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Role of oestrogen receptors on the modulation of NADPH-diaphorase-positive cell number in supraoptic and paraventricular nuclei of ovariectomised female rats

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ABSTRACT

Modulation of nitric oxide producing system (demonstrated via NADPH-diaphorase histochemical rection) by oestradiol has been established in several structures of the rat brain. The objective of the present study was to explore the possible regulation of NADPH-diaphorase activity by oestradiol in neurons of the supraoptic (SON) and the paraventricular

(PVN) nuclei and the role of oestrogen receptors (ERα and ERβ in this regulation. Adult ovariectomised rats were divided into six groups and injected either with vehicle or a single dose of oestradiol, a selective ERα agonist-PPT, a selective ERβ agonist-DPN, a selective ERα antagonist-MPP or a selective ERβ antagonist-PHTPP. The number of NADPH-diaphorase positive elements in the SON and the PVN was modulated by both ERs but, depending on the nucleus, ERα and ERβ ligands induced different effects. These results suggest that the regulation of nitrergic system by ERs may play a role in the control of oestrogen-dependent physiological mechanisms regulated by the SON and the PVN.

INTRODUCTION

Nitric oxide (NO) is a gaseous neurotransmitter synthesized through the action of the enzyme nitric oxide synthase (NOS). This enzyme is regulated by several cofactors including reduced nicotinamide adenine dinucleotide phosphate (NADPH) (1). The neural isoform of NOS (nNOS) has the same distribution and properties of the enzyme NADPH-diaphorase (NADPH-d) (2), therefore histochemistry for NADPH-d has been considered a reliable method to demonstrate the presence of NO-producing elements (3).

NO-producing system is largely diffused within the central nervous system of mammals (4, 5) and other vertebrates (6). In rodents, NADPH-d activity or nNOS immunoreactivity has been reported in the hypothalamic magnocellular nuclei: paraventricular (PVN) and supraoptic nucleus (SON) (7-9). These two structures are a critical node for the regulation and orchestration of physiological relevant responses (10-12). Neurons in the PVN control three major physiological pathways: i) the hypothalamic-pituitary-axis (HPA), through neurons in the parvocellular subdivision of the nucleus (PaV) (13); ii) neurohypophysial peptide signals, via lateral magnocellular neurons (PaLM) expressing vasopressin (AVP) and medial magnocellular neurons (PaMM) expressing oxytocin (OX) (14) and iii) autonomic regulation, using brainstem and spinal cord projecting neurons in the dorsal parvocellular part (PaDC) (15). The intermingling of these three physiologically distinct signalling systems situates the PVN at the crossroad for integration of diverse output in a coordinate and interrelated fashion (16). On the other hand, in the SON, all neurons are magnocellular and, as well as neurons in PaLM and PAMM, express mainly vasopressin and oxytocin and project to the neurohypophysis (17).

Results of previous studies have shown that magnocellular neurons in the SON and the PVN are targets for 17β-oestradiol (E_2). For instance, a high-dose of E_2 increases the size of

nucleoli (related to an increased transcriptional activity) in SON and PVN neurons of virgin rat (18). In addition, E_2 regulates synaptic plasticity in the SON (19), the expression of OX (20), and the expression of neuropeptides co-localizing in OX- and AVP- expressing magnocellular neurons in ovariectomised rats (21). A direct action of E_2 on AVP and OX neurons is suggested also by early autoradiographic studies demonstrating oestradiol binding sites on magnocellular neurons in the SON and the PVN (22, 23), as well as by more recent immunohistochemical and in situ hybridization investigations showing that SON and PVN neurons express oestrogen receptor β (ERβ) (24-27).

