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Chronic unpredictable stress and long-term ovariectomy affect arginine-vasopressin expression in the paraventricular nucleus of adult female mice.

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Running title: Stress, ovariectomy and arginine-vasopressin in PVN

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ABSTRACT

Arginine-Vasopressin (AVP) may regulate the hypothalamic-pituitary-adrenal axis (HPA) and its effects on depressive responses. In a recent study, we demonstrated that Chronic Unpredictable Stress (CUS) depressive effects are enhanced by long-term ovariectomy (a model of post-menopause). In the present study, we investigated the effects of long-term ovariectomy and CUS on AVP expression in different subdivision of the paraventricular nucleus (PVN) of female mice. Both long-term ovariectomy and CUS affect AVP immunoreactivity in some of the PVN subnuclei of adult female mice. In particular, significant changes on AVP immunoreactivity were observed in magnocellular subdivisions, the paraventricular lateral magnocellular (PaLM) and the paraventricular medial magnocellular (PaMM), the 2 subnuclei projecting to the neurohypophysis for the hormonal regulation of body homeostasis. AVP immunoreactivity was decreased in the PaLM by both the long-term deprivation of ovarian hormones and the CUS. In contrast, AVP immunoreactivity was increased in the PaMM by CUS, whereas it was decreased by ovariectomy. Therefore, present results suggest morphological and functional differences among the PVN's subnuclei and complex interactions among CUS, gonadal hormones and AVP immunoreactivity.

Key words: Vasopressin; depression; menopause; stress; hypothalamus.

1. INTRODUCTION

Evolutionary success depends on our ability to adapt to changing circumstances. The neuroendocrine response to stress is an excellent example of a plastic system that responds to threats to homeostasis and alters its output to meet current and expected future demands.

In recent years, magnocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) have been reported to be involved in the regulation of hypothalamic-pituitary-adrenal axis (HPA) and its behavioral and emotional effects (see for extensive reviews, Landgraf, 2001, Engelmann et al., 2004, Landgraf et al., 2007). In particular, HPA-related stress responses involve the action of several factors including arginine-vasopressin (AVP) in magnocellular nuclei (for a review see Rotzinger et al., 2010). The contribution of centrally released vasopressin in the regulation of blood pressure and cardiovascular functions (Pyner, 2009), water intake (Bolignano and Zoccali, 2010, Moeller and Fenton, 2012) and in the emotional and social behavior is well established (Insel, 2010, Albers, 2012, Bosch and Neumann, 2012). On the other hand, it has been postulated that enhanced action of vasopressin in the brain may contribute to generation of pain, anxiety, panic attacks, aggression and depression (Coccaro et al., 1998, Siegel et al., 2007, Caldwell et al., 2008, Surget and Belzung, 2008). Among the different animal models to study the response to stress, Chronic Unpredictable Stress (CUS) has long been used as a model of emotional stress that can induce depressive and anxiety like-behaviors in rodents (Mineur et al., 2006).

The response to stress depends on gonadal hormones and is sexually dimorphic (Patchev et al., 1995, Viau, 2002). Sex differences exist in the response of the HPA axis to stress: i) women react more robustly than men (Kudielka and Kirschbaum, 2005) and ii) in both sexes, activation of the HPA axis may inhibit reproductive functions

(Cameron, 1997). Conversely, the sex differences in HPA function are in part due to differences in the circulating gonadal steroid hormone milieu (Handa et al., 1994). A recent magnetic resonance imaging study demonstrated sex differences in the activation of brain circuitries sensible to stress. In women the activation varies upon the hormonal cycle, whereas in man activated circuitries are similar to those observed in the early follicular menstrual phase of women (when estrogen and progesterone are low, Goldstein et al., 2010).

Recently, we have focused our attention on the inter-regulation between the stress response and gonadal hormones. We used an animal model of post-menopause (long-term ovariectomy), field in which we could study the emotional behavioral effect of the CUS. In a recent study (Lagunas et al., 2010), we demonstrated that CUS emotional effects on depressive and anxiety like-behaviors are enhanced by long-term gonadal hormones deprivation.

Based on these findings, the aim of the present study was to investigate the response of the different PVN subnuclei to the CUS in long-term ovariectomized female mice using corticosterone plasma levels and AVP immunoreactivity as parameter of stress response.

