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Ana Luísa Aires da Silva

Influence of sex and gonadectomy in the anatomy and neurochemical organization in the dorsal parvicellular division of the hypothalamic paraventricular nucleus

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Nome: Ana Luísa Aires da Silva

Endereço electrónico: m04090@med.up.pt

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Assinatura: Ana Luísa Aires da Silva

INFLUENCE OF SEX AND GONADECTOMY IN THE ANATOMY AND NEUROCHEMICAL ORGANIZATION OF THE DORSAL PARVICELLULAR DIVISION OF THE HYPOTHALAMIC PARAVENTRICULAR NUCLEUS

ANA LUÍSA AIRES DA SILVA*

**Corresponding author*: Department of Anatomy, Porto Medical School, University of Porto, Alameda Hernâni Monteiro, 4200-319, Porto, Portugal

Tel: ++351-22-5513616; fax: ++351-22-5513617.

E-mail address: m04090@med.up.pt

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Abbreviations:

ACTH, adrenocorticotrophic hormone

ANOVA, analysis of variance

AR, androgen receptor

BNST, bed nucleus of stria terminalis

b.w., body weight

CRH, corticotrophin-releasing hormone

DAB, diaminobenzidine

DHT, 5α-dihydrotestosterone

ER, estrogen receptor

ERα, estrogen receptor-α

ERβ, estrogen receptor-β

HPA, hypothalamo-pituitary-adrenal

MPN, medial preoptic nucleus

PBS, phosphate-buffered saline

PVN, paraventricular nucleus of the hypothalamus

PVNdp, dorsal parvicellular division of the paraventricular nucleus of the hypothalamus

PVNmp, medial parvicellular division of the paraventricular nucleus

VP, vasopressin

3β-diol, 5α-androstan-3β,17β-diol

Abstract — The production of glucocorticoids by the adrenal cortex is modulated by the paraventricular nucleus (PVN) via the hypothalamo-pituitary-adrenal (HPA) axis and the autonomic-related descending projections originating from the dorsal parvicellular (PVNdp) and lateral parvicellular divisions of the PVN. Females produce more corticosterone than males in basal conditions and in response to stress, and these differences have been ascribed to gender-specific features of the HPA axis. However, no sex-related differences have been detected in the levels of ACTH, the trophic hormone to the adrenal cortex. Thus, we have speculated that sex differences in the activity of the pre-autonomic projections of the PVN might contribute for the sexually dimorphic activity of the adrenal cortex. To test this hypothesis, we have estimated the volume of the PVNdp, the total number and somatic size of its neurons, and the total number of its neurons producing vasopressin (VP) and corticotrophin-releasing hormone (CRH) in intact and in gonadectomized male and female rats. It was found that the volume and total number of neurons of the PVNdp were not influenced by sex or gonadectomy. However, intact females had larger neurons than intact males, a difference that was abolished by gonadectomy. It was also found that the number of CRH neurons was not influenced by sex or gonadectomy, as opposed to the number of VP neurons, which was higher in intact females than in intact males and, in both sexes, decreased by gonadectomy. Since this decrease was greater in females than in males, it abolished the sex differences noticed in intact rats. Therefore, our study shows that the anatomy and neurochemistry of the PVNdp present gender differences, and that these differences are related to circulating levels of sex steroid hormones. This suggests that the PVNdp might be important in the establishment of the sexually dimorphic production of corticosterone.

Key words: Dorsal parvicellular part of the paraventricular nucleus; Corticotrophin-releasing hormone; Vasopressin; Sexual dimorphism; Gonadectomy

The adrenal cortex produces two important classes of steroid hormones, the mineralocorticoids and the glucocorticoids. Glucocorticoids (cortisol in humans and most mammals and corticosterone in rats, mice, and lower vertebrates) are essential for the maintenance of homeostasis and enable the organism to respond to and manage stress. Their production is governed by parvicellular neurons of the hypothalamic paraventricular nucleus (PVN), which exert a dual control over adrenal steroidogenesis: via its neuroendocrine division, the central component of the hypothalamo-pituitary-adrenal (HPA) axis, and via its autonomic-related descending division (for a review, see Bornstein and Chrousos, 1999; Tsigos and Chrousos, 2002).

The core endocrine reaction to stress is the activation of the HPA axis, which includes the neurons of the medial parvicellular division of the paraventricular nucleus (PVNmp) that project to the median eminence, where they release corticotrophin-releasing hormone (CRH). CRH is the major secretagogue of the adrenocorticotrophic hormone (ACTH), produced by the anterior pituitary corticotropes under the synergistic influence of CRH and vasopressin (VP). The VP that acts as a secretagogue of ACTH is also produced by neurons in the PVNmp, where half of the CRH-containing neurons also express VP. By acting on the adrenal cortex, ACTH directly regulates the production of glucocorticoids. In addition to multiple peripheral and central actions, glucocorticoids exert a negative feedback on the hypothalamic neurosecretory cells and pituitary corticotropes, thus controlling the activity of the HPA axis (for a review, see Antoni, 1986).