Modulation of nNOS expression or NADPH-d activity by oestradiol has been established in several structures of the rodent brain (for a review see (28). Studies performed in nuclei involved in reproductive behaviours such as the medial preoptic area (MPA), arcuate nucleus (ARC), ventromedial hypothalamic nucleus (VMH), bed nucleus of stria terminalis (BST), bed nucleus of the accessory olfactory tract (BAOT) and the anteroventral subdivision of the medial amygdala (MeAV), have shown changes in the expression of nNOS throughout the oestrous cycle (29-31). In most nuclei, nitric oxide activity increased when E_2 levels were higher during oestrus (for a review see (32).

ERs are important for the differentiation of the limbic-hypothalamic nNOS system. In fact, significant changes of nNOS immunoreactivity in PVN and ARC nuclei have been observed in male mice knockout for ERα (ERαKO), when compared with wild-type male mice (33). In addition, perinatal exposure to bisphenol A (an endocrine disrupter acting mainly through the ERα) may induce alteration in part of limbic-hypothalamic nNOS system (34). On the other hand, oestradiol, acting through ERβ, increases eNOS expression and decreases the number of nNOS immunoreactive neurons in the PVN in rat hypothalamic slice cultures (35). Observations made on double mutant mice in which males lacked functional ERα, androgen receptor (AR), or both, demonstrated that ER□ and AR interact to regulate nNOS in male and female brain in a site-specific manner (36). The importance of AR for the masculinization of rat nNOS system was further demonstrated by the observation of changes in positive cell number in *Tfm* male rats (characterized by a no functional AR) (37).

Although the oestradiol-mediated modulation of nNOS has been shown overall in the PVN, there is no information about a possible differential regulation of its diverse sub-regions. Since the PVN of rodents consists of several distinct sub-nuclei with different cytological and neurochemical features (38, 39), it is important to determine whether oestradiol differentially modulates nNOS activity in the different PVN sub-regions. On the other hand, regulation of

nitric oxide system by oestradiol and progesterone has been reported in the SON during late pregnancy or in ovariectomised animals exposed to a steroid hormone replacement schedule that mimics late pregnancy (40).

The aim of the present study was, therefore, to demonstrate the possible regulation of nitric oxide producing system by oestradiol in the SON and in the different subnuclei of PVN in adult ovariectomised female rats, as well as to clarify the role of ERα and ERβ in this process by using selective ER ligands.

MATERIAL AND METHODS:

Animals and experimental treatments

Wistar albino female rats from our in-house colony (UNED, Madrid) 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, the European Union legislation (Council Directives 86/609/EEC and 2010/63/UE) and the Spanish Government Directive (R.D. 1201/2005). 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 were bilaterally ovariectomised at the age of 3 months under isoflourane anaesthesia. They were then housed in plastic cages, with food and water *ad libitum*, and randomly assigned to the different treatments. Seven days after surgery 4 rats per group received one i.p. injection of either vehicle (corn oil), 17β-oestradiol (50μg/kg) (Sigma, Madrid, Spain), the selective ERα agonist PPT (4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)*tris*phenol; Biogen Cientifica, Madrid, Spain; 1 mg/kg), the selective ERβ agonist DPN (2,3-bis(4-hydroxyphenyl)-propionitrile; Biogen Cientifica; 1mg/kg), the ERα antagonist MPP (1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; Biogen Cientifica; 1 mg/kg), or the ERβ antagonist PHTPP (4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol; Biogen Cientifica; 1 mg/kg). Doses of ER ligands were based on previous studies in vivo (41,42).

Twenty-four hours after the injection the animals were deeply anaesthetized 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 0.1M phosphate buffer, pH 7.4). Brains were

quickly removed and immersed for 4–6 hours at 4 ºC in the same fixative solution and then rinsed with phosphate buffer. Brains were placed 72 hours in a 30% sucrose solution in PBS at 4 ºC, frozen in liquid isopentane at −35 ºC and stored in a deep freezer at −80 ºC 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 stereotaxic rat brain atlas (43). Sections were collected in a cryoprotectant solution (44) at -20 °C. Every fourth section (one section every 100μm) was histochemically stained for NADPH-d histochemistry.

