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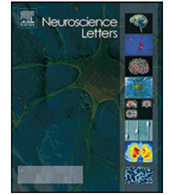

Estrogen receptor α is involved in the estrogenic regulation of arginine vasopressin immunoreactivity in the supraoptic and paraventricular nuclei of ovariectomized rats

Daniela Grassi [a,b,d], Miguel A. Amorim [a], Luis M. Garcia-Segura [a], GianCarlo Panzica [b,c,d,*]

[a] Instituto Cajal, CSIC; E-28002 Madrid, Spain
[b] Laboratory of Neuroendocrinology, Department of Anatomy, Pharmacology, and Forensic Medicine, Neuroscience Institute of Turin (NIT), University of Torino, I-10126 Torino, Italy
[c] National Institute of Neuroscience-Italy (INN), Torino, Italy
[d] Scientific Institute of the Cavalieri-Ottolenghi Foundation, Torino, Italy

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ABSTRACT

The ovarian hormone estradiol regulates the expression of arginine vasopressin gene and the release of arginine vasopressin by magnocellular hypothalamic neurons. Magnocellular neurons express estrogen receptor β and are contacted by afferent neurons that express estrogen receptor α. In this study we have assessed the effect of selective ligands for estrogen receptors to determine the subtype of estrogen receptor involved in the regulation of arginine vasopressin immunoreactivity in the supraoptic and paraventricular nuclei of ovariectomized rats. The volume fraction occupied by arginine vasopressin immunoreactive material was significantly increased in both nuclei in the animals treated with estradiol compared to the animals injected with vehicle. A similar result was obtained with an estrogen receptor α selective agonist. In contrast, the administration of an estrogen receptor β selective agonist did not significantly affect arginine vasopressin immunoreactivity. This finding suggests that estradiol may regulate arginine vasopressin levels on the supraoptic and paraventricular nuclei by acting on afferent neurons expressing estrogen receptor α.

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The function of magnocellular hypothalamic neurons is regulated by estradiol (E_2). This hormone, at a high dose, increases the size of nucleoli in supraoptic and paraventricular neurons of virgin rats [17], suggesting an effect on transcriptional activity. In addition, E_2 regulates synaptic plasticity in the supraoptic nucleus (SON) [25] and the expression of various neuropeptides colocalizing in oxytocinergic and vasopressinergic magnocellular neurons of ovariectomized rats [9].

The actions of E_2 on magnocellular secretory neurons contribute to the regulation of oxytocin (OT) and arginine vasopressin (AVP) release. The expression of the OT gene in the SON and paraventricular nucleus (PVN) of ovariectomized rats is regulated by E_2 [3,11,13,18]. E_2 is also able to regulate AVP gene expression in the PVN of male mice [11] and it increases AVP plasma levels in ovariectomized female rats [6,20], although this effect may depend on the hormonal dose [14]. Finally, E_2 may regulate the release of OT and AVP in hypothalamo-neurohypophysial explants [22,23,27].

Autoradiographic studies formerly demonstrated E_2-binding sites in AVP and OT magnocellular neurons of rat PVN [15,16]. These studies were later confirmed by the identification of estrogen receptor beta (ERβ) in the rat SON and PVN [19], in particular in vasopressinergic and oxytocinergic cells [1,7,8]. Therefore, the effects of E_2 on magnocellular hypothalamic neurons may be exerted in part through the action of ERβ expressed in these cells. In addition, the activity of AVP neurons may be affected by afferent neurons expressing ERα [21,26].

In this study we have explored whether E_2 regulates AVP immunoreactivity in the SON and PVN of ovariectomized rats. In addition, we have assessed the effect of selective ligands for estrogen receptors (ERs) to determine whether the hormonal regulation is exerted by ERα or ERβ.

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 (86/609/EEC) legislation. Experimental

* Corresponding author at: Laboratory of Neuroendocrinology, Department of Anatomy, Pharmacology, and Forensic Medicine, Neuroscience Institute of Turin (NIT), University of Torino, c.so M.D'Azeglio 52, I-10126 Torino, Italy.
Tel.: +39 011 6707970; fax: +39 011 2367970.
E-mail address: giancarlo.panzica@unito.it (G. Panzica).

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procedures were approved by our Institutional Animal Use and Care Committee. Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary.