Instead, the influence of the PVN on the sensitivity of the adrenal cortex to ACTH and on the diurnal variation in resting plasma corticosterone levels does not primarily operate through the HPA axis, but more via sympathetic input to the adrenal gland (for a review, see Bornstein and Chrousos, 1999; see also Motawei et al., 1999; Ulrich-Lai et al., 2006). The pre-autonomic PVN neurons are mainly located in the dorsal parvicellular (PVNdp) and lateral parvicellular divisions of the PVN, and their axons project to the intermediolateral column of the spinal cord where they contact sympathetic preganglionic neurons, which then innervate the adrenal gland through the thoracic splanchnic nerve (for reviews, see Tóth et al., 1997; Bornstein and Chrousos, 1999). This paraventricular-spinal projection is largely vasopressinergic and oxytocinergic (Sawchenko and Swanson, 1982; Cechetto and Saper, 1988), with over 40% of the spinally projecting PVN neurons expressing VP or oxytocin mRNA (Hallbeck and Blomqvist, 1999).

A variety of experimental studies has shown that the stress response is sexually dimorphic, with females producing more epinephrine and corticosterone than males after exposure to a stressful situation (see for example, Shanks et al., 1994; Weinstock et al., 1998; Lund et al., 2004; Figueiredo et al., 2007). The male-female differences in corticosterone levels are also apparent under basal conditions, particularly at proestrus when females are under the influence of high circulating levels of estrogens (see for example, Atkinson and Waddell, 1997; Silva et al., 2009). These sex differences can be ascribed, partially at least, to the presence of significantly more VP- and CRH-producing neurons in the female than in the male PVNmp (Silva et al., 2009). However, in basal as well as in stress conditions, no consistent sex-related differences in ACTH levels have been detected, and the gender differences and cycle-associated fluctuations in plasma corticosterone concentrations are not accompanied by corresponding shifts in ACTH levels (for a review, see Bornstein et al., 2008). This dissociation between corticosterone and ACTH levels, led us to hypothesize that the gender-specific pattern of adrenal

steroidogenesis might rely, partially at least, on the presence of sex differences in the anatomy and/or neurochemistry of the descending part of the PVN. To address this possibility we focused on the PVNdp because retrogade transport studies have provided clear evidence that most PVN cells projecting to the spinal cord are concentrated in this division (Swanson and Kuypers, 1980; Zhang et al., 2000). Thus, our first aim was to estimate, using stereological methods, the volume of the PVNdp and the total number and volume of its neurons in rats of both sexes. The PVNdp contains VP- and CRH-positive neurons (Swanson and Kuypers, 1980; Sawchenko and Swanson, 1982; Kiss et al., 1996), and there is evidence that, in addition to oxytocinergic fibers, the hypothalamo-spinal projection is composed of VP- and CRH-containing fibers (Sawchenko and Swanson, 1982; Cechetto and Saper, 1988). Therefore, our second aim was to evaluate whether, or not, the number of VP- and CRH-immunoreactive neurons in the PVNdp differs between the sexes. Finally, to examine the possibility that sex-differences in the expression of VP and CRH by PVNdp neurons is reflective of gonadal hormone influences, we have also assessed the effects of gonadectomy on the morphology and neurochemistry of the PVNdp in males and in females.

EXPERIMENTAL PROCEDURES

Animals and treatments

The studies were carried out in male and female Wistar rats aged 6 months. Throughout the experiment, rats were maintained under standard laboratory conditions (12-h light/dark cycles,

lights on at 7:00 AM, and temperature of 22 ºC) and had ad libitum access to solid food and water. The estrous cycle was monitored by daily collection of vaginal smears and histological examination every fourth week and at the day of killing. Only females exhibiting regular (4-5 days) estrous cycles were used.

At 5.5 months of age, males and females were randomly assigned to two groups each. Rats from one group were submitted to gonadectomy and the remaining to sham-gonadectomy. Thirteen days later, half of the rats in each of the four groups were stereotaxically injected in the lateral ventricle with colchicine. Two days later, rats were anaesthetized by intraperitoneal injection of a solution (3 ml/kg b.w.) containing 1% sodium pentobarbital and 4% chloral hydrate in physiological saline and intracardially perfused with a solution for conventional histological procedures or for immunocytochemistry. Rats were all killed between 1:00 PM and 2:00 PM. Each of the groups analyzed comprised 6 animals each. The intact female group was formed by pooling rats at random stages of the estrous cycle.

