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ORIGINAL PAPER

Modulatory role of locus coeruleus and estradiol on the stress response of female rats

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Abstract The activity of the hypothalamic-pituitary-adrenal axis is modulated by the norepinephrinergic system and, in females, also by the ovarian hormones. We investigated the role of ovarian steroids and the locus coeruleus (LC) on stress-induced corticosterone secretion in female rats. Ovariectomized rats without hormonal replacement (OVX) or treated with estradiol (OVE) or estradiol plus progesterone (OVEP) were subjected to jugular cannulation. Immediately after that, each hormonal treatment group was subjected to LC lesion or sham surgery or no brain surgery. After 24 h, blood samples of all 9 groups were collected before and after ether inhalation. Other four groups (OVX control, sham and lesioned, and OVE) were perfused for glucocorticoid receptor (GR) immunocytochemistry in hippocampal CA1 neurons and paraventricular nucleus (PVN). Estradiol replacement decreased while LC lesions increased stress-induced corticosterone secretion. The effect of LC lesion was potentiated with the removal of ovarian steroids. Since GR expression of lesioned animals decreased in the hippocampus, but not in PVN, we suggest that the effect of LC lesion on corticosterone secretion could be due to a reduction in the efficiency of the negative feedback system in the CA1 neurons. However, this mechanism is not involved in the estradiol modulation on corticosteroid secretion, as no change in GR expression was observed in estradiol-treated animals.

Keywords Corticosterone · Glucocorticoid receptors · Hippocampus · Ovariectomy · Paraventricular nucleus · Locus Coeruleus

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis and the central noradrenergic system are key systems for the organization of stress responses. Glucocorticoids released in response to stress exert a negative feedback response on the paraventricular nucleus (PVN) and pituitary [1]. This negative feedback, responsible for the termination of the stress response, can also be exerted indirectly through the hippocampus, which subsequently inhibits PVN neurons [2]. Although norepinephrine (NE) from the brainstem nuclei is important for glucocorticoid secretion, the pathways involved in this control are not clear. PVN is innervated by NE terminals from brainstem A1, A2, and locus coeruleus (LC) nuclei [3]. Stress induces an increase in the firing rate of LC neurons [4] as well as NE turnover in several areas innervated by their axons, including the PVN [5] and the hippocampus [6]. However, while hippocampal NE seems to arise exclusively from LC [7], the PVN is sparsely innervated by LC neurons [8] suggesting that PVN noradrenergic innervation arises primarily from

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other nuclei. In addition, although corticotrophin-releasing hormone (CRH) activates NE neurons of LC [9], the PVN is not the major source of CRH to the LC [10].

In female rats ovarian hormones can also modulate the HPA axis activity. Indeed, neurons in the PVN [11] and hippocampus [12] express estrogen receptors, and estradiol stimulates the transcription of CRH [13] and increases adrenal sensitivity to ACTH [14, 15]. Female rats show higher resting HPA axis activity [16] and a more robust responses to several stressful stimuli [17] compared to males. These gender-related differences may be due to the influence of ovarian steroids in the HPA axis. However, the effects of ovarian steroids on stress responses are still controversial. Some studies indicate a stimulatory role of estradiol, since it increases both the basal and stress-induced corticosterone secretion [18]. Moreover, ovariectomy reduces both basal [16] and stress-response levels of corticosterone and ACTH, an effect that is reversed by estradiol treatment [19]. Conversely, other studies indicate an inhibitory role for estradiol, since it decreases CRH-mRNA in the PVN [20] and CRH synthesis in the entire hypothalamus [21].

Beyond a direct effect on the HPA axis, ovarian hormones may modulate stress-responses by acting on brainstem noradrenergic nuclei, such as LC. In fact, mRNA for estradiol [22] and progesterone receptors [23] have been detected in these nuclei. In addition, LC neurons express estradiol and progesterone receptors [24] and accumulate estradiol [25]. In turn, estradiol increases the expression of both tyrosine hydroxylase and dopamine beta-hydroxylase [26] and induces changes in the number of neurons that express receptors for estradiol and progesterone during the estrous cycle in the LC. Thus, ovarian steroids may indirectly modulate the activity of HPA axis through LC.

The present study aimed to investigate the role of ovarian steroids and LC as well as their interaction on basal and stress-induced corticosterone secretion in ovariectomized rats.

Results

Corticosterone levels were measured in decapitated female OVX rats in order to determine the effect of surgical stress following jugular cannulation performed 24 h before the experiment on basal corticosterone secretion. Our results showed that there was no difference between corticosterone levels of decapitated versus cannulated OVX rats (182.5 ± 34.07 vs. 228.7 ± 23.09 ng/ml). Table 1 shows the basal corticosterone levels in all groups studied (ovariectomy and integrity of central nervous system). There were no significant changes in the corticosterone concentrations except for the lesioned OVX values, which were higher than all other groups, including the decapitated animals.

