Testosterone enhances the NADPH-diaphorase staining in the paraventricular nucleus of the hypothalamus

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Summary

Nitric oxide is involved in mechanisms which underlie neuroendocrine events in reproduction. Since gonadal steroids have profound effects on reproductive function, it is of interest to examine whether nitric oxide synthase activity is altered by gonadal steroid hormones in specific nuclei of the hypothalamus. In the paraventricular nucleus of the hypothalamus, changes of nitric oxide synthase/NADPH-diaphorase staining have been shown to occur after an important number of experimental conditions. Based on previous studies demonstrating that NADPH-diaphorase activity in the paraventricular nucleus of the hypothalamus is modulated by estradiol, a possible androgen influence on this activity was investigated in the neuronal populations (magno- and parvicellular) of this nucleus. Cryostat sections were cut and processed for the histochemical detection of the NADPH-diaphorase activity. Following orchidectomy (14 days), numerical data displayed a decrease in the number of NADPH-neurons, especially in the posterior magnocellular and the medial parvicellular subdivisions ($p < 0.05$), which was reversed after daily treatment with testosterone. These data indicate that paraventricular NADPH-diaphorase neurons are influenced by testosterone.

Key words: Testosterone - Nitric oxide synthase - Hypothalamus - Neuroendocrine - Rat.

Introduction

One of the more surprising findings in the last decade has been the demonstration that the toxic gaseous environmental pollutant nitric oxide (NO) is produced in situ in human and other species. This molecule, has, among other well-known functions, an important role as critical neurotransmitter and biological mediator of the neuroendocrine axis (Brann et al., 1997). In the hypothalamus, this highly reactive free radical gas is produced from L-arginine by a calcium- and calmodulin-dependent and NADPH-requiring neuronal enzyme, nitric oxide synthase (NOS) (Bredt and Snyder, 1992). Neuronal NOS is a constitutive protein that can rapidily and transiently synthesize and release small amounts of NO in response to different stimuli.

Different studies have shown the distribution of neuronal NOS within the mammalian central nervous system, including the hypothalamus (for a recent review see Sánchez et al., 1998a). These studies have revealed the presence of a large number of NOS containing neurons in the magnocellular and parvocellular subdivisions of the paraventricular nucleus (PVN) (Sánchez et al., 1994, 1996a, 1998a, b; Vanthalo and Soinila, 1995; Crespo et al., 1998).

On the other hand, over the last decade it has been shown that estradiol has a stimulatory effect upon NOS activity in the ventromedial nucleus of the hypothalamus (Okamura et al., 1994; Ceccatelli et al., 1996). In addition, important changes in the NOS activity of the PVN have been noticed following ovariectomy and/or estradiol treatment as evaluated by means of the NADPH-diaphorase (ND) histochemical technique (Sánchez et al., 1998b) and in situ hybridi-

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zation (Ceccattelli et al., 1996). However, no information is available about a possible repercussion of changes in the circulating levels of testosterone.

Thus, the aim of the present study is to elucidate the effect of orchidectomy (14 days) and testosterone treatment upon the ND-histochemical expression in the PVN.

Material and Methods

Animals and histology. Twenty adult male Wistar rats (275-325 g) divided into four groups were used for the present study. 1.- Normal animals (n=5), without receiving any treatment. 2.- Orchidectomized rats (14 days) (n=5) under ketamine anaesthesia (Ketolar, 50 mg/kg body weight, i.p.). 3.- Orchidectomized rats (14 days) (n=5) receiving a daily dose (i.m) of testosterone (5 µg/100g). 4.- Sham operated group (n=5), in which the testicles, after visualization were left in their location. Animals were deeply anaesthetized with ketamine (Ketolar, 50 mg/kg body weight, i.p.). After packing the heads with ice, the animals were perfused through the ascending aorta with 100 ml Ringer solution followed by 500 ml fixative solution containing 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.3 (PB). The brains were removed and the hypothalamic regions were dissected out, postfixed 4 h at 4°C in the same fixative solution and cryoprotected with 30% sucrose (v/v) in PB at 4°C overnight. Thirty-micrometer coronal sections were cut using a cryostat and serially collected in cold (4°C) PB.

