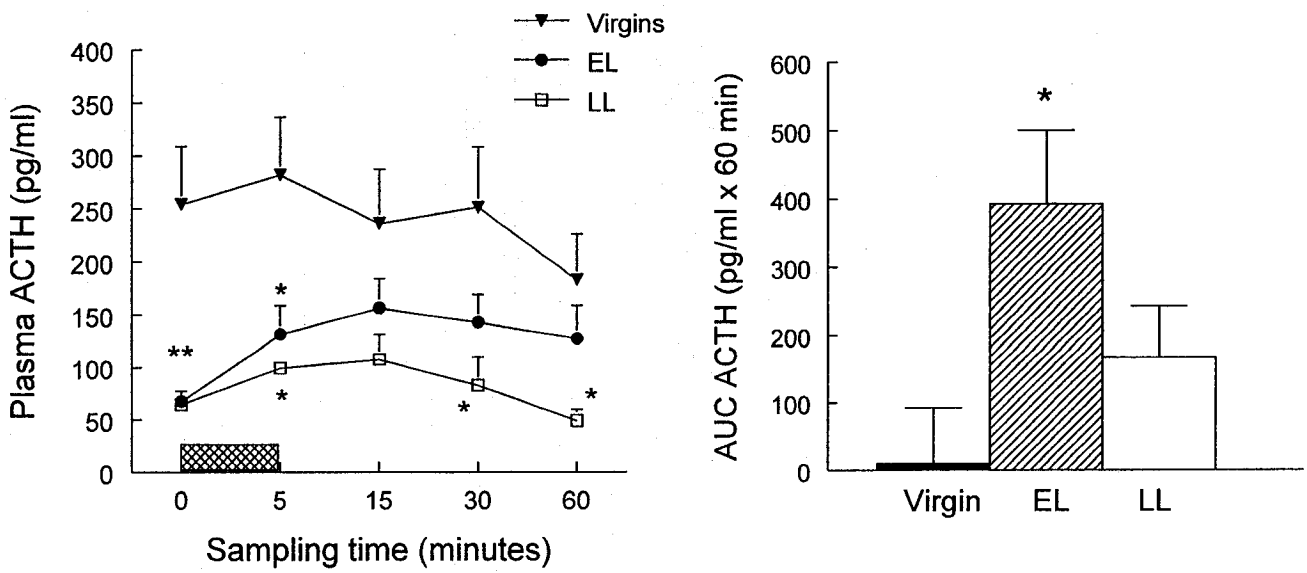
Table 2: Effect of pup separation on plasma corticosterone response to a male intruder in early lactating (EL) females

Separation Paradigm	0 min.	5min	15min	30 min.	60 min.
48h separation					
EL + pups (48h) (n=14)	23.33 ± 2.14	25.96 ± 1.94	30.49 ± 1.47	30.05 ± 1.39	23.82 ± 2.29
EL – pups (48h) (n=5)	13.88± 2.13(a)	27.32 ± 5.11	33.44 ± 2.62	26.66 ± 4.49	14.65 ± 3.92
ANOVA: group p=0.173					
Time p=0.001					
Group x time p=0.022(*)					
2.5h separation					
AM: EL + pups (2.5h) (n=4)	16.96 ± 1.89	27.36 ± 5.79	33.79 ± 2.91	34.27 ± 2.09	12.39 ± 1.07
EL – pups (2.5h) (n=4)	13.60 ± 4.71	23.53 ± 6.5	27.32 ± 5.24	17.64 ± 7.58	12.05 ± 3.95
ANOVA: group p=0.316					
Time p=0.001					
Group x time p=0.06					
PM: EL + pups (2.5h) (n=5)	10.46 ± 0.83	23.27 ± 2.59	29.46 ± 1.25	28.47 ± 2.96	10.89 ± 0.97
EL – pups (2.5h) (n=4)	9.69 ± 1.97	19.41 ± 5.26	36.87 ± 2.03	27.35 ± 5.86	8.90 ± 2.17
ANOVA: group p=0.658					
Time p=0.001					
Group x time p=0.501					

Statistical analysis (ANOVA) was performed on repeated measures across time and independent subjects. *, $p<0.05$

(a) $P < 0.05$ compared to EL+pups

Figure 1


Virgins
EL
LL
Plasma ACTH (pg/ml)
400
350
300
250
200
150
100
50
0
0
5
15
30
60
Sampling time (minutes)
**
*
*
*
*
AUC ACTH (pg/ml x 60 min)
600
500
400
300
200
100
0
*
Virgin
EL
LL

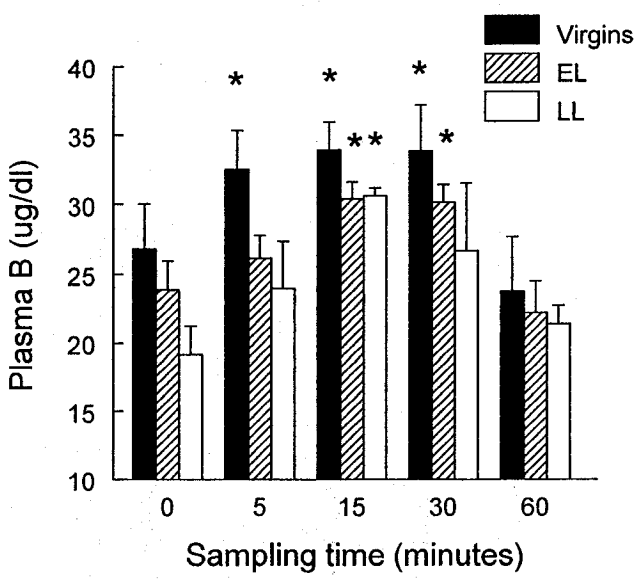

Virgins
EL
LL
Plasma B (ug/dl)
40
35
30
25
20
15
10
*
*
*
**
*
0
5
15
30
60
Sampling time (minutes)

Figure 2

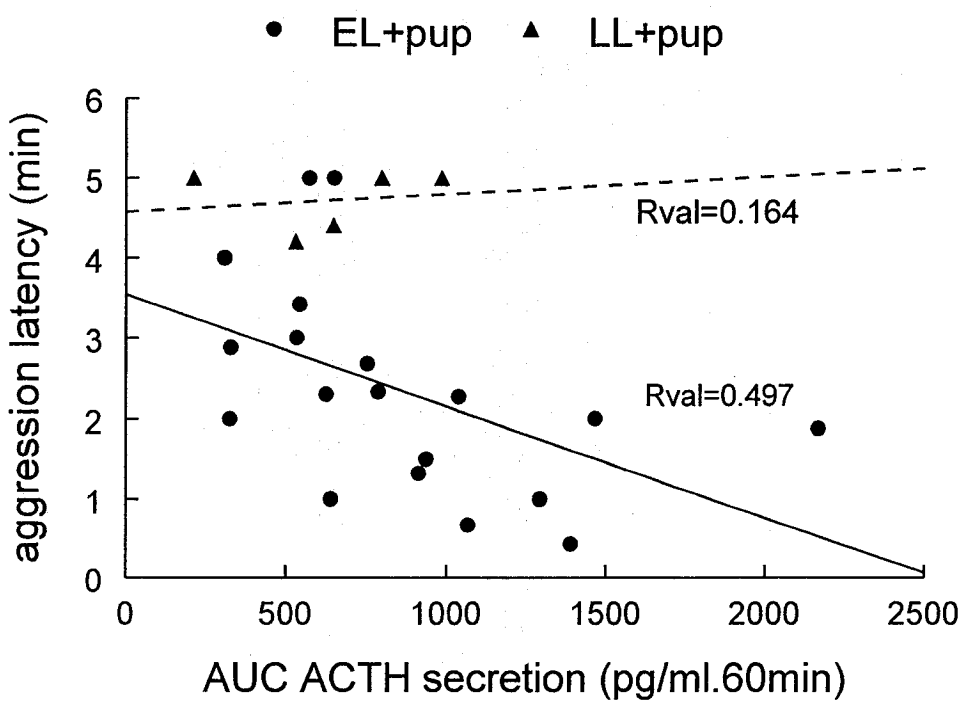
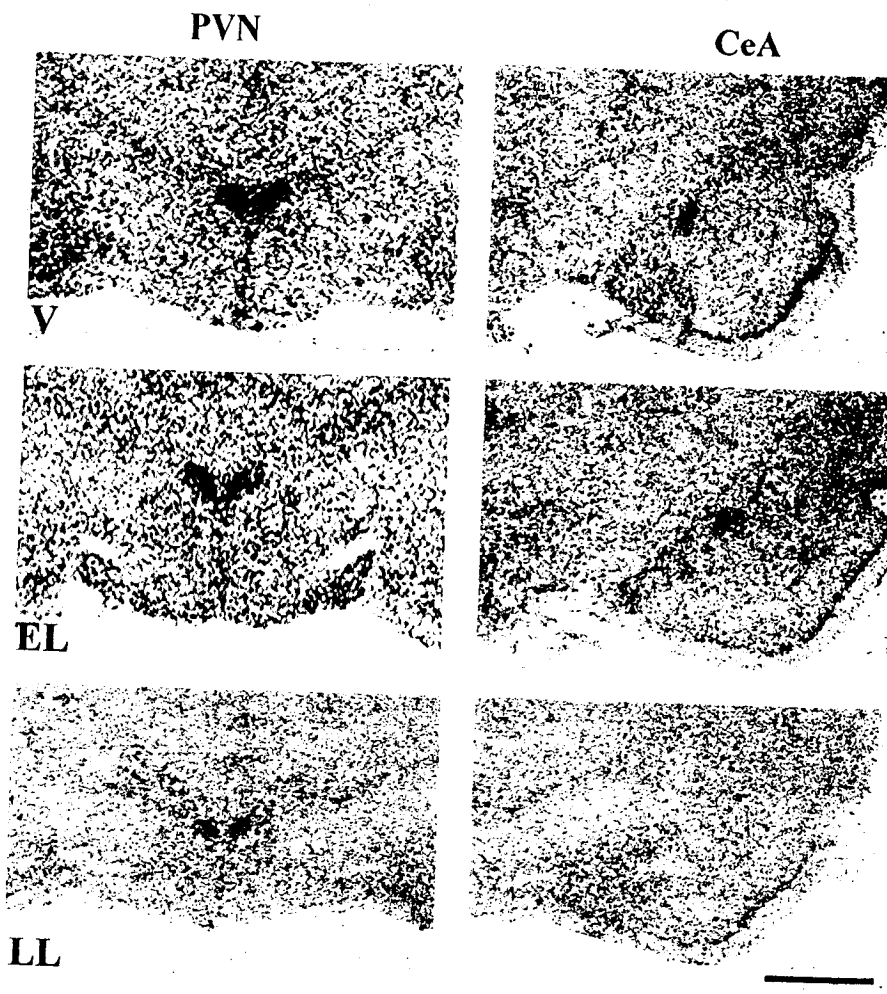