Table 1 Corticosterone secretion prior to stress in all studied groups (ovariectomy and integrity of central nervous system)

Corticosterone basal secretion (ng/ml)		
OVX	Decapitated	182.5 ± 34.07 (10)
	Intact	228.7 ± 23.09 (13)
	Sham	186.8 ± 22.00 (13)
	Lesioned	712.7 ± 33.44 (15) * $P < 0.001$
OVE	Intact	248.7 ± 39.91 (13)
	Sham	170.5 ± 45.55 (13)
	Lesioned	248.1 ± 47.61 (13)
OVEP	Intact	243.3 ± 22.41 (13)
	Sham	243.2 ± 22.41 (13)
	Lesioned	317.4 ± 39.38 (13)

OVX Ovariectomized rats, *OVE* Ovariectomized rats treated with estradiol for 3 days, *OVEP* Ovariectomized rats treated with estradiol for 3 days and progesterone on the fourth day. The number of animals is shown between parentheses

Figure 1 shows the effects of ovarian steroids on corticosterone secretion before and after stress in rats without manipulation of the central nervous system. The stress response of the OVX females was moderately higher than OVE and OVEP groups. The significant main effects for the group (OVEP, OVE, and OVX; $F_{2,34} = 5.83$), times ($F_{5,170} = 38.02$), and interaction between groups and time ($F_{10,170} = 2.93$) were determined. Post hoc Newman–

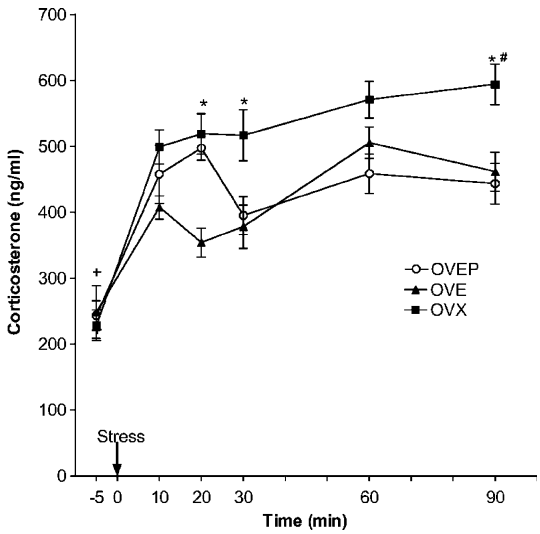

Fig. 1 Effect of ovariectomy and steroid replacement on corticosterone secretion in rats submitted to stress (1 min of exposure to ether). OVX: Ovariectomized rats; OVE: Ovariectomized rats treated with estradiol for 3 days; OVEP: Ovariectomized rats treated with estradiol for 3 days and progesterone on the fourth day. +: pre-stress vs. post-stress; *: OVX vs. OVE; #: OVX vs. OVEP. The number of animals in each group ranged from 10 to 17

Keuls test revealed that at 20, 30, and 90 min after ether stress, corticosterone secretion in OVX animals was significantly higher compared to other groups. Comparing the groups revealed that plasma corticosterone of OVX females was found to be higher than OVE group at times 20, 30, and 90 min after stress, and higher than the OVEP group only at time 90, although a slight trend ($P < 0.10$) was detected at times 30 and 60.

The localization of the LC and photomicrographs showing an intact and lesioned LC are presented in Fig. 2. Only results from animals in which LC lesion was at least 70% of the nucleus are shown. Figure 3 shows the effect of LC lesions on corticosterone secretion in three different hormonal conditions (OVEP, OVE, and OVX groups). In all hormonal conditions, LC lesion increased plasma corticosterone levels. In the OVEP group (Fig. 3a), the effects of LC lesion (corticosterone in lesioned females was higher than in sham operated ones; $F_{1,32} = 11.07$) and time (post-stress corticosterone levels were higher than pre-stress; $F_{5,160} = 19.83$) were found to be significant. However, no significant interaction was detected between LC lesion and time ($F_{5,160} = 0.62$). In the OVE group (Fig. 3b), the effects of LC lesion (corticosterone in lesioned females was higher than in sham operated ones; $F_{1,16} = 8.44$) and time (post-stress corticosterone levels were higher than pre-stress; $F_{5,80} = 10.98$) were found to be significant. However, no significant interaction was drawn between LC lesion and time ($F_{5,80} = 1.06$). In the OVX group (Fig. 3c), a clearer effect of LC lesion on plasma corticosterone before and after stress was observed. The effects of LC lesion ($F_{1,26} = 71.47$), time ($F_{5,130} = 7.51$), and interaction between LC lesion and time ($F_{5,130} = 5.38$) were shown to be significant. Because of the interaction observed in this group, the post hoc Newman–Keuls test was applied, and it revealed that within the sham operated group, all times after stress were different from the pre-stress level. However, within the LC lesioned group, plasma corticosterone before versus after stress did not differ. In OVX lesioned animals, plasma corticosterone concentration was higher than the sham operated animals at each time point.

Figure 3d represents the area under the curve (AUC) of corticosterone in all groups studied, and more clearly shows the effects observed in Figs. a, b, and c. The