NADPH-diaphorase histochemistry

The presence of NADPH-d, a specific marker for nitric oxide-producing neurons (2, 45), was detected by histochemistry performed on free-floating sections. Before the reaction, the sections collected in the cryoprotectant solution were washed for 30 min at room temperature in PBS 0.01M, pH 7.3–7.4 and then overnight at 4 ºC in phosphate buffer 0.1M, pH 7.4. The following day, free floating sections were washed at first in TRIS buffer 0.1M, pH 8.0 for 15 min at room temperature, then in TRIS buffer, pH 8.0 containing 0.5% Triton X-100 for 10 min. The sections were incubated for 30 min at 37 °C in a solution of 0.8 mM nitrobluetetrazolium (Sigma, Madrid, Spain) and 1 mM reduced NADPH (Sigma, Madrid, Spain) in TRIS buffer, 0.1 M, pH 8.0, containing 0.5% Triton X-100.

Quantitative analysis

The quantitative analysis of NADPH-d staining was performed on coded sections without knowledge of the experimental group. All NADPH-d positive cells, either strongly or faintly labelled, were considered for quantification. Selected fields were acquired by a digital camera (Olympus DP25) connected to a Nikon eclipse E600 microscope using a x20 objective. The total number of NADPH-d positive cells within three coded sections was studied in the SON and the PVN. In order to take account 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 and that the grid overlap the total area of the PVN. Cell counting was performed for each square box within the boundaries of the nucleus. To better elucidate the difference of nNOS expression in the diverse functional subparts of the PVN we decided to group the boxes corresponding to the same sub-regions for the statistical analysis to evaluate possible differences with the diverse hormonal treatments. To this aim, we delimited the boundaries of different PVN regions (Fig.

1) following the detailed anatomical description provided by Armstrong et al (46) based on Nissl-stained material.

Statistical Analysis

Data were analyzed by one-way ANOVA (with the treatments as independent variable) followed by Dunnett'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 represented as the mean±SEM.

RESULTS

In the present study, NADPH-d positive cells in the SON, in the overall PVN, and in its subdivisions (PaDC, PaLM, PaMM and PaV) have been assessed. NADPH-d staining was present in SON and in all PVN subdivisions. Qualitative observation of NADPH-d positive cells in the hypothalamic sections revealed differences in the pattern of histochemical staining when comparing the different experimental groups in the total SON and PVN as well as in the different PVN sub-regions. Representative examples of NADPH-d positive cells in the SON and the PVN are shown in Fig.2 and Fig 3. These qualitative differences were confirmed by the quantitative analysis.

1- Quantitative analysis of NADPH-diaphorase positive cells in the supraoptic nucleus (SON)

In the SON, one-way ANOVA showed significant effect of the hormonal treatments on NADPH-d positive cells [$F_{(6,21)}= 24.70$; $P<0.0001$]. The Dunnett's test demonstrated that E_2 treatment had no significant effect, whereas treatment with either the ERα agonist-PPT or the ERβ agonist-DPN induced a significant decrease in the number of NADPH-d positive cells. In contrast, treatment with either ERα antagonist-MPP or the ERβ antagonist-PHTPP caused a significant increase in the number of NADPH-d positive cells (Table. 1, Fig 4a).

2- Quantitative analysis of NADPH-diaphorase positive cells in the PVN

In the whole PVN, one-way ANOVA showed a significant effect of the hormonal treatments on NADPH-d positive cells [$F_{(6,21)}= 272.0$; $P<0.0001$]. The Dunnett's test demonstrated that E_2 treatment had no significant effect, whereas the treatment with the ERα agonist-PPT or the ERβ antagonist-PHTPP induced a significant increase, while the treatment with the ERβ

agonist-DPN or the ERα antagonist-MPP caused a significant decrease, in the number of NADPH-d positive cells (Table 1, Fig 4b).

After this general analysis, we repeated the one-way ANOVA analysis for each subdivision of the PVN.