All procedures were carried out in compliance with the European Communities Council Directives of 24 November 1986 (86/609/EEC) and Portuguese Act nº. 129/92. All efforts were made to minimize the number of animals used and their suffering.

Surgery

Orchidectomy. Rats were anaesthetized by intramuscular injection of 2% xylazine (132 µl/kg b.w.; Sigma-Aldrich Company Ltd., Madrid, Spain) and 10% ketamine (500 µl/kg b.w.; Laboratórios Pfizer, Seixal, Portugal). After anaesthesia, rats were laid on the back with the tail towards the investigator. The skin of the scrotum was cleaned and a small incision was made in the median plane through the skin at the tip of the scrotum. The subcutaneous tissue was removed and the testes exposed. An incision of about 5 mm was made at the tip of each testis, and the

epididymus tail was pulled out together with testis, head of the epididymus, vas deferens and spermatic blood vessels. Blood vessels and vas deferens were isolated, ligated, and then severed distal to the ligature allowing removal of the testis and epididymus. The remaining piece of the vas deferens and the fat was pushed back into the sac, and the incisions were closed with a single suture.

Ovariectomy. After being anaesthetized, as described above, rats were laid on their back with the tail towards the investigator. A median incision, of about 1 cm, was made through the skin at the middle point of linea alba. Entrance to the peritoneal cavity was gained through incision of abdominal wall muscles. The ovaries were pulled out through the muscle incision by grasping the periovarian fat. Using pointed scissors, the junction between the Fallopian tube and the uterine horn, together with all accompanying blood vessels and fat, was severed with a single cut and the returned into the abdominal cavity. The muscle and skin incisions were closed with single sutures.

Stereotaxy. Rats were anaesthetized by sequentially injecting, at intervals of 10 min, solutions in physiological saline of 0.25% acepromazine (176 µl/kg b.w., subcutaneous; Laboratórios Vitória, Amadora, Portugal), followed by 2% xylazine (132 µl/kg b.w., intramuscular) and, finally, 10% ketamine (500 µl/kg, intramuscular). They were then placed on a stereotaxic apparatus with bregma and lambda in the same horizontal plane. After exposing the calvaria by performing a midline incision in the skin of the skull, holes were drilled unilaterally, 1.1 mm posterior to the bregma and 1.7 mm lateral to the midline. Then, a 10-µl Hamilton syringe (901N; Hamilton Bonaduz AG, Bonaduz, Switzerland) was lowered into the right lateral ventricle until 5.2 mm from the surface of the skull. Colchicine (C9654, Sigma-Aldrich) was

dissolved in 0.25% physiological saline (50 nmol/μl, pH 7.4) and injected gradually (2 μl every 2 min) until the total amount of 10 μl was delivered. The needle was left in place for an additional 10 min before being slowly withdrawn.

Tissue preparation

Convention histological procedures. After anaesthesia, rats were perfused with a solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer (pH 7.2). The brains were removed from the skulls, weighed and postfixed for 15 days in fresh fixative. After removal of the frontal and occipital poles, the blocks containing both the right and left hypothalami were dehydrated through a graded series of ethanol solutions, embedded in glycomethacrylate (hydroxyglycomethacrylate, Technovit 7100, Kulzer, Wrhreim, Germany), and sectioned in the coronal plane at 40 μm, as described elsewhere (Madeira et al., 1997; Leal et al., 1998). The sections were collected, mounted serially, and stained with a Giemsa solution modified for use in glycomethacrylate-embedded material (West et al., 1991). Finally, the sections were coverslipped with Histomount mounting medium.

Immunohistochemistry. Forty-eight hours after the injection of colchicine, rats were anaesthetized as described above and perfused with a fixative solution containing 4% paraformaldehyde in phosphate buffer (pH 7.6). Brains were removed, immersed in the same fixative solution for 2 h, and maintained overnight in a solution of 10% sucrose in phosphate buffer. The blocks containing right and left hypothalami were mounted on a Vibratome and serially sectioned at 50 μm in the coronal plane. Alternate sections were separately collected in phosphate-buffered saline (PBS) in order to obtain two independent sets of sections from each

brain; each set was then processed independently for CRH and AVP immunostaining using the avidin-biotin technique with diaminobenzidine (DAB) as the chromogen, as previously described (Madeira et al., 1993, 1997). The antiserum against CRH (Peninsula Laboratories, Belmont, CA) and the antiserum against VP (gift from Dr. R. Buijs, The Netherlands Institute for Brain Research, Amsterdam) were used at the dilution of 1:5000.