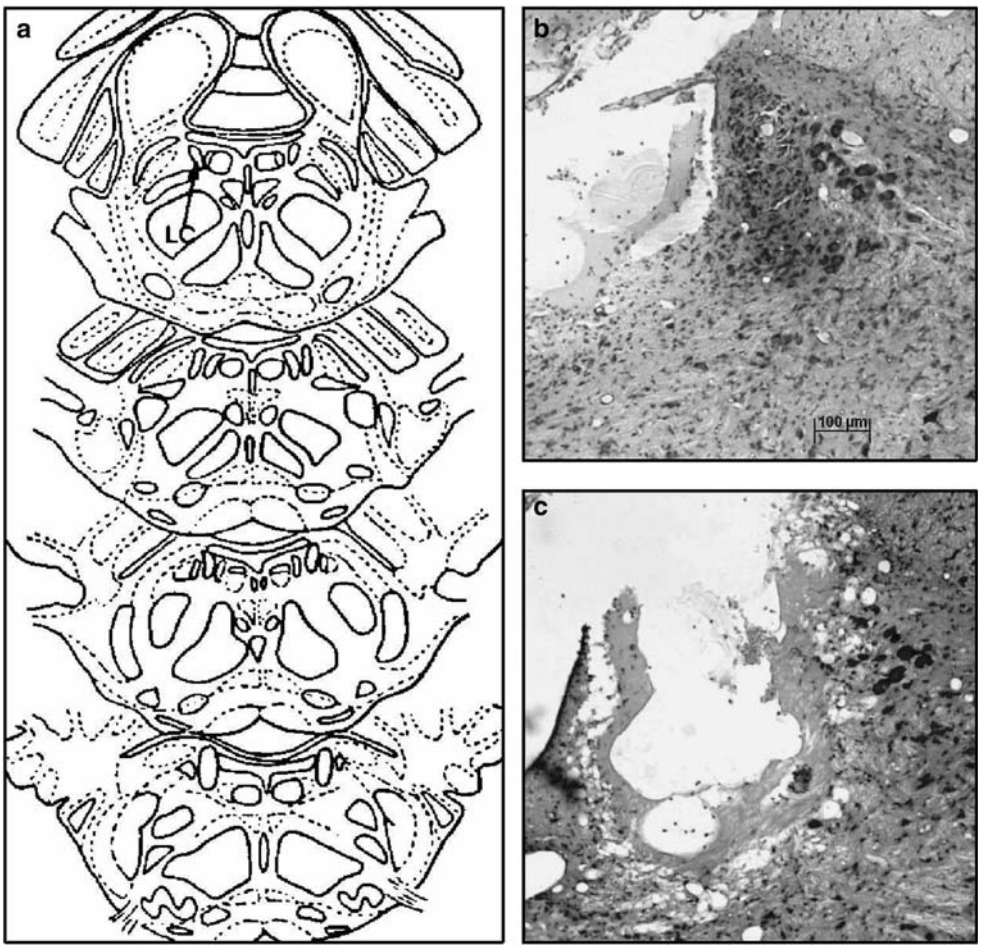

Fig. 2 Schematic drawing showing the localization of the LC (**a**) and photomicrographs with the aspect of an intact (**b**) and a lesioned nucleus (**c**)

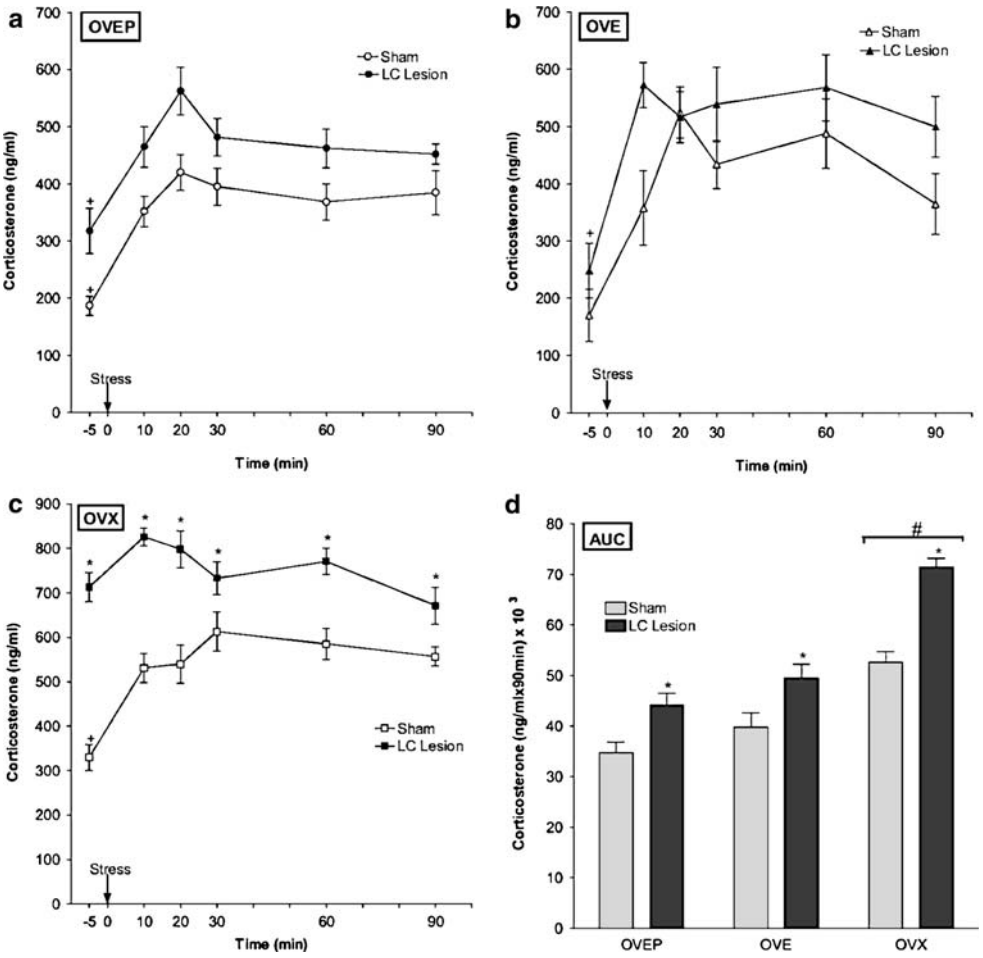

Fig. 3 Effect of locus coeruleus lesion on corticosterone secretion in ovariectomized rats submitted to stress (1 min of exposure to ether). **a** OVEP: rats treated with estradiol for 3 days and progesterone on the fourth day. **b** OVE: rats treated with estradiol for 3 days. **c** OVX: rats without steroid replacement. **d** Total corticosterone secretion represented as area under curve of **a**, **b**, and **c** graphs. +: pre-stress vs. post-stress; *: Sham vs. LC lesion; #: OVX vs. OVE and OVEP. The number of animals in each group ranged from 9 to 17