EL+pup
LL+pup
aggression latency (min)
0
1
2
3
4
5
6
Rval=0.164
Rval=0.497
0
500
1000
1500
2000
2500
AUC ACTH secretion (pg/ml.60min)

Figure 3


PVN
CeA
V
EL
LL

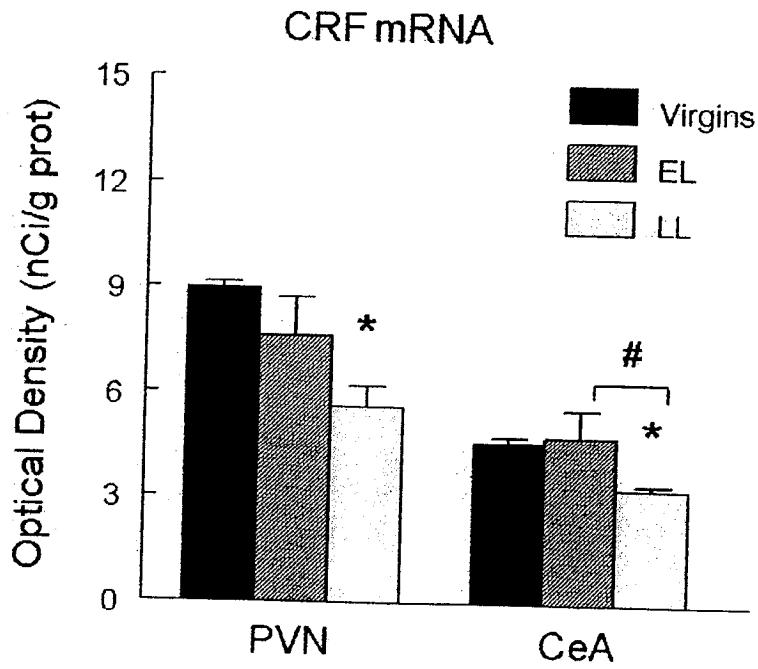

CRF mRNA
Optical Density (nCi/g prot)
15
12
9
6
3
0
Virgins
EL
LL
*
#
*
PVN
CeA

Figure 4

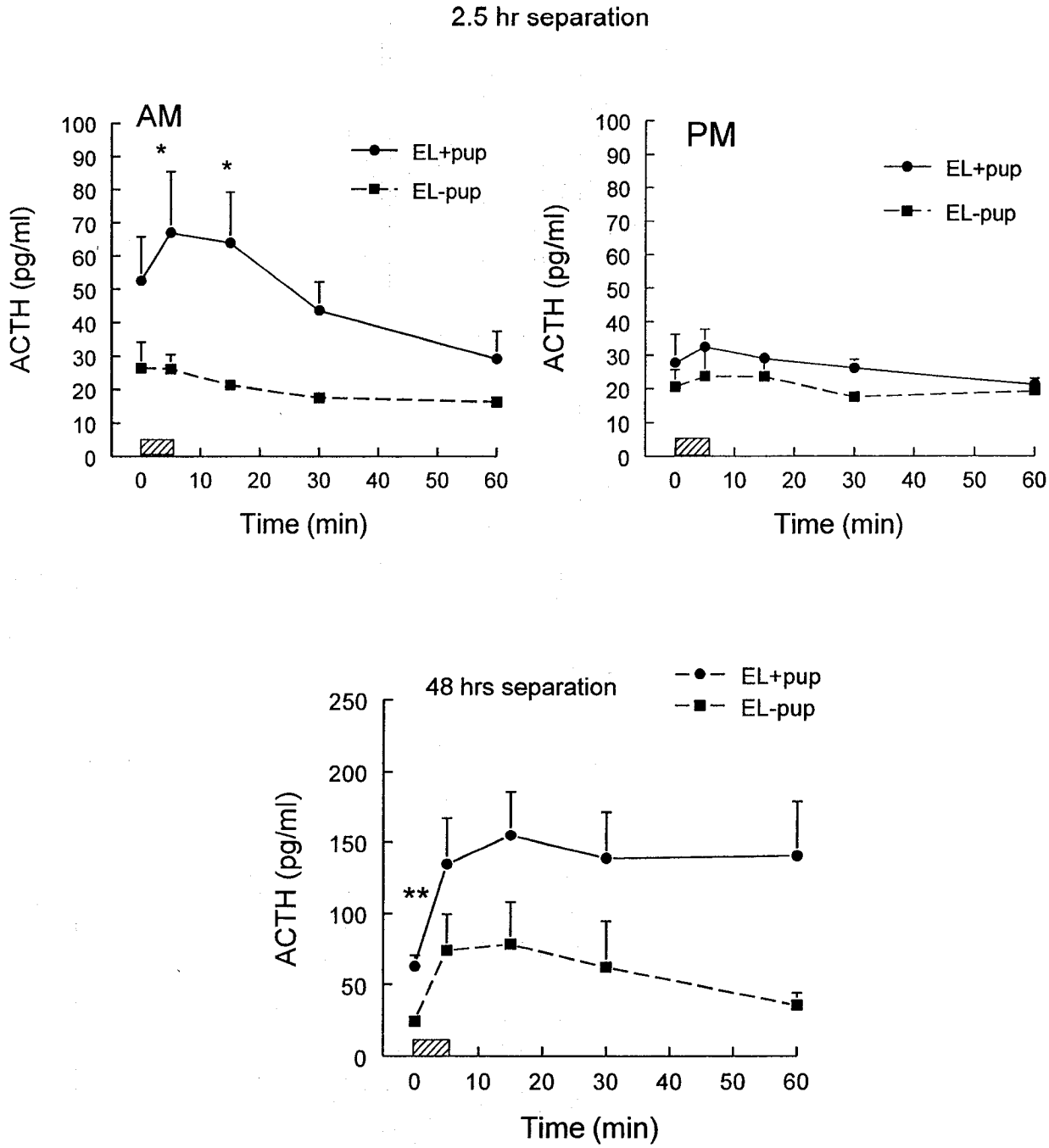

2.5 hr separation
AM
PM
EL+pup
EL-pup
ACTH (pg/ml)
Time (min)
*
*
48 hrs separation
**

Figure 5

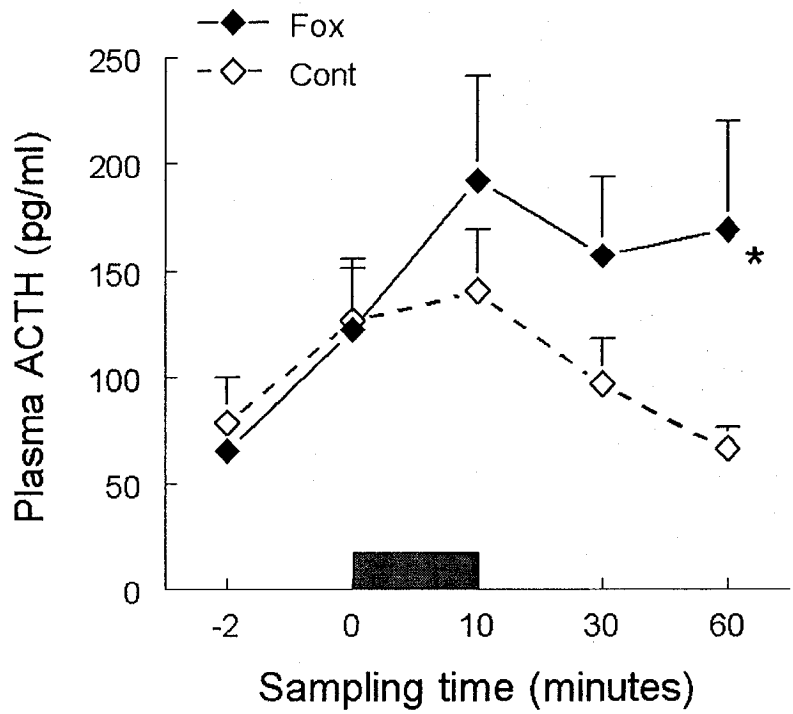
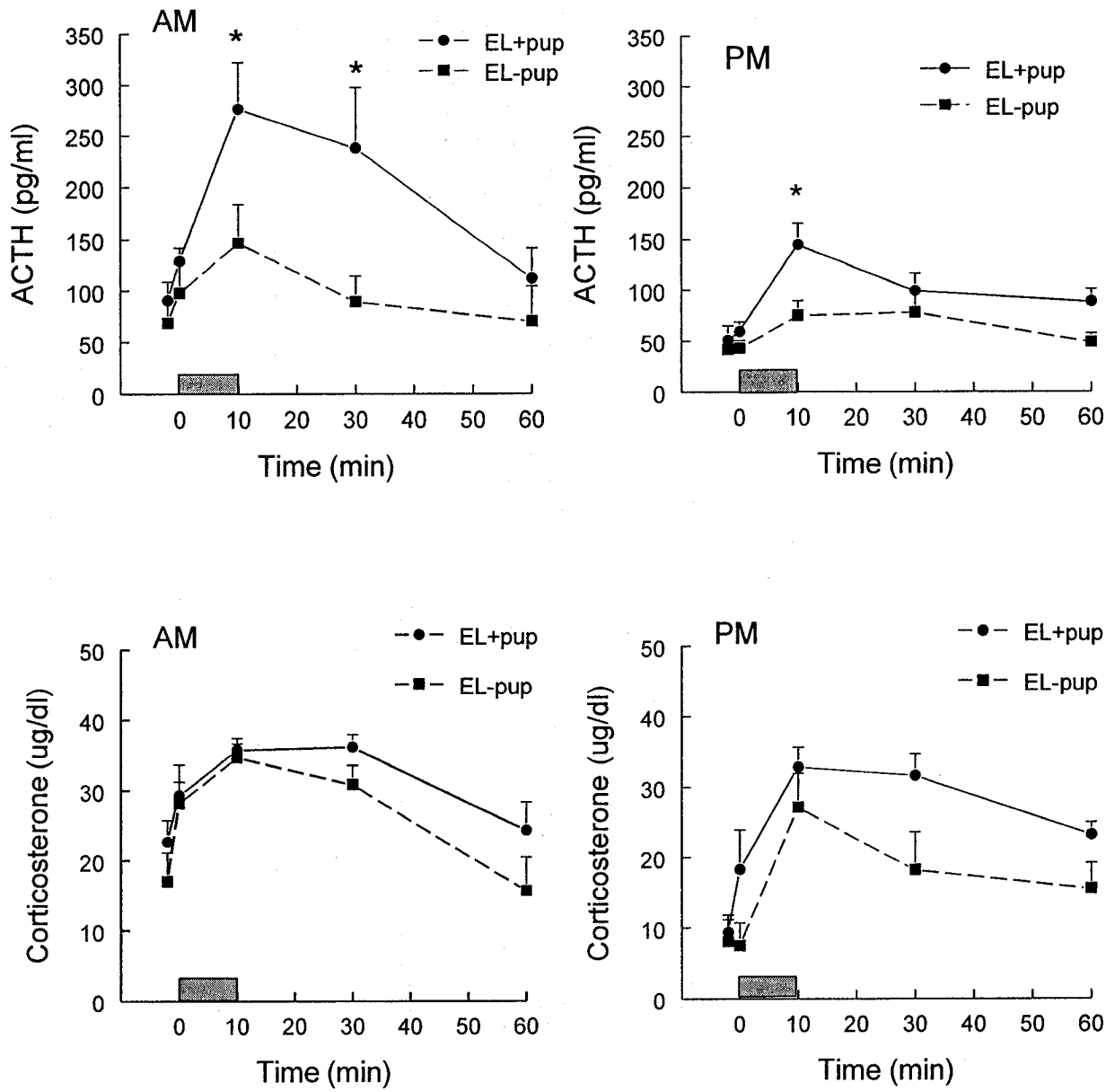