2. RESULTS

2.1 Serum corticosterone assay

Serum corticosterone levels were not significantly different between control animals and ovariectomized (OVX) animals (not submitted to CUS). However, plasma corticosterone levels were significantly increased in OVX-CUS animals compared to controls (Fig. 1). Data were analyzed by a two-way ANOVA with CUS and the

ovariectomy as the two independent variables. The results of two-way ANOVA showed no effect of CUS on serum corticosterone levels [$F_{(1,12)}$= 3.52, p= 0.09], a significant effect of ovariectomy [$F_{(1,12)}$= 8.71, p= 0.016] and a significant effect for the interaction of the two variables [$F_{(1,12)}$= 8.64, p= 0.016]. The Bonferroni's test detected a significant increase of corticosterone levels in OVX females exposed to CUS in comparison to the other groups ($p<0.05$).

2.2 AVP expression in the PVN.

The rodents' PVN consists of several distinct subnuclei with different cytological, neurochemical and circuitry features: the paraventricular dorsal cap (PaDC), the paraventricular lateral magnocellular (PaLM), the paraventricular medial magnocellular (PaMM) and the paraventricular medial parvocellular (PaMP) subnuclei (Benarroch, 2005, Ferguson et al., 2008). We decided, therefore, to measure AVP immunoreactivity in the four different subnuclei, separately.

Qualitative observation of AVP immunopositive staining revealed differences in the immunoreactivity pattern among the different experimental groups (Fig. 2). These qualitative differences were confirmed by the morphometric analysis (Fig. 3).

For each different PVN subnucleus, we analyzed the data by two-way ANOVA with CUS and ovariectomy as independent variables and the volume occupied by AVP immunoreactivity as dependent variable.

In the PaDC, the two-way ANOVA showed a significant effect of stress [$F_{(1,12)}$= 13.62, p= 0.003], no significant effect of ovariectomy [$F_{(1,12)}$= 3.06, p= 0.105] and a significant effect of the interaction of the two variables [$F_{(1,12)}$= 19.72, p= 0,0008]. The post-hoc Bonferroni's test showed that the volume occupied by AVP immunoreactive material

was significantly decreased in ovariectomized females compared to all the other groups ($p<0.01$ vs Control, $p<0.05$ vs CUS, $p<0.001$ vs CUS+OVX).

In the PaLM, the two-way ANOVA showed a significant effect of stress [$F_{(1,12)}= 6.04$, $p= 0.03$], of ovariectomy [$F_{(1,12)}= 42.25$, $p<0.0001$] and of interaction of the two variables [$F_{(1,12)}= 11.69$, $p= 0.005$]. The Bonferroni's test showed a significant decrease of AVP immunoreactivity in all groups compared to control female mice [$p<0.01$ vs CUS, $p<0.001$ vs OVX and CUS+OVX].

In the PaMM, the two-way ANOVA showed a significant effect of stress [$F_{(1,12}=52$, $p<0.0001$] and ovariectomy [$F_{(1,12}= 16.96$, $p= 0.001$]. No significant effect of interaction was detected [$F_{(1,12}= 2.93$, $p= 0.112$]. The Bonferroni's test showed a significant decrease of AVP immunoreactivity in OVX group compared to all the other groups ($p<0.01$ vs Control, $p<0.001$ vs CUS, and $p<0.001$ vs OVX+CUS). AVP immunoreactivity significantly increased in CUS group compared to controls ($p<0.01$) and OVX ($p<0.001$).

In the PaMP, the two-way ANOVA showed a significant effect of stress [$F_{(1,12}= 5.47$, $p=0.037$], but no effect of ovariectomy [$F_{(1,12}= 0.12$, $p= 0.74$] and of the interaction [$F_{(1,12}= 2.12$, $p= 0.17$]. The post hoc Bonferroni's test reported no significant differences among the four experimental groups.