significant main effects for ovariectomy (OVEP, OVE, OVX; $F_{2,108} = 58.59$), LC lesion (sham operated and LC lesion; $F_{2,108} = 29.19$), and interaction between them ($F_{4,108} = 5.18$) are shown. A post hoc Newman–Keuls test showed that corticosterone levels were: (1) higher in the OVX group compared to the OVEP and OVE groups, and (2) higher in LC lesioned OVX, OVE, and OVEP groups than in their respective sham-operated groups.

In order to verify if the stereotaxic surgery performed 24 h before the experiment affected corticosterone secretion, we compared corticosterone basal pre-stress levels of control (no stereotaxic surgery, Fig. 1) animals with a sham lesion. The results showed that the mean ± SEM of plasma corticosterone at −5 min in the OVEP control group (243.3 ± 22.4 ng/ml) was not different (Student *t*-test) from the sham animals (186.7 ± 16.7 ng/ml). Also, in the OVE group, plasma corticosterone in control animals (248.7 ± 39.9 ng/ml) did not differ from that observed in sham animals (170.5 ± 45.5 ng/ml). However, in the OVX group, sham lesioned rats showed higher basal plasma corticosterone concentration (329.7 ± 23.3 ng/ml) compared with control animals (228.7 ± 23.1 ng/ml), although much lower than the lesioned rats. Total corticosterone secretion (measured by AUC) was not statistically different between control and sham groups in all studied conditions (data not shown).

Figure 4 shows the effect of LC lesion (OVX control, sham, and lesioned groups) and estradiol replacement (OVE group) on the number of GR-ir neurons in the PVN and hippocampal CA1 area. Representative schemes and photomicrographies from the areas utilized for counting the GR immunoreactive (GR-ir) neurons in PVN and hippocampus CA1 of an OVX-control rat are shown in Fig. 4a and 4b. In the OVX animals, LC lesion decreased the number of hippocampal CA1 GR-ir neurons ($F_{3,20} = 9.71$), but not in the PVN. However, in both areas, the number of GR-ir neurons in OVX rats with no brain surgery was not different from OVE animals (Fig. 4c and d).

Discussion

We initially tested the influence of ovarian steroids on basal and stress-induced plasma corticosterone.

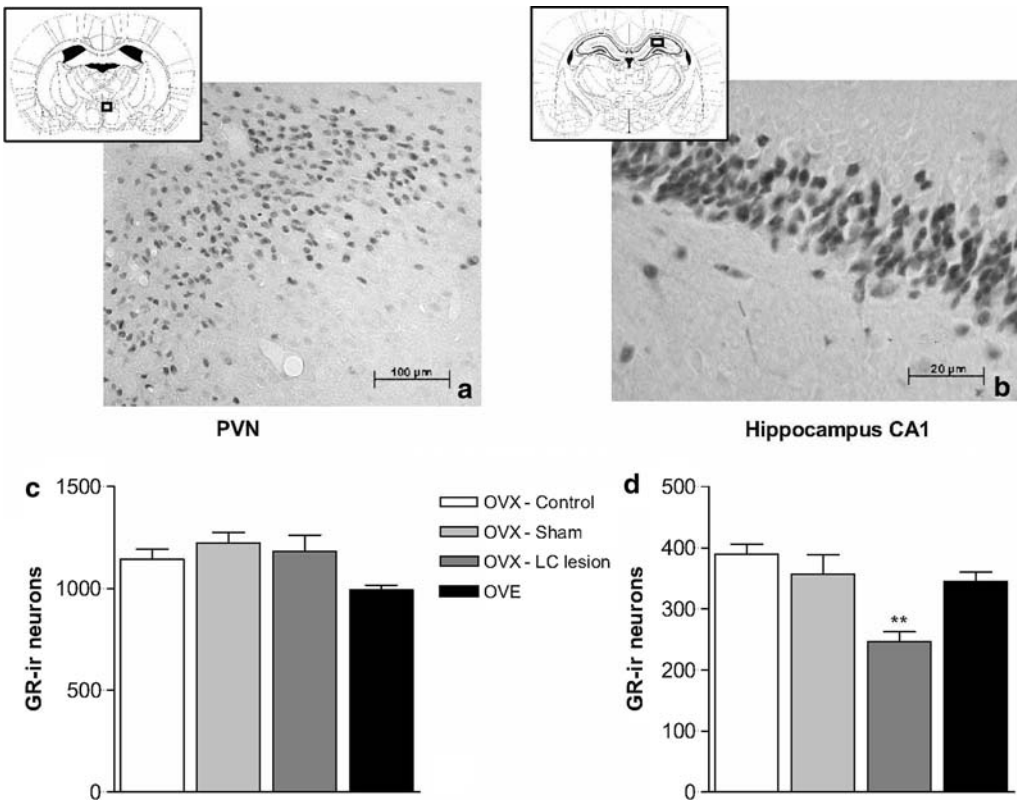

Fig. 4 Representative schemes and photomicrographies of glucocorticoid-immunoreactive (GR-ir) neurons in PVN (**a**) and hippocampus (**b**) of one ovariectomized rat. In C and D: the number of GR-ir neurons in the PVN (**c**) and CA1 region of the hippocampus (**d**) in ovariectomized animals submitted to Locus Coeruleus lesion, sham operation or without brain manipulation (control) and ovariectomized rats treated with estradiol for 3 days. **LC lesion vs. sham and control. The number of animals was 5 in all groups