Fox
Cont
250
200
150
100
50
0
Plasma ACTH (pg/ml)
-2
0
10
30
60
Sampling time (minutes)
*

Figure 6


AM
PM
EL+pup
EL-pup
ACTH (pg/ml)
Corticosterone (ug/dl)
Time (min)
0
10
20
30
40
50
60
0
50
100
150
200
250
300
350
0
10
20
30
40
50
*

IV. COMPLEMENTARY RESULTS AND METHODS

The most important and significant results of my Master's project are presented in the article. However, other experiments were conducted during the course of my project, leading to the results presented in the manuscript. The additional results are presented in this section of the thesis.

1. Predator odor in the home cage

The results of this section are part of a pilot experiment which allowed us to set experimental conditions for subsequent studies.

In these experiments, we investigated the neuroendocrine response of lactating females exposed to predator odor (fox urine) in their home cage. To determine the influence of the stage of lactation, females were tested in early (PPD 3-4) or late (PPD 14-15) lactation and compared to virgin females. In order to examine the effect of pups' presence on the neuroendocrine response, we tested early lactating females with their litters and after 2.5 hours of separation from their pups.

All females were kept undisturbed in their home cage and the predator odor was presented to the rats in the form of three Q-tips absorbed with fox urine (predator odor) or saline (control). They were fixed onto the plexiglass lid of the cage so that the Q-tips were hanging in the cage and did not touch the cage bedding. Females were exposed to the odor for 10 minutes and blood samples were taken either before or at several times during and after stress onset. The basal blood sample was taken before exposing the females to the predator or control odor (at t=0 min) and subsequent blood samples were taken at times t=5, 10, 30 and 60 minutes following the onset of stress. We determined plasma ACTH and corticosterone levels by radioimmunoassay. Brains were collected 60 minutes following the onset of stress for determination of CRF mRNA expression in the PVN and the central nucleus of the amygdala by *in situ* hybridization. Testing with the fox urine and the control odor never occurred at the same time or in the same room in order to prevent control animals from being biased by the fox urine.

1.1 Virgin females

As shown in figure 1a, there was no specific ACTH response of virgin females to exposure to predator odor compared to the controls. Repeated measures ANOVA revealed no significant differences between the two groups (overall group effect: P=0.2063, time effect: P=0.5924 and group x time effect P=0.3737) or no significant differences between individual time points. Virgin females in the control group displayed elevated basal ACTH levels compared to females exposed to the fox odor, but the difference was not significant. These results point out to a greater variability in basal ACTH levels found earlier in virgin females singly housed (43). The high baseline ACTH levels along with the high variability observed in the control group could explain the lack of specific response in the virgin animals.

The lack of response to the fox odor in virgin females might be explained by a lack of activation of the HPA axis in specific stages of the oestrous cycle. It was previously demonstrated that the highest adrenocortical response to stress in virgin females occurred mainly during the proestrous stage of the cycle (124). However, since we did not determine the stage of the oestrous cycle we cannot strongly affirm that the lack of response was due to the effects of the cycle in our virgin females.

The lack of specific effect of fox odor on the adrenocortical response of these virgin females is further illustrated by corticosterone secretion (figure 1b). There was no significant differences between the two groups (Repeated measures ANOVA; overall group effect: P=0.6379, time effect: P=0.8850 and group x time effect: P=0.9010).

The elevated basal corticosterone levels in our females might explain why we observed not specific response to the fox odor. These high basal levels could be due to lack of handling or to the procedure we used. Indeed, in our experiments, animals were handled on the day of surgery (two days before the testing) and again on the day of testing when the jugular catheter was extended for blood sampling. Although we left a long interval between handling and the first basal

sampling, residual corticosterone secretion might still have occurred. The blood sampling procedure might be responsible for increasing the basal corticosterone levels. Generally, low basal corticosterone levels are obtained by decapitation. In our experiments, a jugular catheter was implanted in the animals and corticosterone levels were obtained by blood sampling through the catheter. This procedure might have increased the arousal level in virgin females and elevated basal corticosterone values.

Finally, it is possible that the corticosterone response to a predator odor is gender specific since studies measuring the hormonal response to a predator odor have generally used male rats (125, 126). The nature of the odor used (cat, weasel or fox) and the duration of exposure might also be responsible for the differences observed between our results and data from other studies.

1.2 Lactating females: stage of lactation

We compared the plasma ACTH response to fox odor in the home cage as a function of the stage of lactation (figure 2a). In this experiment, lactating females were tested with their pups present in the cage at the time of testing. In this serie of experiments, we noticed variations in basal ACTH levels between groups. In order to analyze our data, values were normalized to account for the differences in basal levels between the groups (virgins: 48.2 ± 11.8 pg/ml; early lactating females: 149.5 ± 34.1 pg/ml; late lactating females: 240.9 ± 99.7 pg/ml).

Repeated measures ANOVA revealed no significant differences between the groups (overall group effect: $P=0.1047$, time effect: $P=0.8056$ and group x time effect: $P=0.4239$). However, plasma ACTH levels were significantly different between virgin and early lactating females at the 5 minute ($P<0.01$) and at the 10 minute ($P< 0.01$) time points and between virgin and late lactating females at the same time points (5 minutes: $P<0.01$; 10 minutes: $P<0.01$).

In this experiment, the virgin females showed a good ACTH response to the fox odor compared to the two other groups indicating that the fox odor produced an effect on the hormonal response. The early lactating females showed no response to the fox urine. Since testing occurred in the home cage, it is possible

that the females did not perceive the predator odor (immobile object) as a threat to their nest. These results are in opposition to the data presented in the manuscript where we observed a strong ACTH response to the fox odor in early lactating females. The hormonal response to fox odor might therefore be context dependent. This will be discussed in section A4.
The group of late lactating females showed a small ACTH response 30 minutes after the onset of stress. Although the response was highly variable, this delayed response might be attributed to strong behavioral reactions such as burial of the cup and freezing that were observed in these animals while the fox urine was present in the cage. For example, some females went off the nest and smelled the Q-tip while others demonstrated a clear freezing behavior.

In order to better integrate and compare the responses of the virgin and lactating females, we calculated the area under the curve (AUC) for the plasma ACTH response to the fox odor with the normalized data (figure 2b). We established that an AUC of 250 pg/ml x 60 minutes corresponded to no response as observed in the early lactating female group. Virgin females exhibited a significantly greater response compared to the early lactating females who did not respond ($P<0.05$). In these experimental conditions, the late lactating females displayed a delayed response to the fox odor compared to the early lactating females, but the difference between the two groups was not significant ($P=0.1639$).

The expression of CRF mRNA levels in the PVN and central nucleus of the amygdala (CeA) 60 minutes after the onset of exposure to predator odor is depicted in figure 2c. The values shown is this graph represent CRF mRNA expression after stimulation. We did not collect brains for measurement of basal levels of expression, thus we cannot compared the magnitude of the stress response between the groups. In the CeA, expression of CRF mRNA was slightly increased in early lactating females compared to virgin females and late lactating mothers, however this difference was not significant. We suggest that the elevated CRF mRNA expression in the CeA of early lactating females is related to the way they perceive the stressor. The salience of the predator odor stimulus

might be greater for early lactating females compared to virgins or late lactating females. It is possible that early lactating females perceive the stimulus as being more threatening and dangerous because their pups are younger and more vulnerable to danger.

In the PVN, we did not detect significant variations in CRF expression after stress. This is surprising because earlier studies have documented a decrease in CRF mRNA expression in the PVN of middle lactating females (PPD 7-10) after some stressors (5).

1.3 Lactating females: effect of pup separation

In order to determine if the presence of the pups at the time of stress could modulate the neuroendocrine response to fox odor in early lactating females, we examined the effect of pup separation on the ACTH response and on the expression of CRF mRNA in the PVN and CeA. We chose to separate females from their litter for 2.5 hours because mechanisms of neuronal regulation are likely to remain unchanged during this period of time. Except for the important decline in prolactin levels which occurs within 3 minutes of pup separation (127), we posited that the neuroendocrine status of lactating females who have been separated from their pups for 2.5 hours is likely to remain comparable to that of suckled females.

