Original Paper

Developmental Neuroscience

Dev Neurosci 2010;32:71–80
DOI: 10.1159/000280102

Received: October 16, 2009
Accepted after revision: January 22, 2010
Published online: March 25, 2010

Gender-Dependent Effects of Early Maternal Separation and Variable Chronic Stress on Vasopressinergic Activity and Glucocorticoid Receptor Expression in Adult Rats

G.M. Renard M.A. Rivarola M.M. Suárez

Cátedra de Fisiología Animal, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Córdoba, Argentina

Key Words

Arginine vasopressin · Fos · Glucocorticoid receptors · Maternal separation · Variable chronic stress · Sexual dimorphism · Hippocampus

Abstract

The aim of the present study was to investigate the influence of early maternal separation on Fos, arginine vasopressin (AVP) and glucocorticoid receptor (GR) expression in the medial parvocellular portion of the paraventricular hypothalamic nucleus (PaMP), and GR expression in the hippocampus of adult male and female rats subjected to variable chronic stress (VCS). Male and female Wistar rats were isolated 4.5 h daily, during the first 3 weeks of life. At 48 days of age, the rats were exposed to VCS. Nonmaternally separated (NMS) females had a higher number of activated AVP neurons than NMS male rats. Maternally separated (MS) females subjected to VCS also showed a higher number of Fos/AVP double-labeled neurons than males with the same treatment. Males and females subjected to early maternal separation and VCS, compared with the MS animals, showed a decrease in the expression of GR in the PaMP. As regards GR expression in the hippocampus, MS animals subjected to VCS as adults, both males and females, showed an increase in GR expression in the subfields CA1, CA2 and CA3. The increase in AVP-immunoreactive neurons coexpressing Fos in response to stress in females exposed to early maternal separation suggests that perhaps early life stress results in a more reactive neuroendocrine stress response in females. Furthermore, our results demonstrate that the different anatomical levels of the hypothalamic-pituitary-adrenal axis have different roles related to its stress response and support the evidence of regional specificity in GR regulation.

Introduction

Neuroendocrine responses to stress, particularly those mediated by the hypothalamic-pituitary-adrenal (HPA) axis, can differ significantly between males and females in many species. On the other hand, postnatal life environment is important to the development of the HPA axis. Early maternal separation affects the responsiveness of the HPA axis to stressors and it may alter this responsiveness in a gender-dependent way [Kelly et al., 1999; Wigger and Newman 1999; Barna et al., 2003; Slotten et al., 2006]. One of the structures that regulates the activity of the HPA axis is the medial parvocellular portion of the paraventricular hypothalamic nucleus (PaMP). These parvocellular neurons project to the external zone of the

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0378–5866/10/0321–0071$26.00/0

Accessible online at:
www.karger.com/dne

Georgina M. Renard
Cátedra de Fisiología Animal, Facultad de Ciencias Exactas, Físicas y Naturales
Av. Vélez Sársfield 299, Córdoba, X5000JJC (Argentina)
Tel. +54 351 433 2098 2090, Fax +54 351 433 2097
E-Mail georgmrenard@yahoo.com.ar

median eminence, and, during stress, release several peptides, foremost among which are corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), that stimulate ACTH release. This produces the biosynthesis and release of glucocorticoids from the adrenal cortex [Scott and Dinan, 1998; Aguilera and Rabadan-Diehl, 2000; Armstrong, 2004]. CRH neurons in the parvocellular region of the paraventricular nucleus (PVN) of rats have been shown to produce AVP in response to stress [Helmreich et al., 1999], suggesting that the importance of AVP in regulating ACTH secretion is greater during stress. Other studies have demonstrated via intracerebral microdialysis that an ethologically relevant emotional stressor caused a significant increase in AVP release within the PVN [Wotjak et al., 1996]. On the other hand, Zelena et al. [2007] showed that somatic and endocrine changes elicited by chronic mild stress (different mild stimuli for 6 weeks) do not depend on the presence of AVP. The HPA response to stress is, in part, determined by the ability of the glucocorticoids to regulate ACTH release (i.e. glucocorticoid negative feedback), via actions on the synthesis and release of CRH and AVP. Negative feedback, or end product inhibition, is an important regulatory mechanism in neuroendocrine systems.

Corticosterone actions in the brain are mediated by glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). GRs occur everywhere in the brain but are most abundant in hypothalamic CRH neurons and pituitary corticotropes. MR and GR coexpression is found in the hippocampus, the amygdaloid and lateral septal nuclei, and some cortical areas. The occupancy of GRs with higher levels of corticosterone mediates feedback actions aimed to restore disturbances in homeostasis [De Kloet et al., 1998]. GR can interfere with transcription factors and repress stress-induced responses, such as peptide synthesis (including that of CRH and AVP), and thereby terminate ongoing stress reactions [De Kloet et al., 2005]. In previous studies, we analyzed sex differences in the pituitary-adrenal activity as well as behavioral responses of adult rats subjected to early maternal separation and variable chronic stress (VCS). Female rats showed higher basal ACTH levels than males, evidencing a sexual dimorphism in this hormone. Meanwhile, maternally separated (MS) animals subjected to VCS showed a decrease in ACTH levels in both sexes. On the other hand, the stress protocols produced sex-dependent effects on corticosterone levels, showing an increase in females and a decrease in males [Renard et al., 2007]. Hence, it is possible that the sex differences observed in the pituitary-adrenal response could be explained by sex differences in the activation of neurons producing AVP in response to stress. Furthermore, it could be due to a differential regulation of the HPA activity via GR underlying the observed gender-dependent responses. With this in mind, AVP and GR expression were investigated in the PaMP of male and female rats subjected to early maternal separation and to VCS as adults, in an attempt to gain new insights into the neuronal circuits modulating the HPA axis response. To study whether these stress protocols were associated with differential neuronal activation, we employed Fos immunohistochemistry in the PaMP. In this work, the effects of the treatments were evaluated 24 h after the last stressor was applied, so that only chronic, but not acute stress effect on Fos expression was investigated. GR expression was also investigated in the hippocampus of male and female rats subjected to the same treatments, since this brain structure is one of the most important in the regulation of the neuroendocrine response to stress and is at the same time affected by stress responses [Heuser and Lammers, 2003; Herman and Cullinan, 1997; Jacobson and Sapolsky, 1991]. These data may contribute to the understanding of the mechanism underlying gender-related differences in animals subjected to early emotional experiences and their relationship with the development of the HPA axis.

Material and Methods

Animals

Both male and female Wistar rats were used. All animals were subjected to the same conditions: they were housed in a temperature-controlled room (22 ± 2°C) under artificial illumination (12-hour light/12-hour dark; light on at 07:00 a.m.). Water and food were given ad libitum. Experiments were performed in full accordance with protocols approved by the Animal Care and Use Committee of Córdoba University, Argentina.

