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RESEARCH****Research Report****Nitric oxide has a role in attenuating the neuroendocrine response to anaphylactoid stress during lactation****Nela Monasterio, Teresa Morales***

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ABSTRACT

Stress increases nitric oxide (NO) production in the paraventricular nucleus of the hypothalamus (PVH). Lactation diminishes the response to stress and increases basal NO production markers in the PVH of the dam. This study investigated whether lactation modified the anaphylactic reaction to egg white (EW) injection, and if nitric oxide regulates the neuroendocrine response to this stressor. The activational response of PVH to EW was assessed by c-Fos immunohistochemistry, and NO production was determined by histological staining of NADPH-diaphorase and neuronal nitric oxide synthase (nNOS) and by measuring the concentration of total nitrates and nitrites (NOx) in the hypothalamus of lactating and diestrus rats. EW injection significantly increased the number of Fos-positive neurons in the parvocellular subdivision of the PVH in diestrus, but not in lactating rats. Similarly, EW injection increased the number of NADPH-diaphorase- and nNOS-positive cells in the PVH of diestrus rats, but it did not alter the already increased basal number of NO-positive cells in lactating rats. Furthermore, the total concentration of NOx in the hypothalamus, the circulating level of corticosterone and interleukin-6 increased significantly after EW in diestrus, but not in lactating rats, compared to their corresponding controls. Intracerebral administration of L-NAME, a general NOS inhibitor, reversed the attenuation of the activational response to EW in the PVH of lactating rats. The present results show that lactation diminishes the anaphylactoid reaction to EW compared to that in diestrus rats. This attenuation was absent after L-NAME treatment, suggesting that sustained NO production in the PVH during lactation may limit the neuroendocrine response to stress.

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1. Introduction

Acute exposure to stress normally initiates a cascade of reactions that activates various brain regions, triggers hormone secretion, and generates chemical responses, including immune responses that mediate the body's coping mechanisms

(McEwen, 1998). For this, activation of the hypothalamo-pituitary-adrenal (HPA) axis is crucial, and the paraventricular nucleus (PVH) is the hypothalamic element that serves as an integrative center (Sawchenko et al., 1996). The PVH is a complex nucleus subdivided into parvocellular and magnocellular parts with neurons projecting to the median eminence, the posterior

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pituitary lobe, and other CNS regions including autonomic areas. The activational response of the HPA axis involves cells located within the parvocellular subdivision of the PVH and is influenced by the reproductive state of females. During late pregnancy and lactation the classical neuroendocrine stress response of the HPA axis to a variety of stressors is diminished (Da Costa et al., 1997; Higuchi et al., 1989; Lightman and Young, 1989; Shanks et al., 1997, 1999; Stern et al., 1973; Thoman et al., 1970; Windle et al., 1997). The reduced activity of afferent pathways to the PVH (Toufexis et al., 1998) and the differential modulation of receptor-mediated PVH activation (Fenelon et al., 1993) explain in part this hypo-responsiveness occurring during lactation. Also, the PVH of lactating dams undergoes both morphological and neurochemical changes (Johnstone et al., 2000), including a suckling-dependent increase of NADPH-diaphorase (Otukonyong et al., 2000; Popeski et al., 1999), which is commonly used as an activational marker for neuronal nitric oxide synthase (nNOS) (Ceccatelli and Eriksson, 1993), the principal enzyme that synthesizes NO in the nervous system.

Besides the neuroendocrine stress response, lactation modifies the immune activation induced by stress (Shanks et al., 1997), suggesting that the bidirectional communication between the immune and endocrine systems is modified by reproduction (Mouihate et al., 2008). The stress response of lactating dams to immune challenges has been studied in an experimental model of infection by using lipopolysaccharide injection (Popeski et al., 1999). Another experimental model of immune stress is the systemic injection of egg white (EW),

which induces anaphylactic reactions (Földes et al., 2000; Selye, 1949). In male rats, EW injection activates the HPA axis, hypophysiotropic neurons of the PVH, and circuits providing afferent control to neurons related to hypothalamic stress and/or involved in central autonomic regulation (Földes et al., 2000). This model of anaphylaxis is a systemic allergic reaction, and the response involves cellular and humoral immunity (Elenkov and Chrousos, 1999, 2006). Systemic allergic reactions are triggered when allergens cross-link preformed IgE bound to the high-affinity receptor FcεR1 on mast cells (Rozniecki et al., 1999). Once activated, mast cells release mediators that cause capillary leakage, edema, and smooth muscle contraction, which collectively may lead to severe symptoms of anaphylactic shock (Ewan, 1998; Silver et al., 1996). This syndrome with all of the symptoms of anaphylaxis induced by systemic injection of EW (Földes et al., 2000) has been referred to as the anaphylactoid reaction (Selye, 1949), because it occurs after the primary exposure to foreign protein and is characterized by edema in the paws and legs and around the snout and pruritus (Kovács, 2002).

NO is synthesized by a number of NOS isoenzymes, which catalyze the oxidation of L-arginine to NO and citrulline. NOS is expressed in hypothalamic cells, including both magnocellular and parvocellular neurons of the PVH. NADPH-diaphorase and NOS mRNA increase in the PVH of male or cycling-female rats following osmotic stimuli, or neurogenic or physiological stress (Calka et al., 1994; Calka and Block, 1993; Calza et al., 1993; Kadowaki et al., 1994; Okamura et al., 1994). Moreover, increased

