Estrogen receptor beta and G protein-coupled estrogen receptor 1 are involved in the acute estrogenic regulation of arginine-vasopressin immunoreactive levels in the supraoptic and paraventricular hypothalamic nuclei of female rats

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Estrogen receptor beta and G protein-coupled estrogen receptor 1 are involved in the acute estrogenic regulation of arginine-vasopressin immunoreactive levels in the supraoptic and paraventricular hypothalamic nuclei of female rats

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ABSTRACT

The ovarian hormone 17β-estradiol is known to regulate the release, expression and immunoreactivity of arginine-vasopressin (AVP) in the supraoptic and paraventricular hypothalamic nuclei of rodents. Previous studies have shown that estrogen receptor α is involved in the effects of chronic estradiol administration on arginine-vasopressin immunoreactivity in the female rat hypothalamus. In this study we have examined the effect of an acute administration of estradiol or specific agonists for estrogen receptors α, β and G protein-coupled estrogen receptor 1 on the immunoreactivity of arginine-vasopressin in the hypothalamus of adult ovariectomized female rats. Acute estradiol administration resulted in a significant decrease in the number of arginine-vasopressin immunoreactive neurons in the supraoptic and paraventricular nuclei after 24 hours. The effects of the specific estrogen receptors agonists suggest that the action of estradiol on arginine-vasopressin immunoreactivity is mediated in the supraoptic nucleus by G protein-coupled estrogen receptor 1 and in the paraventricular nucleus by both estrogen receptor β and G protein-coupled estrogen receptor 1. Thus, in contrast to previous studies on the effect of chronic estrogenic treatments, the present findings suggest that estrogen receptor β and G protein-coupled estrogen receptor 1 mediate the acute effects of estradiol on arginine-vasopressin immunoreactivity in the hypothalamus of ovariectomized rats.

Key words: 17β-estradiol; GPER; PPT; DPN; G1; hypothalamus

1. INTRODUCTION

In addition to its role in reproduction, the ovarian hormone 17β-estradiol (E2) exerts numerous homeostatic actions, including the regulation of the levels of arginine-vasopressin (AVP) in plasma (Skowsky et al., 1979; Stone et al., 1989; Ho and Lee, 1992). Since the pioneering work of Sar and Stumpf (Sar and Stumpf, 1980 and Sar and Stumpf 1981), using autoradiographic analysis of [3H] E2 binding sites in the rodent brain, it is known that AVP neurons in the supraoptic (SON) and paraventricular (PVN) nuclei are direct targets of E2. More recent studies have shown that SON and PVN neurons express estrogen receptors (ERs) (Alves et al., 1998; Suzuki and Handa, 2004; Suzuki and Handa, 2005; Grassi et al., 2017) and that E2 regulates AVP release, expression and immunoreactivity in SON and PVN (Greer et al., 1986; Liu et al., 1995; Swenson et al., 1998; Sladek et al., 2000; Nomura et al., 2002; Somponpun and Sladek, 2002; Almeida-Pereira et al., 2016).

In a previous study (Grassi et al., 2010), the role of ERα and ERβ in the regulation of AVP immunoreactivity in the SON and PVN was assessed after chronic E2 administration and we demonstrated that the observed changes in the AVP immunoreactivity in both nuclei are mediated by ERα and not by ERβ. However, the possible effects of acute changes in E2 levels on AVP immunoreactivity have not been yet determined. In addition, recent findings indicate that the membrane associated G protein-coupled estrogen receptor 1 (GPER) is expressed in neurons of the SON and PVN (Grassi et al., 2017). GPER may thus confer a previously unexplored additional level of complexity in the estrogenic regulation of AVP neurons in the SON and PVN. Therefore, in the present study we have examined the effects of the acute administration of specific agonists for ERα, ERβ and GPER on AVP immunoreactivity in the SON and PVN of ovariectomized rats.

2. RESULTS

2.1 Estrogenic regulation of AVP immunoreactivity in the SON

Ovariectomized rats were injected with either vehicle (controls), 17β-estradiol (E2), the ERα agonist PPT, the ERβ agonist DPN or the GPER agonist G1. AVP immunoreactivity was assessed in the hypothalamus. Qualitative differences in AVP immunoreactivity in the SON were observed between the different experimental groups (Fig. 1). Animals injected with E2 showed a decreased immunostaining compared to controls (Fig. 1). No obvious differences in AVP immunostaining were detected between control animals and the animals injected with the ERα agonist PPT or the ERβ agonist DPN. In contrast, animals injected with the GPER agonist G1 showed a reduced AVP immunoreactivity compared to control animals (Fig. 1).