Separation Procedures

On postnatal day 1, litters were culled to 8 pups (4 females and 4 males if possible) and randomly assigned to either maternal separation or nonmaternal separation procedure. The rat pups were daily deprived of their mother for 4.5 h during the first 3 weeks of life [Ogawa et al., 1994]. Each separation consisted of removing the mother from the home cage. The dam was placed alone in a cage in the same room. After 4.5 h, the mother was returned to the home cage. Separations were carried out between 08:00 a.m. and 12:30 p.m. Nonmaternally separated (NMS) rats remained undisturbed in the maternal cage, except for a bedding change twice a week, until the weaning age at postnatal day 30. After weaning, animals were housed by sex in standard cages. All the subjects were handled daily by the same researcher from weaning until they were perfused, to minimize stress reactions to manipu-

lation [Loyber et al., 1977]. Handling consisted of picking up each animal from its home cage by placing the hand over the animal's back, with the thumb and forefinger pressing its forelegs towards its head, then the animal was placed in another cage for 1 min and finally, put back in its home cage. Unrelated subjects were used to avoid confounding litter effects (each experimental group was made up of subjects from at least 3 litters).

Variable Chronic Stress

At 48 days of age, the rats were exposed to a 24-day variable stressor paradigm (modified Katz's stress model) [Katz et al., 1981]. Individual stressors are listed in table 1. The type of stressor and the day on which it was applied were chosen by using a random number table, except for that on day 24. In this case, noise was used as a stressor on the day preceding the sacrifice, to avoid the unpredictability associated with this chronic stress model [García Marquez and Armario, 1987]. The following stressors were used: (a) 4 h of noise produced by an alarm bell (85 dB); (b) ether anesthesia until loss of reflex; (c) 2 intraperitoneal injections of isotonic saline at different times; (d) 24 h of food deprivation; (e) 1 h of restraint inside a 6-cm diameter metal cylinder. The stressors used in this paradigm did not affect the rat's body weight.

Table 1. Variable chronic stress (Katz's modified model)

Day	Stressor	Hour
1	Ether anesthesia	12:00 a.m.
2	Restraint	10:00 a.m. to 11:00 a.m.
3	Noise	09:00 a.m. to 01:00 p.m.
4	Two saline injections	11:00 a.m. and 03:00 p.m.
5	Ether anesthesia	10:00 a.m.
6	Fasting	for 24 h
7	Rest day	
8	Ether anesthesia	09:30 a.m.
9	Noise	09:30 a.m. to 01:30 p.m.
10	Two saline injections	11:30 a.m. and 03:30 p.m.
11	Restraint	03:00 p.m. to 04:00 p.m.
12	Noise	10:00 a.m. to 02:00 p.m.
13	Fasting	for 24 h
14	Rest day	
15	Ether anesthesia	01:00 p.m.
16	Restraint	03:00 to 04:00 p.m.
17	Noise	09:30 a.m. to 01:30 p.m.
18	Two saline injections	12:00 a.m. and 04:00 p.m.
19	Noise	09:30 a.m. to 01:30 p.m.
20	Fasting	for 24 h
21	Rest day	
22	Ether anesthesia	11:45 a.m.
23	Restraint	10:00 to 11:00 a.m.
24	Noise	09:00 a.m. to 01:00 p.m.

Animals were subjected to various stressors for 24 days.

Perfusion and Immunohistochemical Procedures

At the time of perfusion, all the animals were 2.5 months old. Rats were sacrificed between 09:00 and 12:00 a.m., at diestrus in the case of the female rats, in order to avoid unwanted variability in circulating hormone levels linked to diurnal fluctuations and the estrus cycle. Diestrus was determined by examination of vaginal smears. Twenty-four hours after the last stressor, rats were deeply anesthetized with chloral hydrate (0.54 g/kg i.p.) and transcardially perfused with heparinized saline followed by a 4% paraformaldehyde in 0.1 M phosphate buffer (PB) solution. On the day of perfusion, the phase of the estrous cycle was verified immediately after death of the animals. Only females that were in diestrus were considered for this work, which is consistent with our previous studies.

Brains were removed and fixed overnight in 4% paraformaldehyde-PB solution and then stored at 4°C in 20% sucrose-PB solution. Immunoreactions were performed with the free-floating method. Forty-micrometer coronal sections were cut using a freezing microtome. Brains were processed for Fos-like immunoreactivity (Fos-LIR) and AVP immunoreactivity (AVP-IR) double-labeling or were used for immunoreactivity localization of GR. Immediately before immunostaining, sections were placed in a mixture of 10% H_2O_2/10% methanol until oxygen bubbles ceased appearing. They were then incubated in 10% normal horse serum (NHS) in PB for 1 h to block sites of nonspecific binding of serum products. Free-floating sections were carefully matched for anatomical location to assure comparability of regions of interest across conditions.

Fos-LIR and AVP-IR Double-Labeling

The sections from each brain were first processed for Fos-LIR using an avidin-biotin-peroxidase procedure. For the immunostaining of c-Fos protein, free-floating sections were incubated for 48 h at 4°C in an antibody raised in rabbits against a synthetic 14-amino-acid sequence corresponding to residue 417 of human Fos (Ab-5, batch No. 60950101; Oncogene Science, Manhasset, N.Y., USA) diluted 1:10,000 in a solution of PB containing 2% NHS and 0.3% Triton X-100. As we cannot exclude cross-reactivity of this c-Fos antibody with other proteins of the Fos family (such as Fos-B, Fra-1 and Fra-2), we consider the term 'Fos-like immunoreactivity' (Fos-LIR) to be more appropriate for the interpretation of Fos protein immunostaining. After being washed in PB, the sections were incubated in biotin-labeled anti-rabbit immunoglobulin and avidin-biotin-peroxidase complex (ABC kit; Vectastain, Vector Laboratories, 1:200 dilution in 1% NHS-PB) for 2 h at room temperature. The peroxidase label was detected using 3,3′-diaminobenzidine (Sigma), intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a blue-black nuclear reaction product. The Fos-labeled sections, also processed for immunocytochemical localization of AVP, were incubated for 72 h at 4°C with its antibody: polyclonal rabbit anti-vasopressin antibody (Chemicon International; 1:10,000 dilution). After incubation, the sections were rinsed and incubated with biotin-labeled anti-rabbit immunoglobulin and the avidin-biotin-peroxidase complex for 1 h at room temperature. Cytoplasmic AVP-IR was detected with unintensified diaminobenzidine hydrochloride, which produces a brown reaction product. Finally, free-floating sections were mounted on gelatinized slides, air-dried overnight, dehydrated, cleared in xylene, and placed under a coverslip with DePeX mounting (Fluka, Buchs, Switzerland).