Stereological analyses

The subdivisions of the PVN were delineated and named according to Swanson and Kuypers (1980). Therefore, the dorsal parvicellular division (PVNdp) was identified as a distinct lens-shaped group of horizontally oriented medium-sized cells, dorsal to the posterior magnocellular and medial parvicellular parts (Fig. 1A). All sections in which the PVNdp was visualized were used for the estimation of the volume of, and total number of neurons in the PVNdp. The estimates were obtained either from the right or left nucleus, and,thus represent unilateral values. The volume was estimated by using the principle of Cavalieri and point-counting techniques (Gundersen and Jensen, 1987), and the total number of Giemsa-stained neurons by applying the optical fractionator method (West et al., 1991; Madeira et al., 1997; Leal et al., 1998). In each section, the fields of vision were systematically sampled using a step size of 70 μm along the *x*-axis and 70 μm along the *y*-axis. The disector used had a counting frame area of 1687 μm^2 at the tissue level and a fixed height of 15 μm. The estimations were performed, at magnification of 2000×, using the CAST— Grid system software (Olympus DK, Denmark) and a Heidenhain MT-12 microcator (Heidenhain, Germany).

The same stereological method was employed to estimate the total number of CRH- (Fig. 1B) and VP-immunoreactive neurons (Fig. 1C) in the PVNdp. The sampling schemes used were

as described above, with the following modifications. The step size was 50 μm along the *x*- and the *y*-axes and the height of the disector was 10 μm. Immunostaining of the perikaryal cytoplasm with a relatively unstained nucleus was the criteria for identification of CRH- and VP-containing neurons.

Statistical analyses

To discern main effects, data from all parameters estimated were analyzed by using a two-way analysis of variance (ANOVA). Sex and gonadectomy were used as the independent variables. Whenever significant effects were detected, the Tukey HSD post hoc test was performed to examine differences between the groups. Differences were considered to be significant if $P<0.05$.

RESULTS

PVNdp volume

ANOVA tests revealed that the volume of the PVNdp (Fig. 2A) was not significantly influenced by sex ($F_{1,20}=0.48$, $P=0.500$) or gonadectomy ($F_{1,20}=1.86$, $P=0.188$); no significant interaction between these variables was also detected ($F_{1,20}= 0.03$, $P=0.872$).

Total number of PVNdp neurons

As revealed by ANOVA, there was no significant effect of sex ($F_{1,20}$=0.26, P=0.613) or gonadectomy ($F_{1,20}$=0.66, P=0.427) on the total number of PVNdp neurons (Fig. 2B). In addition, no significant interaction between sex and gonadectomy was noticed ($F_{1,20}$=3.56, P=0.073).

Mean somatic volume of PVNdp neurons

ANOVA detected a significant influence of sex ($F_{1,20}$=10.42, P=0.004) in the somatic volume of PVNdp neurons (Fig. 2C). Intact females had larger neurons than intact males. Although no significant effect gonadectomy ($F_{1,20}$=3.84, P=0.064) and no significant interaction between sex and gonadectomy ($F_{1,20}$=1.41, P=0.249) have been detected, the sex difference noticed in intact rats was no longer apparent after gonadectomy.

Total number of CRH- and VP-immunoreactive neurons

The total number of CRH-immunoreactive neurons (Fig. 3A) was not significantly influenced by sex ($F_{1,20}$=0.13, P=0.719) or gonadectomy ($F_{1,20}$=0.36, P=0.558). No significant interaction between these variables ($F_{1,20}$=0.02, P=0.878) was also detected.

Conversely, the total number of VP-immunoreactive neurons (Fig. 3B) varied as a function of sex ($F_{1,20}$=5.80, P=0.025) and gonadectomy ($F_{1,20}$=240.02, $P<5x10^{-4}$); a significant interaction between these variables was also detected ($F_{1,20}$=24.03, $P<5x10^{-4}$). Intact females had significantly more VP neurons than intact males. Gonadectomy was associated with a significant decrease in the number of VP-immunoreactive neurons in males (50%) and in females (70%), and annulled the sex differences observed in intact groups.

DISCUSSION

The results of the present study show that the volume of the PVNdp and the total number of its constituent neurons do not differ between the sexes and, in males as well as in females, are not altered by gonadectomy. A sex-related difference was however noticed in the somatic volume of PVNdp neurons of intact rats, with females having significantly larger neurons than males. Together, these results show that the structural organization of the PVNdp is sexually dimorphic because the presence of larger neurons in females is not associated with a larger PVNdp, despite the existence of a similar number of neurons in both sexes. This suggests that PVNdp neurons are less packed and the neuropil is more abundant in males than in females, which might indicate that the afferent fibers to this PVN division are more numerous in males than in females. Further research will be however required to clarify this point.