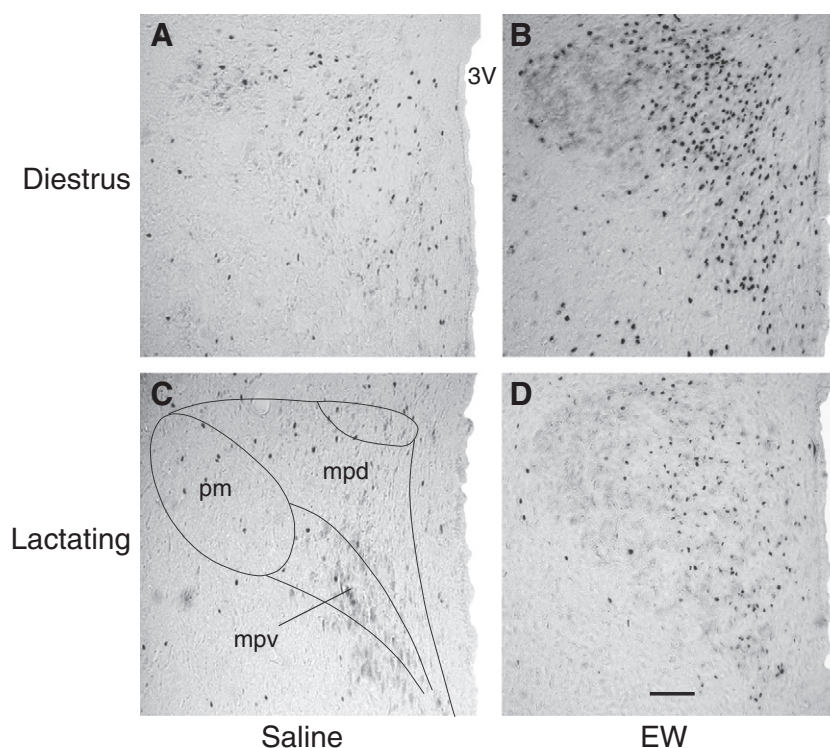


Fig. 1 – Anatomical localization of activational Fos response induced by immune challenge in the PVH of diestrus and lactating rats. Bright-field microphotographs showing c-Fos-immunoreactive cell nuclei in saline- (A, C) or EW-injected (B, D) rats. Immune stress caused cell activation mainly in the parvocellular subdivision of the PVH of diestrus and lactating rats (B, D). Abbreviations: 3 V, third ventricle; mpd, dorsal zone of the medial parvocellular subdivision; mpv, ventral zone of the medial parvocellular subdivision; pm, posterior magnocellular. Scale bar: 100 μ m.

Table 1 – Number of Fos-positive cells in the PVH.

	Parvocellular PVH subdivision					Magnocellular PVH subdivision				
	Saline (ip)	Egg white	Saline (icv)	LNAME	LNAME +EW	Saline (ip)	Egg white	Saline (icv)	LNAME	LNAME +EW
Diestrus	14.9±2.3	201.3±11.8 ^{a,b}	28.8±7.3	166.2±24.7 ^{a,b}	64±4.3 ^a	6.4±0.7	16.2±1.8 ^{a,b}	7.2±1	53.2±5.6 ^{a,b}	34.77±4.0 ^{a,b}
Lactating	40.8±12.1	80.3±9.8 ^a	56.5±12.7	62.5±10.2	47.2±4.6	5.3±1.3	9.1±1.5	14.8±2.5	30.8±2.7 ^{a,b}	25.15±3.3 ^{a,b}

Values represent mean±SEM; p<0.01, n=6.
^a Significantly different from their corresponding control (p<0.01).
^b Significantly different from egg white-treated lactating group (p<0.01). Analysis of variance.

NO markers have been documented in the PVH after an immunological stress (Rivier and Shen, 1994). However, the role of NO in the neuroendocrine stress response is not clear (Orlando et al., 2008). Blockade of NO generation by NOS antagonists markedly affects the ACTH and corticosterone response to stress, and both inhibitory and stimulatory effects of NO on the HPA axis have been reported (Gadek-Michalska and Bugajski, 2008; Masood et al., 2003, 2004; O'Shea and Gundlach, 1996; Okere et al., 1999; Popeski and Woodside, 2001; Summy-Long et al., 1993; Yasin et al., 1993). Of particular interest is the unexpected expression of NO markers, such as NADPH-diaphorase, in the PVH of lactating dams, since these animals have a diminished response to virtually any stress stimulus (Lightman et al., 2001; Neumann, 2001; Walker et al., 1995).

Given the neuroendocrine and immune alterations occurring during lactation, the aim of this study was to investigate the influence of NO on the activational response of the PVH to an immunological challenge, and to elucidate whether the already high expression of the NO-markers, nNOS and its functional indicator NADPH-diaphorase, in the PVH of lactating rats is diminishing the neuroendocrine response to the immune stress.

2. Results

2.1. Fos expression in the PVH induced by anaphylactoid challenge

Fos activation after EW treatment was localized to the parvocellular subdivision of the PVH of diestrus and lactating rats (Figs. 1B,D). Control animals, either diestrus or lactating, showed scattered Fos-ir throughout the PVH (Figs. 1A,C). The parvocellular subdivision of the PVH of diestrus rats showed a strong activational response with an increase of 13.4 fold (p<0.01), whereas in lactating rats the increase was 2.0 fold (p<0.01) relative to the corresponding control (Table 1). The Fos activation induced by EW in the PVH of lactating rats was significantly lower than that of EW-treated diestrus rats (p<0.01). The intracerebroventricular (icv) injection of the NOS inhibitor L-NAME into diestrus rats increased the number of c-Fos-positive profiles by 5.7 fold (p<0.01) compared to the icv-saline-injected diestrus group. EW-treatment after L-NAME decreased, instead of further increasing, Fos activation, which was even below (p<0.01) the amount shown by EW-injected diestrus rats. None of the various treatments significantly

changed the number of activated cells in the parvocellular-PVH of lactating rats (Table 1).

In the magnocellular subdivision of the PVH, which contains oxytocin and vasopressin neurons, the increase in the number of Fos positive cells after EW was 2.5-fold in the

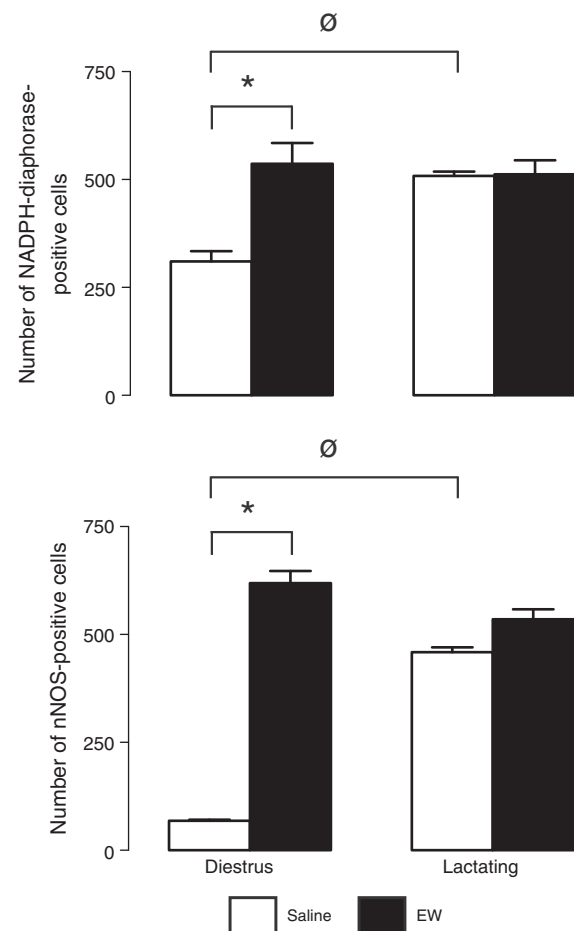


Fig. 2 – Number of NADPH-diaphorase- and nNOS-positive cells detected in the PVH after immune challenge. The PVH of diestrus rats showed increased NADPH-diaphorase and nNOS label after EW injection compared to the corresponding control; no change was observed in lactating rats. Values represent mean±SEM. *Significantly different from corresponding control (p<0.005–0.001, n=6); Ø significantly different from diestrus control group (p<0.05–0.005).

diestrus group ($p < 0.01$), while in the EW-lactating group there was no significant difference compared to corresponding ip-saline controls. The number of Fos positive profiles in the magnocellular subdivision of the PVH of lactating rats was increased more by icv-saline than by ip-saline injection ($p < 0.05$). L-NAME administration alone, or before EW challenge increased Fos in the magnocellular-PVH of both groups ($p < 0.01$), in comparison to the corresponding icv-saline-

injected rats. This increase was more pronounced in the diestrus (7.4 fold in LNAME, 4.8 fold in LNAME+EW) than in the lactating animals (2.1 fold in LNAME, 1.7 fold in LNAME+EW). Interestingly, in both lactating and diestrus rats, the Fos expression induced by EW occurred largely (~90%) in the parvocellular subdivision of the PVH, the known location of corticotropin-releasing hormone (CRH)-containing, stress-related neurosecretory cells (Table 1).