Histochemical procedure. Sections were washed in 0.1 M Tris-HCl buffer pH 8.0, and processed for ND histochemistry as previously described (Alonso et al., 1992a, b; Arévalo et al., 1992, 1993; Sánchez et al., 1994, 1996a, b, 1998a, b; Crespo et al., 1998). Briefly, sections were incubated for 60-90 min at 37°C in an incubation solution made up of 1 mM reduced ß-NADPH (Sigma #N1630), 0.3 mM nitro blue tetrazolium (Sigma #N6876) and 0.08% Triton X-100 in 0.1 M Tris-HCl buffer, pH 8.0. The course of the reaction was controlled under the microscope. When the histochemical reaction was concluded, sections were washed in 0.1 M Tris-HCl buffer, pH 8.0, dehydrated in ethanol series and mounted with coverslips using entellan.

Numerical data. Calculation of the number of positive cells was carried out with an image analyzer system MIP-2 (IMCO 10). By means of a graphic tablet, we counted for all sections of each individual animal the number of the stained neurons. All the counts were always carried out by the same author (FS). Only cells in which the nucleus was present were considered. Total numbers of stained neurons were corrected according to the Abercrombie's formula by a factor of 0.750 (Sánchez et al., 1994; 1996 a; 1998 b). The mean and the standard error of the mean for each group were calculated by using the corrected average for each individual animal.

Results

In order to facilitate putative comparisons in the chemoarchitecture of the PVN, for the present analysis the nomenclature and nuclear boundaries proposed in previous papers from our group have been considered (see Sánchez et al., 1994, 1996a, 1998a, b).

All neuronal types of the nucleus (magno- and parvicellular) displayed positive labelling. In the normal animals most of the stained neurons were located in the posterior magnocellular subdivision, forming a dense cluster of ND active neurons (Figs. 1a and 3a). A few stained cells were present in the commissural magnocellular subdivision.

In the parvicellular subdivisions, positive neurons were located in the anterior medial and periventricular parvicellular subdivisions (Figs. 1a and 3a), In addition, in the dorsal subdivision a small cluster of ND-neurons located close to the dorsal part of the third ventricle was evident (Figs. 1a and 3a). In the lateral subdivision ND-stained neurons were also present (Fig. 2a).