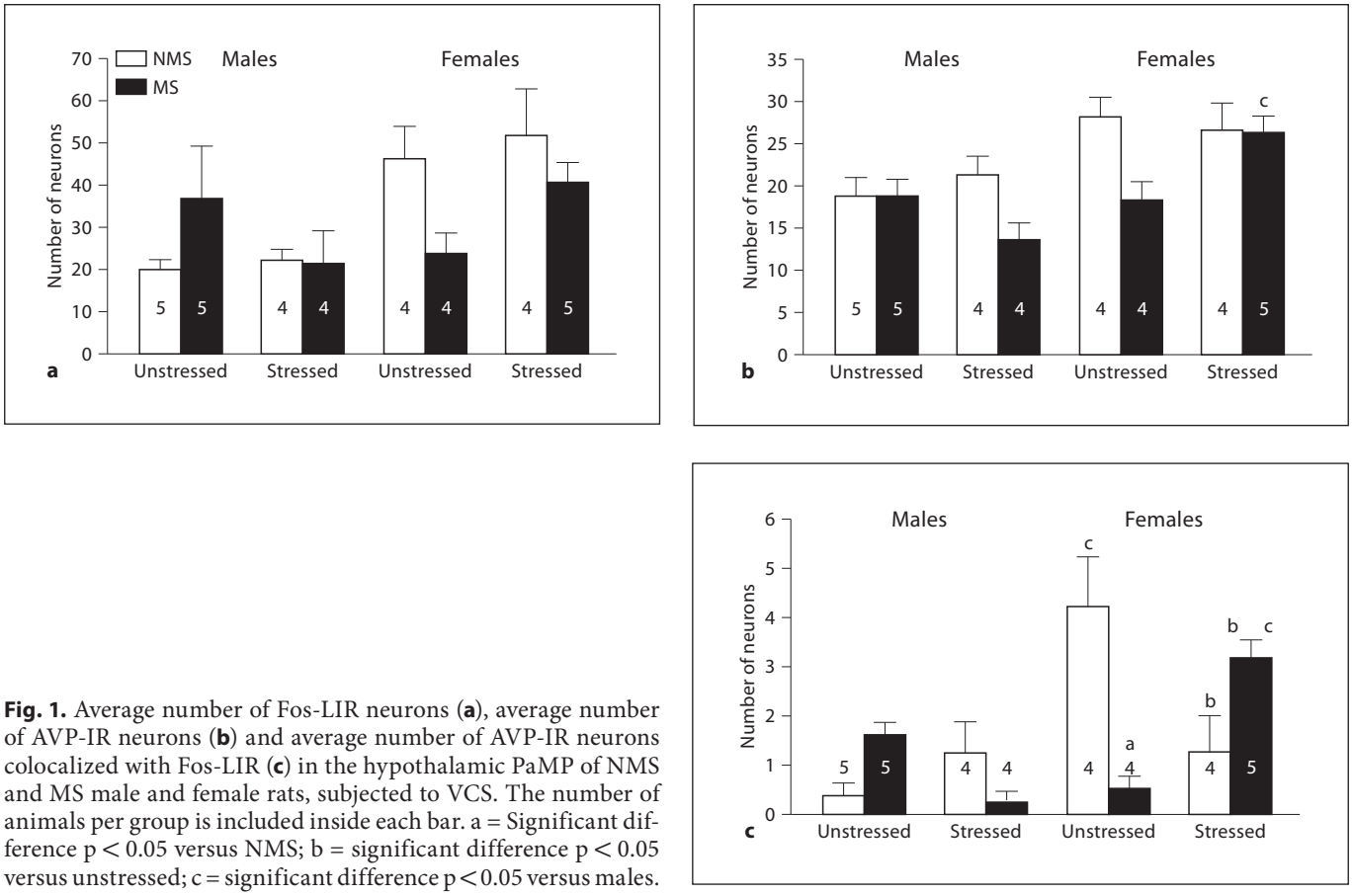

Fig. 1. Average number of Fos-LIR neurons (**a**), average number of AVP-IR neurons (**b**) and average number of AVP-IR neurons colocalized with Fos-LIR (**c**) in the hypothalamic PaMP of NMS and MS male and female rats, subjected to VCS. The number of animals per group is included inside each bar. a = Significant difference $p < 0.05$ versus NMS; b = significant difference $p < 0.05$ versus unstressed; c = significant difference $p < 0.05$ versus males.

GR Immunoreactivity

Sections processed for immunocytochemical localization of GR were washed 3 times for 5 min in PB, and blocked with 10% NHS in 0.1 M PB for 1 h at room temperature. Primary antibody was diluted 1:500 in PB containing 2% NHS and 0.3% Triton X-100 and incubated for 72 h at 4°C [rabbit anti-GR antibody (P-20, sc-1002); Santa Cruz Biotechnology, Inc.]. After incubation, sections were rinsed with PB and incubated in the appropriate biotinylated secondary antiserum and avidin-biotin-peroxidase complex (ABC kit; Vectastain, Vector Laboratories) for 2 h at room temperature. GR immunoreactivity was detected and mounted like AVP-IR. To test validity of the GR immunohistochemistry used in the present study, we omitted the primary antibody of GR.

Cytoarchitectural and Quantitative Analysis

The brain nuclei exhibiting Fos-LIR, AVP-IR and GR immunoreactivity were identified and delimited according to the rat brain atlas of Paxinos and Watson [1997]. The numbers of AVP-IR and Fos-LIR nuclear profiles in the sections were counted at the medial parvocellular subnuclei of the paraventricular hypothalamic nuclei (distance from the bregma of the corresponding plates: –1.80 mm). GRs were counted at the PaMP and the dorsal hippocampus (bregma: –2.80 to –3.60 mm). Five sections per rat were analyzed at the level of the hippocampus (CA1–CA3 and dentate gyrus). The images were analyzed using a computerized system that included a Zeiss microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device.