Ovariectomized rats presented higher corticosterone secretion after ether stress, when compared to rats that were treated with steroid hormones, mainly estradiol treated females. Since the results observed in females treated with estradiol and progesterone were similar to those observed in females treated only with estradiol, we may conclude that the mild inhibitory effect on the corticosterone response to ether stress was mainly due to estradiol. On the other hand, progesterone does not seem to play a major role in this response.

The role of estradiol on corticosterone secretion is controversial, as studies have designated it to be both stimulatory and inhibitory. For example, Burgess and Handa described a stimulatory role for estradiol on HPA axis activity following ether stress [18]. One reason for the higher activity of the HPA axis after treatment with estradiol may be the high dose (500 µg/100 g of body weight) administered [16]. Alternatively, these results could be related to the length of the estradiol treatment. For example, a physiological dose of estradiol (75 pg/ml) applied for 21 days led to hyperactivation of the HPA axis in response to stress [18].

Conversely, inhibition of the HPA axis activity occurs when a physiological dose of estradiol is applied for periods of time less than 7 days [27]. The estradiol dose used in the present study, which is currently utilized in our laboratory, results in a plasma concentration similar to that observed in the proestrus afternoon in our animals (data not shown). Although this dose did not change basal secretion, it inhibited stress-induced corticosterone secretion. These data are strengthened by previous studies that have shown that estrogen antagonists increased corticosterone response to restraint stress [28]. Furthermore, in female mice, ovariectomy does not change basal corticosterone, but significantly increases LPS-induced corticosterone secretion, and steroid replacement is able to counter this effect [29].

We also studied the influence of LC lesion on corticosterone basal and stress-induced release. Previous studies have shown that the noradrenergic system exerts a stimulatory effect on the stress response [30], thus a decreased corticosterone response to ether stress in the LC lesioned rats would be expected. However, results of experiment II showed that LC lesion induces an overall increase of

corticosterone secretion. LC lesion in all groups induced a significant increase in corticosterone secretion that was potentiated by ovariectomy (Fig. 3d: OVX LC lesion group compared with OVX sham group). In addition, these ovariectomized lesioned rats did not show a further increase in plasma corticosterone after ether exposure, but maintained the already high corticosterone throughout the experiment. Thus, the expected stimulatory role of LC on corticosterone secretion was not observed. In fact, although LC neurons have been related to stress responses [4], we recently demonstrated that LC lesion does not affect the prolactin response to ether stress in female rats under different hormonal conditions [31]. Therefore, the role of norepinephrinergic neurons of LC on the stress response does not seem to be consistently excitatory.

Corticosterone levels did not return to those of pre-stress throughout the experiment. Two hypothesis could be raised to explain this result: (1) females are more reactive to stress than males (response to stress is stronger) and take a longer time to recover to the baseline levels [17]. and/or (2) since the corticosterone circadian increase starts at 15:00 h in our colony of rats (unpublished data of our laboratory) and the blood samples were collected from 15:00 to 16:30 h, the return to pre-stress levels of corticosterone was masked.

One could argue that the increased secretion of corticosterone is due to the stereotaxic surgery performed 24 h before the experiment. In the present study, although the OVX group showed increased basal levels of corticosterone in the sham lesioned rats compared to the control rats (with no stereotaxic surgery), no difference in basal corticosterone was observed between control and sham animals in the OVE and OVEP groups. Also, although basal corticosterone levels in the OVX sham animals were 44% higher than the OVX control levels, the corticosterone levels of OVX lesioned animals were 120% higher than the OVX sham animals. Additionally, no difference was observed when we compared the corticosterone levels of jugular cannulated rats with those of decapitated rats, demonstrating that jugular cannulation performed 24 h prior to the experiment does not influence the basal corticosterone. These data are supported by studies using animals submitted [32] or not [14] to previous surgery and suggest that the effect of LC lesion on corticosterone secretion cannot be primarily due to surgical stress.

On the other hand, it is well known that corticosterone levels are higher in females than in males [16, 17]. A recent study with ovariectomized rats [33] demonstrated that 24 h after jugular vein cannulation the basal levels of corticosterone were even higher around (400 ng/ml) than those found in our study. In fact, when compared, it is remarkable that the OVX lesioned animals have significantly more pre-stress corticosterone than all other groups, suggesting that both LC and endogenous ovarian steroids may hold the corticosterone secretion under control, maybe preventing an overreaction of HPA axis.