The morphometric analysis of AVP immunoreactivity confirmed the qualitative observations. One-way ANOVA showed a significant treatment effect ($F_{4,17}$ = 9.32; P=0.0004). The post hoc Bonferroni's test showed that the number of AVP positive cells was significantly decreased by the treatment with either E2 (P<0.01), or the selective GPER agonist G1 (P<0.01), compared to control animals (Fig. 2). In contrast, the ERα agonist PPT and the ERβ agonist DPN had no significant effects on the number of AVP immunoreactive cells (Fig. 2).

2.2 Estrogenic regulation of AVP immunoreactivity in the PVN

Qualitative differences in AVP immunoreactivity in the PVN were also observed between the different experimental groups (Fig. 3). Compared to controls, AVP immunoreactivity was decreased in the PVN of animals treated with E2, the ERβ agonist DPN or the GPER agonist G1 (Fig. 3). ANOVA analysis confirmed a significant treatment effect ($F_{4,15}$=30.45; P<0.001). Post-hoc analysis revealed significant differences between the control and the E2 (P<0.001), DPN (P<0.001) and G1 (P<0.001) groups (Fig. 4).

The PVN was subdivided in its four different sub-regions: the paraventricular dorsal cap (PaDC), the paraventricular lateral magnocellular subdivision (PaLM), the paraventricular medial magnocellular subdivision (PaMM) and the paraventricular parvocellular subdivision (PaV) (Fig. 5). ANOVA analysis revealed a treatment effect in the PaDC ($F_{4,15}$=8.57; P<0.0008), PaLM ($F_{4,15}$=27.73; P<0.0001) and PaMM ($F_{4,16}$=4,56; P=0.0120). In contrast, no significant effect of treatment was detected in PaV ($F_{4,15}$=2.74; P=0.0680) (Fig. 6).

Compared to control values, the number of AVP immunopositive cells was significantly decreased in the PaDC by treatments with either E2 (P<0.05), the ERβ agonist DPN (P<0.05) or the GPER agonist G1 (P<0.05). Similar results were obtained in the PaLM, where the number of AVP immunoreactive cells was significantly decreased by E2 (P<0.001), DPN (P<0.001) and G1 (P<0.001) (Fig. 6). In the PaMM, although estradiol treatment resulted in a significant (P<0.05) decrease in the number of AVP immunoreactive cells compared to control values, the effects of ER agonists did not reach statistical significance (Fig. 6).

3. DISCUSSION

The findings of the present study indicate that an acute administration of E2 to ovariectomized rats resulted in a significant decrease in the number of AVP immunoreactive neurons in the SON and PVN. This finding extends the results of previous studies showing that AVP neurons in the SON and PVN are regulated by E2. Thus, chronic E2 administration potentiates c-fos immunoreactivity in AVP neurons in the SON and/or PVN of ovariectomized rats submitted to water deprivation (Vilhena-Franco et al., 2016), hypovolemic shock (Mecawi et al., 2011) or hypertonic extracellular volume expansion (Vilhena-Franco et al., 2011). In addition, chronic E2 administration to ovariectomized rats increases AVP expression in the SON (Garcia et al., 2017) and AVP immunoreactivity in the SON and PVN (Grassi et al., 2010).

Surprisingly, the short-term effects of acute E2 administration observed in the present study, decreasing AVP immunoreactivity in the SON and the PVN, are opposite to the effects of chronic E2 treatment, which increases AVP immunoreactivity in these hypothalamic nuclei (Grassi et al., 2010). This different effect of chronic (Grassi et al., 2010) and acute (present study) estradiol treatments has been observed in female rats from the same strain that were ovariectomized at the same age (3 months) and the administration of estradiol was in both cases initiated seven days after ovariectomy. This suggests that acute and chronic actions of estradiol involve different mechanism to regulate AVP immunoreactivity in SON and PVN neurons.

The physiological implications of the different regulation of AVP immunoreactivity in the hypothalamus by acute and chronic E2 treatments remain to be determined. E2 has vasodilator activity and low E2 levels after menopause represent a risk factor for the development of hypertension (Mendelsohn and Karas, 1999; Alecrin et al., 2004; Lima et al., 2005; Maas and Franke, 2009; Khalil, 2013). However, supraphysiological dosages of estrogens in first generation oral contraceptives were shown to increase blood pressure in young women (Oelkers, 1996) and chronic treatment with low doses of estradiol increased blood pressure in female rats (Subramanina et al., 2017). The effects of E2 in the control of blood pressure involve both ERα, ERβ and GPER (Traupe et al., 2007; Meyer et al., 2011) and are mediated by actions at different levels, including the regulation of AVP expression in magnocellular hypothalamic neurons (Pietranera et al., 2015).