Representative sections in control and experimental groups were acquired at exactly the same level, with the aid of the Adobe Photoshop Image Analysis Program (version 5.5). The number of Fos-LIR- and AVP-IR-positive cells in the PaMP was counted manually under microscopy. Colocalization of Fos-LIR and AVP-IR in the same sections was also counted. In dual-immunolabeling experiments, positive cells were identified as those expressing both black nuclear and brown cytoplasmic reaction products. The number of GR-immunoreactive (GR-ir) cells was counted using ImageJ software. Sections were viewed at a magnification of ×40 and the number of GR-labeled cells was quantified in the PaMP and in the CA1, CA2 and CA3 subfields and dentate gyrus of the hippocampus. Investigators were blinded to the grouping while taking the photomicrographs and performing the image analysis, within grids of defined size that where placed over each area. All images used in the analysis were taken on the same microscope and with the same optical settings. Counts of GR-labeled cells were obtained from each area of interest, maintaining constant background intensity across different sections and animals such that a GR-labeled cell was counted only if it reached a defined

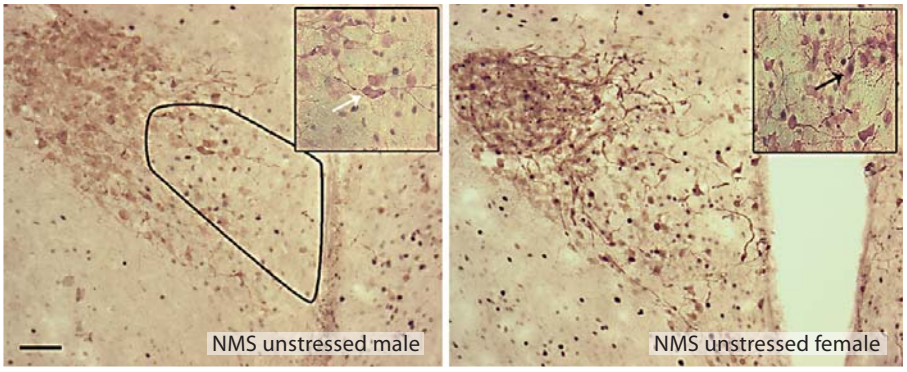

Fig. 2. Representative photomicrographs of AVP-IR and Fos-LIR dual-stained neurons in the hypothalamic PaMP of NMS unstressed male and female rats. Bar scales: 100 μm. The insets on the right are shown at a magnification of ×40. The white arrow points to the AVP-IR neuron, the black arrow points to colabeled Fos/AVP.

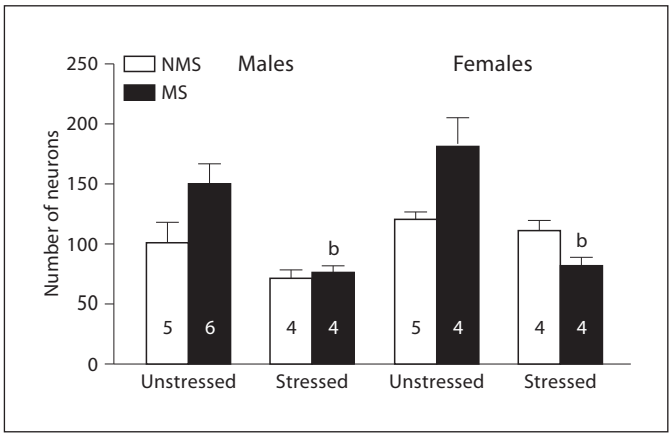

Fig. 3. Average number of GR-ir neurons in the hypothalamic PaMP of NMS and MS male and female rats, subjected to VCS. The number of animals per group is included inside each bar. b = Significant difference $p < 0.05$ versus unstressed.

darkness threshold above background. The counting procedure was done in 4 or 5 animals from each condition. Data were analyzed by three-way ANOVA (factors: rearing condition × stress × sex) followed by a Tukey post hoc test. For all comparisons, p values of less than 0.05 were considered statistically significant.

Results

Fos-LIR and AVP-IR

Figure 1 shows Fos-LIR and AVP-IR in the PaMP of male and female rats subjected to early maternal separation and VCS as adults. There were no significant effects of maternal separation, VCS and sex on the number of Fos-LIR cells in the PaMP (fig. 1a).

MS females, subjected to VCS, showed a higher number of AVP-IR cells [$F(1, 27) = 17.63$; $p < 0.001$] than males subjected to the same treatment (fig. 1b). With regard to AVP neurons showing Fos-LIR, MS females and stressed females showed decreased vasopressinergic activity in the PaMP ($p < 0.05$) compared with NMS females. On the other hand, adult MS females, subjected to VCS, showed an increase in the number of Fos-LIR/AVP-IR neurons ($p < 0.05$) compared with adult MS females without VCS (fig. 1c). Regarding sex differences, NMS females had a higher number of AVP neurons showing Fos-LIR [$F(1, 27) = 15.20$; $p < 0.001$] compared with NMS male rats (fig. 2). Furthermore, MS females subjected to VCS also showed a higher number of Fos/AVP double-labeled neurons [$F(1, 27) = 15.20$; $p < 0.001$] compared with males with the same treatment (fig. 1c).

GR Immunoreactivity

Medial Parvocellular Portion of the Paraventricular Hypothalamic Nucleus

The effect of early maternal separation and VCS on the number of GR-ir neurons in the PaMP in male and female rats is displayed in figure 3. There were no significant sex-dependent effects on the expression of GR in the PaMP. In contrast, both male and female rats showed an increased tendency towards the expression of GR in MS rats compared to NMS animals, although this did not reach statistical significance. In addition, males and females subjected to early maternal separation and VCS showed a decrease in GR expression compared with the MS animals [$F(1, 28) = 27.22$; $p < 0.001$] (fig. 4).

Dorsal Hippocampus

Figure 5 shows the number of GR-ir neurons in the subfields CA1, CA2 and CA3 and in the dentate gyrus of the hippocampus of early MS male and female rats subjected to VCS as adults. There were no significant effects of maternal separation on the number of GR-ir neurons