As described earlier, the ovariectomy or the LC lesion separately determined higher corticosterone secretion in response to the stressor and the combination of both procedures led to even further corticosterone secretion, especially the basal levels. Thus, we inferred that the glucocorticoid negative feedback was affected. Corticosteroid negative feedback of the HPA axis in stressful situations is mediated through GRs, which are located in several areas such as the hippocampus, the PVN, and the pituitary. These receptors are activated when glucocorticoid concentration is high, inhibiting CRH release [34]. This modulatory pathway is demonstrated by studies showing that the treatment of animals with a GR antagonist (RU38486) increases corticosterone secretion in response to laparotomy [35]. High corticosterone, as well as CRH and ACTH levels, has also been observed in GR knockout rats compared to wild-type animals [36], suggesting a less efficient negative feedback in the knockouts.

The PVN expresses a large amount of GR [37] and it is a target for glucocorticoids to exert negative feedback. It has also been proposed that extra-hypothalamic structures such as the hippocampus may be involved in the glucocorticoid negative feedback during stressful situations, exerting a potent inhibitory effect and thus inducing the termination of the stress response [2]. In fact, the hippocampus is known to influence the negative feedback of glucocorticoids on PVN activity during stress [38]. In the hippocampus, GR-ir cells are preferentially found in CA1, CA2, and the dentate gyrus [39]. Corticosterone infusion in the lateral ventricle activates the expression of GR in several brain areas, including the hippocampus [40]. In addition, hippocampal stimulation decreases while the lesion increases glucocorticoid secretion [41].

Thus, we tested the hypothesis that estradiol and LC neurons could inhibit corticosterone release by potentiating the negative feedback mechanism through an increase in GR synthesis. The importance of estradiol in maintaining the efficiency of the glucocorticoid negative feedback has been shown in previous studies. In males, a downregulation of GR is observed after chronic stress, although this effect is not observed in females since GR mRNA expression remains unchanged [42]. In this study, it is suggested that estradiol prevents this downregulation, since OVX rats have lower GR mRNA expression in the hippocampus. In fact, it was described that estradiol may even increase GR mRNA [27] and the expression of GR [43], which subsequently increases the efficiency of the glucocorticoid negative feedback. Besides increasing the synthesis, estradiol also increases glucocorticoid binding to GR in the

spinal cord [44] in a dose-dependent manner as low doses of estradiol facilitate GR translocation from the cytoplasm to the nucleus, while at high doses, GRs are dispersed in the cytoplasm [43]. However, we did not observe any change in the number of GR positive neurons in the hippocampus or PVN of estradiol-treated rats. A likely mechanism for this inhibitory action of estrogens on corticosterone secretion may be the decrease of CRH synthesis in the PVN caused by estradiol, which has been suggested previously [21].

Another possibility is that the increased baseline levels of corticosterone in the OVX lesioned rats could be due to a reduced action of NE in the PVN or hippocampus. In this regard, it is important to consider that there are noradrenergic inputs to PVN from several nuclei [45] and that LC has just a minor participation in these inputs, which arise mainly from A2 and A1 noradrenergic groups [46]. NE content in the medial preoptic area, medial basal hypothalamus, and the PVN 6 or 24 h after LC lesion was decreased and this decrease was much smaller in the PVN compared to other areas [47, 48]. Nevertheless, we tested the influence of the LC on the PVN by studying the effect of LC lesion on GR synthesis in the neurons of this nucleus, and found that there was no change in the number of GR-ir cells in this region (Fig. 4). This result suggests that LC lesion may not alter the negative feedback efficiency on PVN neurons and reinforces the hypothesis that the LC-PVN pathway does not seem to be directly involved in the regulation of the HPA axis.

Because the influence of LC on GR synthesis does not seem to occur through PVN neurons, this control could be exerted by the hippocampus given that, while LC weakly innervates PVN, its neurons are the sole NE source to the hippocampus [7]. In fact, since NE increases the number and the activation of GRs in this area [49], it is possible that LC neurons can influence the efficiency of hippocampal negative feedback. Although we did not measure NE content in the hippocampus after LC lesion, it is known that the lesion of the dorsal noradrenergic bundle, originated exclusively in the LC, virtually eliminates all hippocampal NE [50]. Our data from experiment III showed that LC lesion induced a decrease in the number of GR-ir in the hippocampal CA1 region. This result indicates a stimulatory role of NE from LC in hippocampal GRs synthesis and suggests that NE depletion in lesioned animals may have impaired the glucocorticoid negative feedback mechanism in the hippocampus, resulting in increased corticosterone secretion. Therefore, since NE is known to stimulate the hippocampal neurons [49] and the hippocampus inhibits PVN [2], we hypothesize that LC activation could also provoke an inhibition of the HPA axis. Apparently, this route was predominant in the present case, where rats were submitted to ether stress. However, other pathways to limbic structures, for example, may also be involved in this complex feedback control since LC has numerous efferent projections to a vast number of brain regions [51]. It is important to note that our conclusions are restricted to the effects of ether stress, since the involvement of NE on ACTH and corticosterone secretion is dependent on the nature of the stressor [52].

Based on these data, we may suggest that LC NE induces GR synthesis in the CA1 hippocampus, facilitating the glucocorticoid stimulatory action in this area, which increases the efficiency of negative feedback in PVN neurons and decreasing CRH, ACTH, and glucocorticoid release. It also seems likely that the stimulatory role of LC on hippocampus superimposes the stimulatory influence of LC on PVN. Moreover, the estrogens could act synergistically with LC, albeit through a different mechanism such as decreasing CRH synthesis in PVN [21] and ACTH secretion [14]. Therefore, the increase of corticosterone secretion becomes more evident when both the LC and estradiol are absent.