In the PaDC, one-way ANOVA revealed a significant effect of the hormonal treatments on the number of NADPH-d positive cells [$F_{(6,21)}$= 274.2; P<0.0001]. The Dunnett's test demonstrated that E_2 treatment as well as treatment with the ERα agonist-PPT or the ERβ antagonist-PHTPP induced a significant increase in the number of NADPH-d positive cells. In contrast, the treatment with the ERα antagonist-MPP or the ERβ agonist-DPN caused a significant decrease in the number of NADPH-d positive cells (Table 1, Fig 5A).

In the PaLM, one way ANOVA revealed a significant effect of the hormonal treatments on NADPH-d positive cells [$F_{(6,21)}$= 158.5; P<0.0001]. The Dunnett's test demonstrated that the treatment with E2, the ERα agonist-PPT or the ERβ antagonist-PHTPP resulted in a significant increase in the number of NADPH-d positive cells. In contrast, the treatment with the ERα antagonist-MPP or the ERβ agonist-DPN induced a significant decrease in the number of NADPH-d positive cells (Table 1, Fig 5B).

In the PaMM and in the PaV, one way ANOVA revealed a significant effect of the hormonal treatments on NADPH-d positive cells [$F_{(6,21)}$= 180.7; P<0.0001 and $F_{(6,21)}$= 134.8; P<0.0001, respectively]. The Dunnett's test demonstrated that E2 treatment had no significant effect, whereas the treatment with the ERα agonist-PPT and the ERβ antagonist-PHTPP induced a significant increase in the number of NADPH-d positive cells in both PaMM and PaV. In contrast, treatment with the ERβ agonist-DPN or the ERα antagonist-MPP decreased the number of NADPH-d positive cells in both sub-nuclei (Table 1, Fig 5C and Fig 5D).

DISCUSSION

The activity of nNOS in the SON and the PVN participates in the control of body fluid homeostasis (47, 48). Previous studies have determined that oestradiol regulates the activity of nNOS in the SON and PVN (7-9). In the preoptic area, the regulation exerted by oestradiol on the activity of nNOS is mediated by ERs, which modulate the interaction of nNOS with the NMDA receptor and the consequent phosphorylation of nNOS (49-51). In this study we have assessed the role of ERα and ERβ on the regulation of nNOS in the SON and PVN. Present findings indicate that, in the SON and the PVN, the number of NADPH-d positive cells is modulated by both ERα and ERβ. However, the regulation exerted by ERs on

NADPH-d activity is different in the two magnocellular hypothalamic nuclei. In the SON, the number of NADPH-d positive cells is decreased by agonists and increased by antagonists of ERα and ERβ transcriptional activity, suggesting that the activation of both ERs may downregulate the number of cells expressing NADPH-d. In contrast, in the PVN considered as a whole and in the different PVN subdivisions (PaDC, PaLM, PaMM and PaV), the number of NADPH-d positive cells is increased by the ERα agonist and the ERβ antagonist and decreased by the ERβ agonist and the ERα antagonist. Thus, in the PVN, ERα positively regulates the number of NADPH-d positive cells while ERβ exerts a negative regulation.