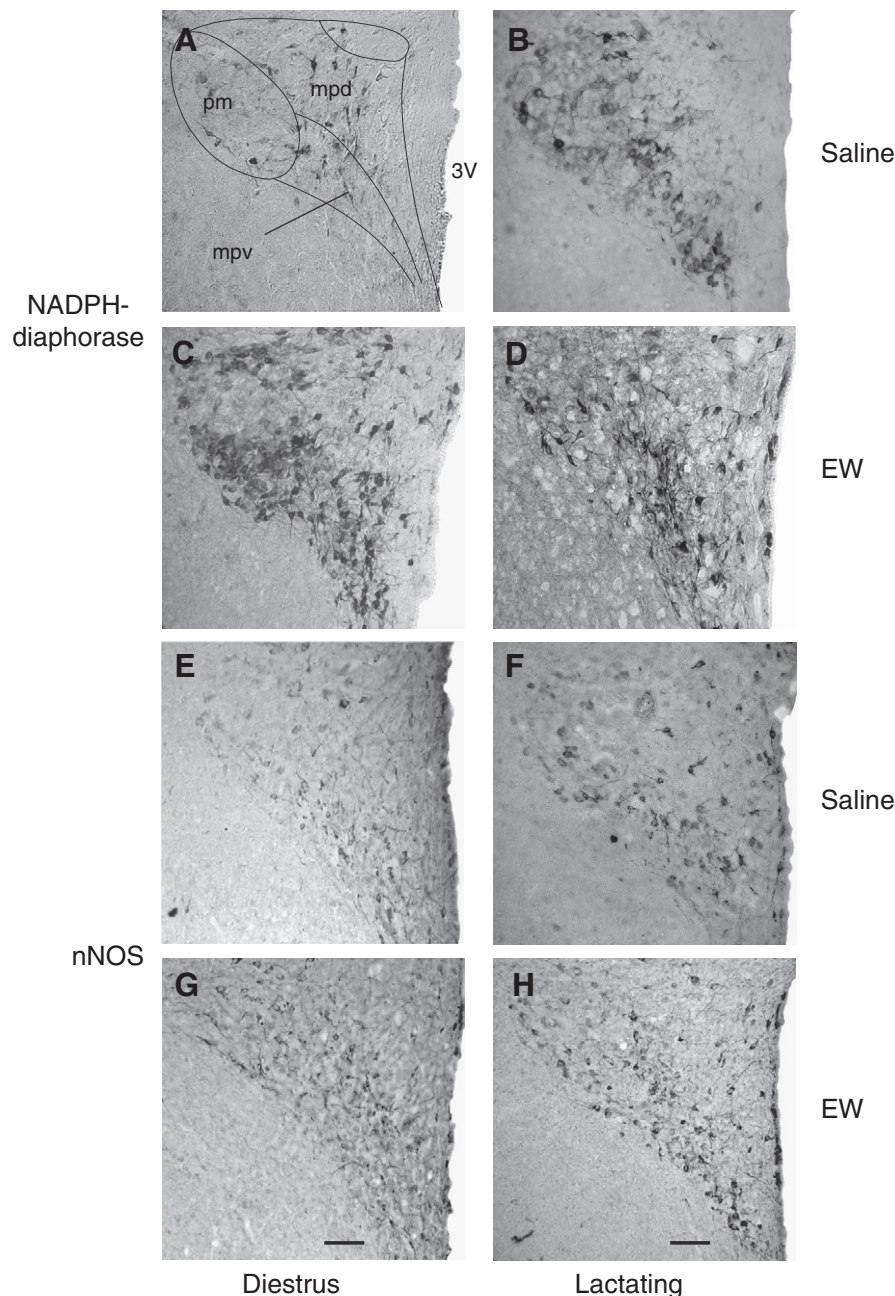


Fig. 3 – Representative microphotographs of the PVH of diestrus and lactating rats after saline (A, B, E, F) or EW injection (C, D, G, H). Staining for NADPH-diaphorase was localized in the ventral and dorsal zones of the medial parvocellular subdivision of the PVH of diestrus rats (A, C), and it increased after EW treatment but did not change in lactating (B, D) rats. The anatomical location of nNOS expression in the PVH was similar to that of NADPH-diaphorase, and changes in nNOS-positive cells displayed a pattern similar to that of the diaphorase label. *Abbreviations:* 3 V, third ventricle; mpd, dorsal part of the medial parvocellular subdivision; mpv, ventral part of the medial parvocellular subdivision; pm, posterior magnocellular. *Scale bar:* 100 μ m.