3. DISCUSSION

Our results show that CUS did not affect plasma corticosterone levels in gonadally intact females, but it increased plasma corticosterone levels in OVX females submitted to CUS compared to control females (Fig. 1). These findings are in concordance with our previous results demonstrating that gonadally intact females submitted to CUS did not show differences in anxiety and depression-like behavior compared to control

group. All these data suggest that the gonads carry out a protective effect against the stress-dependent anxiety and depression-like behavior, as reported also in other studies (Lagunas et al., 2010, Vega-Rivera et al., 2013). Since the corticosterone secretion is normally regulated by the light-dark cycle, and the exposure to constant light disrupts this circadiam rhythm (Park et al., 2013), the manipulations of this cycle on the last day of CUS could have altered the corticosterone levels. However, we have not observed differences between the CUS group and controls, therefore it seems unlikely that this created a significant problem. The significant difference between OVX animals submitted to CUS and controls, with a significant increase of plasma corticosterone levels in OVX animals submitted to CUS, could be related with the lack of estrogen inhibition of the HPA axis response on PVN (Lund et al., 2006, Weiser and Handa, 2009), and also with the decreased levels of estrogen receptor β mRNA expression in the PVN of OVX animals (Jin et al., 2005, Barker and Galea, 2009, Liu et al., 2011).

The present findings indicate that both long-term ovariectomy and CUS may affect AVP immunoreactivity in the PVN of adult female mice. In particular, significant changes of the AVP immunoreactive material compared to control animals were observed in magnocellular subdivisions, the PaLM and the PaMM, the 2 subnuclei projecting to the neurohypophysis for the hormonal regulation of body homeostasis. On the other hand, the parvocellular subnuclei, the PaDC and the PaMP, were not influenced.

Long-term gonadal hormones' deprivation induced a significant decrease of AVP immunoreactivity in the PVN and in particular in the PaLM, not influencing the other magnocellular and parvocellular subnuclei. Similarly, De Vries et al. (1984) demonstrated that during the next 2 weeks from the ovariectomy no effect of ovariectomy could be detected upon the AVP innervation of dorsal vagal complex that

is derived from the parvocellular division of the PVN (Sawchenko and Swanson, 1982) in mice. More over, it has been shown that administration of estradiol (E_2) to postmenopausal women increases both circulating levels of AVP (Bossmar et al., 1995) and rapid intrahypothalamic AVP release (Wang et al., 1995, Burbach et al., 2001).

In our females, CUS significantly affects AVP immunoreactivity in the PVN but only in the magnocellular subnuclei, PaLM and PaMM, with no significant effects in the parvocellular subdivisions. Moreover, CUS affects AVP immunoreactivity in a different way within the magnocellular subnuclei: in the PaLM, CUS induced a decrease of AVP immunoreactivity, while in the PaMM, CUS caused an increase in both gonadally intact (CUS) and ovariectomized animals (OVX+CUS).

The difference within magnocellular and parvocellular neuronal subpopulations could be due to the differential expression of estrogen receptors (ER) in the PVN. In the rat and mouse, hypothalamus ERβ is the predominant ER isoform found in the PVN (Mitra et al., 2003, Merchenthaler et al., 2004). Interestingly, E_2 has been shown to activate the Mitogen-Activated Protein Kinases (MAPK) pathway via Extracellular signal-Regulated Kinases (ERK) 1/2 in the PVN and the SON of mice (Abraham et al., 2004). In the PVN, this is potentially mediated via either ERβ (Grassi et al., 2013) and/or G protein-coupled receptor 30 (GPR30). In the hypothalamus intense GPR30 expression was found in the PVN. In the rat PVN, GPR30 immunoreactivity is prominent in the magnocellular PVN with low-level labelling in the parvocellular PVN. Dual labelling of hypothalamic sections revealed that GPR30 immunoreactivity was present in AVP magnocellular neurons and 50–70% co-expressed GPR30 and AVP in the PVN (Hazell et al., 2009). In addition, looking in detail into the picture of Hazell's paper (Fig. 4 A-B-C), the higher levels of AVP and GPR30 co-expression are localized in the part of

magnocellular subdivision that we identify as PaLM suggesting an involvement of GPR30 in the regulation of AVP in the PaLM.

Present results, showing that CUS selectively affects AVP immunoreactivity in the PaLM and PaMM subdivisions are in agreement with previous studies indicating that the magnocellular neurosecretory system plays a role in some, but not all, chronic stress paradigms (Albeck et al., 1997, Guccione et al., 2002, Seale et al., 2004). On the contrary, no effects were detectable in AVP immunoreactive levels in the parvocellular PVN subdivision. This is in agreement with (Pinnock and Herbert, 2001) who demonstrated that the AVP response to both acute and repeated stress was unaltered in the low-dose corticosterone-treated rats compared to control animals in the parvocellular PVN, suggesting that a return of AVP to lower levels is necessary to allow this peptide to respond to repeated stress.