Female rats were bilaterally ovariectomized at the age of 3 months under isoflourane 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 for 3 consecutive days with vehicle (corn oil), 17β-E_2 (50 μg/kg), the selective ERα agonist PPT (4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; 1 mg/kg), or the selective ERβ agonist DPN (2,3-bis(4-hydroxyphenyl)-propionitrile; 1 mg/kg). Twenty-four hours after the third injection of the estrogenic compound or vehicle, 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 removed and immersed for 4–6 h at 4 °C in the same fixative solution and then rinsed with phosphate buffer. Brains were placed overnight in a 30% sucrose solution in PBS, frozen in liquid isopentane at −35 °C, and stored in a deep freezer at −80 °C until sectioning. Brains were serially cut in the coronal plane at 25 μm thickness with a cryostat. The plane of sectioning was oriented to match the drawings corresponding to the transverse sections of the rat brain atlas [11]. Sections were collected in a cryoprotectant solution at −20 °C. Every fourth section (one section every 100 μm) was immunohistochemically stained for AVP after an overnight washing in PBS, pH 7.3, at 4 °C.

The following day, sections were first washed for 30 min at room temperature in PBS containing 0.2% Triton X-100 and then treated for 20 min with a solution of PBS containing methanol/hydrogen peroxide to quench endogenous peroxidase activity [24]. Sections were incubated for 30 min with normal goat serum (1:300, Vector Laboratories, Burlingame, CA, USA) and then incubated overnight at room temperature with a rabbit polyclonal AVP antibody (Chemicon) diluted 1:100,000, in 0.1 M PBS, pH 7.3–7.4, containing 0.2% Triton X-100. A biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) was then used at a dilution of 1:200 for 60 min at room temperature. The antigen–antibody reaction was revealed by incubation with avidin-peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories) for 60 min. The peroxidase activity was visualized with a solution containing 0.187 mg/mL 3,3-diaminobenzidine (Sigma, Milan, Italy) and 0.003% hydrogen peroxide in 0.05 M Tris–HCl buffer pH 7.6. Sections were mounted on chromallum coated slides, air dried, cleared in xylene, and cover slipped with Entellan (Merck, Milan, Italy).

The morphometric analysis of AVP immunostaining was performed on coded sections without previous knowledge of the experimental group from which the sections were obtained. Due to the intensity of the immunoreaction and the number of immunostained processes surrounding cell bodies it was impossible to