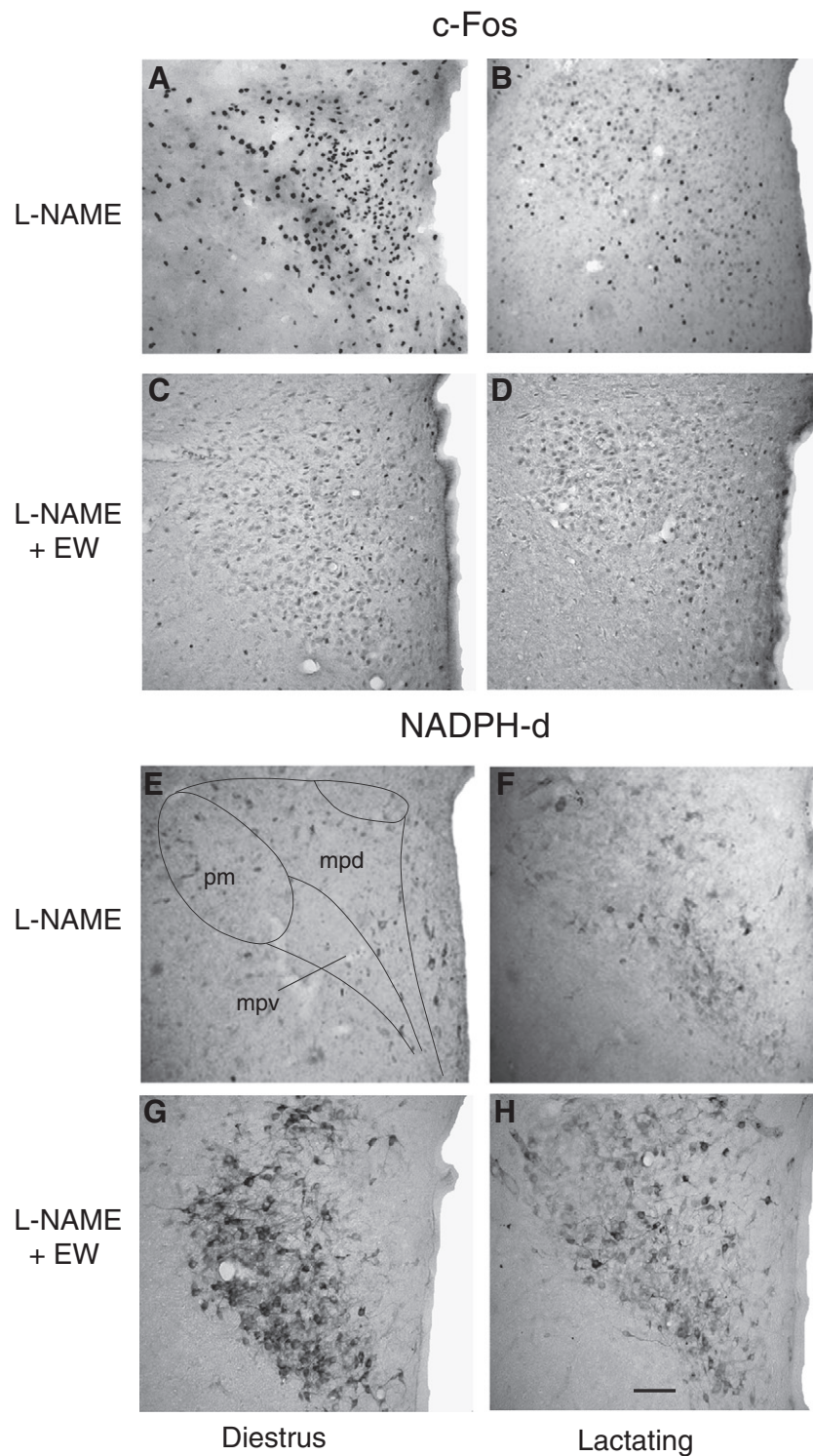


Fig. 4 – Representative microphotographs of the PVH of diestrus and lactating rats after icv L-NAME (A–D), or icv L-NAME followed by EW injection 24 h later (E–H). L-NAME alone induced Fos expression (A, B) and decreased NADPH-diaphorase (NADPH-d) staining in the PVH of diestrus and lactating rats (E, F). A Fos response (C, D) and a strong NADPH-diaphorase signal (G, H) were observed in diestrus and lactating rats treated with L-NAME and EW. Abbreviations: 3 V, third ventricle; mpd, dorsal part of the medial parvocellular subdivision; mpv, ventral part of the medial parvocellular subdivision; pm, posterior magnocellular. Scale bar: 100 μ m.

2.2. NADPH-diaphorase and nNOS after EW

The number of NADPH-diaphorase-positive cells ($p < 0.05$) and of nNOS-positive neurons ($p < 0.001$) increased significantly in the PVH of diestrus rats after EW injection as compared to their respective controls (Fig. 2). In lactating rats, EW injection did not modify the number of diaphorase-stained or nNOS-positive cells in the PVH. Further-

more, the number of diaphorase- and of nNOS-labeled cells in the PVH of lactating rats that received saline was significantly greater than in the diestrus control group ($p < 0.01$; $p < 0.05$, respectively, Fig. 2).

NADPH-diaphorase-labeled cells were located mainly in the ventral zone of the medial parvocellular part (Fig. 3B) of the lactating control group, while diestrus rats showed only a few stained cells in the dorsal zone of the medial