Materials and methods

Animals

Female Wistar rats weighing 250–300 g provided by the Animal Center of the Ribeirão Preto Campus—University of São Paulo, were maintained in collective cages (5 per cage) in a quiet environment in our animal facilities under controlled temperature (24 ± 0.5°C) and lighting (lights on from 6:00 to 18:00 h). Food and water were supplied ad libitum. All procedures were in accordance with the parameters established by the Brazilian Council of Animal Experimentation (COBEA).

Experimental design

Experiment I The effect of ovarian steroids on stress-induced corticosterone secretion

One week after ovariectomy, rats were divided into different groups according to the hormonal treatment to which they were assigned. (1) Primed with estradiol from the 7th to 9th day and given progesterone on the 10th day after ovariectomy (OVEP); (2) primed with estradiol over three consecutive days, from the 7th to 9th day after ovariectomy (OVE); and (3) without hormonal replacement (OVX). All rats underwent cannulation of the jugular vein 24 h before the stress response test, which began at 15:00 h on the 8th day in the OVX group and on the day of the last hormone

Table 2 Experimental design for experiment I

Group	Day 0	Day 7	Day 8	Day 9	Day 10
OVX	Ovariectomy	Jugular cannulation	Stress	–	–
			Blood sampling	–	–
OVE	Ovariectomy	17-β-estradiol	17-β-estradiol	17-β-estradiol	–
			Jugular cannulation	Stress	–
				Blood sampling	–
OVEP	Ovariectomy	17-β-estradiol	17-β-estradiol	17-β-estradiol	Progesterone
				Jugular cannulation	Stress
					Blood sampling

OVX Ovariectomized rats without hormonal replacement, *OVE* Ovariectomized rats treated with Estradiol Benzoate, *OVEP* Ovariectomized rats treated with Estradiol Benzoate + Progesterone

injection in all other groups. At this time, rats were subjected to 1 min of ether vapor inhalation in a closed container. Blood samples were collected 5 min before and 10, 20, 30, 60, and 90 min after ether exposure. Next, samples were centrifuged and plasma was separated and frozen for later corticosterone measurement. The experimental designed is summarized in Table 2. In order to verify the effect of surgical stress of jugular cannulation performed 24 h before the experiment on basal corticosterone secretion, one group of OVX rats was decapitated at 15:00 h and a trunk blood sample was taken for corticosterone measurement.

Experiment II The effect of LC lesion on stress-induced corticosterone secretion in ovariectomized rats with or without hormonal replacement

Rats were ovariectomized and divided into three groups (OVEP, OVE, and OVX) as described in Table 1. All rats underwent LC lesion or sham operation at the time of jugular cannulation and the animals were submitted to the same blood-sampling schedule described in experiment I. At the end of the experiment, the rats were decapitated and the brains from lesioned animals were removed for histological verification of the LC lesion.

Experiment III The effect of LC lesion or estradiol replacement on the number of GR positive neurons in the PVN and hippocampal CA1 region of ovariectomized rats

Rats were submitted to LC lesion or sham surgery 24 h before the perfusion of the brain, which was performed at 15:00 h on day seven after ovariectomy. In the control group, no brain surgery was performed. Another group of ovariectomized animals without any central nervous system manipulation was perfused after three consecutive days of estradiol treatment (beginning on the 7th day after ovariectomy). All brains were then removed and processed for immunocytochemistry of GR in the PVN and hippocampal CA1 region.

Anesthesia

For ovariectomy and perfusion, the animals were anaesthetized with ketamine (100 mg/kg; Agner, São Paulo, SP, Brasil) and xylazine (14 mg/kg; Coopers do Brasil, São Paulo, SP, Brasil). For jugular cannulation and stereotaxic surgeries, the animals were anesthetized with an intraperitoneal injection of 1 ml per 100 g of body weight of a 2.5% saline solution of tribromoethanol (Aldrich Chem. Comp. Inc., Milwaukee, WI, USA).

Ovariectomy

The animals underwent bilateral gonadectomy by removing the ovaries through small lateral incisions. After the suture of the incisions, the animals were treated with 0.2 ml i.m. of pentabiotics (5,000 UI; Wyeth do Brasil, São Paulo, SP, Brasil).

Hormone replacement

17-β-estradiol injections (5 μg/0.2 ml/animal s.c.; Sigma, Saint Louis, MO, USA) were performed at 09:00 h and progesterone injections (2.5 mg/0.2 ml/animal s.c.; Sigma, Saint Louis, MO, USA) at 10:00 h. The vehicle used for the steroids was corn oil.

Cannulation of the jugular vein

After anesthesia, a silastic cannula was inserted through the external jugular vein into the right atrium, as described previously [53]. The cannula was kept filled with heparinized saline until the time of blood sampling. This technique allows taking blood samples manually from the animal undisturbed and is used in the literature as a normal procedure of a blood sampling method for corticosterone [33].

LC lesion

Anesthetized rats were positioned in a stereotaxic instrument (David Kopf), with the head at the zero point on the incisor bar. The dorsal surface of the skull was exposed and 2-mm diameter holes were made bilaterally, 1.2 mm lateral to the midline and 3.4 mm caudal to the lambda. A stainless steel monopolar electrode, 200 μm in diameter and insulated except for the tip, was angled 15° (antero-posterior) and lowered 6.8 mm below the surface of the skull. A 2.0 mA constant anodal current was applied for 10 s, clamping the tail with the cathode ending. The sham animals were submitted to the same surgical procedures except that the electrode was lowered 2.0 mm dorsal to that used for lesions and current was not applied. After surgery, the electrode was removed, the skin was sutured, and the animals were placed in individual cages in a silent room for recovery.