Our results further show that the PVNdp is also sexually dimorphic with respect to the number of neurons of some of its chemically-identified subpopulations. Even though there were no sex differences in the number of CRH-producing neurons, intact females had significantly more VP-producing neurons than intact males. VP is one of the main neurotransmitters of the paraventricular-spinal projection, and there is evidence that it acts as an excitatory neurotransmitter at synapses of neurons of the descending part of the PVN (reviewed in Pyner, 2009; see also, Motawei et al., 1999). In addition, it was also shown that the electric activation of splanchnic nerves stimulates adrenal steroidogenesis and increases the adrenal sensitivity to ACTH (reviewed in Bornstein and Chrousos, 1999). Therefore, the presence of sex differences in the number of VP neurons suggests that the PVNdp might be implicated in the establishment of the male-female differences in corticosterone production that occur under basal conditions

(Critchlow et al., 1963; Silva et al., 2009) and in response to stress (for example, Shanks et al., 1994; Paulmyer-Lacroix et al., 1996; Figueiredo et al., 2007).

The sex differences noticed in the PVNdp are, in part at least, dependent on the circulating levels of gonadal steroids. In fact, gonadectomy annulled the male-female difference noticed in the size of PVNdp neurons due to a slight, although not significant, decrease in the somatic volume of the neurons in ovariectomized rats. This variation probably reflects reduced neuronal activity since gonadectomy was associated to a significant decrease in the number of VP neurons in the PVNdp of male and female rats. Due to the greater reduction in the number of VP neurons in females than in males (to 30% and 50% of the numbers in intact male and female rats, respectively), the sex-related difference noticed in intact rats was not apparent in gonadectomized rats. The reduction in the number of VP neurons in the PVNdp of ovariectomized rats is in line with the decreased corticosterone production and VP synthesis observed in the main component of the neuroendocrine part of the PVN, the PVNmp, after ovariectomy (Seale et al., 2004a,b). However, the finding of a decrease in the number of VP neurons in castrated male rats was rather unexpected. In effect, there is evidence that orchidectomy increases the basal and stress-induced corticosterone and ACTH levels and enhances VP mRNA expression in the PVNmp, and that these effects are reversed by testosterone administration (Bingaman et al., 1994; Seale et al., 2004a,b). Thus, although not ruling out a possible modulatory function, the results obtained in this study show that the VP conveyed by the hypothalamo-spinal projection is not likely to play a determining role in corticosterone production in males, as opposed to what happens in females. In contrast to VP neurons, the number of CRH neurons was not modified by gonadectomy in rats of both sexes. This fact, together with the lack of sex differences in the number of CRH neurons in the PVNdp suggest that, as opposed to VP neurons, sex steroids are not directly regulating CRH neurons via a traditional genomic mechanism, at least. The lack of sensitivity of CRH neurons to

sex steroid hormones, also noticed in ERβ knockout male mice (Nomura et al., 2002), might be related to the absence of a significant expression of estrogen receptors by CRH neurons in the PVNdp (Stern and Zhang, 2003; Miller et al., 2004), as opposed to what happens to VP-producing PVNdp neurons (Stern and Zhang, 2003).

The comparison of the results herein reported with data available in the literature indicates that the sensitivity to gonadal steroids and the mechanisms through which these steroids modulate neuronal activity differs between the spinal-projecting part and the neuroendocrine part of the PVN. In the latter, CRH and VP neurons are both activated by estrogens and inhibited by testosterone, whereas in the PVNdp CRH neurons are not sensitive to sex steroids and VP neurons are activated by estrogens as well as by testosterone. The influence of sex steroids upon brain structure and function are mediated by androgen receptors (ARs) and two classes of estrogen receptors (ERs), the ERα and the ERβ. Interestingly, the expression of ARs in the PVN is confined to the descending part of the PVN, namely to the PVNdp and lateral parvicellular division (Bingham et al., 2006). In addition, and in contrast to ERα, ERβ is abundant in the rat PVN (Miller et al., 2004) and, similar to ARs, is expressed solely by neurons of its spinal-projecting cell groups (Isgor et al., 2003; Stern and Zhang, 2003; Bingham et al., 2006). Because ARs and ERs are virtually absent from the neuroendocrine division of the PVN, it is believed that the influence of sex steroids in the morphology and function of these neurons is indirect and mediated by the neural afferents to this PVN division. In fact, the PVNmp receives abundant projections from a variety of brain regions, including the medial preoptic nucleus (MPN) and bed nucleus of stria terminalis (BNST), which are richly endowed with ARs (Williamson and Viau, 2007). Given that the efferent fibers from the MPN are mainly GABAergic (Simerly et al., 1986), it has been suggested that the activation by testosterone of GABA neurons in the MPN might lead