The plasma ACTH response of females tested with their pups (EL+pups) and without their pups (EL-pups, 2.5hrs) is shown in figure 3a. There was no ACTH response to fox odor when females were tested in their home cage. Moreover, the presence or absence of the pups did not affect ACTH secretion in this experimental setup. This is interesting in view of data reported in our manuscript showing the effect of pup presence on the ACTH response to fox odor in a novel environment. Repeated measures ANOVA analysis of the ACTH response revealed a significant time effect ($P=0.0056$) but no significant overall group effect ($P=0.9044$) or group x time effect ($P=0.5844$).

Despite the lack of ACTH response and the lack of variations in CRF mRNA expression in the PVN in lactating females tested with their pups, we found a significant increase in CRF mRNA expression in the CeA of early lactating females tested with their pups compared to females separated from their litter (One-way ANOVA $P<0.05$) (figure 3b). This is suggestive of a specific effect of the presence of pups on CRF mRNA expression in the CeA or of a dissociation between the neuroendocrine response (CRF mRNA in the PVN and plasma ACTH) and CRF mRNA expression. It is possible that the expression of CRF in the PVN and CeA is not always functionally related and that inputs to the PVN, other than the afferents from the CeA, influence the expression of CRF mRNA in the PVN. Alternatively, somatosensory inputs from pups have been shown to reach the amygdala directly, thus modulating the expression of CRF in this structure without affecting the PVN.

1.4 Summary

In these experiments, we did not observe a significant response to fox odor in lactating females nor consistently in virgin females. This could be due to our experimental conditions, such as the use of Q-tips, which may have affected the concentration of the predator odor in the cage. In addition, the fact that the stimulus was immobile may have affected the way the females perceived the stimulus: as less threatening, invasive and stressful.

The lack of significant response in these experiments might also be due to the fact that testing occurred in the home cage, suggesting that the neuroendocrine response to fox odor might be context dependent. Most studies looking at the endocrine response to a predator odor transfer animals in a novel environment for testing. These studies have shown a significant increase in plasma corticosterone levels following exposure to the predator odor in the novel environment (125, 128). In our studies, it is clear that presentation of the fox odor in a novel environment was able to trigger large neuroendocrine responses that we did not observe in the home cage irrespective of the reproductive status of the animal. Similarly, a large body of literature demonstrates that the context in which

a stress occurs or a drug is administered is critical to elicit specific responses. For example, sensitization to the psychostimulant methylphenidate (a drug used for the treatment of attention-deficit/hyperactivity disorders) in rats occurs mainly when the drug is administered in a novel environment (129) and not when given in the drug-related environment. Likewise, behavioral sensitization to amphetamine generally occurs when the drug is administered in a novel cage (130) and not in the home cage. These results indicate that the context is an important factor in determining the behavioral and endocrine response to various stimuli. In our experiments, the home cage and nest is probably considered as a "safe place" by the mother, explaining why we observed no significant neuroendocrine response to stress in females tested in their home cage.

In view of the above results, we have decided to change our experimental conditions in order to increase the stressfulness of the fox odor stimulus. In the following experiments (described in the manuscript) we combined this stressor to a novel environment and administered these two stimuli simultaneously. The results from the neuroendocrine response to fox odor combined to a novel environment are shown in the article.

2. Differential contribution of the novel environment and the predator odor to the stress response

Results from our article demonstrated that lactating females showed a greater hormonal response to fox urine combined to a novel environment when pups were present in the cage. However, we were unable to dissociate the contribution of novelty versus predator odor to the adrenocortical responses since the two stressors were administered simultaneously. We hypothesized that the contribution of novelty to the response might be less important in lactating females compared to virgin females because previous studies have demonstrated that lactating females display a reduced endocrine and behavioral response to the open field compared to their cycling counterparts (52, 64, 90).

The purpose of these complementary experiments was to study the differential contribution of both stressors on the hormonal response by presenting them

sequentially. We reasoned that the immediate arousing effects of transferring to a new cage and retrieving pups in a new nest might have declined 10 minutes after transfer to the novel environment and that additional responses to predator odor would be observed. In order to do so, we tested females in a novel environment for 10 minutes followed by exposure to fox odor in this same environment for another 10 minutes. A basal blood sample was taken in the home cage (t= -2 minutes) after which females were transferred to the novel cage where a second blood sample was immediately taken (t = 0). Females were left free to explore the new cage for 10 minutes after which a blood sample was taken (t = 10 minute) and females were then exposed to fox urine for 10 minutes. Additional blood samples were taken at times 20, 40 and 70 minutes following the onset of the transfer to the novel cage. In these experiments, we changed the method of presentation of the fox odor. We used a cotton wetted with fox urine and introduced it into a styrofoam cup. The cup containing the odor was placed in the cage at the onset of testing and for a duration of 10 minutes.

In this experiment, we compared virgin females and the early lactating females (PPD 4-5) in the presence of their pups. The plasma ACTH response to the novel environment and the fox odor stressor for early lactating and virgin females is shown in figure 4a. Repeated measures ANOVA revealed a significant overall group effect ($P=0.0057$) but no significant time effect ($P=0.2304$) or group x time effect ($P=0.4606$).

Basal ACTH levels in early lactating females were significantly greater than those of virgin females ($P<0.05$). This is likely to be due to the fact that 2 animals had to be reconnected before the start of the experiment, contributing to the rise in ACTH baseline.

Only the early lactating females showed an additional increase in ACTH levels following exposure to fox urine. Plasma ACTH levels declined steadily 10 minutes after exposure to the openfield and fox odor did not elicit a response in the virgin group. It is unlikely that the elevated ACTH response to the novel environment modulates the response to the fox odor stressor in virgin females. The novel environment stressor is a mild stress and is not sufficient to deplete pituitary ACTH releasable pool. The ACTH pituitary stock is large enough to

respond to two successive stressors. Plasma ACTH levels between virgins and early lactating females were significantly different at 40 minutes ($P<0.05$).

Figure 4b shows the delta value for the plasma ACTH response to the novelty component and the fox odor part of the stressor in virgin and early lactating females. This graph allows a better visualization of the responses to the novel cage and to the fox odor separately. For each animal, the delta values for the novel cage were calculated by subtracting the ACTH values at t=10 min from that at t=0 min. For the delta value for the fox odor component, we subtracted the values at t=20 min from those at t=10 min. The average and SEM were calculated for each group.

The early lactating females displayed a trend towards a greater ACTH response to the novel environment component of stress (delta value = 52.2 ± 50.1) compared to the virgin females (delta value = 24.6 ± 23.8). This is surprising since reports in the literature have shown that early lactating females exhibit reduced anxiety and ACTH response to a novel environment compared to virgin females (52, 64). One-way ANOVA analysis revealed no significant difference between the two groups ($P=0.6319$). Also unexpectedly, the virgin females did not further respond to the fox odor stimulus (delta value = -30.9 ± 16.8) whereas the early lactating females showed only a small response to the fox urine (delta value = 7.91 ± 30.3). Again, there was no significant difference between the two groups (one-way ANOVA; $P=0.2939$) consistent with our data presented in the manuscript.

The plasma corticosterone response to the combined stressor is depicted in figure 4c. The virgin females show a peak corticosterone secretion immediately after the transfer into the novel cage (t=0 min) and another peak 70 minutes after the transfer into the testing cage. The pattern of secretion in early lactating females was different and showed a steady increase in corticosterone secretion over time. Repeated measures ANOVA revealed a significant time effect ($P=0.004$) and group x time effect ($P=0.0316$) but no overall group effect ($P=0.4686$).

We were surprised by the results obtained in these last experiments since we did not expect the early lactating females to respond strongly to the novel environment nor the lack of response to fox odor in virgin females. Since these experiments were performed only once with a small number of animals per group (n=5), we need to repeat them in order to validate these results.

Figure legends

Figure 1

Plasma ACTH (A) and corticosterone (B) responses to fox odor (n=4) and control odor (n=4) in virgin females. Females were exposed to the fox odor (or control) in their home cage for 10 minutes. Repeated measures ANOVA across time and within subjects revealed no significant group effect (P=0.2063), time effect (P=0.5924) or group x time effect (P=0.3737) for the ACTH response. Similarly, there was no difference between the groups for the corticosterone response (repeated measures ANOVA; overall group effect: P=0.6379, time effect: P=0.8850 and group x time effect: P=0.9010). All values are represented as mean ± SEM.

Figure 2

Plasma ACTH response to fox odor in virgin females (n=4), early lactating (n=4) and late lactating females (n=4) with normalized values to the baseline (A). Females were exposed to fox odor for 10 minutes in their home cage. There were no significant differences between the groups (repeated measures ANOVA; overall group effect: P=0.1047, time effect: P=0.8056 and group x time effect: P=0.4239). However, plasma ACTH levels were significantly different between virgin and early lactating females at the 5 minute ($^{**}P<0.01$) and at the 10 minute ($^{**}P< 0.01$) time points and between virgin and late lactating females at the same time points (5 minutes: $^{**}P<0.01$; 10 minutes: $^{**}P<0.01$).

The area under the curve (AUC) (figure 2B) is calculated for the ACTH response to fox odor over 60 minutes in virgin females, early and late lactating females. All

values are represented as means ± SEM. Values normalized to baseline in each group were used to calculate the AUC. Early lactating females were significantly different from the virgins ($^{*}P<0.05$). Horizontal bar indicates that an AUC of 250 pg/ml x 60 minutes corresponds to no response.

Figure 2C depicts the expression of CRF mRNA in the PVN and CeA in virgins, early lactating and late lactating females. Expression of CRF mRNA levels was measured by *in situ* hybridization 60 minutes after the onset of the fox odor stressor. Bars represent mean optical density (nCi/g proteins) ± SEM of 3-4 coronal sections/animal and 4 animals/group. One-way ANOVA revealed no significant differences between the groups.

Figure 3

Effect of litter separation (2.5 hours) on the plasma ACTH (A) and CRF mRNA expression (B) responses of early lactating females to fox odor for 10 minutes. Females were tested in the presence (EL+pups) or absence (EL-pups) of their pups in their home cage. For the plasma ACTH response, repeated measures ANOVA revealed a significant time effect ($P=0.0056$) but no significant group effect ($P=0.9044$) or group x time effect ($P=0.5844$). All values represent mean ± SEM.