It is not surprising that the nitrergic system of the SON and the PVN is downregulated by the ERβ. In fact, several neuroanatomical studies have reported the presence of ERβ (but not ERα) in rat SON and PVN (25, 27) and ERβ is downregulating the number of NOS expressing cells in the PVN of hypothalamic slices (35). On the other hand, the effects of ERs (in particular that of ERα) could be mediated by ERs-sensitive neurons projecting to SON and PVN, as the ERα expressing neurons located in the subfornical organ (SFO), the organum vasculosum laminae terminalis (OVLT), the anteroventral periventricular nucleus (AVPe) and the medial preoptic nucleus (MPO) (52-56). Therefore, the final effect of ER agonists and antagonists on the nitrergic system in the SON and the PVN may depend on a different balance in the activation of locally expressed ERβ and afferent neurons regulated by ERα. The different effect of ERs' ligands in the SON and the PVN may be due to the different composition of the afferent inputs to these nuclei, predominantly glutamatergic and GABAergic neuronal groups surrounding the PVN and the SON respectively (15, 57).
Interestingly, E_2 did not exert the same effect than ERs agonists. Thus, E_2 increased the number of NADPH-d positive cells in the PaDC and the PaLM subdivisions of the PVN, imitating the effect of the ERα agonist. In contrast, no significant effect of E_2 on the number of NADPH-d positive cells was detected in the SON and in the PaMM and the PaV subdivisions of the PVN. Since E_2 is an agonist of both ERs, a different balance in the activation of ERα and ERβ mediated transcription may explain the different effects of E_2 in the different subdivisions of the PVN. However, this cannot explain the lack of effect of E_2 in the SON, because in this nucleus both ERs have the same effect. A possible explanation is the lower dose used of 17β-oestradiol together with its lower affinity for ERs compared to the dose and affinity of the synthetic ER ligands (58, 59). In addition, possible differences in the half-life of 17β-oestradiol and the synthetic ER ligands may also contribute to the differences.

CONCLUSIONS

In conclusion, present results indicate that the nitrergic system in the SON and the PVN is modulated by ERα and ERβ with regional specificity. Since these two nuclei are implicated in the control of relevant physiological mechanisms, the regulation exerted by ERs may have a significant impact in the control of body homeostasis.

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FIGURE LEGENDS

Figure 1, Schematic representation of the anatomical subdivisiones of the PVN (red lines) showing the localization of the boxes used for the morphometric analysis (black lines). PaDC - Paraventricular Dorsal Cap; PaLM - Paraventricular Lateral Magnocellular subdivision; PaMM - Paraventricular Medial Magnocellular subdivision; PaV - Paraventricular Parvocellular subdivision.

Figure 2, Representative microphotographs of NADPH-diaphorase positive cells in the supraoptic nucleus in animals treated with vehicle (Control), 17β-oestradiol (E2), the ERα agonist PPT, the ERα antagonist MPP, the ERβ agonist DPN or the ERβ antagonist PHTPP. Scale bar, 100 μm. * Optic Chiasm.

Figure 3, Representative microphotographs of NADPH-diaphorase positive cells in the paraventricular nucleus in animals treated with vehicle (Control), 17β-oestradiol (E2), the

ERα agonist PPT, the ERα antagonist MPP, the ERβ agonist DPN or the ERβ antagonist PHTPP. Scale bar, 100 µm. * 3rd Ventricle.

Figure 4, Number of NADPH-diaphorase expressing cells in the supraoptic nucleus (SON) (A) and in the whole paraventricular nucleus (PVN) (B) in animals treated with vehicle (Control), 17β-oestradiol (E2), the ERα agonist PPT, the ERα antagonist MPP, the ERβ agonist DPN or the ERβ antagonist PHTPP. * Significant difference ($P<0.05$) versus control values. Data are reported as mean+SEM.

Figure 5, Number of NADPH-diaphorase expressing cells in the subdivisions of the paraventricular nucleus in animals treated with vehicle (Control), 17β-oestradiol (E2), the ERα agonist PPT, the ERα antagonist MPP, the ERβ agonist DPN or the ERβ antagonist PHTPP. (A), Paraventricular Dorsal Cap (PaDC); (B), Paraventricular Lateral Magnocellular subdivision (PaLM); (C), Paraventricular Medial Magnocellular subdivision (PaMM) and (D), Paraventricular Parvocellular subdivision (PaV). * Significant difference ($P<0.05$) versus control values. Data are represented as mean+SEM.

Table 1. Schematic summary of the effect of the treatments on the number of NADPH-diaphorase expressing cells in the supraoptic nucleus (SON), in the whole paraventricular nucleus (PVN) and in the PVN sub-regions: Paraventricular Dorsal Cap (PaDC); Paraventricular Lateral Magnocellular subdivision (PaLM); Paraventricular Medial Magnocellular subdivision (PaMM) and Paraventricular Parvocellular subdivision (PaV). The green up-arrows indicate a significant increase and red down-arrows a significant decrease in the number of NADPH-diaphorase positive cells.