Differences in AVP immunoreactivity in magnocellular hypothalamic neurons induced by acute and chronic E2 treatments may potentially reflect changes in AVP synthesis and/or changes in AVP release, given that the hormone regulates both the expression and the release of AVP (Sladek et al., 2000; Nomura et al., 2002; Somponpun and Sladek, 2002; Grassi et al., 2013; Pietranera et al., 2015). It should be noted that, at least in some circumstances, increased AVP immunoreactivity in

hypothalamic magnocellular neurons is associated with increased AVP release. For instance, this has been observed for the direct actions of angiotensin II in the brain, which participate in the upregulation of blood pressure and that are antagonized by E2 (Almeida-Pereira et al., 2016, 2018). Central angiotensin II administration increases AVP immunoreactivity in the PVN and in parallel increases AVP levels in plasma (Almeida-Pereira et al., 2016). Interestingly, subcutaneous injections of E2 block both effects of central angiotensin II administration (Almeida-Pereira et al., 2016). Thus, at least in this case, a decrease in AVP immunoreactivity induced by E2 in magnocellular neurons is associated with a decrease in AVP release. New experiments are needed to determine whether acute and chronic estradiol treatment have opposite effects in AVP expression and/or release.

Our findings, using subtype selective ER agonists, suggest that the acute effect of E2 on AVP immunoreactivity in the hypothalamus of ovariectomized rats is mediated by GPER in the SON and by ERβ and GPER in the PVN. This is in contrast with the effect of chronic E2 in AVP immunoreactivity in the SON and the PVN, which is mediated by ERα (Grassi et al., 2010). Since the acute effects of E2 are opposite to the effects of chronic E2 treatment, our present and previous findings indicate that the acute and chronic effects of E2 on AVP immunoreactivity in the hypothalamus of ovariectomized rats are mediated by different ER subtypes.

Studies in cell lines have shown that ERα, ERβ and GPER have different effects on AVP transcription, being activated by ERα and inhibited by ERβ and GPER (Shapiro et al., 2000; Grassi et al., 2015; Grassi et al., 2013a). However, these differences cannot be directly translated to AVP regulation in the SON and the PVN in vivo, since in rats ERα is not expressed in AVP neurons in these hypothalamic nuclei (Shughrue et al., 1997; Merchenthaler et al., 2004; Suzuki and Handa, 2005). In contrast, several studies have reported ERβ expression by AVP neurons in the SON and the PVN (Gruber et al., 1986; Alves et al., 1998; Suzuki and Handa, 2005). GPER

immunoreactivity has been also detected in the rat SON and PVN (Brailoiu et al., 2007; Grassi et al., 2017) and GPER expression has been reported in AVP neurons in the SON and PVN of mice (Hazell et al., 2009).

The absence of ERα expression in the SON and PVN of rats indicate that the ERα-mediated effects of E2 on AVP immunoreactivity in these hypothalamic nuclei could be indirect, involving other neuronal populations, while E2 may directly activate ERβ and GPER in the AVP neurons of the SON and PNV. The existence of parallel indirect and direct mechanism of action of E2 on SON and PVN AVP neurons may contribute to the different effects of each receptor subtype on AVP immunoreactivity. For instance, ERβ and GPER may exert a direct inhibitory effect on AVP transcription in SON and PVN neurons GPER (Shapiro et al., 2000; Grassi et al., 2013b; Grassi et al., 2015) while ERα could have a promoting effect through neuronal inputs arising from neurons expressing this ER subtype (Grassi et al., 2010).

Another possible factor contributing to the differential involvement of each ER subtype in the effects of acute and chronic E2 treatments is the dissimilar consequences that these treatments may have on ER expression or ER signaling. Although this possibility has not been fully explored, it is known that chronic E2 treatment exerts a differential regulation of the expression of ERα and ERβ in different brain regions and neuronal populations (Osterlund et al., 1998; Prange-Kiel et al., 2003; Bohacek and Daniel, 2009). For instance, 17β-estradiol increases the expression of ERα, but decreases the expression of ERβ in hippocampal neurons in vitro (Patisaul et al., 1999). Chronic E2 treatment also affects ER expression in the SON and the PVN (Patisaul et al., 1999; Suzuki and Harada, 2004). Patisaul et al. showed that chronic 17β-estradiol administration decreases the expression of ERβ in PVN neurons of ovariectomized rats (Patisaul et al., 1999). Furthermore, Suzuki and Harada treated ovariectomized rats with estradiol benzoate, for 3 days before they have been euthanized, and observed a significant decrease in the expression of ERβ in the SON and the

PVN (Suzuki and Harada, 2004). Therefore, it is plausible that chronic E2 treatment, by decreasing ERβ expression in the SON and PNV, could favor the up regulation of AVP immunoreactivity mediated by ERα. However, to our knowledge, there is no available information on the effects of E2 treatments on the expression of ERα and GPER in the SON and the PVN.