to inhibition of hypophysiotrophic neurons in the PVNmp, and thus to decreased production of VP and CRH (Williamson and Viau, 2008). In contrast to neurons in the PVNmp, neurons in the PVNdp of males express ARs (Bingham et al., 2006) and receive relatively few afferents from other AR-positive brain areas (Williamson and Viau, 2007). Thus, it is possible that, in this particular division of the PVN, the increased gene expression resulting from activation of ARs by testosterone overrides the inhibitory influences conveyed by neural afferents from other androgen-sensitive brain regions. Although, in males, PVNdp neurons also express the ERβ, the activating effects of testosterone on VP expression seem to be mediated by ARs because they are compatible with the increased corticosterone production and c-fos expression by PVN neurons resulting from the administration of the non-aromatizable androgen 5α-dihydrotestosterone (DHT; reviewed in Handa et al., 2009). Lending further support to this possibility are the results from earlier studies (reviewed in Williamson et al., 2005) showing a decrease in the number of VP-positive cells after castration and a reversal of this effect by testosterone in regions that express ARs, such as the BNST **(**Williamson and Viau, 2007). However, a possible role for the ERβ in mediating the inhibitory effects of testosterone on neuronal activation in the PVN has recently emerged from a study showing that the DHT metabolite 5α-androstan-3β,17β-diol (3β-diol), a selective ERβ agonist, suppresses the hormonal response and c-fos mRNA expression in the PVN (Lund et al., 2006). It deserves to be mentioned that in females the activation of the ERβ enhances the activity of the HPA axis (Isgor et al., 2003), which is consistent with reduction in the number of VP neurons herein reported.

In summary, our study shows that the anatomy and neurochemistry of the PVNdp differs between male and female rats, and that these sex differences are dependent on the circulating levels of sex steroid hormones. This indicates that the PVNdp might play a significant role in the

in the establishment of the sexually dimorphic pattern that characterizes corticosterone production. Further studies, namely the analysis of the oxytocinergic population of the PVNdp, will be however required to shed light on the nature of the influence exerted by this PVN division on the activity of the adrenal cortex.

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FIGURE LEGENDS

Fig. 1. Photomicrographs of Giemsa (A) and immunostained (B, C) coronal sections through the PVN of an intact male rat. The PVNdp is delineated by a continuous line. This division contains neurons that produce CRH (B) and VP (C). F, fornix; mp, medial parvicellular division of the PVN; pm, posterior magnocellular division of the PVN; V, third ventricle. Scale bars=50 µm in A, B and C.

Fig. 2. Graphic representation of the estimates obtained from Giemsa-stained sections of the PVNdp of intact (I) and gonadectomized (Gdx) male and female rats. (A) Volume of the PVNdp. The volume does not differ among the groups studied. (B) Total number of PVNdp neurons. The total number of PVNdp neurons does not differ among the groups studied. (C) Mean somatic volume of PVNdp neurons. The volume is larger in intact females than in the remaining groups. $^{*}P$=0.02.

Fig. 3. Graphic representation of the estimates obtained from immunostained sections of the PVNdp of intact (I) and gonadectomized (Gdx) male and female rats. (A) Total number of neurons immunoreactive for CRH. The number of CRH-immunoreactive neurons does not differ among the groups studied. (B) Total number of neurons immunoreactive for VP. The number VP-immunoreactive neurons is larger in intact females than in intact males. In addition, intact males and females have more VP-immunoreactive neurons than castrated and ovariectomized rats. Male vs females, $^{*}P$=0.0003; gonadectomized vs intact ^{+}P=0.0002.