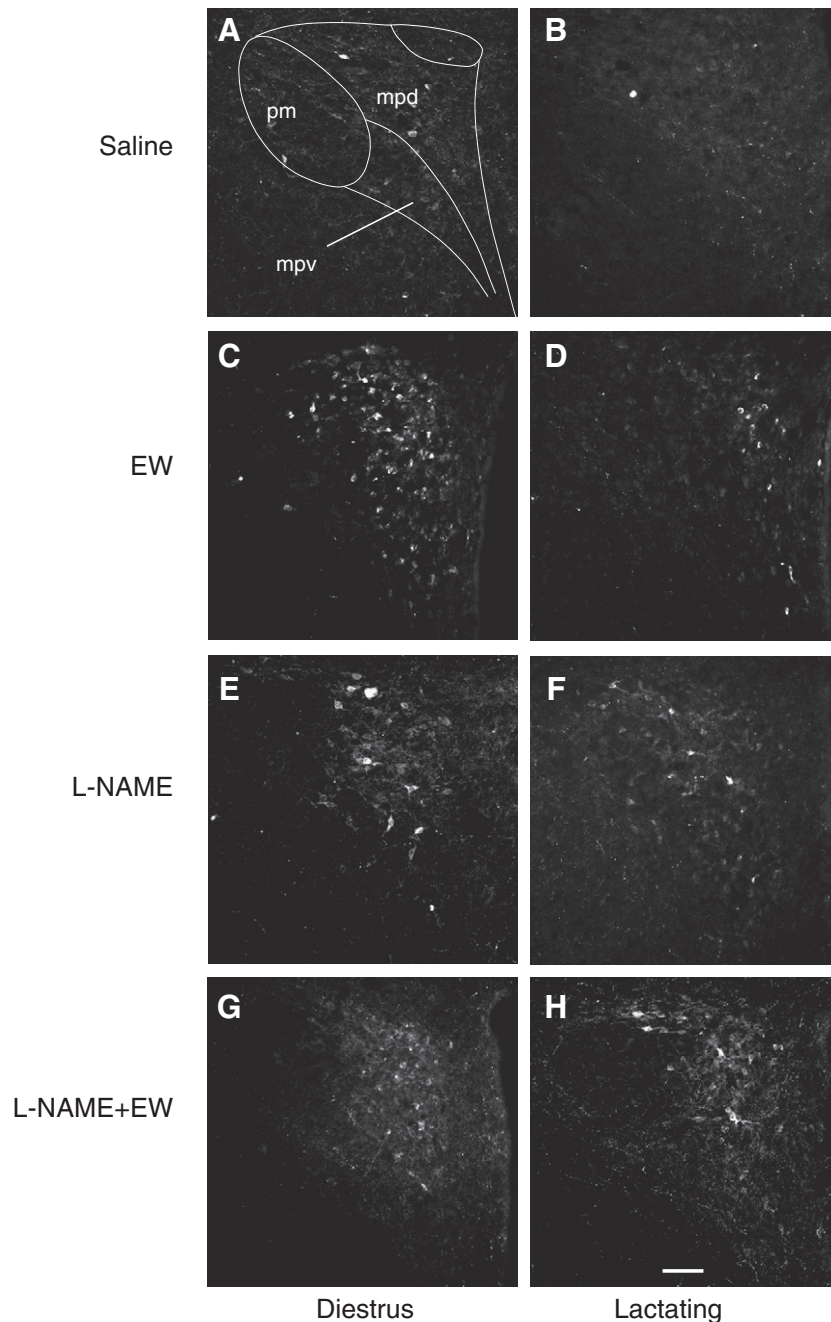


Fig. 5 – Representative microphotographs showing CRH immunostaining in the PVH of diestrus and lactating rats after NO inhibition. In diestrus animals EW injection increased CRH expression in comparison to their controls (A, C), while it had little effect in lactating rats (B, D). L-NAME icv injection augmented the number of CRH-stained neurons in the PVH of both groups of rats compared to their controls (E, F). L-NAME followed by EW injection induced the greatest increase in CRH stained neurons in PVH of lactating rats (H) giving a pattern similar to that observed in diestrus rats (G). *Abbreviations:* mpd, dorsal part of the medial parvocellular subdivision; mpv, ventral part of the medial parvocellular subdivision; pm, posterior magnocellular. Scale bar: 100 μ m.

parvocellular part (Fig. 3A). Cells densely stained for this NO-marker were observed in the ventral zone of the parvocellular part of the PVH of the rats in the two physiological conditions after immune challenge (Figs. 3C, D). Only a few NADPH-diaphorase-positive neurons were found in the magnocellular part of the PVH of lactating rats (Figs. 3B,D). A similar distribution of the nNOS-positive cells was observed in the PVH of lactating and diestrus animals corresponding to the ventral and dorsal zones of the medial parvocellular part of the PVH (Figs. 3E–H).

2.3. L-NAME effects on c-Fos, NADPH-diaphorase, and CRH distribution in the PVH

The icv injection of L-NAME induced c-Fos expression (Figs. 4A,B) in both subdivisions of the PVH in both diestrus and lactating rats. This NO-blocker was effective in diminishing NADPH-diaphorase staining in the PVH, regardless of the hormonal condition (Figs. 4E,F). An EW challenge given 24 h after icv L-NAME induced c-Fos-positive signal in the PVH of rats in both hormonal stages (Figs. 4C,D), but this response was different depending on the subdivision of the PVH. In diestrus rats, both parvo- and magnocellular subdivisions exhibited Fos activation after icv L-NAME, whereas Fos signal increased only in the magnocellular part of the PVH of lactating rats. In spite of icv L-NAME, EW treatment largely restored the NADPH-diaphorase staining in the PVH of diestrus and lactating rats (Figs. 4G,H).

With respect to CRH induction by the immune challenge, the PVH of diestrus rats showed an increase in CRH immunostaining in response to EW compared to their control group (Figs. 5A,C). In lactating rats, EW treatment caused a minimal increase of CRH staining in comparison to the corresponding control group (Figs. 5B,D), in accordance with what has been previously shown for other stressors (Lightman and Young, 1989; Shanks et al., 1999; Stern et al., 1973; Thoman et al., 1970; Windle et al., 1997). L-NAME-injected animals displayed greater CRH staining in the PVH relative to the controls for both hormonal conditions (Fig. 5E), and it was more evident in the lactating rats (Fig. 5F). Also, CRH staining in the PVH of lactating rats increased when EW was injected after L-NAME (Fig. 5H), and it resembled the pattern shown by L-NAME+EW-injected and EW-injected diestrus rats (Fig. 5C,G). Furthermore, the PVH of L-NAME+EW diestrus animals (Fig. 5G) exhibited less CRH-positive label than the PVH of diestrus EW-treated rats (Fig. 5C).

2.4. Effect of EW and nitric oxide inhibition on total hypothalamic nitrates and nitrites (NOx)

In order to have a better sense of the EW-induced changes in NO, the levels of total nitrates and nitrites (NOx) were assessed in supernatants from homogenates of the whole hypothalamus as a general indicator of total NO production. In agreement with our observations described above, NOx levels were enhanced after EW injection in the hypothalamus of diestrus, but not of lactating rats (Fig. 6). The EW injection significantly increased the amount of total NOx in the hypothalamus supernatants of diestrus rats compared to all the other diestrus groups (saline, sham-operated, L-NAME and L-NAME+EW) ($p < 0.01$, Fig. 6). L-NAME treatment of

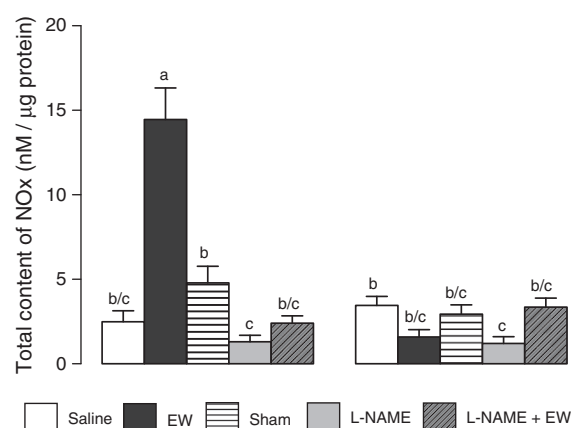


Fig. 6 – Effect of EW and L-NAME on the total content of hypothalamic nitrates/nitrites (NOx). EW injection increased the concentration of total hypothalamic NOx above the level present in diestrus animals receiving the various other treatments. In contrast, values of the NOx content in the hypothalamus of lactating rats were as low as in the control or L-NAME-treated diestrus rats; EW or L-NAME alone further decreased the hypothalamic NOx values in lactating rats, but not in L-NAME+EW rats. Values represent mean $\pm$ SEM of NOx in nM/ μ g protein; $p < 0.01$ – 0.05 , $a \neq b \neq c$.

diestrus rats lowered the hypothalamic NOx concentration in comparison to their sham control ($p < 0.05$, Fig. 6), but not compared to L-NAME+EW treated rats. On the other hand, in lactating rats hypothalamic NOx decreased after EW treatment compared to the corresponding saline group ($p < 0.05$). Injection of L-NAME into lactating dams reduced the total hypothalamic NOx concentration in comparison to the sham control ($p < 0.05$), and surprisingly total hypothalamic NOx was restored to the basal level in the lactating L-NAME+EW group (Fig. 6).

2.5. Corticosterone levels in response to EW challenge

The basal level of circulating corticosterone was higher in lactating than in the diestrus rats ($p < 0.05$; Fig. 7). EW treatment did not alter corticosterone levels in lactating rats but did increase them in diestrus animals relative to their respective saline controls ($p < 0.01$). The icv injection of L-NAME provoked a rise in corticosterone levels of both lactating and diestrus groups in comparison to their corresponding control or EW-treated group ($p < 0.05$; Fig. 7). Diestrus rats treated with EW after L-NAME had the same corticosterone plasma concentration as those treated with L-NAME alone, but in lactating rats EW+L-NAME lowered corticosterone to the level observed in the control or EW-treated groups ($p < 0.05$; Fig. 7).