Further studies should determine the role of the expression of different ER subtypes in specific neuronal populations, the different transcriptional regulation of AVP by ERα, ERβ and GPER and the differential regulation of the expression of each ER form by chronic E2 on the regulation of AVP immunoreactivity in the SON and PVN. All these factors may together contribute to the different effects of acute and chronic E2 treatments on AVP regulation in these hypothalamic nuclei.

In summary, in contrast to the previously observed ERα-dependent up regulation of the number of AVP immunoreactive cells in the SON and PVN by chronic estrogenic treatments, the present findings indicate that ERβ and GPER mediate acute effects of estradiol decreasing the AVP immunoreactivity in the hypothalamus of ovariectomized female rats.

4. MATERIALS AND METHODS

4.1 Animals and experimental treatments

Wistar albino female rats from our in-house colony were kept on a 12:12-h light–dark cycle and received food and water ad libitum. Animals were handled in accordance with the guidelines published in the "NIH Guide for the care and use of laboratory animals", the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience, and following the European Union (2010/63/UE) and the Spanish legislation (L6/2013; RD53/2013). Experimental procedures were approved by our Institutional Animal Use and Care Committee (UNED, Madrid). Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary.

Female rats (n=20) were bilaterally ovariectomized at the age of 3 months under isoflurane anesthesia. They were then housed in plastic cages and randomly assigned to the different treatments. Seven days after surgery 4 rats per each group received one i.p. injection of either vehicle (corn oil), 17β-estradiol; BiogenCientifica, Madrid, Spain; (50μg/kg), the subtype-selective ERα agonist PPT (4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol; BiogenCientifica, Madrid, Spain; 1 mg/kg), the selective ERβ agonist DPN (2,3-*bis*(4-Hydroxyphenyl)-propionitrile; BiogenCientifica, Madrid, Spain; 1mg/kg), or the selective GPER agonist G-1 (((±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone; BiogenCientifica, Madrid, Spain; 2,4 nmol/animal). Doses of the different ligands were based on a previous *in vivo* study [Waters et al., 2009; Santollo et al., 2010; Grassi et al., 2013b; Grassi et al., 2013c; Dennis et al., 2009; Grassi et al., 2017].

Twenty-four hours after the injection of the estrogenic compounds all the animals were deeply anesthetized with pentobarbital (Normon Veterinary Division, Madrid, Spain, 50 mg/kg) and perfused through the left cardiac ventricle with 50 ml of saline solution (0.9% NaCl) followed by 250 ml of fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were quickly removed and immersed for 4–6 hours at 4ºC in the same fixative solution and then rinsed with phosphate buffer. Brains were placed for 72 hours in a 30% sucrose solution in PBS, frozen in liquid isopentane at −35ºC, and stored in a deep freezer at −80ºC until sectioning. Brains were serially cut in the coronal plane at 25 μm thickness with a cryostat, obtaining 4 series of adjacent serial sections. In each series, each section was 100 μm distant from the following one. The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the rat brain atlas of [Paxinos and Watson, 2013]. Sections were collected in multiwell plates with a cryoprotectant solution [Watson et al., 1986] and kept at -20°C.

4.2 Immunohistochemistry

The presence of AVP was detected by immunohistochemistry performed on free-floating sections

according to the following steps. Before the reaction, the sections collected in the cryoprotectant solution were washed overnight at 4ºC in PBS 0.1M, pH 7.3–7.4. The following day, free floating sections were first washed for 30 min at room temperature in PBS 0.1M, pH 7.3–7.4, containing 0.2% Triton X-100 and 0.2% BSA. Sections were then treated for 10 min with a solution of PBS 0.1M, pH 7.3–7.4, containing methanol/hydrogen peroxide (PBS/methanol 1:1 with 0.3% hydrogen peroxide) to quench endogenous peroxidase activity. Sections were washed for 30 min at room temperature in PBS 0.1M, pH 7.3–7.4, containing 0.2% Triton X-100 and 0.2% BSA and then incubated for 72 hours at 4°C with a rabbit polyclonal AVP antibody (Chemicon, Merck, Germany, reference AB1565) diluted 1:100,000 in 0.1M PBS, pH 7.3–7.4, containing 0.2% Triton X-100, 0.2% BSA and 3% normal goat serum. A biotinylated goat anti-rabbit secondary 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, Rockford, IL, USA) for 90 min. The peroxidase activity was visualized with a solution containing 0.187 mg/mL 3,3- diamino-benzidine (Sigma, Madrid, Spain) in PBS 0.1M, pH 7.3–7.4. The sections were washed in the PBS 0.1M, pH 7.3–7.4 and collected on chrome alum-gelatin coated slides, air dried, cleared in xylene, and cover slipped with DEPEX (VWR International Eurolab, Barcelona, Spain) for quantitative analysis.