In conclusion, our results indicate that magnocellular subdivisions, PaLM and PaMM, known to be the part of the PVN sensible to the ovarian hormone-dependent regulation of AVP system (Wang et al., 1995), are differentially regulated by the exposure to CUS. As previously discussed this is probably related to differences in the distribution of nuclear or membrane-bound estrogen receptors (Merchenthaler et al., 2004, Hazell et al., 2009), or to differences in estrogen-sensitive afferents to PVN (Grassi et al., 2010). It is interesting to underline that the specific stimulation of GPR30 in male and ovariectomized female mice induced anxiogenic effects (Kastenberger et al., 2012). In our previous experiment, we demonstrated an enhancement of anxiety and depressive-like behaviors in OVX mice submitted to CUS (Lagunas et al., 2010) and now, the present findings show a correlation between these altered behaviors and modifications in the levels of AVP immunoreactivity in some PVN's subnuclei, in

agreement with the involvement of this system in depression and anxiety (Landgraf, 2001, Surget and Belzung, 2008, Rotzinger et al., 2010).

4. EXPERIMENTAL PROCEDURE

Female C57BL6 mice (Harlan, Barcelona, Spain) housed in groups, with unlimited access to food and water, under controlled light schedule (12 hours light:12 hours dark) were used for this experiment. The animals were handled following the European Union guidelines (Directive 86/609/EEC). Our Institutional Animal Use and Care Committee (CSIC) approved the experimental procedures. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum.

4.1. *Chronic unpredictable stress protocol (CUS)*

At the age of two months, female mice were assigned to two different groups: intact and ovariectomized. Mice belonging to the second group were bilaterally ovariectomized under isoflurane anesthesia. Four months after ovariectomy, the groups were subdivided into 2 subgroups: stressed and no stressed mice. The stressed groups were submitted to the CUS protocol to define the effect of chronic unpredictable stress in physiological and long-term gonadal hormones' deprivation conditions. The stressed animals were submitted to CUS protocol during 4 weeks. The stress procedure was a modified version of a previously described model (Moreau et al., 1992). The stress consisted of repeated psychological stressors, such as: social isolation, predator proximity, presence of an intruder, continuous room and cage changes, cage tilting, damp sawdust and lights on for a long period (see Table 1). None of the stressors involved food or water deprivation. Control and CUS animals were housed in the same animal facility but in separate rooms.

In summary, we obtained a total of 4 experimental groups: (i) (Control) intact not stressed mice (n=24); (ii) (CUS) intact stressed mice (n=25); (iii) (OVX) ovariectomized not stressed mice (n=24) and (iv) (OVX+ CUS) ovariectomized stressed mice (n=24). At the end of CUS protocol, at the age of 7 months, animals were assessed for forced swimming test and elevated plus-maze test (Lagunas et al., 2010). Some animals (n=4) from all experimental groups were not behaviorally assessed and their brains were used for vasopressin immunohistochemical staining.

4.2. Determination of serum corticosterone.

Blood samples were collected immediately before the perfusion by tail nick and collected into EDTA-coated capillary tubes and kept on ice. The blood was rapidly placed in microfuge tubes and allowed to clot for 1 h. After centrifugation at 3500 rpms serum was stored at -70 °C until assayed.

The serum corticosterone levels were measured using a kit of enzyme-immunoassay (EIA) for the quantitative determination of corticosterone in mouse serum (IDIS OCTEIA) according to the manufacturer's instructions. The sensitivity of the measurement was 0.55 ng/mL. The intra- and inter-assay coefficients of variation were 3.8% and 7.7%, respectively. The results are presented as ng/mL.