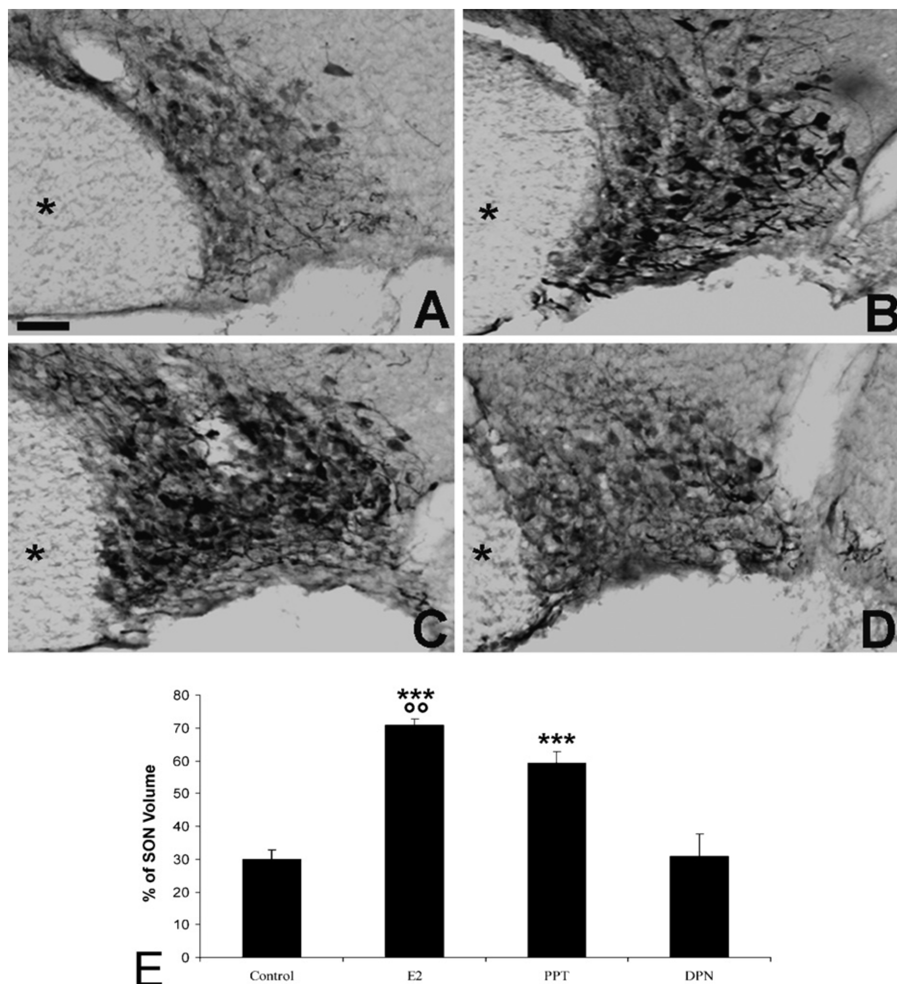

Fig. 1. Changes of AVP immunoreactivity in the SON. (A) Control animal; (B) E_2 treated animal; (C) PPT treated animal and (D) DPN treated animal. Scale bar, 90 μm. *Optic chiasma. (E) Volume fraction of SON occupied by AVP immunoreactive material in Control animals ($N = 4$) and in animals treated with E_2 ($N = 3$), PPT ($N = 3$) or DPN ($N = 3$). ***$P < 0.001$ in comparison to Control animals, °°$P < 0.01$ in comparison to PPT animals.

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distinguish each singular cell. This prevented the possibility of a direct cell counting. Therefore, we estimated the percentage of the volume occupied by AVP immunoreactive material with the point counting method of Weibel [2,28] on stained sections throughout the entire rostrocaudal extension of the SON (5–6 sections) and PVN (3–4 sections) in the right side of the hypothalamus. A grid of 20 square boxes of 8 μm^2 each for SON and 20 square boxes of 8 μm^2 each for PVN, and a 20× objective were used. The total number of points of intersection of the lines of the grids falling on the SON or the PVN (reference volume) and the number of points falling on immunoreactive material were counted. This method gives the volume fraction of immunoreactive material versus the total volume of the SON and the PVN and does not differentiate between immunoreactive neuronal somas and immunoreactive neuronal processes.

Data were analyzed via one-way ANOVA, followed, if significant, by a post hoc analysis with the Tukey's test. The SigmaStat 2.03 program was used for calculating probability values. *N* was the number