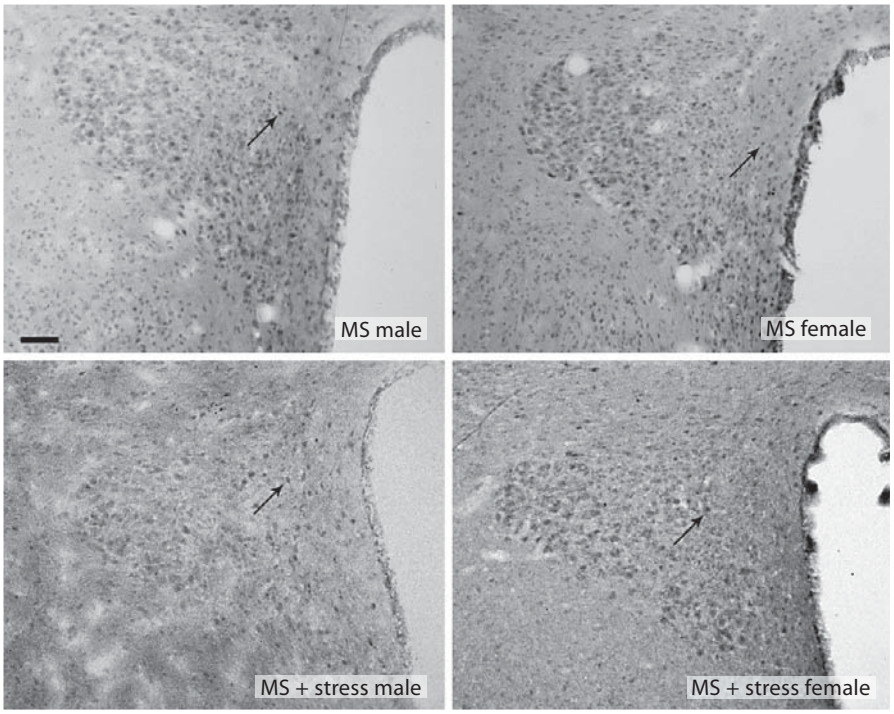

Fig. 4. Representative photomicrographs of GR-ir neurons in the hypothalamic PaMP of MS male and female rats, subjected to VCS as adults. Bar scales: 100 μm. The arrows point to examples.

in the hippocampus of male and female rats. VCS produced an increase in the number of GR-ir neurons in the subfields CA2 [$F(1, 24) = 52.82$; $p < 0.0001$] and CA3 [$F(1, 24) = 62.15$; $p < 0.0001$] of the hippocampus in male rats compared with unstressed males. In the MS animals subjected to VCS as adults, both males and females showed an increase in the expression of GR in the subfields CA1 [$F(1, 24) = 73.23$; $p < 0.0001$], CA2 [$F(1, 24) = 52.82$; $p < 0.0001$] and CA3 [$F(1, 24) = 62.15$; $p < 0.0001$] of the hippocampus (fig. 6), compared to MS unstressed animals. Regarding sex differences, MS females subjected to VCS showed a smaller number of GR-ir neurons in the subfield CA1 [$F(1, 24) = 28.53$; $p < 0.0001$] compared to males subjected to the same treatment. On the other hand, NMS females subjected to VCS showed a decrease in the expression of GR in the subfield CA3 [$F(1, 24) = 20.54$; $p < 0.0001$] compared with NMS stressed males.

Discussion

The primary focus of these studies was to investigate the gender-dependent effects on Fos, AVP and GR expression in the PaMP of MS rats subjected to VCS as adults. We found that the PaMP has a vasopressinergic activity that is dependent on the animal's sex. Another interesting finding is that the combination of early-life stress and VCS as adults produced a neuronal activity pattern in vasopressinergic neurons that is opposite in males and females. In males, the activity of AVP neurons turned back to baseline, whereas in females the exposure to both protocols caused a marked increase in such activity. Since AVP is a hypothalamic hormone that, together with the CRH, stimulates ACTH release from the pituitary gland, a higher number of AVP neurons expressing Fos-LIR in females is in accordance with a previous work, in which female rats exhibited elevated ACTH levels compared to males, evidencing a sexual dimorphism [Renard et al., 2007]. The gender-dependent activity of vasopressinergic neurons in NMS animals could be due to the excitatory effects of estrogens and the inhibitory effects of androgens on HPA axis function [McCormick et al., 2002; Rhodes and Rubin, 1999]. It has been demonstrated that in male and female rats, the administration of estrogens increases the secretion of basal corticosterone and the ACTH and corticosterone response to physical and psychological stressors [Rhodes and Rubin, 1999]. In ovariectomized females with the corresponding hormonal replacement, estrogens also showed stimulant effects on AVP biosynthesis in parvocellular neurons [Viau and Meaney 2004]. On the other hand, gonadectomized males treated with estradiol showed an increase in c-fos mRNA, CRH hRNA and