2.6. Changes in circulating levels of IL-6 induced by EW

To determine the magnitude of the peripheral inflammatory response caused by EW, the plasma concentration of interleukin 6 (IL-6) was determined in samples collected at the time of sacrifice. EW challenge alone or after L-NAME,

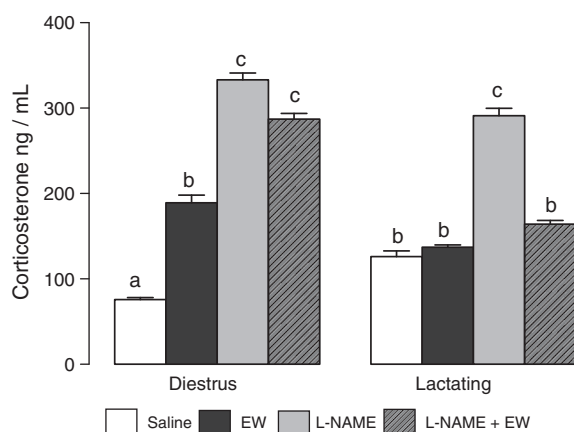


Fig. 7 – Basal and stimulated circulating corticosterone concentration. Immune stress induced by EW injection elicited higher circulating corticosterone levels in diestrus rats. The basal corticosterone concentration was higher in lactating than in diestrus rats, and did not change after immune challenge. L-NAME icv administration induced an increase over corresponding controls in both groups. When L-NAME was given before EW challenge only diestrus animals displayed a rise in corticosterone when compared to controls. Values represent mean $\pm$ SEM; $p < 0.01$, $a \neq b \neq c$.

significantly increased IL-6 levels in the diestrus rats in comparison to the corresponding control ($p < 0.05$). Lactating rats did not show significant changes in the circulating level of IL-6 after EW treatment (Fig. 8), but this level increased in response to icv L-NAME ($p < 0.05$), and increased further in L-NAME+EW in comparison to the lactating saline group ($p < 0.01$; Fig. 8).

3. Discussion

The present report provides evidence that lactation suppresses HPA axis responsiveness to anaphylactoid reactions in comparison to diestrus rats and that NO has a role in this suppression. In diestrus rats, EW induced an intense anaphylactoid reaction including edema in the paws and legs and around the snout, along with significantly greater Fos activation (Monasterio et al., 2008), nNOS and NADPH-diaphorase staining within the PVH, higher hypothalamic NOx, and corticosterone and IL-6 plasma concentration in comparison to their controls. The same stimulus resulted in a markedly reduced cellular activation in the PVH of lactating rats, and no change in their plasma corticosterone or IL-6 concentration, and minor visible symptoms of an evident anaphylactoid reaction, such as edema in the paws and snout. This difference in the reactivity to immune stress between the two conditions could be explained by the lack of IL-6 response to EW during lactation, suggesting that lactation affects some but not all of the possible mediators of the afferent pathways to the PVH.

The intensity of the Fos activational response to EW varied depending on the reproductive status of the female rats; however, the response to EW was located within the parvocel-

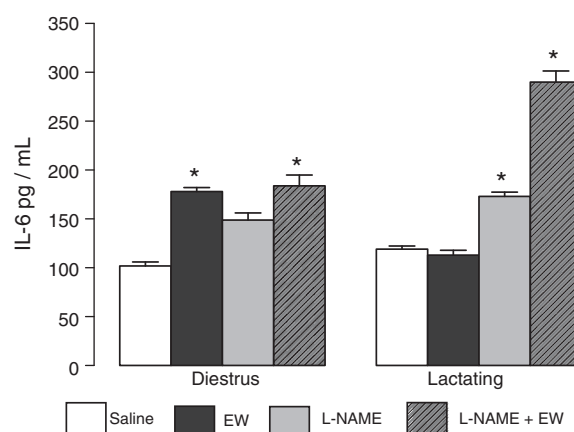


Fig. 8 – Basal and stimulated interleukin-6 (IL-6) circulating concentration. Immune challenge increased the circulating concentration of IL-6 in diestrus rats in comparison to their control. No significant change in IL-6 concentration was detected after EW treatment in lactating rats. Administration of L-NAME induced an increase in IL-6 in lactating but not in diestrus rats. When L-NAME was administered before EW, cytokine level increased over and above the level observed with L-NAME alone in lactating animals, and diestrus rats also showed a significant increase over the controls. Values represent mean $\pm$ SEM. *Significantly different from corresponding control ($p < 0.05$).

lular subdivision of the PVH (~90% of the total), where CRH-secreting neurons are found (Sawchenko and Swanson, 1983). Although attenuated, the PVH of lactating rats did show some Fos activation (Table 1), suggesting that the stress response is not totally absent in this phase. EW challenge has been shown to stimulate Fos activation in a similar fashion in male rats, where 66% of activated cells were located in the parvocellular subdivision of the PVH (Földes et al., 2000). This finding emphasizes the importance of this hypothalamic nucleus as a target for gonadal hormones affecting activational responses to stress.

The present results indicate the complexity of the influences of the physiological condition upon the neuroendocrine stress response. EW induced a full stress response in diestrus rats, while lactating dams showed an attenuation of the markers analyzed, including peripheral IL-6 and the hypothalamic NOx. Moreover, icv injection of the NO-blocker L-NAME counteracted the attenuated response of the HPA axis of lactating rats, as measured by Fos and CRH in the PVH, hypothalamic NOx, and circulating IL-6 and corticosterone.

These results are in agreement with previous reports (Lightman et al., 2001; Mouihate et al., 2008; Shanks et al., 1999) showing that lactation modifies immune responses, e.g., it blocks the inflammatory response to endotoxins. IL-6 is an interleukin that acts as both pro- and anti-inflammatory cytokine, depending on the stimulus and the phase of the response observed. Since IL-6 appears in the circulation prior to other cytokines, the present analysis relied on circulating IL-6 determination to evaluate the general immunological response to an acute challenge (Ericsson et al., 1997). The fact that IL-6 did not change in lactating rats after EW but did

increase after icv L-NAME indicates a diminished activation of the immune system during this phase and suggests that NO might be involved in this regulation. Thus, the attenuated neuroendocrine response, i.e., circulating corticosterone, to the immune stressor could be partially explained by the lack of peripheral stimulation, and further studies are needed to understand how central inhibition of NO can influence circulating level of this steroid. This study also highlighted the need for a wider analysis of immune activation caused by immune stimuli or any other stressor in the lactating rat. Therefore, the release of other interleukins and immunological mediators, such as histamine, must be determined to understand better how the reproductive condition of the female rats changes the immune response induced by stress.