4.3. Immunohistochemistry

The animals (seven month-old) were deeply anesthetized with pentobarbital (Normon Veterinary Division, Madrid, Spain, 50 mg/kg) and perfused through the left cardiac ventricle with saline solution (0.9% NaCl) and then with fixative solution (4% paraformaldehyde in 0.1M PBS, pH 7.4). Brains were quickly dissected, immersed for 4–6 h at 4ºC in freshly prepared fixative solution and then rinsed with 0.1M PBS. Brains were serially cut in the coronal plane at 50µm thickness with a Vibratome (VT

1000 S, Leica Microsystems, Wetzlar, Germany). The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the mouse brain atlas (Paxinos and Franklin, 2001). Sections were collected in a cryoprotectant solution (Watson et al., 1986) at -20°C. Every forth section (one section every 200 µm) was stained for AVP immunohistochemistry according to our standard protocol (Plumari et al., 2002, Grassi et al., 2010). Briefly, free-floating sections were washed overnight at 4ºC in PBS 0.1M then in PBS containing 0.2% Triton X-100 and 0.2% BSA, and finally treated with a solution of PBS/methanol (1:1) with 0.3% hydrogen peroxide (to quench endogenous peroxidase activity). The sections were incubated for 48 hours at 4°C with a rabbit polyclonal AVP antibody (Chemicon, Millipore Ibérica, Madrid, Spain) diluted 1:10,000 in PBS-Triton X-100-BSA buffer, containing 3% normal serum goat. A biotinylated goat anti-rabbit antibody (Thermo scientific, Pierce, Rockford, IL, USA) was then used at a dilution of 1:300 for 120 min at room temperature. The antigen–antibody reaction was revealed by incubation with avidin-peroxidase complex (Thermo scientific, Pierce) for 90 min. Peroxidase activity was visualized with a solution containing 0.187mg/ml 3,3-diamino-benzidine (Sigma, Madrid, Spain) in PBS. Sections were mounted on chromallum-coated slides, air-dried, cleared in xylene, and cover slipped.

4.4. Morphometrical analysis

The morphometric analysis of AVP immunostaining was performed on coded sections without previous knowledge of the experimental group. The quantification of AVP immunoreactivity was achieved within the PVN on 3 coded sections (covering the entire rostrocaudal extension of the right PVN) by estimating the percentage of volume occupied by AVP immunoreactive material with the Weibel's point counting method (Weibel, 1979), using a grid of 20 square boxes of 8 μm^2 each and a x20 objective

(Grassi et al., 2010). Since rodents' PVN consists of several distinct subnuclei with different cytological and neurochemical features (Ferguson et al., 2008), quantification was separately performed on four different subnuclei of the PVN [paraventricular dorsal cap (PaDC), paraventricular lateral magnocellular (PaLM), paraventricular medial magnocellular (PaMM) and paraventricular medial parvocellular (PaMP) subnuclei] (see Fig. 2). The PVN subdivisions were identified following the anatomical description based on Nissl-stained material (Silverman and Pickard, 1983, Armstrong, 1985). Percentage of the volume fraction occupied by immunoreactive material versus the total volume of each PVN subnucleus was considered for statistical analysis. In figure 3, volume fraction values were normalized to control values.

4.5 Data analysis

Statistical analysis of the experimental data was carried out using SPSS (version 17.0). Two-way analysis of variance (ANOVAs) with Bonferroni post hoc test was conducted to evaluate effects of the ovariectomy, the CUS and the potential interactions. The level for statistical significance was set at $p < 0.05$.

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FIGURES' CAPTIONS

Fig.1. Corticosterone levels in serum (ng/mL±S.E.) of intact mice (Control), ovariectomized mice (OVX), intact mice submitted to stress (CUS) and ovariectomized mice submitted to stress (OVX+CUS). Different letters denote significant differences ($p<0.05$) between pairs.

Fig.2. Representative microphotographs of AVP immunostaining within paraventricular nucleus (PVN) in control mice (control), ovariectomized mice (OVX), control mice submitted to stress (CUS) and ovariectomized mice submitted to stress (OVX+CUS). PaDC - Paraventricular Dorsal Cap; PaLM - Paraventricular Lateral Magnocellular subdivision; PaMM - Paraventricular Medial Magnocellular subdivision; PaMP - Paraventricular Medial Parvocellular subdivision.

Scale bar, 100 μm. * 3rd Ventricle.

Fig.3. Volume fraction of Paraventricular Dorsal Cap (PaDC), Paraventricular Lateral Magnocellular (PaLM), Paraventricular Medial Magnocellular (PaMM) and Paraventricular Medial Parvocellular (PaMP) subdivisions occupied by AVP immunoreactive material in A) ovariectomized animals (OVX), B) control animals submitted to stress (CUS) and C) ovariectomized animals submitted to stress (OVX+CUS). The results are normalized to control animals' value equal 100. Different letters denote significant differences ($p<0.05$) between pairs.