	SON	overall PVN	PaDC	PaLM	PaMM	PaV
Estradiol			↑	↑		
PPT	↓	↑	↑	↑	↑	↑
MPP	↑	↓	↓	↓	↓	↓
DPN	↓	↓	↓	↓	↓	↓
PHTPP	↑	↑	↑	↑	↑	↑

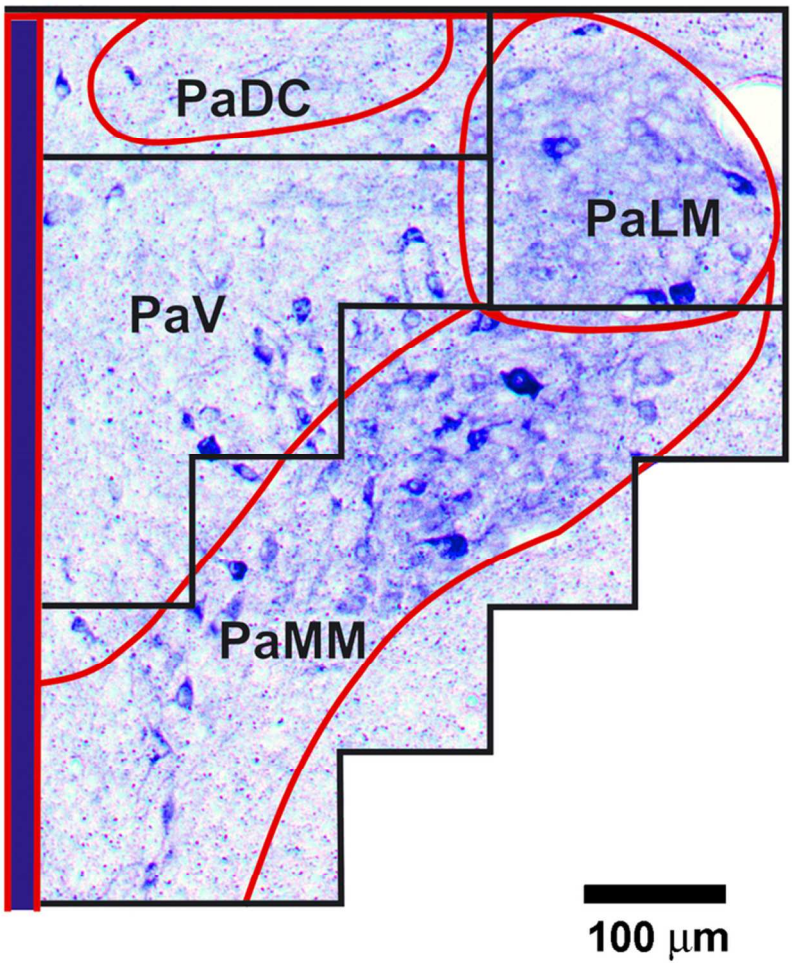

Figure 1, Schematic representation of the anatomical subdivisiones of the PVN (red lines) showing the localization of the boxes used for the morphometric analysis (black lines). PaDC - Paraventricular Dorsal Cap; PaLM - Paraventricular Lateral Magnocellular subdivision; PaMM - Paraventricular Medial Magnocellular subdivision; PaV - Paraventricular Parvocellular subdivision.