ICV-L-NAME decreased the hypothalamic NOx concentration, whereas it induced Fos in the PVH; furthermore, CRH label was evident in the PVH of both, lactating and diestrus rats treated with L-NAME suggesting that NO may exert an inhibitory action(s) within this nucleus that would be reversed by the NO-blocker. When EW challenge was applied after L-NAME to diestrus rats, the hypothalamic concentration of NOx and the stain for NADPH-diaphorase increased indicating NO production, but CRH and Fos in the PVH as well as circulating corticosterone declined in the diestrus animals. In lactating rats in which NO was blocked by L-NAME, the HPA axis recovered full activity as indicated by Fos and CRH in the PVH and increased corticosterone levels. However, when EW treatment was given after L-NAME, the markers for the HPA axis activation and NO production increased further over those in lactating animals treated with L-NAME alone. These results indicate the complexity of the regulation of NO and the importance of the local redox balance.

Neuroendocrine and autonomic actions of the NO within the PVH have been described (Stern, 2004) and are most likely mediated by direct and/or indirect modulation of the electrical activity of major output neurons, including magno- and parvocellular neuroendocrine and parvocellular pre-autonomic neurons. In this sense, GABA, the major inhibitory neurotransmitter in the PVH, has been previously shown to evoke sympatho-inhibitory responses within the PVH (reviewed by Stern, 2004), and the density of GABAergic inputs that innervate the PVH is markedly increased during lactation and is accompanied by specific electrical activity (Gies and Theodosis, 1994). The data presented here support the hypothesis that increased NO during lactation may act, together with other neurotransmitters, such as GABA and neuropeptides (Stern, 2004; Vega et al., 2010) within the PVH to reduce HPA axis activity, indicating that NO is an important mediator of the EW-induced PVH response in female rats. Taken together, these results suggest that the high levels of NO production during lactation may be necessary in order to maintain an attenuated response to stress. Therefore, the activity of constitutive NO systems involved in the EW-induced HPA axis stimulation may also be regulated differently depending of the hormonal status of the animal (Okamura et al., 1994; Popeski et al., 1999).

Corticosterone exerts negative feedback on the CNS, but it also functions as regulator of immune function (Rabin et al., 1990). In this study, circulating corticosterone increased in diestrus rats in response to EW injection, and the higher basal level in lactating rats did not change after the immune stress.

This result was expected and agrees with results from others showing that during lactation, the daily rhythm of corticosterone secretion is lost, and the plasma concentration of corticosterone is not increased by stress (Lightman and Young, 1989). The chronically high corticosterone level could explain the diminished acute inflammatory response to EW during lactation (Selye, 1949). Reduced plasma concentrations of circulating pro-inflammatory cytokines, including TNF- α , IL-1 β , and IL-6, along with increased levels of anti-inflammatory cytokines and changes in the production of other inflammatory mediators including prostaglandins and systemic NO occur during pregnancy (Mouihate et al., 2008; Ericsson et al., 1997). NO can also influence immune responses within the brain. For example, NO generated by nNOS participates in the reduction of lipopolysaccharide-induced fever in pregnant rats by inhibiting the release of norepinephrine and attenuating COX-2 expression in the brain (Lacroix and Rivest, 1998; Matsumura et al., 1998; Shanks et al., 1999).

Nitrites and nitrates (NOx) are NO metabolites: they are considered to be an indicator of total NO production and they accumulate after stress. In this study, total hypothalamic NOx increased significantly in EW-treated diestrus rats but decreased in EW-treated lactating dams, suggesting differential NOS activation in the hypothalamus of the two groups (Bredt et al., 1990) depending of the NO level already present. This suggests that when elevated, NOS activity is suppressed by an acute stressor, in this case EW.

Numerous studies indicate that NO participates in the control of neuroendocrine processes, including the CRH system. NO seems to perform dual functions in this system: it may augment, possibly via cGMP, the stimulatory effect of IL-1, further increasing CRH release (Seo and Rivier, 2001). In contrast, NO may prevent the over-stimulation of this neuronal circuit by oxidizing the redox-sensitive site of the NMDA receptor and thus downregulating CRH and NO release (reviewed by Riedel, 2000). Which of these opposing effects that predominate seems to depend mainly on the redox balance set by the physiological and hormonal status of the animal (Gadek-Michalska and Bugajski, 2008). Consistent with earlier work (Otukonyong et al., 2000; Popeski et al., 1999), we found a higher number of cells stained for NADPH-diaphorase in the PVH of lactating than of diestrus rats. Increases in NADPH-diaphorase staining in this area have also been observed in response to other stimuli that activate both the oxytocin and vasopressin systems, such as suckling (Otukonyong et al., 2000; Popeski et al., 1999), osmotic challenge, and water deprivation (Bains and Ferguson, 1994; Calka and Block, 1993; Torres et al., 1993; Yasin et al., 1993). Colocalization of CRH, vasopressin, and oxytocin with NO has been reported (Nylén et al., 2001).

In summary, actions of NO in the hypothalamus, primarily in PVH neurons (Bredt et al., 1990; Vincent and Hope, 1992), include modulation of electrical activity and modification of afferent transmission by presynaptic action upon their afferent nerve terminals (Bains and Ferguson, 1994). NO has also been shown to act within the PVH and possibly to alter the mechanisms regulating the neuronal activity resulting in milk ejection (Okere et al., 1999). The present data and previous results from other laboratories (Lightman et al., 2001; Shanks et al., 1997, 1999) support the hypothesis that endocrine-immune interactions are altered during lactation, and that NO is regulating the PVH

response to stress. Regardless of the mechanism, the dampened stress effects during lactation may be of adaptive value to the mother and offspring.

4. Experimental procedures

4.1. Animals

Pregnant (18–19 days) adult Wistar rats (250–300 g) were housed individually under controlled temperature and lighting conditions (12:12 h light/dark cycle, lights on at 06:00 h), with food and water available *ad libitum*. One day after parturition, litter sizes were culled to 8–10 per dam. Mothers were kept undisturbed with their litters except for cage cleaning, and they were used 10–12 days postpartum. Virgin rats were pair housed in the same animal room and were used on diestrus day 1, determined by following vaginal smears for at least three cycles. To minimize potential effects of handling on Fos, NOS, and CRH expression, all animals were handled daily at the same time of day for 5 days prior to the day of the experiment. The Institutional Animal Care and Use Committee of the Institute for Neurobiology at the National University of Mexico approved all experimental protocols, and animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

4.2. Acute immune stress induced by egg white injection

An anaphylactoid reaction was induced by intraperitoneal (ip) injection of fresh filtered egg white solution (EW, 0.5 ml/100 g b.w., 50% v/v in sterile saline). Control groups for each condition (diestrus and lactating) received the same volume of isotonic saline. Rats were injected at 10:00 h and euthanized 3 h later (around 13:00 h). Experimental and control groups contained 6 animals each.