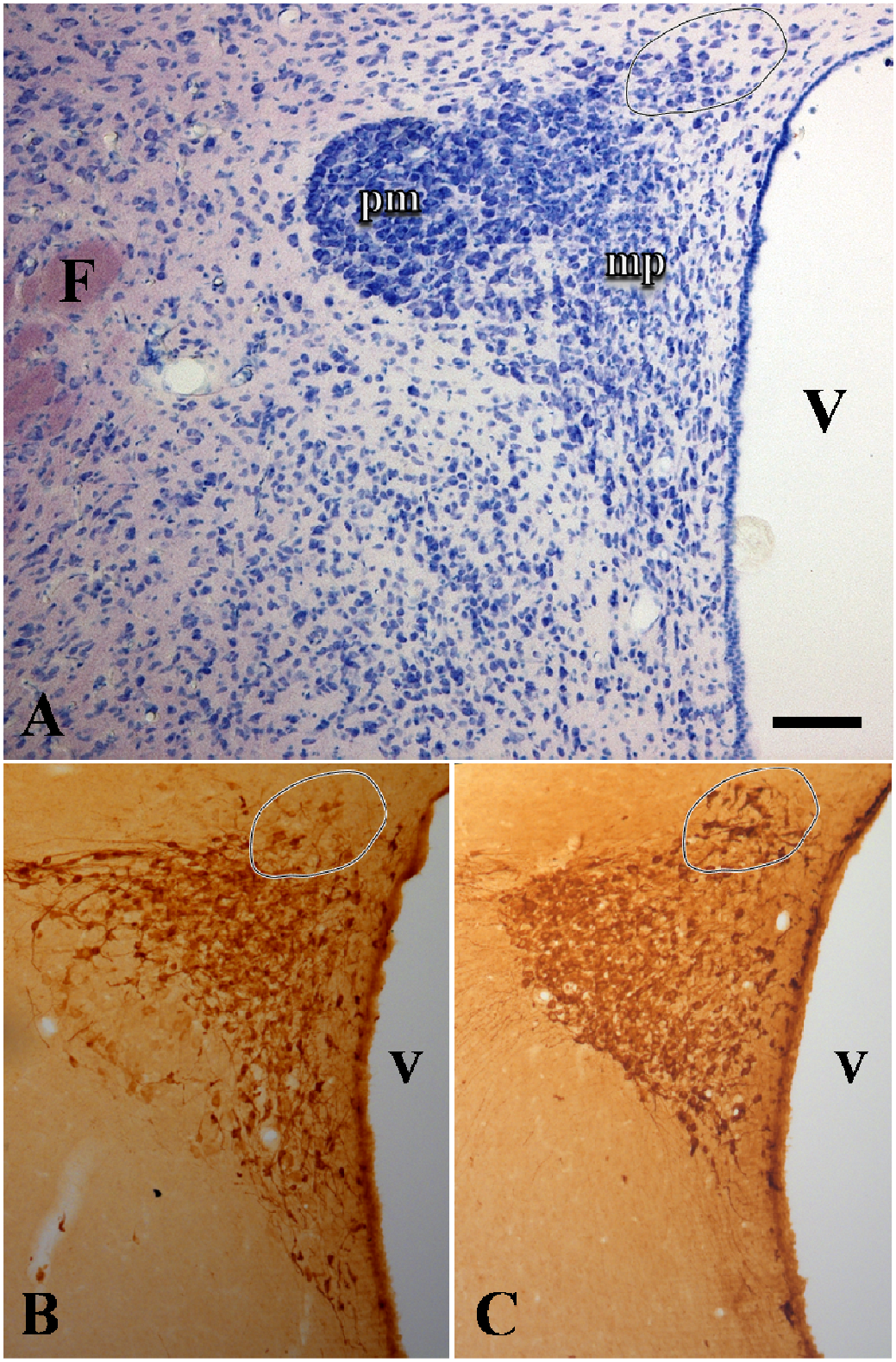

FIG. 1

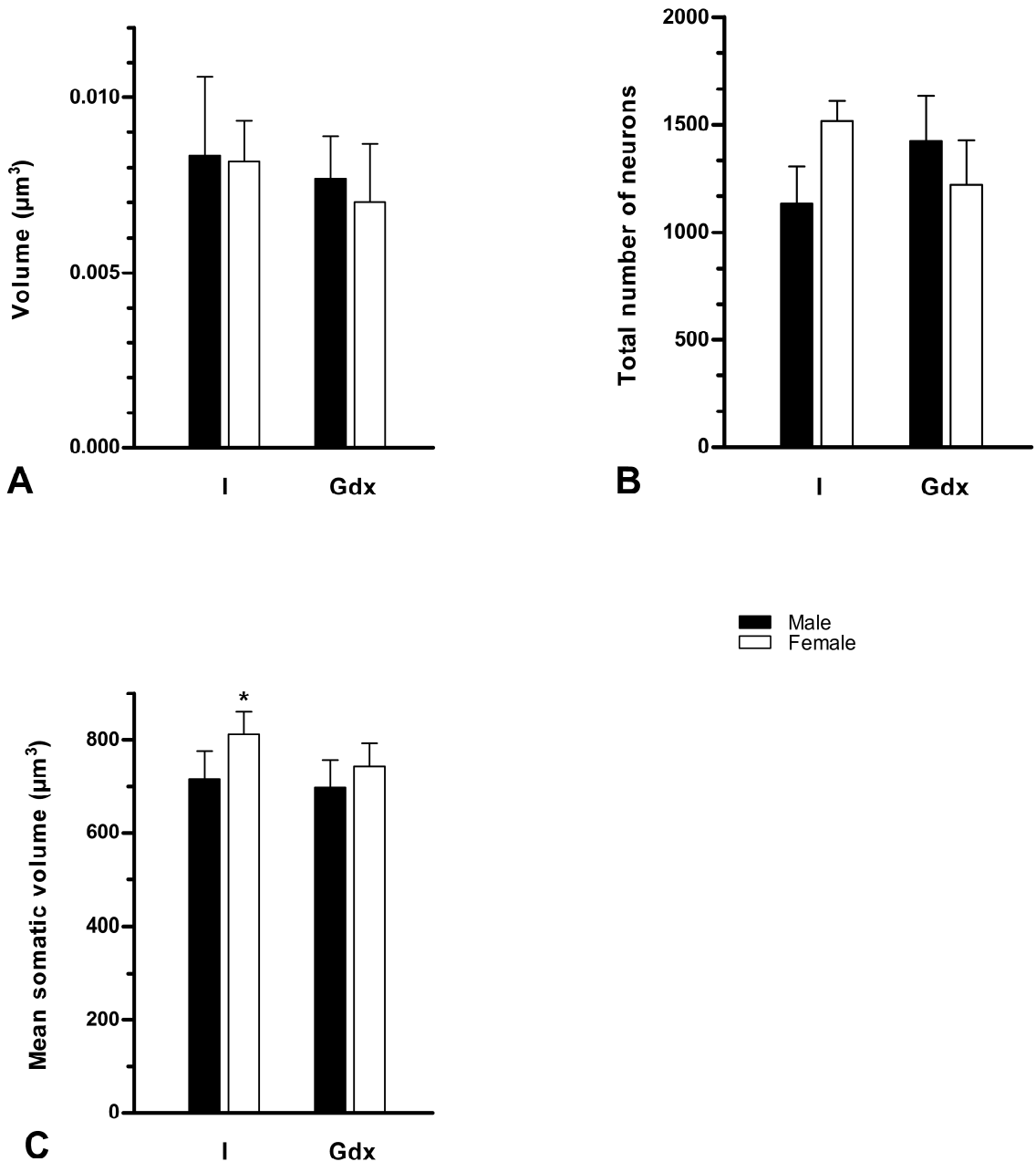
Volume (µm³)
0.010
0.005
0.000
A
I
Gdx
Total number of neurons
2000
1500
1000
500
0
B
I
Gdx
Male
Female
Mean somatic volume (µm³)