Ether exposure

All animals were exposed for 1 min to ether vapors inhalation in a closed container with a cotton wool soaked in ether. After ether exposure, the animals were returned to their original cage.

Blood sampling

Thirty minutes before starting blood sampling, an extension was connected to the jugular cannula and animals remained undisturbed until the experiment. Five minutes before and 10, 20, 30, 60, and 90 min after ether exposure, 600 μl blood samples were collected in heparinized vials and kept on ice. After each sample, the same volume of sterile saline was injected. The blood samples were centrifuged at 4°C for 10 min, at 1200 g, and plasma was stored at −70°C, for posterior corticosterone measurement by radioimmunoassay.

Radioimmunoassay

Plasma samples were extracted using ethyl ethanol and corticosterone was measured in the resuspended pellet by radioimmunoassay. The primary antibody and standard were provided by Sigma Inc. (USA) and the ^{3}H labeled hormone was obtained from Amersham (Piscataway, NJ, USA). The lower limit for detection was 21.39 ng/ml and the intra-assay and inter-assay error were 3.9 and 14.4%, respectively.

Histological analysis of LC lesions

The extension and location of the lesions were histologically determined in all lesioned animals. After removing and fixing the brains, 13-μm-thick frontal sections were cut throughout the LC using a microtome, and were stained by the Nissl technique and examined microscopically. Only animals bearing lesions in more than 50% of the LC were used. The histological aspect of the LC lesion was similar to that previously described [48, 54].

Immunocytochemistry

Anesthetized rats were perfused through the heart with 0.01 M phosphate buffered saline (PBS), pH 7.4, and 4% paraformaldehyde (PFA). The brains were quickly removed, postfixed in 4% PFA for 2 h, and cryoprotected with 30% sucrose in 0.1 M phosphate buffer at 4°C, where they were kept for approximately 48 h. Tissue sections (30 μm) were cut on a cryostat throughout the length of the PVN and hippocampus. The sections of each area were placed in separate culture dishes with 0.01 M PBS, rinsed with 0.1 M glycine and were incubated for 30 min in 0.4% triton (TX-100), followed by 1 h in 1% hydrogen peroxide (H_2O_2), and then, incubated in buffer with 1% bovine serum albumin (BSA). The sections were incubated overnight with rabbit anti-GR M-20 (sc-1004; 0.06 μg/ml; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) in PBS containing 0.3% TX-100 and 1% BSA. After the buffer washes, sections were incubated for 1 h with biotinylated anti-rabbit goat IgG (Vectastain ABC-Peroxidase kit; Vector Laboratories, Burlingame, CA, USA) at 1:600 dilution in 0.01 M PBS, and for 30 min with avidin DH-biotinylated horseradish peroxidase complex from the same kit. The final reaction for GR was carried out with a solution containing 3,3′-diaminobenzidine-HCl (DAB 0.2 mg/ml; Sigma, St. Louis, MO, USA) and H_2O_2 (1%, Merck S.A., Rio de Janeiro, RJ, Brazil) in 0.05 M Tris–HCl buffer, pH 7.6. Sections were mounted on glass slides, air-dried, rinsed in ethanol, cleared in xylene, coverslipped with Entellan (Entellan, Merck KgaA, Darmstadt, Germany), and analyzed by light microscopy. No nuclear labeling was observed when GR primary antibody was replaced with PBS containing 0.3% TX-100 and 1% BSA, indicating the specificity of the antibodies for these proteins (data not shown).

Section analysis

The number of GR-immunoreactive (GR-ir) cells were counted in a rectangular area (800 × 700 μm^2) of PVN in five sections, and also in a rectangular area (200 × 150 μm^2) of hippocampal CA1 region in six sections, corresponding to plates 24–26 and 32–35 of the Paxinos and Watson atlas [55], respectively. The labeled cells were counted by a researcher blinded to the experimental group

and considered to be positive when the nucleus was completely stained brown. The total number of GR-ir cells of each animal in both areas was calculated and averaged per group.

Statistical analysis

In experiment I, the mean ± SEM of corticosterone plasma levels in three groups (OVEP, OVE, and OVX) and six times (5 min before, 10, 20, 30, 60, and 90 min after stress) were compared by a two-way ANOVA, for repeated measures. In experiment II, the mean ± SEM of corticosterone plasma levels in two groups (sham-surgery and LC lesion) and six times (as above) were compared by a two-way ANOVA, for repeated measures. The area under the curve for each of the six groups studied (sham and LC lesion of OVEP, OVE and OVX groups) was calculated using the mean ± SEM area under the curve of the individual animals of each group (ng/ml ×90 min) and compared by a two-way ANOVA. In experiment III, the mean ± SEM of the number of GR-ir cells counted in the PVN and CA1 in four groups (OVX without brain surgery, OVX with sham or LC lesion and OVE) was compared by a one-way ANOVA, as well as the pre-stress (basal) corticosterone levels of all groups. The Student-Newman–Keuls test for multiple comparisons was applied when appropriate following the ANOVA. For all experiments, a P-value <0.05 was considered statistically significant.

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