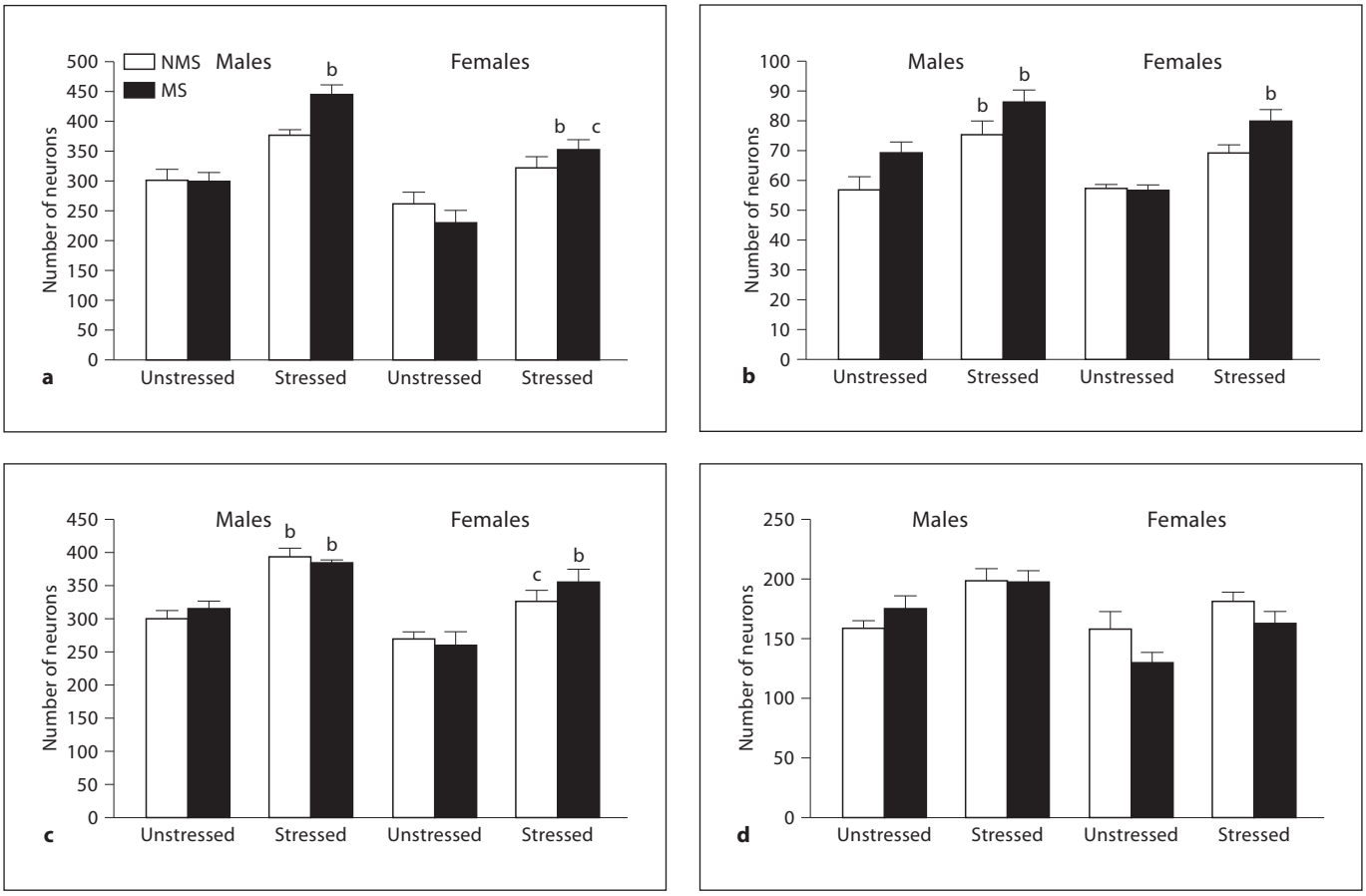

Fig. 5. Average number of GR-ir neurons in the subfields CA1 (**a**), CA2 (**b**) and CA3 (**c**), and in the dentate gyrus (**d**) of the hippocampus of NMS and MS male and female rats, subjected to VCS. The number of animals per group is 4 in all cases. b = Significant difference $p < 0.05$ versus unstressed; c = significant difference $p < 0.05$ versus males.

AVP hRNA expression in the PVN in response to stress; in contrast, treatment with dihydrotestosterone produced a decrease in the same parameters [Lund et al., 2004]. The gonadal and adrenal systems are thus intimately entwined, reinforcing the idea that gonadal status plays a large part in modulating normal, individual variations in stress responsiveness, but also implicating sex steroids in the development of stress-related disorders associated with HPA dysfunction [Williamson et al., 2005]. As regards the effects of early maternal separation and VCS in female rats, this produces an increase in AVP neurons expressing Fos-LIR. In spite of this, previous results showed a decrease in plasma ACTH levels because of the effect of both stress protocols [Renard et al., 2007]. A possible explanation for this loss of correlation between AVP and ACTH may be another mechanism that could be inhibiting the release of AVP to the median eminence. It is noteworthy that Fos labeling in the nerve cell nucleus is not necessarily accompanied by electrical activation. However, because of technical limitations it cannot be shown if AVP is released into the portal vessels. Early MS males subjected to VCS, unlike females, showed a diminution in activity in vasopressinergic neurons in the PaMP. Other authors have reported a decrease in AVP-IR in the PVN of MS males subjected to forced swim stress [Desbonnet et al., 2008]. This is consistent with the diminution observed in ACTH and corticosterone levels in animals with the same treatments [Renard et al., 2007]. Regarding GR immunoreactivity, there were no gender-dependent effects in the PaMP or in the dorsal hippocampus in animals subjected to both physical and emotional stress protocols. Males and females showed a diminution in GR expression in the PaMP and an increase in the CA1, CA2 and CA3 subfields of the hippocampus, when they were

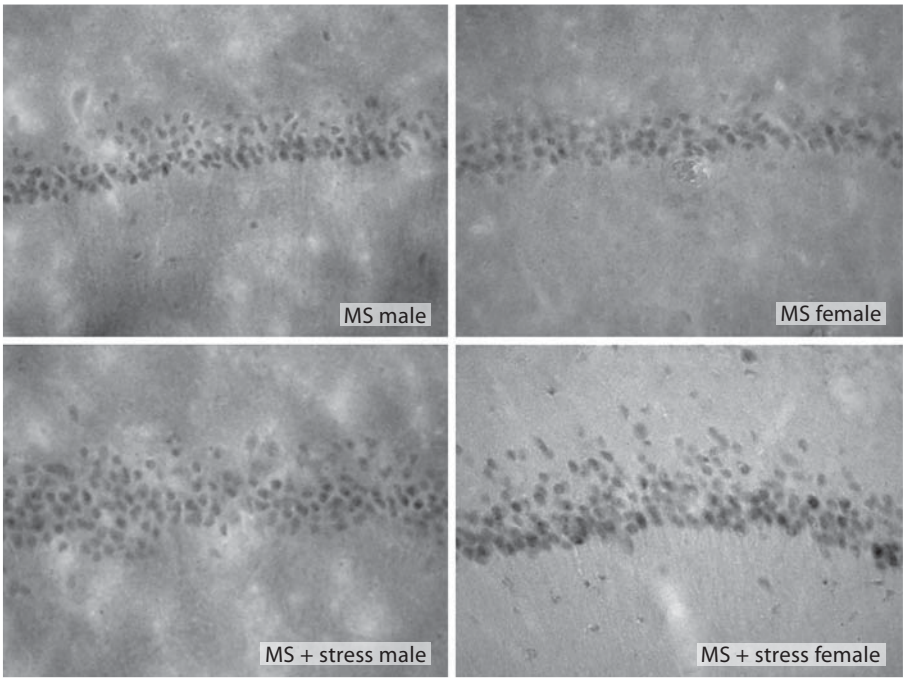

Fig. 6. Representative photomicrographs of GR-ir neurons in the CA1 subfield of the hippocampus of MS male and female rats, subjected to VCS as adults (×40). Note that MS animals exposed to chronic stress show increased GR expression.

subjected to early maternal separation and VCS as adults. However, Karandrea et al. [2000] showed that subjecting animals to a chronic monotypic stress produces a diminution in GR mRNA levels in the hippocampus, without alteration in females. Furthermore, in the hypothalamus, they showed a diminution in GR mRNA levels in females and no changes in males. Nevertheless, this difference could be due to the variations in the stress protocol used. Other investigators have found an increase in GR mRNA density in the frontal cortex of MS male rats subjected to VCS, but they have not found changes in the hippocampus of males with the same treatment [Ladd et al., 2005]. It has also been shown that early postnatal environment affects the differentiation of hippocampal neurons; this effect involves alterations in GR expression in the rat brain, resulting in changes in the sensitivity of the system to the inhibitory effects of glucocorticoids on the synthesis of CRH and AVP in hypothalamic neurons [Meaney et al., 1996]. Moreover, these effects of maternal separation have also been shown to be gender-dependent, with greater decreases in GR levels in the PVN of males compared to females, following 24 h of maternal separation [Avishai-Eliner et al., 1999]. However, there are few studies using GR expression to compare the effects of early maternal separation on the stress response in adult males and females.