800
600
400
200
0
*
C
I
Gdx

FIG. 2

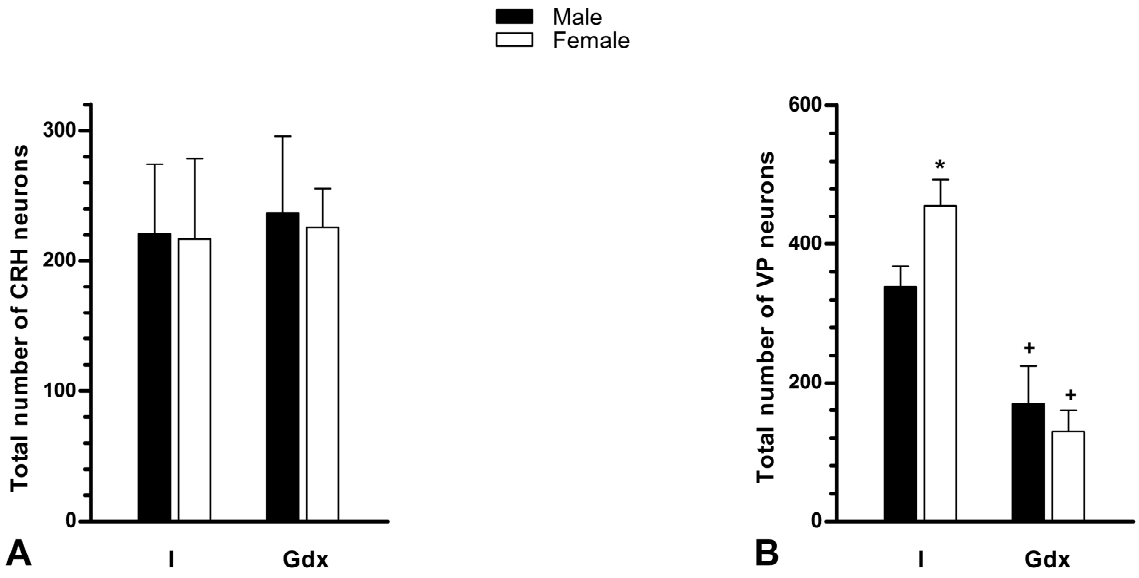
Male
Female
Total number of CRH neurons
300
200
100
0
A
I
Gdx
Total number of VP neurons
600
400
200
0
*
+
+
B
I
Gdx

FIG. 3