Figure 3B shows the expression of CRF mRNA in the PVN and CeA of early lactating females tested with (EL+pups) and without (EL-pups) their litter. Expression of CRF mRNA levels was measured by *in situ* hybridization 60 minutes after the onset of the fox odor stressor. Bars represent mean optical density (nCi/g proteins) ± SEM of 3-4 coronal sections/animal and 3-4 animals/group. Expression of CRF mRNA in the CeA of EL+pups is significantly different from EL-pups (one-way ANOVA $^{*}P<0.05$).

Figure 4

Differential effect of novel environment and fox odor on the plasma ACTH response in virgins (n=5) and early lactating females (n=5) (figure 4a). Females were sampled in their home cage (2- minutes) just prior to transfer in the novel cage (0 minutes). They were left in the novel cage for 10 minutes after which the

fox odor was put in the cage for 10 minutes. Females remained in the novel cage until 70 minutes. Repeated measures ANOVA revealed a significant overall group effect (P=0.0057) but no significant time effect (P=0.2304) or group x time effect (P=0.4606). Basal ACTH levels in early lactating females were significantly different than those in virgin females ($^{*}P<0.05$).

Figure 4b represents the delta value for the plasma ACTH response to fox odor and novel environment in virgins and early lactating females. The delta values for the novel cage were calculated by subtracting the ACTH values at t=10 min from that at t=0 min. For the delta value for the fox odor component, we subtracted the values at t=20 min from those at t=10 min. The average and SEM were calculated for each group.

Figure 4c represents the plasma corticosterone response to the novel environment and predator odor presented sequentially in virgin females (n=5) and early lactating females (n=5). Repeated measures ANOVA revealed a significant time effect (P=0.004) and group x time effect (P=0.0316) but no overall group effect (P=0.4686). All values are represented as mean ± SEM.

Figure 1: Plasma ACTH (A) and corticosterone (B) responses to fox odor in virgin females (home cage)

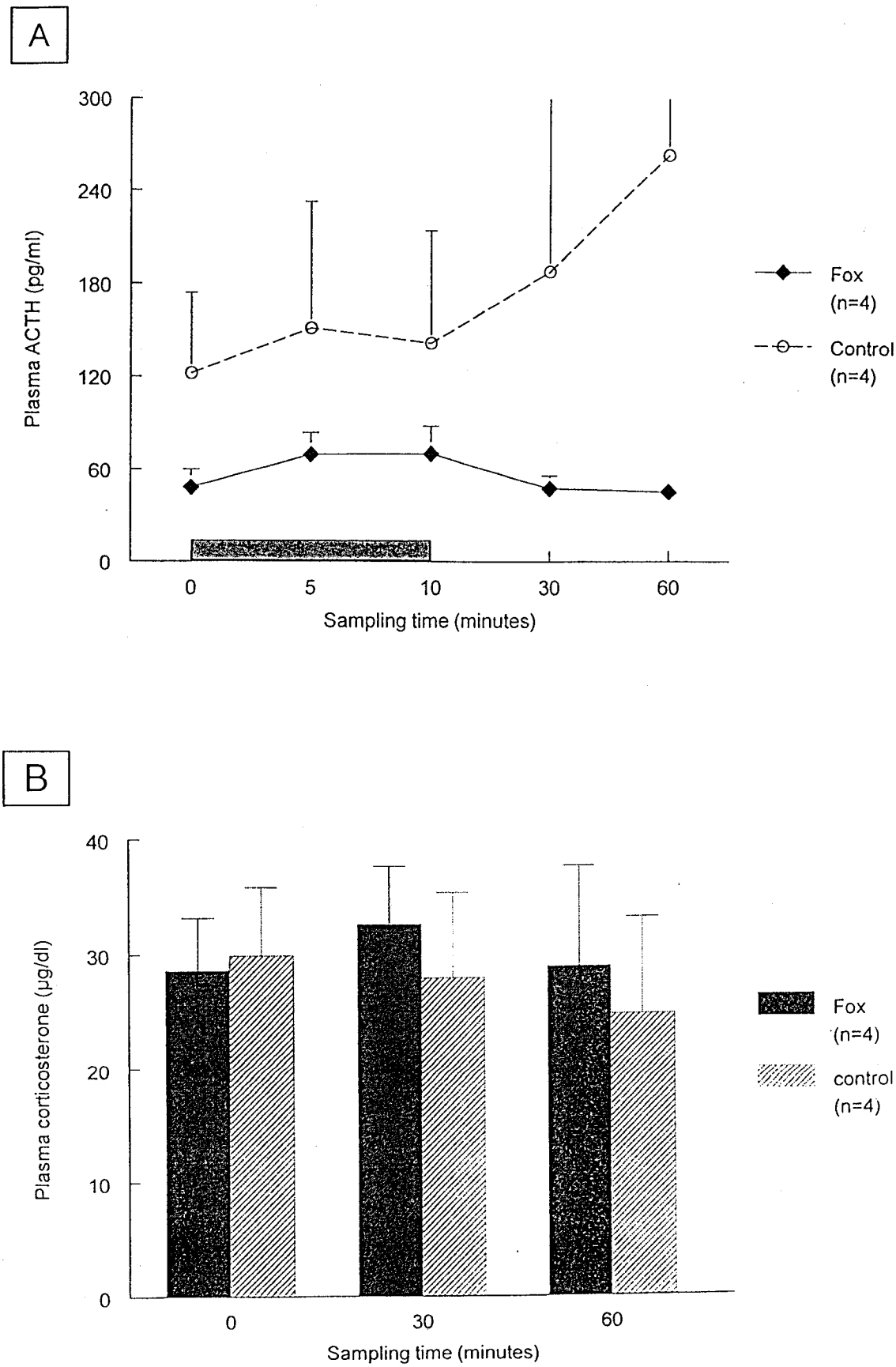


Figure 2a-b: Plasma ACTH response to fox odor as a function of the stage of lactation (home cage)

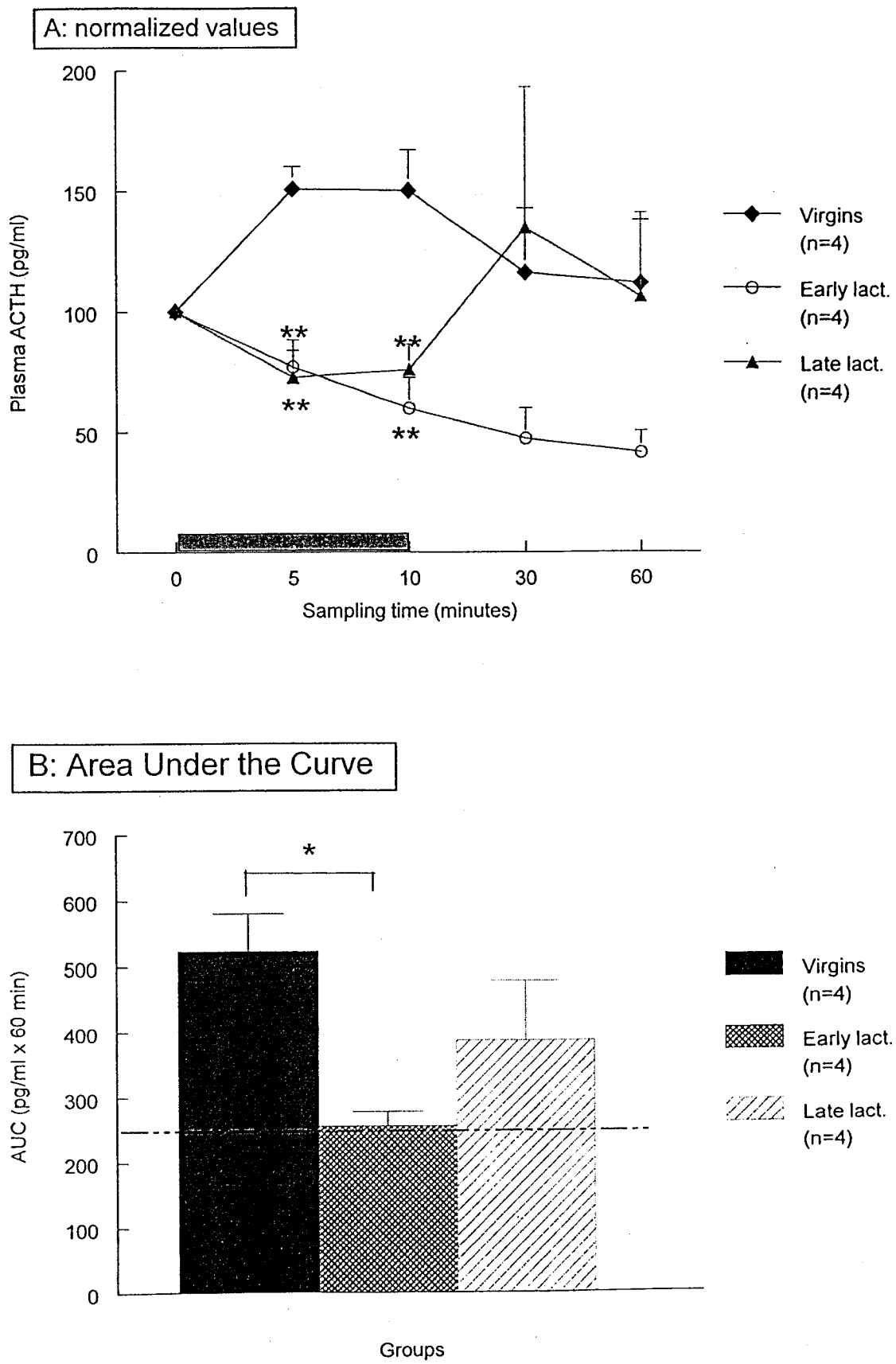


Figure 2c: Stress-induced CRF mRNA expression according to the stage of lactation