4.3 Morphometric analysis

The quantitative analysis of AVP immunostaining was performed on coded sections without knowledge of the experimental group. Selected fields were acquired by a digital camera (Olympus DP25) connected to a Nikon eclipse E600 microscope using a x20 objective. The number of AVP positive cells was assessed in the SON and the PVN, using three coded sections per animal. Each section was 100 µm distant from the following one. Sections were positioned in the anteroposterior axis around bregma -1.3 mm for the SON and around bregma -1.8 mm for the PVN [Paxinos and Watson, 2013]. Data presented are the sum of the total number of immunolabeled cells in the three

sections for each rat. Because in previous studies we did not detect a lateralization of the distribution of AVP positive cells, counts were restricted to the right hemisphere of the hypothalamus. Given the anatomical heterogeneity of the PVN, a grid of 24 square boxes of 8 μm^2 each were used in each slice and the grid was precisely located ensuring the left side of the grid on the wall of the third ventricle. The grid overlaps the total area of the PVN. Cell counting was performed for each square box within the boundaries of the nucleus. The PVN subdivisions were identified following the detailed anatomical description provided for the rat by [Armstrong et al., 1980] based on Nissl-stained material. The boxes corresponding to the same PVN subdivision, along the three coded sections, were grouped for the statistical analysis (see fig 5). In the SON, instead, all AVP positive cells within the anatomical boundaries of the nucleus were counted together.

4.4 Statistical Analysis

Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test, using the SPSS-17.0 software (SPSS Inc, Chicago, USA). A value of $P < 0.05$ was considered statistically significant. Data are presented as the mean±SEM.

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AUTHOR CONTRIBUTIONS

P.C, G.C.P., L.M.G.S. and D.G. designed and supervised the experiments; D.G., N.L., M.M., M.dA. and H.P. performed the experiments; D.G. prepared the figures for publication; D.G. and L.M.G.S. wrote the first draft of the manuscript; all authors read and approved the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that would prejudice its impartiality.

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FIGURE LEGENDS

Fig. 1, Representative examples of AVP immunoreactivity in the supraotic nucleus from ovariectomized rats treated with vehicle (Control), estradiol (E2), the ERα agonist PPT, the ERβ agonist DPN or the GPER agonist G1. *, Optic chiasm. Scale bar 100 μm.

Fig. 2, Morphometric analysis of the number of AVP immunoreactive cells in the supraotic nucleus of ovariectomized rats treated with vehicle (control), estradiol (E2), the ERα agonist PPT, the ERβ agonist DPN or the GPER agonist G1. Data represents the mean±SEM. **, significant differences (P<0.01) versus the control group.

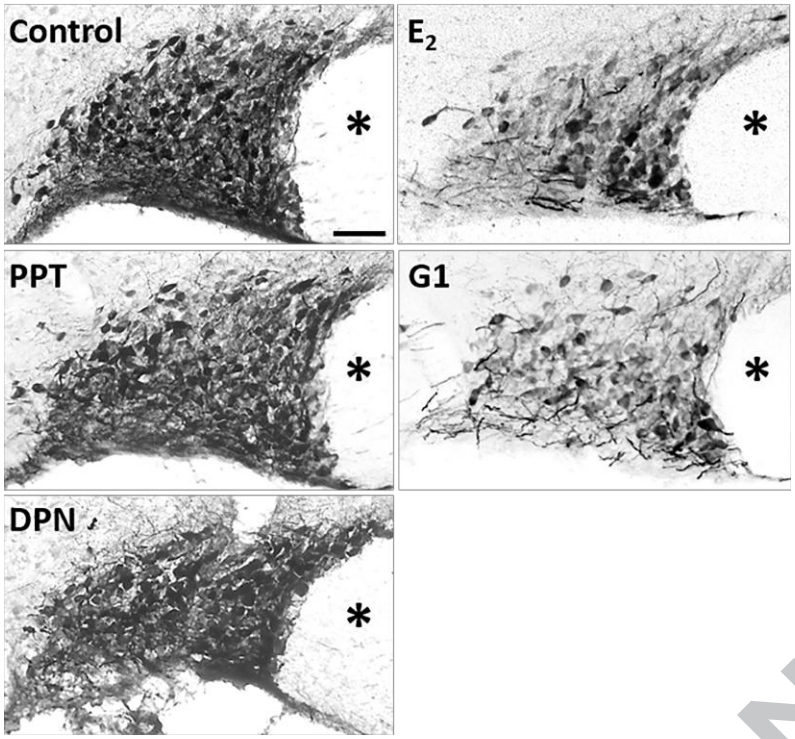
Fig. 3, Representative examples of AVP immunoreactivity in the paraventricular nucleus from ovariectomized rats treated with vehicle (Control), estradiol (E2), the ERα agonist PPT, the ERβ agonist DPN or the GPER agonist G1. *, Third ventricle. Scale bar 100 μm.