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Day		Continuous room and cage changes (8 h once per hour) 9AM-5PM	Introducing in a cage with rat sawdust (1h) 12PM-1PM				Lights on 9AM-7PM
Night	Social isolation 7PM-9AM	Animals were paired with an unfamiliar mouse 7PM-9AM	Predator proximity: introducing a rata into the cage (2h) 7PM-9PM	Damp sawdust 7PM-9AM	Cage tilting (45º) 7PM-9AM	Lights on 7PM-9AM	Lights on 7PM-9AM

Table 1. Schematic representation of chronic unpredictable mild stress (CUS) protocol. Stress protocol was repeated continuously during 4 weeks (Lagunas et al., 2010)

Highligths

- Long-term ovariectomy induces a decrease of AVP-ir in some subnuclei of the PVN
- The CUS significantly affects AVP-ir not in the same direction of ovariectomy
- Magnocellular subdivisions of the PVN are significantly affected by CUS
- Long-term ovariectomy and CUS induce changes in AVP-ir with regional specificity

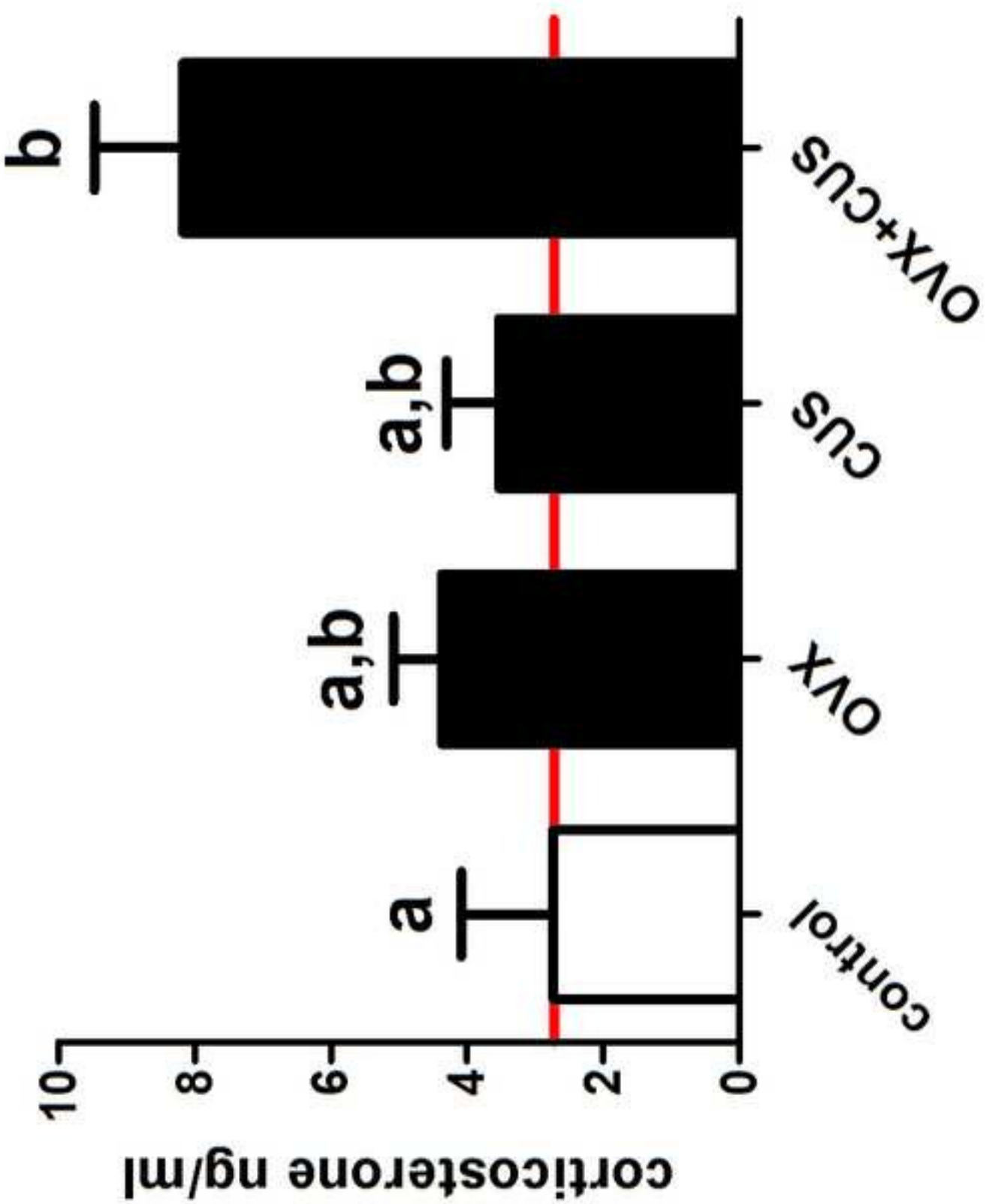
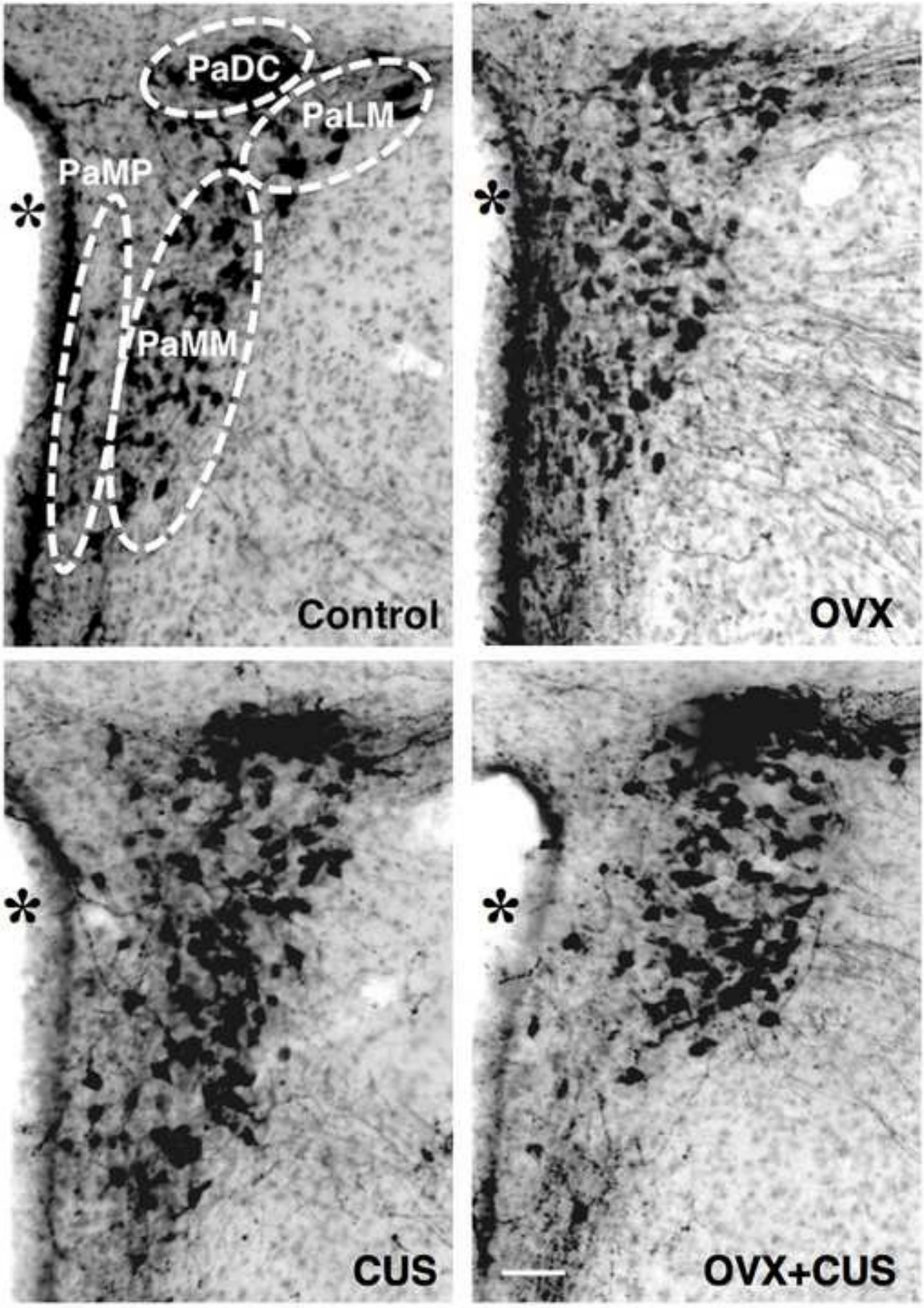