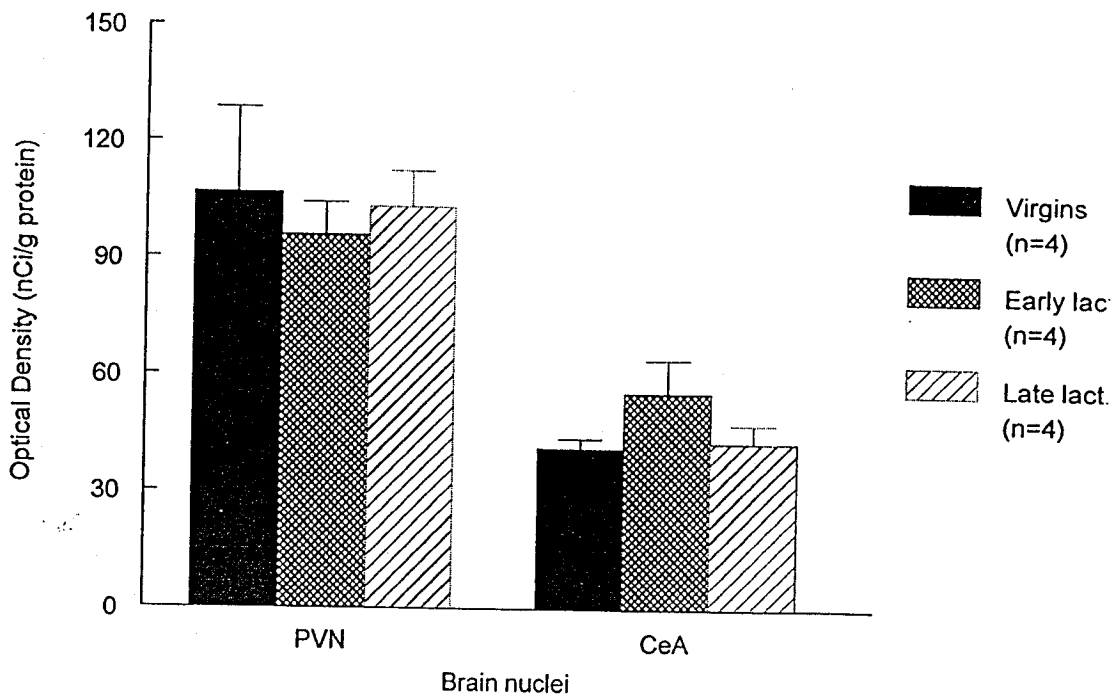


Figure 3: Effect of litter separation on plasma ACTH (A) or CRF mRNA expression (B) in response to fox odor (home cage)

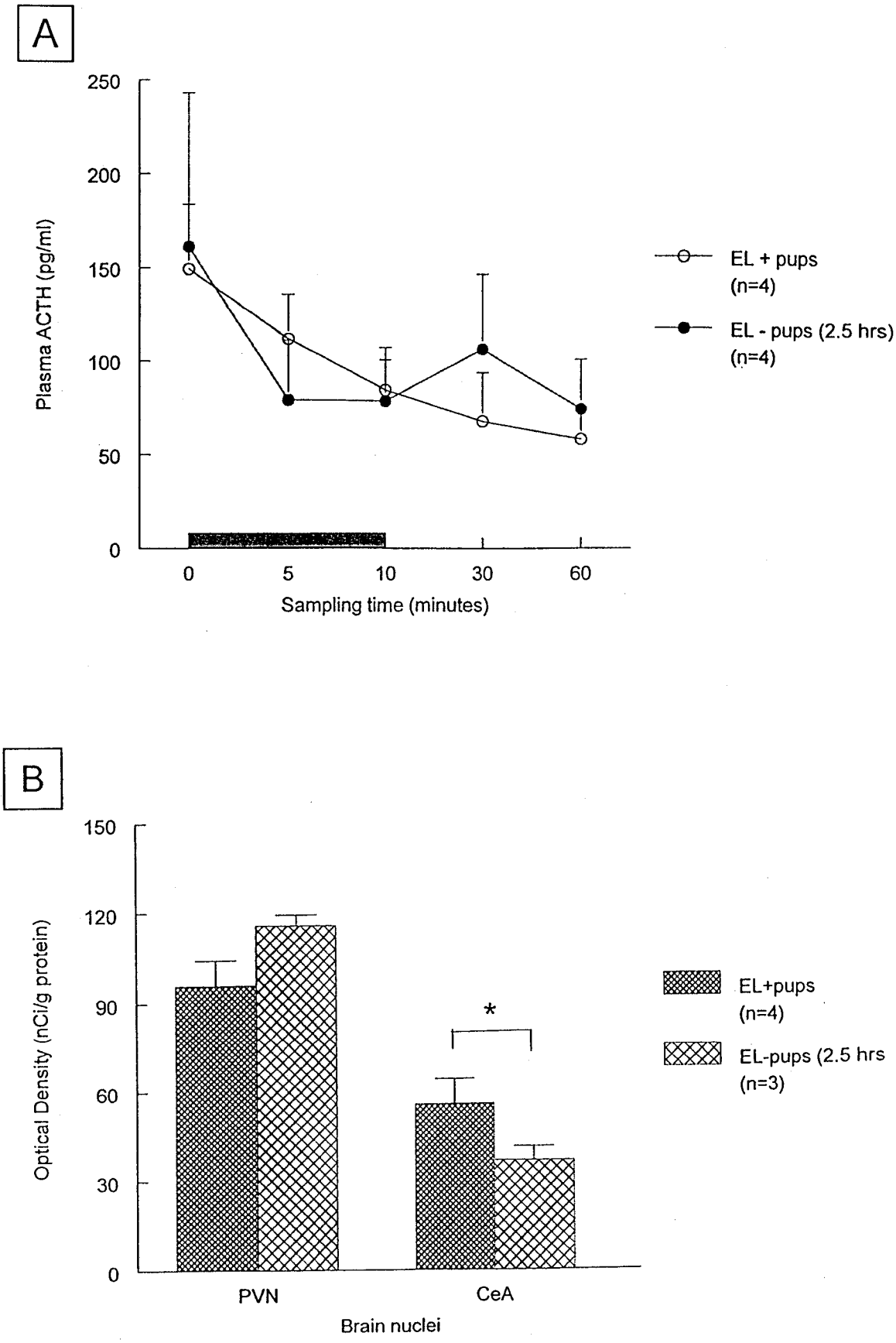


Figure 4a: Differential effects of the novel environment and fox odor on the plasma ACTH response

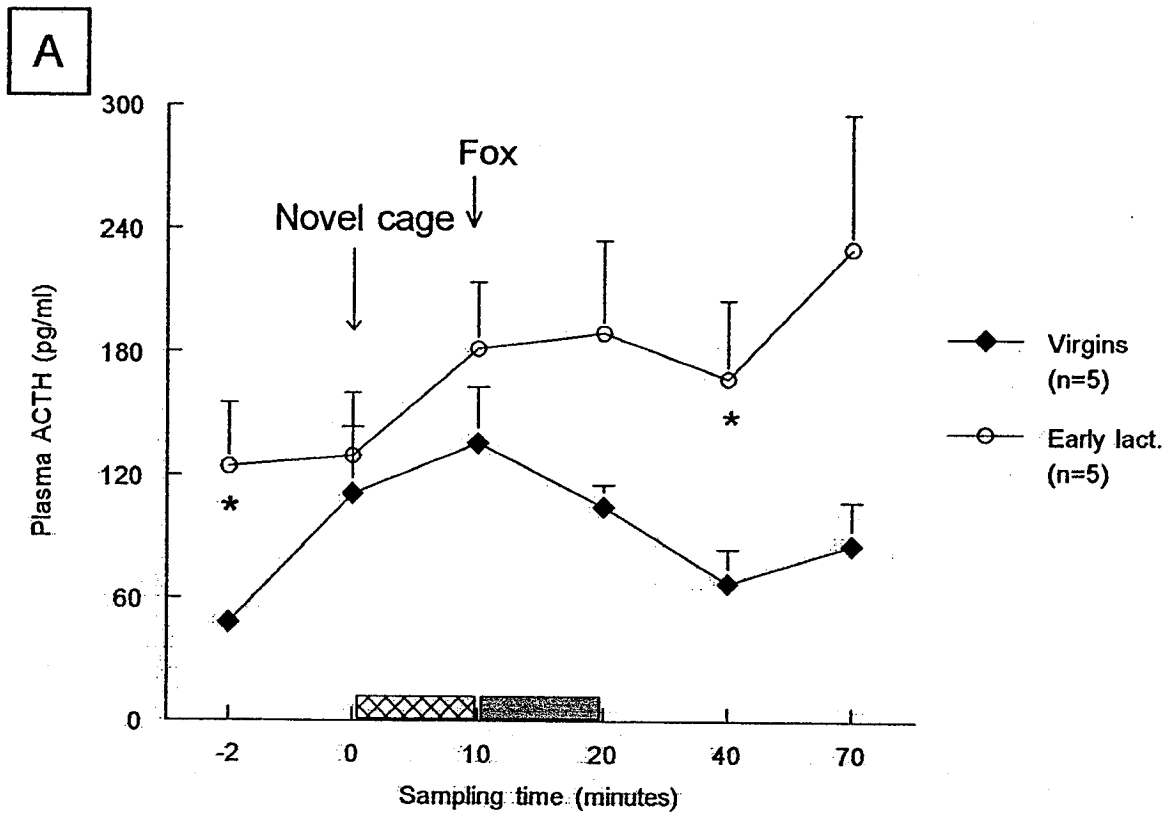


Figure 4b: Delta value for the plasma ACTH response to fox odor and novel environment