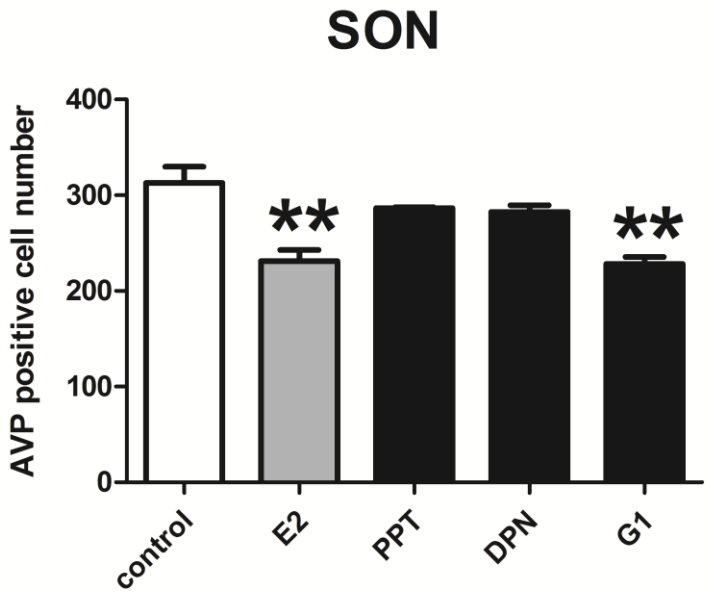
Fig. 4, Morphometric analysis of the number of AVP immunoreactive cells in the paraventricular nucleus of ovariectomized rats treated with vehicle (control), estradiol (E2), the ERα agonist PPT, the ERβ agonist DPN or the GPER agonist G1. Data represents the mean±SEM. ***, significant differences (P<0.001) versus the control group. ^^^, significant differences (P<0.001) versus the estradiol treated group.

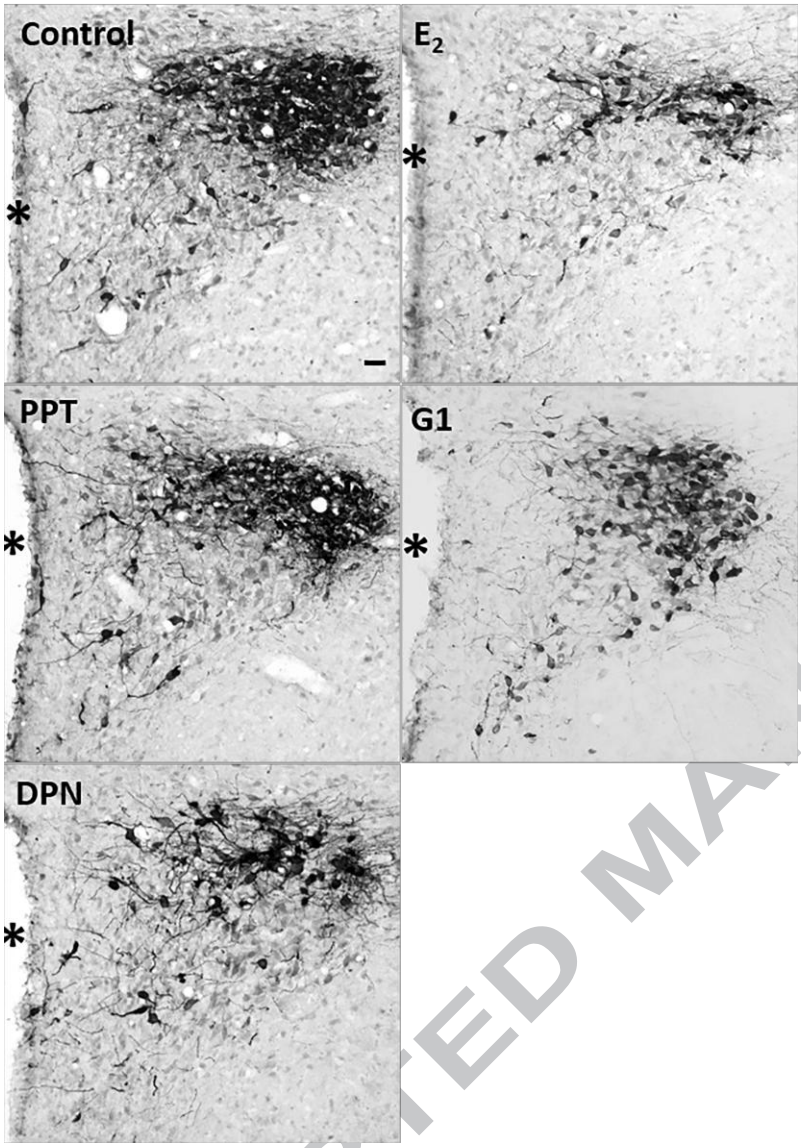
Fig. 5, Schematic representation of the anatomical subdivisions of the paraventricular nucleus: the paraventricular dorsal cap (PaDC), the paraventricular lateral magnocellular subdivision (PaLM),