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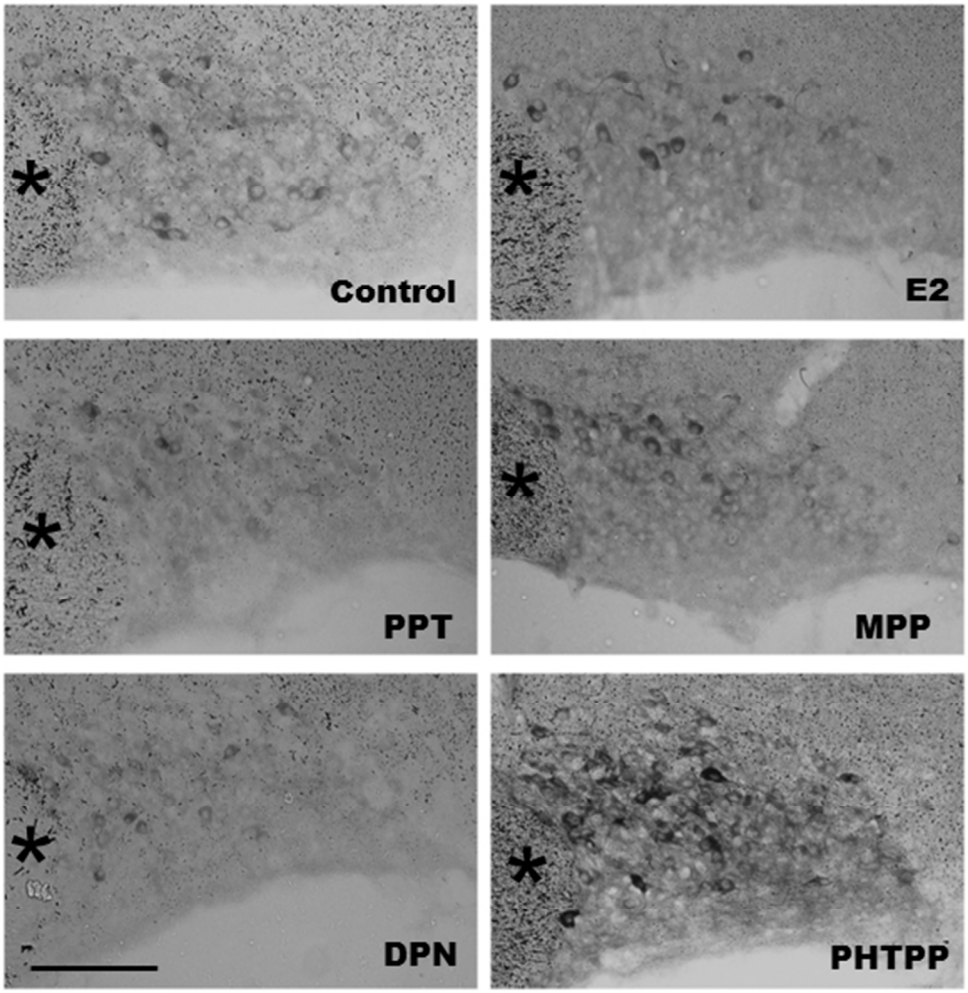

Figure 2, Representative microphotographs of NADPH-diaphorase positive cells in the supraoptic nucleus in animals treated with vehicle (Control), 17β-oestradiol (E2), the ERα agonist PPT, the ERα antagonist MPP, the ERβ agonist DPN or the ERβ antagonist PHTPP. Scale bar, 100 μm. * Optic Chiasm.
158x163mm (96 x 96 DPI)