corticosterone ng/ml
0
2
4
6
8
10
a
a,b
a,b
b
control
OVX
CUS
OVX+CUS

Figure 1



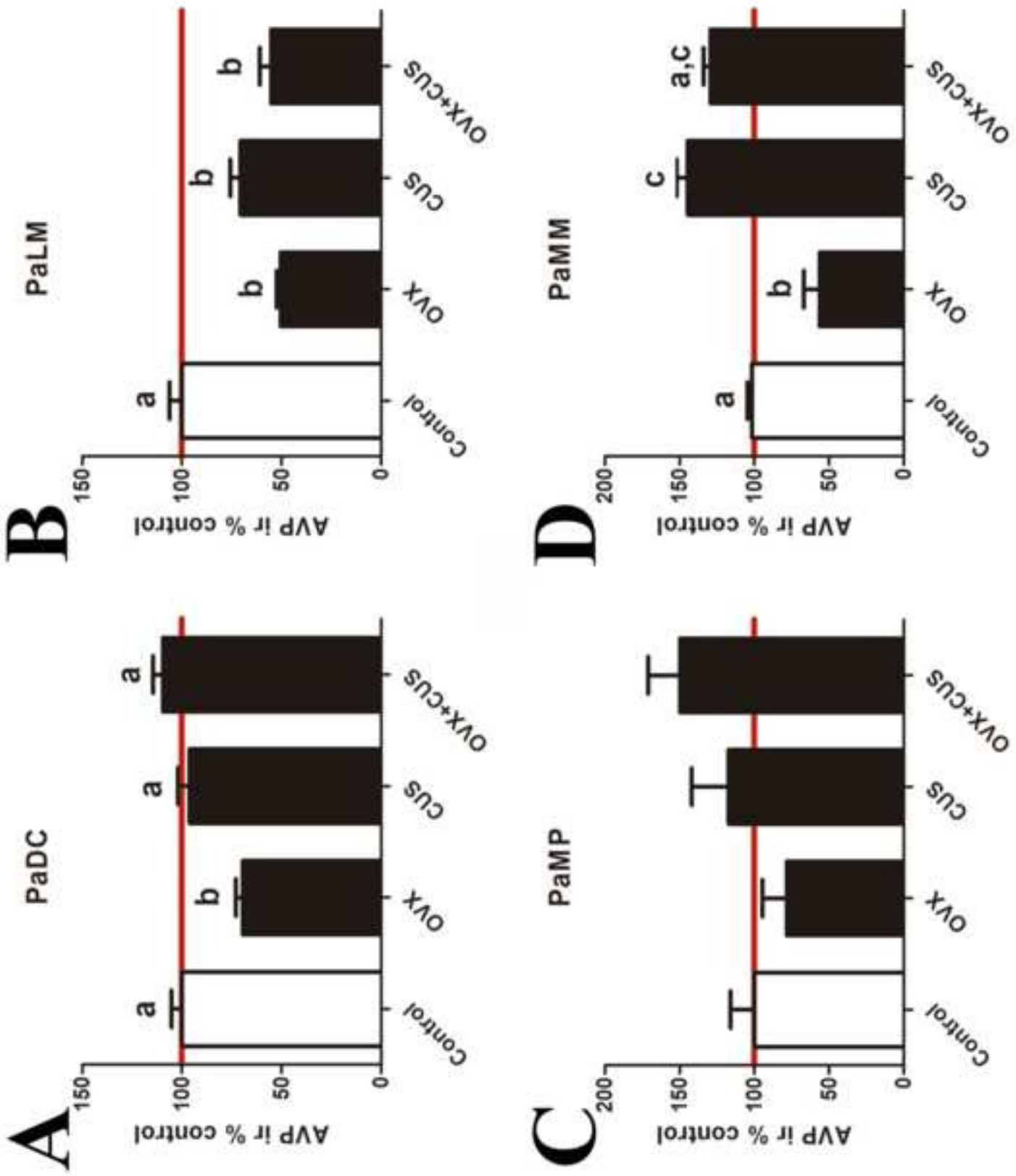


Figure 3