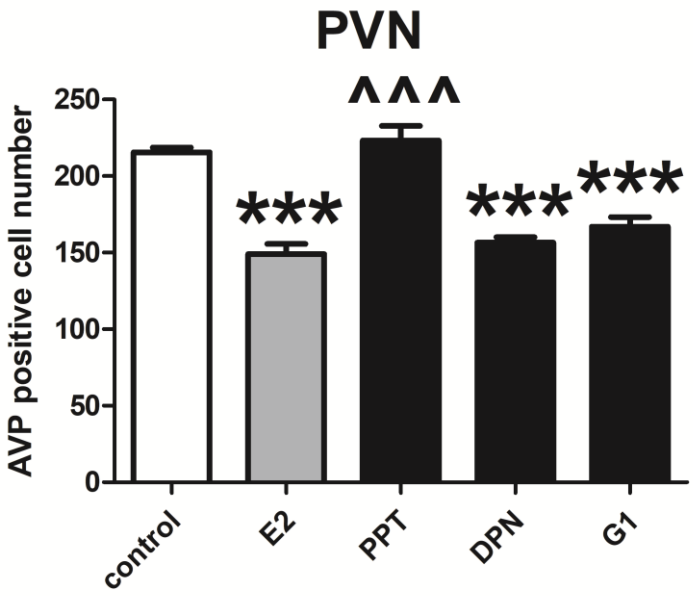
the paraventricular medial magnocellular subdivision (PaMM) and the paraventricular parvocellular subdivision (PaV). Scale bar 100 μm.

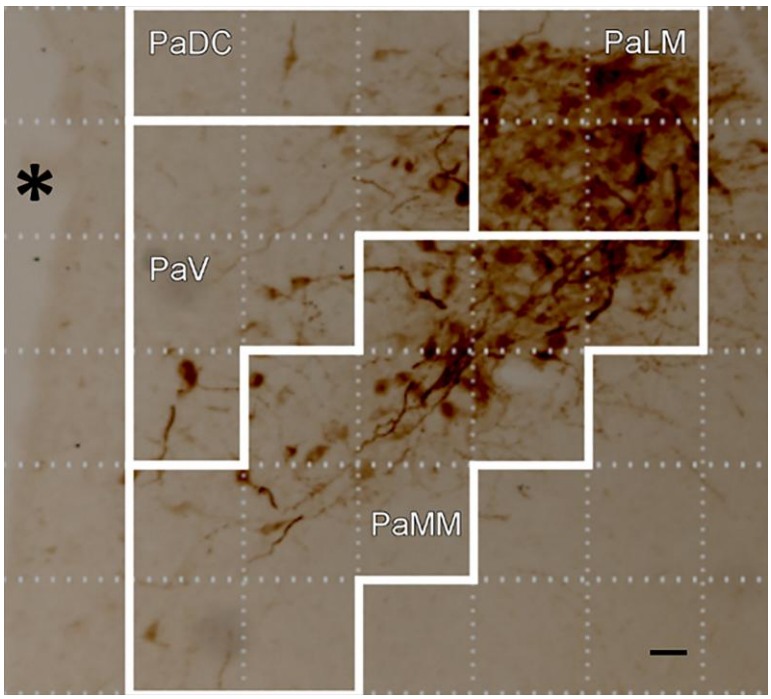
Fig. 6, Morphometric analysis of the number of AVP immunoreactive cells in the different subdivisions of the paraventricular nucleus of ovariectomized rats treated with vehicle (control), estradiol (E2), the ERα agonist PPT, the ERβ agonist DPN or the GPER agonist G1. Data represents the mean±SEM. Significant differences * $p<0.05$ and *** $P<0.001$ versus the control group. Significant differences ^ $P< 0.05$ and ^^^ $P<0.001$ versus the estradiol treated group.