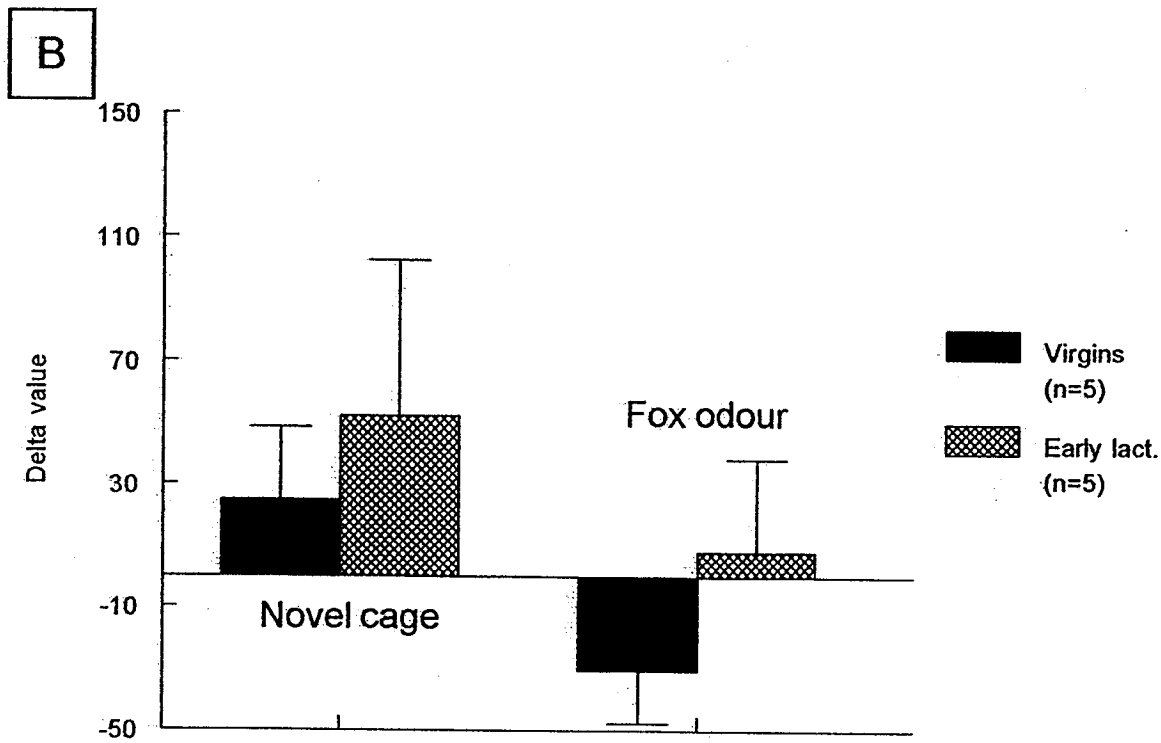
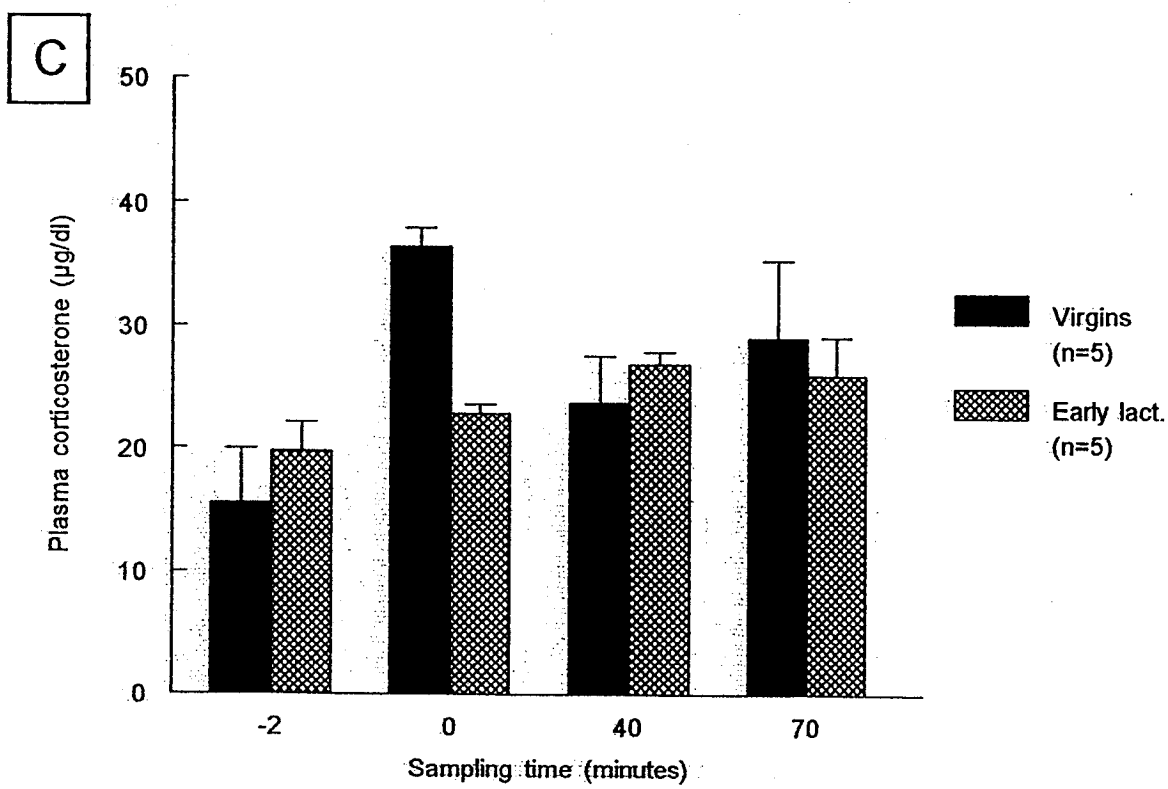


Figure 4c: Contribution of the predator odor and novel environment to the plasma corticosterone response



V. DISCUSSION AND CONCLUSION

1. Summary of findings

The hyporesponsiveness to various physical and psychological stressors during lactation is a well-known fact. In the present studies, we investigated whether this blunted stress response can be eliminated when the mother is exposed to specific stressful situations that are threatening for her litter. The most important finding of this thesis is the ability of pups to modulate the stress response in lactating females. Indeed, we found that when early lactating female rats are exposed to a psychological stressor that puts their pups in danger, they exhibit a large HPA axis response to the stressor only if their litter is present in the same environment. We suggest that by their presence, pups might affect the way the lactating females perceive the stressor. It is thus interesting to start delineating the possible role of several brain structures in modulating the effect of pups on stress responsiveness during lactation.

2. Psychological stressors

Our results are not in line with previous studies that have reported a modulating effect of the presence of pups on the endocrine response to a psychological stressor such as a noise stress. A study reported a reduced corticosterone response to a noise stress in early lactating females (PPD 2-5) tested with their pups compared to females that had been separated from their litter for 72 hours (5). This is typical of the blunted stress response observed in nursing females. In this experiment, the time of separation was relatively long (72 hours). Thus it is difficult to compare both female groups since they correspond to two different hormonal and physiological states. Moreover, in this study, the noise stress is considered as a psychological stimulus, and it probably does not represent a threat to the mothers. We suggest that if nursing females perceive the noise stimulus as a threat, they would display an elevated hormonal response

compared to females that have been separated from their litter prior to the stress. In our studies, we observed a greater hormonal (ACTH) response to the male intruder or the fox odor in mothers tested with their pups compared to females who had been separated from their litter for 2.5 or 48 hours before testing. The 2.5 hours separation paradigm allows to better compare females tested with and without their pups since in this short period of time, endocrine and physiological mechanisms are likely to remain unchanged.

Behavioral responses to a psychological stressor have also been shown to be affected by the presence of pups. In one study, early lactating females (PPD 5-6) froze markedly less in response to a sudden auditory stimulus compared to their cycling counterparts (67). This suggests first that lactating females are less fearful than virgins, an observation we documented earlier (52). However, the decrease in freezing behavior was observed only when lactating animals were in the presence of their litter at the time of stress (67), suggesting that the presence of pups modulates the behavioral response to stress. The reduction in freezing observed in this study would allow the lactating mother to pursue her maternal activities such as nesting and grooming without being disrupted by the noise stress. This diminished fear in lactating females makes ethological sense since it allows the mother to take care adequately of her pups.

In our studies, lactating females exposed to fox odor in a novel cage displayed an altered behavior compared to their cycling counterparts. Mother rats showed an increased exploratory behavior and buried more the odor-containing cup compared to virgin females. These behaviors are indicative of an increased state of stress and alertness.

Although it is difficult to dissociate the stimulatory effects of suckling (43) from the psychological effects of the presence of pups on the stress response, it is likely that a combination of both elements is responsible for the modulation of the HPA axis response to stress when the period of separation is short (2.5 hours in our experiments). Within 2.5 hours of separation, the neuroendocrine status of lactating females is likely to remain similar to that of suckled females, apart from the lower prolactin levels observed in separated females (127). Prolactin acting at the brain level might modulate the neuroendocrine and behavioral response to

stress during lactation. In a recent study by Torner et al (2002), intracerebroventricular infusion of an antisense oligonucleotide against brain prolactin-receptors enhanced the anxiety-related behavior on the elevated plus maze and increased the stress-induced ACTH release (65). These results indicate that prolactin has an anxiolytic effect during lactation and that it might be inhibitory to the HPA response to stress during the peripartum period. In our studies, we observed a larger ACTH response in females tested with their pups (with elevated prolactin in the circulation) compared to mothers tested in the absence of their litter (with reduced plasma prolactin levels). If prolactin is mediating the stress hyporesponsiveness of lactation, it would have been logical to expect the opposite. However, it is possible that this inhibition is superseded by stimulatory pathways to the hypothalamus when lactating females are exposed to a threatening emotional stressor.

Nursing females that have been separated from their litters for 48 to 72 hours are less likely to be comparable to suckled females because several neuroendocrine parameters are in the process of returning to a pre-lactational status. For instance, full normalization of morning basal levels of ACTH and B occurs 24 hours after litter removal (43) and the accumulation of CRF mRNA in the PVN following intraperitoneal saline injection in early lactating females (PPD 7) becomes only fully restored 48-72 hours after separation from the litter (45). Therefore, the variations in the stress response observed in females separated for 48-72 hours could be attributed to the absence of various olfactory, auditory and suckling stimuli provided by pups. In our studies, we used two psychological stimuli that represent ecologically salient stressors for the mother and her litter and that are likely to be processed by the amygdala. We suggest that the increased stress response observed in females tested with their pups is mainly due to the emotionally arousing effects of the stressors used.

3. Effect of maternal activities on the arousal level of lactating females

Previous reports have shown that lactating females are less emotional and fearful compared to virgin females when they are placed in a novel environment such as an open-field (52, 90). However, in our previous experiments (52), we tested lactating females in the absence of their pups and without the arousing effects of maternal activities. In our study, females that were placed in a novel cage with their pups showed increased maternal activities such as retrieval of the litter in the nest, nesting attempts and grooming. As demonstrated in a recent study by Numan et al (1995), activation of regions involved in maternal behavior such as the medial preoptic area (MPOA) and the ventral portion of the bed nucleus of stria terminalis (vBNST) occurs only when the lactating female can physically interact with her litter (104). There are reciprocal connections between the amygdala and the MPOA/vBNST and it has been demonstrated that the medial amygdala influences positively the level of activation of the MPOA/vBNST (100). The amygdala seems to play a central role in the integration of olfactory stimuli and of stressful stimuli with affective properties. More precisely, the central amygdala is involved in the processing of emotional stimuli, and it directly influences the activity of the medial amygdala which is mainly involved in processing olfactory stimulations. With respect to our results, it is possible that the pup-directed activities displayed by the mother increase her arousal level through an activation of the MPOA and vBNST. This would explain, at least in part, the elevated endocrine response following exposure to a predator odor in a novel environment.