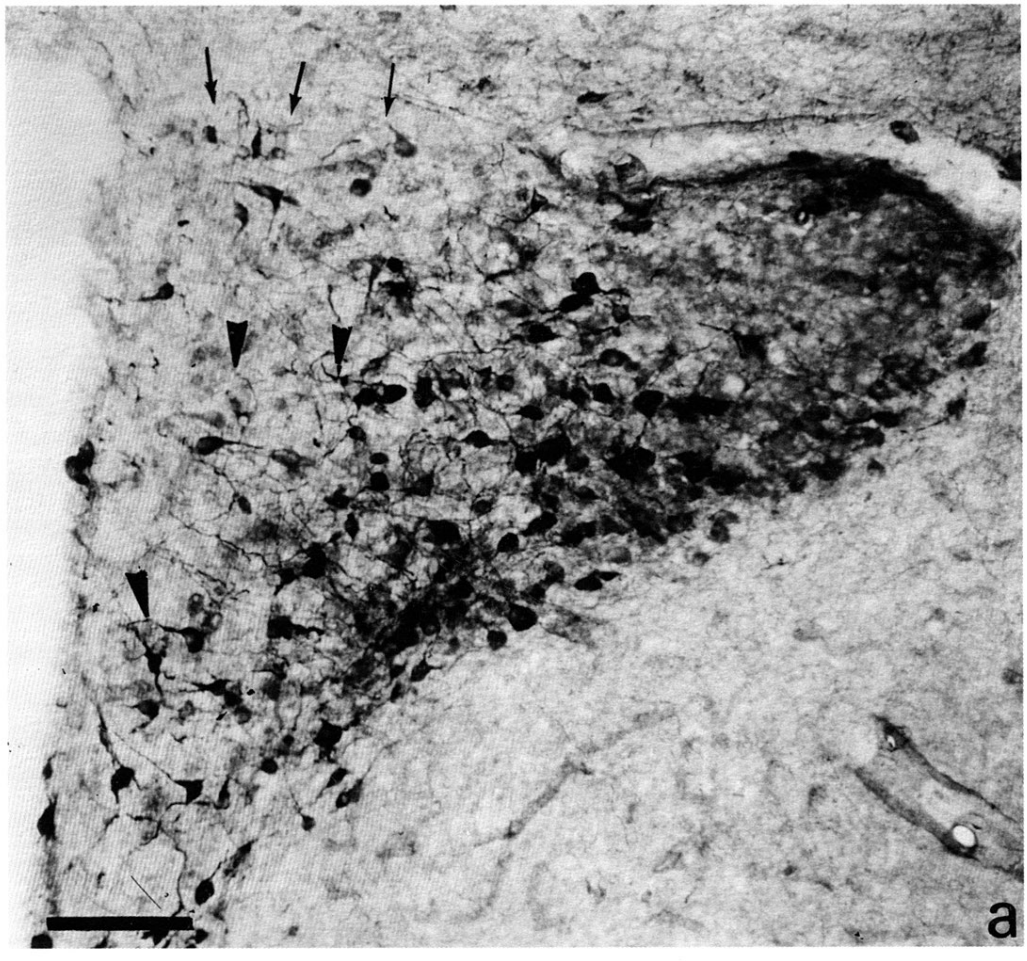
Following orchidectomy a decrease in the number of ND-neurons was observed (Fig. 1b and 2b) both at the level of the magno- and parvicellular subdivisions, although in some of them, such us the dorsal parvicellular one, this decrease was not statistically significant (Table 1). In addition, a decrease in the intensity of staining, when compared to non treated animals, was also observed (Figs. 1b and 2b).

These changes were reversed when orchidectomized animals received a daily dose of testosterone (Fig. 3b. Table 1). In fact, in this group of animals, an increase in the histochemical expression of ND activity in the different subdivisions of the PVN, especially at the level of the posterior magnocellular subdivision (Fig. 3b), was detected.

Cell counts in this group showed a significant statistical increase in the number of ND-neurons in the posterior magnocellular subdivision when compared to the normal and sham groups ($p < 0.05$) (Table 1).

Discussion

NO is one of the neuroactive chemicals produced in the PVN. The presence of this labile gas depends on the presence and activation of its