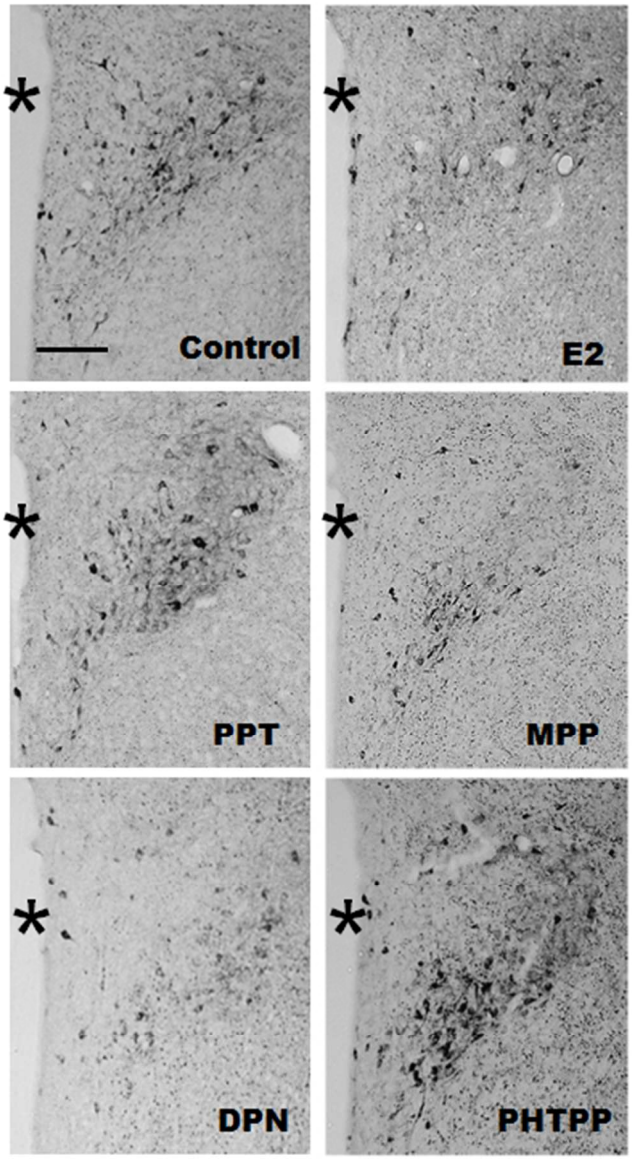

Figure 3, Representative microphotographs of NADPH-diaphorase positive cells in the paraventricular nucleus in animals treated with vehicle (Control), 17β-oestradiol (E2), the ERα agonist PPT, the ERα antagonist MPP, the ERβ agonist DPN or the ERβ antagonist PHTPP. Scale bar, 100 μm. * 3rd Ventricle. 35x63mm (300 x 300 DPI)

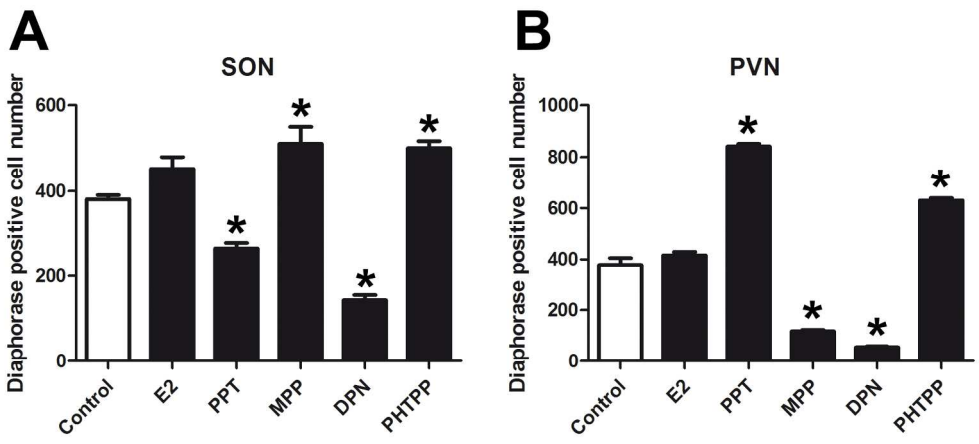

Figure 4, Number of NADPH-diaphorase expressing cells in the supraoptic nucleus (SON) (A) and in the whole paraventricular nucleus (PVN) (B) in animals treated with vehicle (Control), 17β-oestradiol (E2), the ERα agonist PPT, the ERα antagonist MPP, the ERβ agonist DPN or the ERβ antagonist PHTPP. * Significant difference ($P<0.05$) versus control values. Data are reported as mean+SEM.