4. Brain structures involved in the modulation of the neuroendocrine response

In light of our results, it is interesting to speculate on the differential activation of the amygdala following various stressors and on the implication of the central amygdala (CeA) in the discrimination of different stimuli. Because of its involvement in emotional processing, the amygdala could be responsible for distinguishing between stressors of different emotional values.

In our studies, we observed an increased CRF mRNA expression in the CeA of early lactating females exposed with their pups to the fox odor in the home cage compared to females tested without their litter. Even though the rise in CRF mRNA levels was observed in females tested in their home cage and not in a novel environment, the expression of CRF in the CeA might be indicative of a selective activation of this structure during exposure to threatening emotional stimuli. In addition to CRF, it is possible that variations in other neurotransmitters could also explain the difference in response observed in females exposed to a psychological stimulus in their home cage versus a novel environment. For example, levels of the neuropeptide enkephalin rise in the CeA when rats are placed in an environment that they associate with an unpleasant experience (131). Also, studies suggest that serotonin and norepinephrine might play an important role in the stimulatory effect of the CeA on the HPA axis. Depletion of norepinephrine in the CeA or administration of an a-adrenoreceptor blocker in the CeA blocks the HPA response to photic stimulation (132, 133).

The CeA is not the only nucleus of the amygdala involved in the stress response to a predator odor. The medial (MeA) and basolateral (BLA) nuclei receive more direct projections from the olfactory system (38, 111) and might therefore be directly activated after exposure to a stressful olfactory stimulation. These nuclei can also modulate the HPA axis response to stress since the BLA sends afferent projections to the CeA and the MeA sends direct and indirect inputs to the PVN. These indirect inputs are likely to be glutamatergic (150). In addition, the expression of CRF mRNA in the CeA is modulated by cortical inputs. The CeA receives massive direct excitatory glutamatergic inputs from the cerebral cortex (134) whereas the MeA is innervated mainly by the accessory and main olfactory cortex (134). It has been shown that the suckling stimulus along with the elevated progesterone levels during lactation contribute to the deficient cortical activation following NMDA stimulation. Total recovery of cortical activation was observed only after removal of both the suckling stimulus and elevated progesterone levels (135). In light of these studies, we can suggest that the hyporesponse observed during lactation might result in part from a decreased excitatory input from the

cortex to the amygdala, which would then result in a lower activation of the hypothalamic PVN.

5. Context-dependent response

The modulation of the stress response by pups does not seem to occur at all times and appears to be context dependent. Indeed, in our complementary experiments, we found that the presence of pups did not affect the adrenocortical response to fox urine when females were tested in their home cage. When the predator odor was presented in a novel environment, mothers tested in the presence of their pups responded much more than the separated females. Therefore, the context in which the stressor is administered appears to play an important role in determining the magnitude of the stress response and the pup effect.

We suggest that the context of novelty increased the emotional salience of the fox urine stressor. Consequently, lactating females would perceive the stimulus as being more threatening and would show an enhanced adrenocortical response if they are exposed to a predator odor in a novel environment. Accordingly, we found that early lactating females displayed a much larger ACTH response to the fox odor when it was presented in a novel cage compared to when it was being presented in the home cage.

Using fox urine as a stressor, we show that context is a determinant of the magnitude of the stress response and it is also critical for behaviors that are closely associated or triggered by stress, such as relapse to drug seeking behaviors. For example, it is known that intermittent footshock stress reinstates drug-taking behavior in rats, but only when the footshock is given in the environment in which the drug was previously available and not when administered in a "neutral" environment (136).

Based on studies looking at the concept of context-dependency, we could suggest that central noradrenergic activity is important in modulating the context-dependent stress response. It has been shown that activation of the central noradrenergic system is critical to relapse to drug seeking induced by stress and

that the noradrenergic activity stimulates the activity of CRF neurons in the BNST and CeA during relapse to stress-induced drug seeking (137). It is possible that the activation of the noradrenergic system is specific to the context in which the drug is administered. Similarly, it is possible that the context of novelty in our fox odor paradigm causes a specific activation of the central noradrenaline system, which would stimulate positively the CeA and BNST. This would explain in part the enhanced adrenocortical response to the fox odor in lactating females tested in a novel cage compared to females tested in their home cage. Therefore, the context in which a stress occurs or a drug is administered is critical to elicit a specific response.

6. Variations of the stress response according to the stage of lactation

Cascades of endocrine events occurring towards the end of pregnancy trigger the onset of maternal behavior. Progesterone, estrogen, prolactin and OT are the main hormones involved in initiating maternal behavior upon parturition. Plasma progesterone levels in the plasma are high throughout most of pregnancy and start to decline drastically on the 19th day of gestation to reach very low levels at parturition (138). During lactation, progesterone levels rise steadily until about day 10-12 of lactation, from which point progesterone levels begin to decrease (70). On the other hand, estrogen levels remain relatively low during pregnancy and rise around the 16th day of gestation, reaching peak levels on the last day of gestation (139). Within twenty-four hours following birth, levels of estrogen rise and then fall quickly. Then, the concentrations of estrogen in the plasma decline to levels observed in the diestrous stage (around 45 pg/ml) (140). Throughout the second half of pregnancy, prolactin levels remain low but they reach peak levels on the last two days of gestation (141). During lactation, prolactin levels in the blood rise in response to suckling by the young. Similar to prolactin, OT levels remain low throughout gestation and a surge of OT occurs upon parturition (142). During lactation, plasma levels of OT respond more closely to the intensity and frequency of suckling stimulus compared to prolactin. OT concentration rises

rapidly at the onset of the suckling period and it declines rapidly after termination of the stimulus (73).

In our studies, we observed a decrease in the neuroendocrine response according to the stage of lactation. Early lactating females showed a greater neuroendocrine response to the male intruder compared to late lactating females. Concentration of estrogen is low during lactation whereas levels of progesterone in the blood peak around day twelve of lactation from which point they start to decline. It has been demonstrated that estrogen and progesterone act in concert to facilitate the release of ACTH (124) and that prolactin is involved in the inhibition of the HPA axis response to stress during lactation (65). In rats, CRF mRNA expression in the PVN increases on the afternoon of the proestrous stage, a period where estrogen and progesterone are concentrations peak (143). This suggests that ovarian hormones are able to modulate the expression of CRF mRNA, and consequently affect the release of ACTH. Since progesterone levels start to decline after a peak on PPD 12, the stimulatory effect it exerts on the release of ACTH will be also diminished. This could explain in part why late lactating females (PPD 15) responded less to the male intruder compared to early lactating females.

In addition, behavioral changes are seen as a function of lactation. For instance, the amount of time the lactating females spend nesting decreases as lactation proceeds. This is thought to result from the fact that the need for warmth provided by the mother diminishes as pups grow because their body temperature increases (144).

Noradrenergic and adrenergic afferents arising from the medulla constitute the major excitatory fibers to the PVN (16). These inputs facilitate the HPA activity following stress in virgin females (145) but we have previously demonstrated that this facilitatory effect is not present in lactating females (63). Estrogen and progesterone are also involved in the regulation of noradrenergic transmission in the brain and especially in the hypothalamus (146). Therefore, these ovarian hormones could also modulate the activity of the HPA axis through this route.

As pups grow, the need for maternal care decreases and the suckling frequency and intensity is dampened. Therefore, the stimulatory effects of suckling on the

activity of the adrenocortical axis in the mother would be less important at later stages of lactation. It has been suggested that the mother-pup interaction may be associated with increased brain GABAergic activity (147, 148). There is evidence that the levels of GABA in the CSF vary according to the presence of pups. The concentration of GABA in the CSF was measured in lactating females before and after pup separation for 6 hours. Females that were with their litter had a very high concentration of GABA in the CSF compared to females that had been separated from their litter. When females were reunited with their pups, the concentration of GABA was restored in the CSF (149). These results suggest that pup-related stimuli may modulate the neuroendocrine and behavioral stress response of lactating females through a general enhancement of the GABAergic neurotransmission in the brain. Important GABAergic projections from forebrain limbic sites to the hypothalamus modulate HPA activity by affecting the synthesis and release of CRF from the PVN (7). An enhancement of the activity of the GABAergic system due to pup-related stimuli could result in more inhibitory GABAergic afferents to the hypothalamus. This would dampen the synthesis and release of CRF from the PVN.

7. Conclusion

Our results have demonstrated that pups are able to modulate the neuroendocrine and behavioral response to a threatening psychological stressor, in a way that results in a reversal of the stress hyporesponsiveness when lactating females are submitted to a pup-relevant stressor in the presence of their litter. Mechanisms underlying the reversal of the stress hyporesponsiveness during lactation are still unknown. Possible mechanisms include a disinhibition of the amygdala and an increase in brainstem noradrenergic (NA) inputs to the PVN. These NA inputs are known to be reduced during lactation and might underlie the blunted stress responsiveness (51, 52). It is possible that these excitatory afferents to the PVN are increased when females are submitted to a relevant emotional stressor in the presence of their litter. The inputs from the amygdala to the PVN are reduced when lactating females are exposed to a non-

threatening stimulus. The amygdala might be disinhibited when mothers are submitted to a stressor that is relevant to their pups. Finally, alterations in central GABAergic and glutamatergic systems could also explain the reversal of the stress hyporesponsiveness. This reversal of the stress hyporesponsiveness observed when lactating females are submitted to a pup-relevant psychological stressor contributes to alert her selectively to stimuli threatening her pups. We suggest that a filtering mechanism is in place during lactation, allowing the mother to attribute different salient values to the diverse stressors she is exposed to. Consequently, she would only react strongly to the stimuli she perceives as threatening. The amygdala would be a central element of this filtering mechanism, allowing the mother to perceive differentially various stimuli. This makes perfect natural sense since it allows the mother to protect adequately her pups without being distracted by environmental stresses that she perceives as unimportant.

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