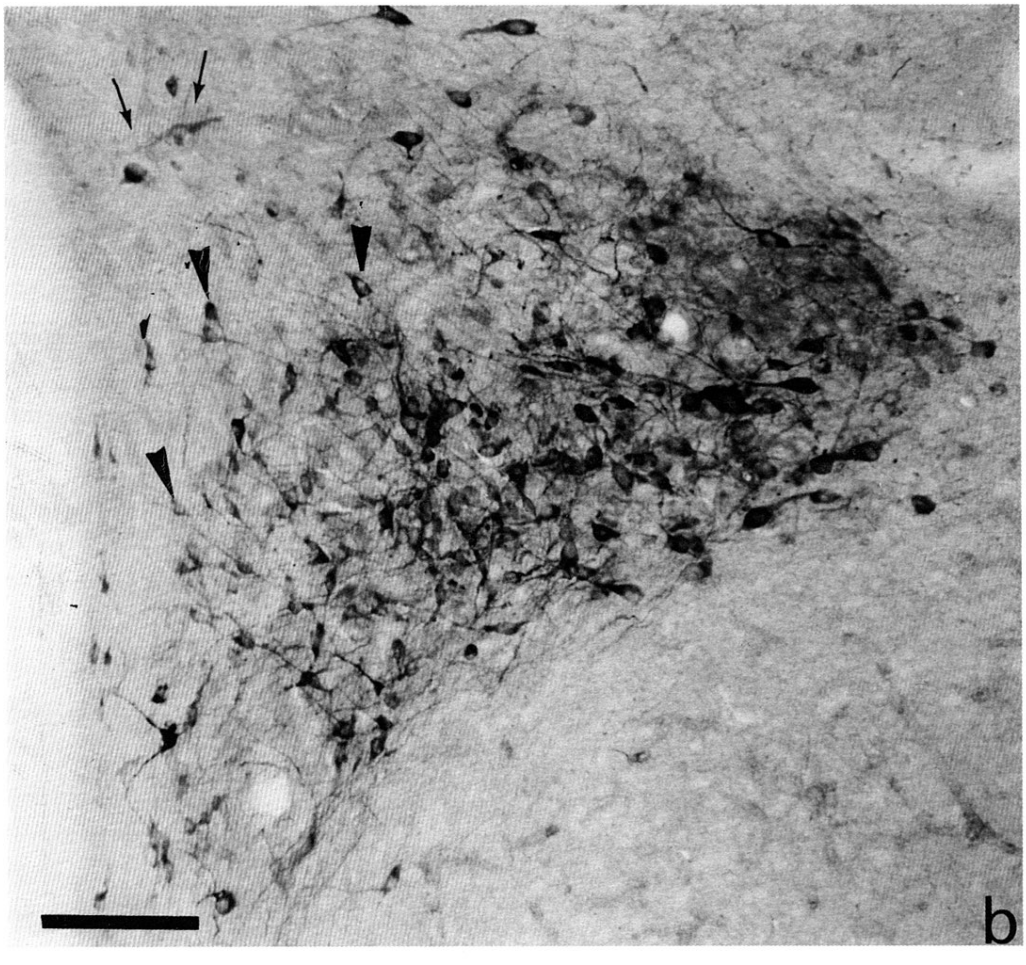


Fig. 1.– a) Normal rat (x125). Note, the preferential location of ND-active neurons in the magnocellular posterior subdivision. A group of neurons is located at the level of the dorsal parvicellular subdivision (arrows). In the medial parvicellular subdivision some cells were also present (arrowheads). **b)** Orchidectomized rat (x125). Note the decrease in the number of neurons as well as in the intensity of staining when compared to Figure 1a. Scale bar: 125 μm.