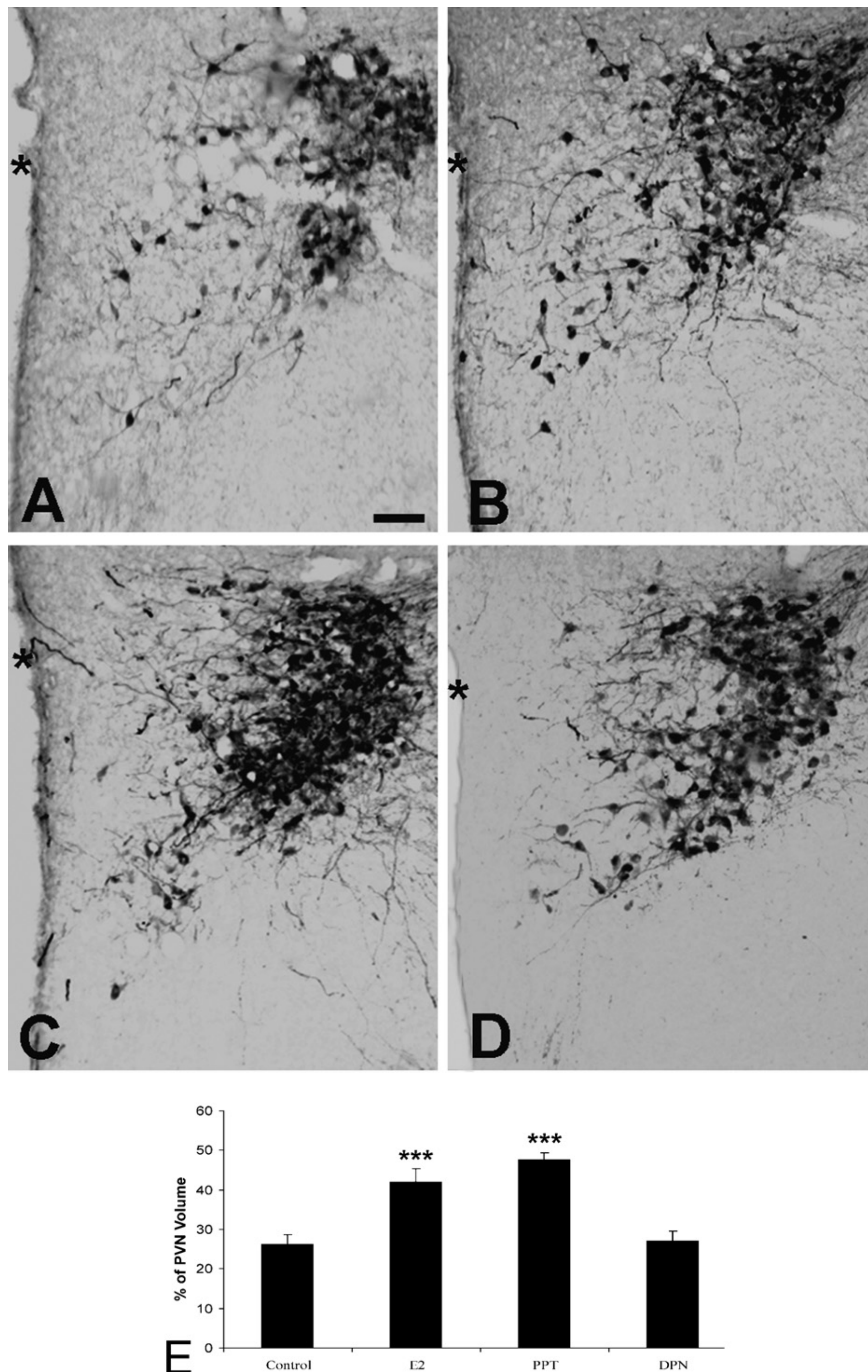

Fig. 2. Changes of AVP immunoreactivity in the PVN. (A) Control animal; (B) E_2 treated animal; (C) PPT treated animal and (D) DPN treated animal. Scale bar, 100 μm. *3rd Ventricle. (E) Volume fraction of PVN occupied by AVP immunoreactive material in Control animals ($N = 3$) and in animals treated with E_2 ($N = 3$), PPT ($N = 3$) or DPN ($N = 3$). ***$P < 0.001$ in comparison to Control animals.

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of animals and is indicated in the figure legends. $P < 0.05$ was considered statistically significant. Data are represented in graphs as the mean ± SEM.

In both nuclei, the visual inspection of the immunostained sections revealed qualitative differences in the pattern of AVP immunoreactivity among the different experimental groups (Figs. 1A–D and 2A–D). These differences were confirmed by the quantitative morphometric analysis. The one-way ANOVA reported strong significant effect of treatment for both SON [$F(3,9) = 59.153$, $P < 0,001$], and PVN [$F(3,8) = 32.718$, $P < 0,001$]. The post hoc Tukey's test showed that the volume occupied by AVP immunoreactive material was significantly increased in both nuclei in the animals treated with E_2 compared to the animals injected with vehicle ($P < 0.001$, Figs. 1E and 2E). A similar result was obtained with the ERα selective agonist PPT ($P < 0.001$, Figs. 1E and 2E). In contrast, the ERβ selective agonist DPN did not significantly affect AVP immunoreactivity (Figs. 1E and 2E). In the SON, the value of volume occupied by immunoreactivity in the E_2 group was also significantly higher than in the PPT one ($P < 0.01$).

Our findings showing that E_2 increases AVP immunoreactivity in ovariectomized female rat SON and PVN extend the results of previous studies showing that magnocellular neurons in these nuclei are targets for E_2, and that, at the same time, this hormone increases the AVP release in vivo and in vitro [6,20,22,23,27]. In contrast, in male mice, Nomura et al. [11] have reported that E_2 decreases AVP mRNA in the PVN and the number of AVP immunoreactive cells in the SON. These different results may reflect sex and species differences (males versus ovariectomized females, mice versus rats) and may also be related with the different distribution of ERs in the magnocellular system of rats and mice [10,19]. In fact, in rats AVP neurons colocalize only with ERβ [1,5], whereas in mice both receptors have been described in magnocellular regions [10].

ERβ seems fundamental to mediate the inhibiting effect of E_2 on the expression of AVP mRNA in PVN of male mice, due to the fact that this effect disappears in ERβ-KO mice [11]. ERβ seems also to regulate AVP release by magnocellular neurons in rats, as demonstrated by the effects of genistein (a selective agonist of ERβ) in decreasing the glutamate-induced release of AVP from hypothalamic explants [23].

In our model (ovariectomized rat females, without any other treatment) the increase of AVP immunoreactivity in SON and PVN due to exposure to E_2, seems to be dependent on ERα. In fact, AVP immunoreactivity increases after PPT (ERα selective agonist) but it shows no variations after DPN (ERβ selective agonist) administration. However, since the affinity of PPT for ERα is not the same than the affinity of DPN for ERβ it is not possible to exclude that DPN at a different dose may affect AVP immunoreactivity. Nevertheless, our findings indicate that ERα is involved in the regulation of AVP neurons in SON and PVN. Since ERα is not expressed in the magnocellular neurons of the rat SON and PVN [8,19], the regulation exerted by E_2 on AVP immunoreactivity via ERα may be mediated by afferent neurons expressing this receptor, such as the subfornical organ, the organum vasculosum laminae terminalis, the anteroventral periventricular nucleus and the medial preoptic nucleus, among others [4,5,12,21,26].

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