It has been well established that MR and GR immunoreactivity were colocalized not only in CA1, but also in the CA2 region of the pyramidal cell layer and granular cell layer of the dentate gyrus. On the other hand, although Han et al. [2005] found expression of both receptors in the parvocellular region of the PVN, colocalization of MR and GR immunoreactivity in the hypothalamus has not been elucidated. A decreased GR/MR ratio was detected in the hippocampus of rats exposed to long-term restraint, in males probably due to persisting down-regulation of GR mRNA, and in females owing to an increase in MR mRNA levels [Karandrea et al., 2000]. Thus, a possible MR/GR interaction could be the basis for the differences in stress responses. Stress exposure in MS males would produce an increase in MR reducing or maintaining the GR/MR ratio, nevertheless further studies are needed to elucidate this point. The diminution in the expression of GR immunoreactivity in the PaMP of females subjected to early maternal separation and VCS can be attributed to the increase in corticosterone levels observed in animals with the same treatment [Renard et al., 2007]. However, in the hippocampus, there is an increase in the expression of GR immunoreactivity in the CA1, CA2 and CA3 subfields. Consonant with our results, Karandrea et al. [2000] reported that stress-induced alterations in GR genes depend on the nervous structures of the HPA axis. On the other hand, the increased colo-

calization of Fos and AVP points towards an increased activation of PaMP cells following VCS in females subjected to early maternal separation. A plausible explanation for this is the reduced expression of GR immunoreactivity, thus affecting the negative feedback control mediated by this receptor. In MS males subjected to VCS, the expression of GR immunoreactivity in the PaMP is diminished, but is increased in the CA1, CA2 and CA3 subfields of the hippocampus. Hence, the inhibition of the HPA axis observed in males subjected to both stress protocols could be regulated at the level of the hippocampus. On the other hand, the diminished colocalization of Fos and AVP in these animals could be due to an enhanced susceptibility to glucocorticoid negative feedback. We can conclude that in females, both stress protocols altered the negative feedback mediated by GR, since we observed higher GR immunoreactivity in the hippocampus and, in turn, increased levels of corticosterone, with their adverse consequences. In contrast, the HPA axis in males seems to be better regulated by the feedback via GR, showing an increase in GR expression at the hippocampus and a decrease in circulating corticosteroids. In line with our results, Desbonnet et al. [2008] reported a sexual dimorphism in the CRH response to stress in animals subjected to maternal separation, with the neuroendocrinological stress system being more reactive in females. In addition to the results found on GR expression, the literature reports a minimal GR expression in the adult CA3 subfield of the hippocampus [De Kloet et al., 2005]. However, our experimental model shows not only the presence of GR in this area, but also its increased expression when animals are stressed or maternally separated and subjected to VCS, suggesting a redistribution of GR in the brain. The present study failed to demonstrate any sex differences in GR expression in the subfields of the hippocampus. However, despite the importance of glucocorticoids in HPA axis regulation, it is also susceptible to inhibition by glucocorticoid-independent mechanisms. The PVN is richly innervated by GABAergic neurons from multiple brain regions, including the bed nucleus of the stria terminalis, dorsomedial hypothalamus, lateral hypothalamic area and neurons scattered in the immediate surroundings of the PVN [Cullinan et al., 1993, 1996]. The degree to which GABAergic inhibitory circuits respond to neural stimuli versus glucocorticoid levels is not clear. However, it is relevant that animals with adrenalectomy can inhibit the ACTH response [Jacobson et al., 1988], indicating the existence of mechanisms that regulate the HPA axis activity in the absence of feedback by glucocorticoids. Therefore, we must consider the possibility that the sex differences shown in the regulation of the axis could be due to gender-dependent neurochemical responses to stress that have been shown in different neurotransmitter systems, such as GABAergic, cholinergic and monoaminergic [Cahill, 2006; Davis and Wilkinson, 2006; Chadda and Devaud, 2005], that may regulate differences in HPA activity. Besides, there is evidence that maternal behavior plays a critical role in HPA response to stress [Caldji et al., 2000] and that maternal care is sex-dependent towards the pups [Moore et al., 1997]. So, male and female pups miss out on different components of maternal care during maternal separation, and after reunion rat dams do not care in the same way for both sexes, which may lead to different long-term effects. On the other hand, the AVP neurons in the parvocellular region project to different areas inside the central nervous system and AVP is a sex-dimorphic modulator of synaptic transmission. Furthermore, this neuropeptide is distributed in different brain areas including the lateral septum, medial amygdaloid nucleus and bed nucleus of the stria terminalis, where male mammals have a greater cell number and fiber density than females [Rhodes and Rubin, 1999]. Taken together, these results offer additional support to the notion that the activity of the HPA axis is sex-dependent in such stress situations as early maternal separation and VCS. The enhanced activity in vasopressinergic neurons in response to stress in females exposed to early maternal separation suggests that perhaps early-life stress results in a more reactive neuroendocrine stress response in females than in males subjected to the same interventions. Furthermore, our results demonstrate that the different anatomic levels of the HPA axis have different roles in its stress response and support the evidence of regional specificity in GR regulation.

Acknowledgements

This work was supported by the Secretaría de Ciencia y Técnica (Argentine National Secretariat for Science and Technology), grant 69/08. This work was made as a PhD student at the Doctorado de Ciencias Biológicas of the Facultad de Ciencias Exactas, Físicas y Naturales of the Universidad Nacional de Córdoba.

References

Aguilera G, Rabadan-Diehl C (2000): Vasopressinergic regulation of the hypothalamic-pituitary-adrenal axis: implications for stress adaptation. Regul Pept 96:23–29.

Armstrong WE (2004): Hypothalamic supraoptic and paraventricular nuclei; in Paxinos G (ed): The Rat Nervous System, ed 3. Amsterdam, Elsevier Academic Press, chapt 15, pp 369–388.

Avishai-Eliner S, Hatalski CG, Tabachnik E, Eghbal-Ahmadi M, Baram TZ (1999): Differential regulation of glucocorticoid receptor messenger RNA (GR-mRNA) by maternal deprivation in immature rat hypothalamus and limbic regions. Brain Res Dev Brain Res 114:265–268.

Barna I, Bálint E, Baranyi J, Bakos N, Makara GB, Haller J (2003): Gender-specific effect of maternal deprivation on anxiety and corticotropin-releasing hormone mRNA expression in rats. Brain Res Bull 62:85–91.

Cahill L (2006): Why sex matters for neuroscience. Nat Rev Neurosci 7:477–484.

Caldji C, Diorio J, Meaney MJ (2000): Variations in maternal care in infancy regulate the development of stress reactivity. Biol Psychiatry 48:1164–1174.

Chadda R, Devaud LL (2005): Differential effects of mild repeated restraint stress on behaviors and GABA(A) receptors in male and female rats. Pharmacol Biochem Behav 81:854–863.