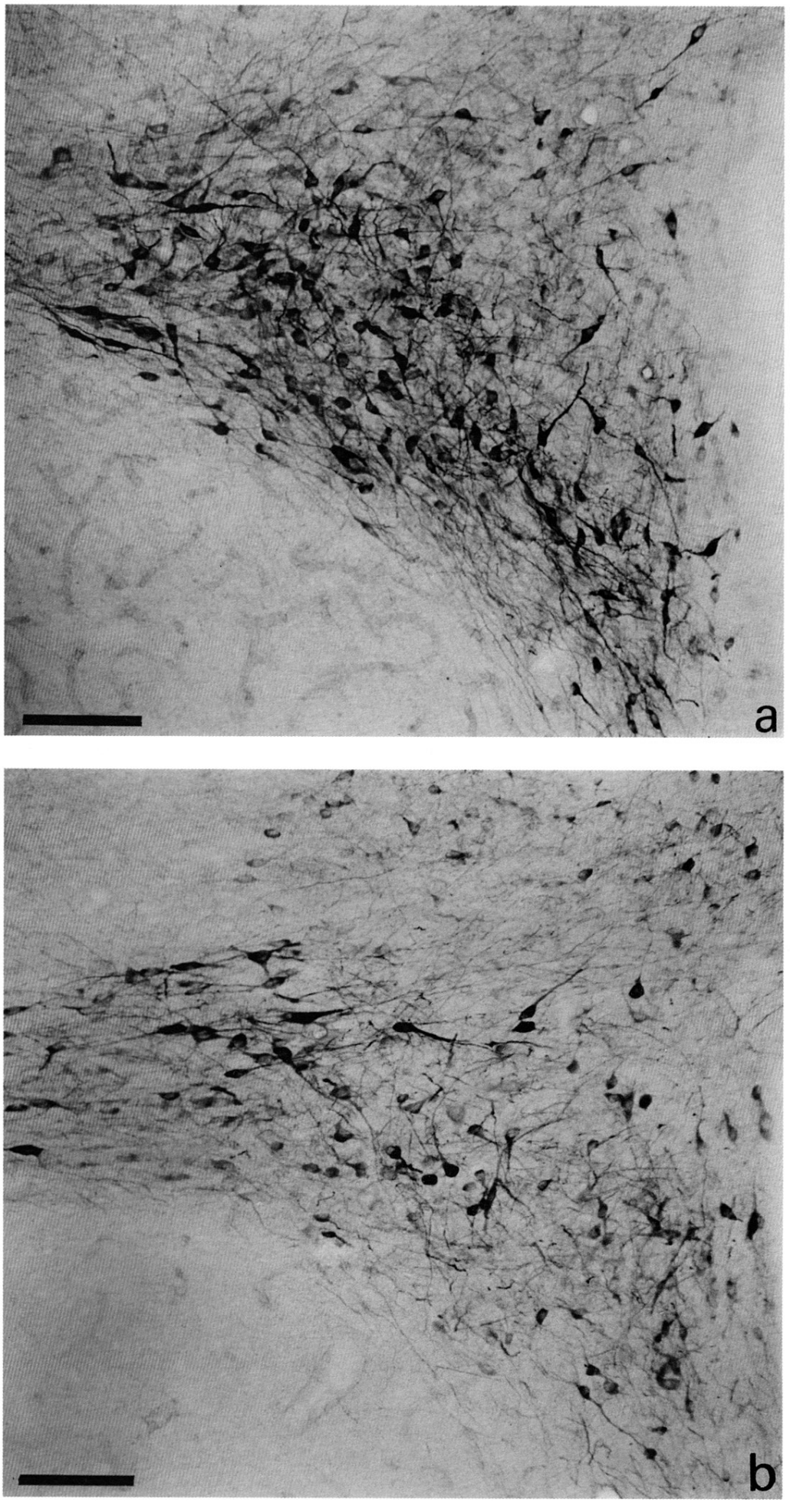


Fig. 2.– a) Normal rat (x125). Note the localization of ND-neurons at the level of the lateral parvicellular subdivision. **b)** Orchidectomized rat (x125). Note the decrease in the number of ND-neurons and in the intensity of staining when compared to normal rat. Scale bar: 125 μm.