4.3. Surgery

Subsets of lactating and diestrus rats were given an intra-cerebro-ventricular (icv) injection of the NOS blocker N ω -nitro-L-arginine methyl ester hydrochloride (L-NAME) to determine the effect of general NOS inhibition on the EW-induced HPA axis activation. L-NAME (25 μ g/1 μ l, Sigma Chemical Co., St Louis, MO, USA) (Ayers et al., 1997; Gadek-Michalska and Bugajski, 2008; Seo and Rivier, 2001) or sterile saline (1 μ l) was stereotactically administered into the right lateral ventricle (AP: –1.2 mm, L: –2 mm, DV: –5 mm, from skull) under ketamine-xylazine (Cheminova de Mexico, Edo.Mexico, Mexico) anesthesia (0.2 ml/100 g b.w., ip). Twenty-four hours after surgery rats were subjected to either EW or saline injection and euthanized 3 h later.

4.4. Tissue preparation

Rats were anesthetized with a lethal dose of urethane (1.5–2 g/kg bw, ip). Animals were immediately perfused via the ascending aorta with saline followed by 350 ml of ice-cold 4% paraformaldehyde in 0.1 borate buffer (pH 9.5). Brains were

removed, postfixed in the same fixative for 3 h, then cryoprotected with 20% sucrose at 4 °C. Five, 1-in-5 series of 30- μ m-thick, frozen coronal sections through the hypothalamus were collected and stored in cryoprotectant (30% ethylene glycol and 20% glycerol in 0.05 M sodium phosphate buffer) at –20 °C until histochemical processing.

4.5. Immunohistochemistry

c-Fos immunoreactivity was localized using antisera raised in rabbits against a synthetic N-terminal fragment of human c-Fos protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Fos immunoreactivity (Fos-ir) was analyzed on free-floating sections using conventional avidin-biotin-immunoperoxidase detection (Sawchenko et al., 1990). Endogenous peroxidase was neutralized by treating tissue for 10 min with 0.3% hydrogen peroxide followed by an 8-min exposure to 1.0% sodium borohydride to reduce free aldehydes. Next, sections were incubated at 4 °C for 48 h with the primary antiserum diluted 1:5000 in phosphate-buffered saline (PBS) supplemented with 0.3% Triton X-100 and 2% blocking serum. The primary antibody was detected using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), and the diaminobenzidine reaction product was developed using a nickel-enhanced glucose oxidase method (Shu et al., 1988). Control experiments, in which the primary antiserum was pre-absorbed overnight with Fos peptide, yielded no evidence of specific nuclear labeling in tissue from control or experimental animals. Some tissue was subsequently processed for NADPH-diaphorase histochemistry, or nNOS (1:5000) or CRH (1:500, Chemicon International, Inc., Billerica, MA, USA) immuno-histochemistry using the protocol described above. The sections were mounted on gelatin-coated slides, dehydrated, and coverslipped with entellan.

4.6. NADPH-diaphorase histochemistry

Neuronal NO synthase produces NO in a NADPH-dependent fashion in response to changes in free intracellular calcium by oxidation of arginine to citrulline (Vincent and Hope, 1992). Because the immunostaining indicates the presence of the protein (nNOS), while NADPH-diaphorase is thought to indicate the activity of the different types of NO synthases, including nNOS, iNOS, and eNOS, both staining methods (NADPH-diaphorase histochemistry and nNOS immunodetection) were employed here to have a better sense of changes in NO markers. Floating brain sections were incubated in 10 ml of 0.1 M phosphate buffer solution, pH 7.4, containing 10 mg β -NADPH (Sigma-Aldrich Inc., USA), 1 mg nitroblue tetrazolium, and 0.3% Triton X-100, at 37 °C for 60 min. The tissue sections were rinsed in 0.1 M ice-cold phosphate buffer to stop the reaction, and were mounted on gelatin-coated slides, dehydrated by graded alcohols, cleared in xylene, and coverslipped with entellan.

4.7. Brain nitrates and nitrites (NOx) assay

Subsets of animals of each experimental condition were killed by decapitation, brains were rapidly removed, and the complete hypothalamus was dissected from each brain and frozen on dry ice for further analysis. The total concentration of NOx, the final

products of NO, was determined using a colorimetric assay as described previously (Stern, 2004). Briefly, brain samples were homogenized in 200 μ l distilled water and centrifuged at 10,000 g for 15 min at 4 °C. Then, 50 μ l of supernatant was mixed with 20 μ l of 0.86 mM h-NADPH, 0.11 mMFAD, and 20 mU of nitrate reductase. Samples were incubated for 1 h at room temperature in the dark. Next, in order to precipitate the proteins, 5 μ l of 1 M ZnSO₄ was added to the samples. They were centrifuged at 6000 g for 5 min at 4 °C, and supernatants were removed. Griess reagent (Sigma-Aldrich Inc., USA; 100 μ l, 1:1 mixture of 1% sulphanilamide in 5% H₃PO₄ and 0.1% N-(1-naphthyl) ethylenediamine) was added to 50 μ l of supernatant and the mixture was incubated for 10 min at room temperature. Absorbance was measured at 595 nm in a microplate reader (Bio Rad, Mod. 680, Hércules, CA, USA) and converted to NO_x content using a nitrate standard curve. Brain supernatant protein was estimated by Bradford method. The data were expressed as nmol NO_x/μg protein.

4.8. Corticosterone and IL-6 determinations

Atrial blood was collected under anesthesia before perfusion (around 13:00 h) to determine serum corticosterone and IL-6 concentrations. After 2 h at room temperature to allow clot formation, serum was separated by centrifugation and stored at –20 °C until assay. Serum corticosterone and IL-6 concentrations were determined using immunoassay kits (Catalog No. 900–097 Assay Designs, Inc. Ann Arbor, MI, for corticosterone; and Catalog No. KRC0061 Biosource Camarillo, CA, for IL-6) that employed a competitive immunoassay for quantitative determinations. The optical density of the samples and controls from the standard curve were read immediately in a microplate reader at 450 nm (Bio Rad, Hércules, CA, USA).

4.9. Cell counting and statistical analysis

The number of cells immuno-labeled for Fos and nNOS, or labeled for NADPH-diaphorase was estimated by counting cells positive for each marker across all sections in one of the tissue series, corresponding to the rostro-caudal extension of the PVH (5–6 sections spaced 150 μ m apart). Images were captured using a bright-field microscope attached to a digital CCD camera (Nikon). Positive label for Fos was counted in the parvocellular subdivision, as well as in the posterior magnocellular subdivision of the PVH as a function of the treatment and hormonal status using IpLab (BD Biosciences — Bioimaging Division) image software. The boundary of the parvocellular or magnocellular subdivision was outlined, and the numbers of positive profiles were recorded after setting a common threshold for all images.

Cell counts were averaged for each group. Statistical differences were determined by one-way ANOVA followed by the Tukey multiple comparisons test. All data are presented as the mean $\pm$ SEM; p values <0.05 were considered significant.

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