Cullinan WE, Helmreich DL, Watson SJ (1996): Fos expression in forebrain afferents to the hypothalamic paraventricular nucleus following swim stress. J Comp Neurol 368:88–99.

Cullinan WE, Herman JP, Watson SJ (1993): Ventral subicular interaction with the hypothalamic paraventricular nucleus: evidence for a relay in the bed nucleus of the stria terminalis. J Comp Neurol 332:1–20.

Davies W, Wilkinson LS (2006): It is not all hormones: alternative explanations for sexual differentiation of the brain. Brain Res 1126: 36–45.

De Kloet ER, Joëls M, Holsboer F (2005): Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6:463–475.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M, (1998): Brain corticosteroid receptor balance in health and disease. Endocr Rev 19:269–301.

Desbonnet L, Garrett L, Daly E, McDermott KW, Dinan TG (2008): Sexually dimorphic effects of maternal separation stress on corticotrophin-releasing factor and vasopressin systems in the adult rat brain. Int J Dev Neurosci 26:259–268.

García Marquez B, Armario A (1987): Chronic stress depresses exploratory activity and behavioral performance in the forced swimming test without altering ACTH response to a novel acute stressor. Physiol Behav 40: 33–38.

Han F, Ozawa H, Matsuda K, Nishi M, Kawata M (2005): Colocalization of mineralocorticoid receptor and glucocorticoid receptor in the hippocampus and hypothalamus. Neurosci Res 51:371–381.

Helmreich DL, Watkins L, Deak T, Maier S, Akil H, Watson SJ (1999): The effect of stressor controllability on stress-induced neuropeptide mRNA expression within the paraventricular nucleus of the hypothalamus. J Neuroendocrinol 11:121–128.

Herman JP, Cullinan WE (1997): Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci 20:78–84.

Heuser I, Lammers CH (2003): Stress and the brain. Neurobiol Aging 24:S69–S76.

Jacobson L, Akana SF, Cascio CS, Shinsako J, Dallman MF (1988): Circadian variations in plasma corticosterone permit normal termination of adrenocorticotropin responses to stress. Endocrinology 122:1343–1348.

Jacobson L, Sapolsky R (1991): The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12:118–134.

Karandrea D, Kittas C, Kitraki E (2000): Contribution of sex and cellular context in the regulation of brain corticosteroid receptors following restraint stress. Neuroendocrinology 71:343–353.

Katz RJ, Roth KA, Carrol RJ (1981): Acute and chronic stress effects on open field activity in the rat: implications for a model of depression. Neurosci Biobehav Rev 5:247–251.

Kelly SJ, Ostrowski NL, Wilson MA (1999): Gender differences in brain and behavior: hormonal and neural bases. Pharmacol Biochem Behav 64:655–664.

Ladd CO, Thrivikraman KV, Huot RL, Plotsky PM (2005): Differential neuroendocrine responses to chronic variable stress in adult Long Evans rats exposed to handling-maternal separation as neonates. Psychoneuroendocrinology 30:520–533.

Loyber I, Perassi NI, Lecuona FA, Peralta ME (1977): Effects of handling normal and bulbectomized rats at adrenal and plasma corticosterone levels. Experientia 33:1393–1394.

Lund TD, Munson DJ, Haldy ME, Handa RJ (2004): Androgen inhibits, while oestrogen enhances, restraint-induced activation of neuropeptides neurons in the paraventricular nucleus of the hypothalamus. J Neuroendocrinol 16:272–278.

McCormick CM, Linkroum W, Sallinen BJ, Miller NW (2002): Peripheral and central sex steroids have differential effects on the HPA axis of male and female rats. Stress 5:235–247.

Meaney MJ, Diorio J, Francis D, Widdowson J, LaPlante P, Caldji C, Sharma S, Seckl JR, Plotsky PM (1996): Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. Dev Neurosci 18: 49–72.

Moore CL, Wong L, Daum MC, Leclair OU (1997): Mother-infant interactions in two strains of rats: implications for dissociating mechanism and function of a maternal pattern. Dev Psychobiol 30:301–312.

Ogawa T, Mikuni M, Kuroda Y, Muneoka K, Mori KJ, Takahash K (1994): Periodic maternal deprivation alters stress response in adult offspring: potentiates the negative feedback regulation of restraint stress-induced adrenocortical response and reduces the frequencies of open field induced behaviors. Pharmacol Biochem Behav 49:961–967.

Paxinos G, Watson C (1997): The Rat Brain in Stereotaxic Coordinates, ed 3. San Diego, Academic Press.

Renard GM, Rivarola MA, Suárez MM (2007): Sexual dimorphism in rats: effects of early maternal separation and variable chronic stress on pituitary-adrenal axis and behaviour. Int J Dev Neurosci 25:373–379.

Rhodes ME, Rubin RT (1999): Functional sex differences ('sexual diergism') of central nervous system cholinergic systems, vasopressin, and hypothalamic-pituitary-adrenal axis activity in mammals: a selective review. Brain Res Brain Res Rev 30:135–152.

Scott LV, Dinan TG (1998): Vasopressin and the regulation of hypothalamic-pituitary-adrenal axis function: implications for the pathophysiology of depression. Life Sci 62:1985–1998.

Slotten HA, Kalinichev M, Hagan JJ, Marsden CA, Fone KC (2006): Long-lasting changes in behavioural and neuroendocrine indices in the rat following neonatal maternal separation: gender-dependent effects. Brain Res 1097:123–132.

Viau V, Meaney MJ (2004): α_1-Adrenoreceptors mediate the stimulatory effects of oestrogen on stress-related hypothalamic-pituitary-adrenal activity in the female rat. J Neuroendocrinol 16:72–78.

Wigger A, Neumann ID (1999): Periodic maternal deprivation induces gender-dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. Physiol Behav 66:293–302.

Williamson M, Bingham B, Viau V (2005): Central organization of androgen-sensitive pathways to the hypothalamic-pituitary-adrenal axis: implications for individual differences in responses to homeostatic threat and predisposition to disease. Prog Neuropsychopharmacol Biol Psychiatry 29:1239–1248.

Wotjak CT, Kubota M, Liebsch G, Montkowski A, Holsboer F, Neumann I, Landgraf R (1996): Release of vasopressin within the rat paraventricular nucleus in response to emotional stress: a novel mechanism of regulating adrenocorticotropic hormone secretion? J Neurosci 16:7725–7732.

Zelena D, Domokos A, Barna I, Csabail K, Bagdy G, Makara GB (2007): The role of vasopressin in chronic stress studied in a chronic mild stress model of depression. Ideggyogy Sz 60:196–200.