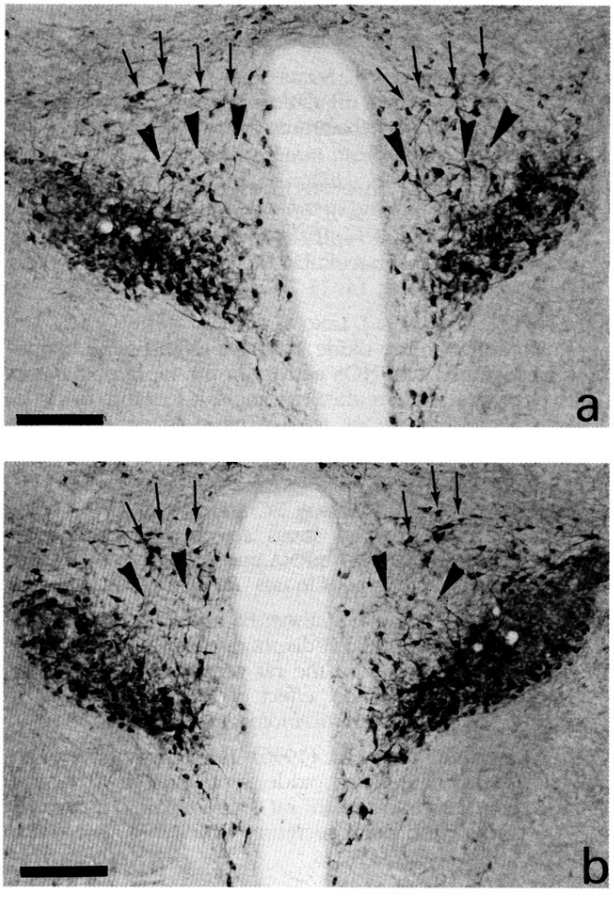


Fig. 3.– a) Normal rat (x50). Panoramic view of the posterior magnocellular, dorsal parvicellular (arrows) and medial parvicellular (arrowheads) subdivision. **b)** Orchidectomized + T rat (x50). Note the slight increase in the number of neurons, especially at the level of the posterior magnocellular subdivision. Scale bar: 250 μm.

synthetic enzyme NOS. NO functions as an important neurotransmitter in the hypothalamus which plays a major role in neuroendocrine regulation, including the control of hypothalamic portal blood flow, the control of pituitary hormone secretion and the regulation of sexual behaviour (Ceccatelli et al., 1993a, b; Brann et al., 1997).

According to the information available NO seems to be essential for basic PVN neurochemical functions modulating the functional activity of the different types of neurons located in the PVN (see Yamada et al., 1996). In addition, since NO is a gas, it is not restricted to action only at synapses as other classical neurotransmitters, and thus, in effect provides a novel mechanism for rapid regulation of hypothalamic neuronal networks regardless of their cytoarchitecture (see Brann et al., 1997)

Nowadays, it is perfectly established that the expression of ND/NOS in the PVN is modulated by a number of experimental conditions including, among others, stress (Calzà et al., 1993; Kishimoto et al., 1996), salt loading (Kadowaki et al., 1994; Villar et al., 1994b; Blázquez et al., 1995), modification of food intake (Ueta et al., 1995b; O' Shea et al, 1996), lactation (Ceccatelli and Eriksson 1993), hypothyroidism (Ueta et al., 1995a), hypophysectomy (Villar et al., 1994a), exposure to low temperature (Sánchez et al., 1998c) and in lesser degree adrenalectomy (Sánchez et al., 1996a).

The hypothalamus is a target for circulating sex steroids. Androgens have potent effects on the central nervous system (see Staub and De Beer, 1997). In males, variation in morphology, behaviour and reproductive tactics can often be explained by varying levels of androgens. Over the last decade several studies have indicated that estradiol has a potent effect upon the expression of NOS in the hypothalamus. In fact, changes in the ND/NOS staining in the ventromedial nucleus of the hypothalamus and the PVN following modifications of the quantitative estradiol levels are currently known (Okamura et al., 1994; Cecattelli et al., 1996; Sánchez et al., 1998b).

Our data demonstrate, for the first time, that the expression of ND activity in the PVN is modulated by testosterone, adding a new dimension to the knowledge of the regulation of the expression of this enzymatic activity in the PVN. These results are in accordance with the idea that sex hormones influence NOS/ND-activity in the hypothalamus (Okamura et al., 1994; Cecattelli et

Table 1.– Number (mean ± S.E.M.) of ND-neurons in the different subdivisions of the PVN in normal (N), sham-operated (Sham), bilaterally orchidectomized (OCS), bilaterally orchidectomized treated with testosterone (OCS+T). * $p < 0.05$ when compared to normal and sham operated animals.

Subdivision	N	Sham	OCX	OCX+T
Magnocellular				
Commissural	86± 4	89± 5	67± 3	92± 4
Posterior	793±32	801±31	670±23*	899±32*
Parcicellular				
Anterior	17± 2	18± 2	11± 2*	23± 2
Medial	32± 3	35± 3	21± 2*	39± 3
Periventricular	39± 3	33± 3	26± 2*	42± 3
Dorsal	15± 2	18± 2	16± 2	21± 2
Lateral	39± 3	42± 3	26± 2*	43±43

al., 1996; Sánchez et al., 1998b) and that ND/NOS neurons located in the PVN respond to a variety of stimuli affecting circulating hormone levels (for a recent review see Sánchez et al., 1998a). However, further studies should be performed to correlate the changes observed in the present study with a specific physiological response.

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