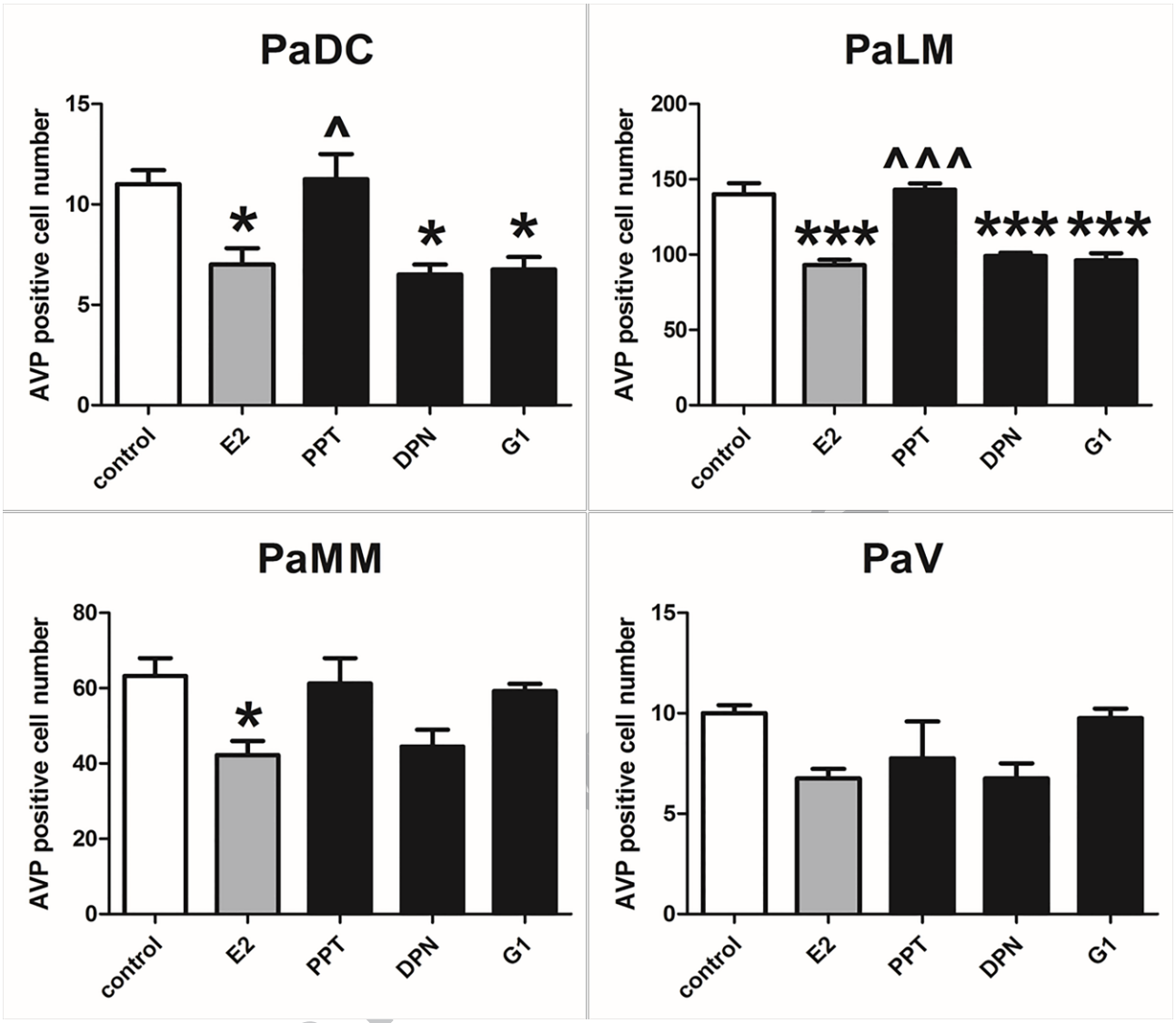












Highlights

Acute estradiol administration decrease the number of AVP immunoreactive neurons in SON and PVN

ERβ and GPER mediate the acute effects of estradiol decreasing the AVP-ir in the PVN

GPER mediates the acute effects of estradiol decreasing the AVP-ir in the SON