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Hypothalamic Changes in Norepinephrine Release in Rats with Estradiol Valerate-Induced Polycystic Ovaries[1]

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ABSTRACT

Chronic anovulation and polycystic ovaries (PCO) can be induced by a single i.m. injection of estradiol valerate (EV, 2 mg in oil) in the rat. Constant exposure to high plasma levels of estradiol provokes a neurotoxic effect on the hypothalamic neurons, including those from the arcuate nucleus. Because of the important participation of hypothalamic norepinephrine (NE) in the regulation of GnRH release and the possible noxious effect of prolonged exposure of these neurons to estradiol, our interest was to study the activity of the noradrenergic neurons innervating the hypothalamus. We analyzed the biosynthesis, content, and release of NE from the noradrenergic nerve terminals of the hypothalamus during the PCO condition. We found a decrease in tyrosine hydroxylase (TH) activity and in the content of dopamine (DA) in the anterior hypothalamus after 2 mo of EV injection, whereas dopamine-β-hydroxylase (DβH) was increased without changes in NE content. No variations in TH activity or in DA and NE contents in the medial hypothalamus were observed, but a decrease in DβH activity was evident. After 2 mo of EV administration, an increase in the electrically induced release of NE from anterior hypothalamic blocks incubated in vitro was detected; this effect was not evidenced in the medial hypothalamus. After 5 mo of EV administration, release of NE increased in anterior hypothalamic blocks but decreased in medial hypothalamic tissue. The inhibitory effect of morphine on NE release found in control animals was increased in the hypothalamus from PCO rats, suggesting an increased number of μ-opioid binding sites in noradrenergic neurons. Together these data indicate increased noradrenergic activity of the nerve terminals from the anterior hypothalamus and decreased activity from catecholaminergic nerve terminals from the medial hypothalamus during PCO. These results agree with similar findings described in women with PCO syndrome as suggested from the analysis of urinary catecholamines metabolites, and gives further support for the involvement of central catecholamines in the maintenance of the syndrome in mammals.

INTRODUCTION

Chronic anovulation and polycystic ovaries (PCO) can be induced by a single i.m. injection of estradiol valerate (EV) in the rat [1]. Recent evidence from our laboratory indicates that an increase in the sympathetic tone of neurons innervating the ovary is observed 30 days after a single EV injection [2]. This activation is established before the appearance of ovarian cysts, and it is correlated with an exaggerated steroid secretory response of the ovary to β-adrenergic agonists [3]. In addition, 60 days after a single EV injection, a progressive multifocal lesion throughout the hypothalamus, including the arcuate nucleus, has been reported [1]. It is hypothesized that the EV-induced increase in ovarian sympathetic and steroid secretory activities, not EV per se, are responsible for such hypothalamic lesions since EV injection cannot produce a similar neural degenerative reaction in ovariectomized rats [4]. Presumably, the hypothalamus of the EV-treated rats is constantly exposed to increased circulating gonadal steroids, especially estradiol, that provoke the neurotoxic effect.

The nature of the neural and chemical components involved in the degeneration of hypothalamic neurons is not known. Desjardins et al. [5] observed a specific reduction in the number of β-endorphin neurons in the arcuate nucleus of the hypothalamus after EV treatment. They suggested that the loss of β-endorphin neurons is accompanied by a compensatory up-regulation of μ-opioids receptors on the nerve terminals or cell bodies of target neurons because of the marked increase in μ-opioid binding sites in the medial preoptic area (MPOA, [6]). The MPOA is a region rich in GnRH cell bodies and in norepinephrine (NE)-containing nerve terminals [7]; both are targets for opioidergic action. Therefore, the possibility exists that the decreased number of opioid neurons found after 60 days of EV administration could affect the activity of GnRH neurons either directly or indirectly via NE release.

In this paper we examined the possibility that EV affects the activity of hypothalamic catecholaminergic nerve terminals and the opioidergic input in them. We analyzed the following: 1) the contents of NE and dopamine (DA), and the activity of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DβH)—the enzymes of catecholamine biosynthesis); these are end-points indicating changes in dopaminergic and noradrenergic turnover rates during the PCO condition; 2) the release of NE from the anterior hypothalamus (AH) and medial hypothalamus (MH) under the PCO condition, which is used as an index of the activity of the catecholaminergic neurons during EV-induced pro-

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gressive hypothalamic lesions; and 3) the effect of morphine (an agonist of opioid receptors) on the in vitro release of NE from hypothalamic explants in PCO rats. A preliminary report has appeared elsewhere [8].

MATERIALS AND METHODS

Animals

Virgin adult cycling Sprague-Dawley rats (200–220 g) derived from a stock maintained at the University of Chile were used. They were allowed free access to pelleted food and tap water, and were housed in quarters with controlled temperature (22°C) and photoperiod (lights-on from 0700–1900 h). Only animals exhibiting regular 4-day estrous cycles were used for the experiments. PCO was induced by administration of a single i.m. injection of EV (Sigma Chemical Co., St. Louis, MO; 2 mg/rat in 0.2 ml corn oil), as described by Brawer et al. [1]. Thirty-five rats received injections of EV, and a similar number of age-matched controls received injections of vehicle alone. Because EV-treated rats are in constant estrus, controls were used during the estrous phase of the cycle. NE-release experiments were performed at 2 and 5 mo after injection of EV, and changes in catecholamine content and activities of the enzymes of its biosynthesis were evaluated 60 days after EV administration. The 2-mo post-EV period was selected because it is during this time that the ovary develops multiple cysts [3, 5], hypothalamic microglial cells and reactive astrocytes increase in size, and hypothalamic β-endorphin neurons decrease in number by 60% [5]. The 5-mo post-EV interval corresponds to the time when neuronal degeneration is fully developed [1]. At the end of each experiment, rats were killed, their brains were rapidly removed and maintained at 4°C, and the hypothalamic regions were dissected out according to procedures described by Glowinski and Iversen [9]. The AH was defined as the region located 1 mm in front of and 1 mm behind the optic chiasma. The MH was defined as the region located 1 mm anterior and 1 mm posterior to the median eminence; this region also includes the medial basal hypothalamus. Tissues from the AH and MH for NE-release experiments were immediately transferred to Krebs-bicarbonate buffer for preincubation. All other tissues (AH and MH) for analysis of catecholamines and enzymatic activities were frozen (−20°C) up to the time of the assay.

Measurement of TH Activity

TH activity was measured in hypothalamic tissue obtained from control (estrous) and EV-treated rats. Tissues were homogenized in 10 volumes of 0.1 M acetate buffer (pH 6.1)/0.2%Triton X-100. Homogenates were centrifuged at 27 000 × *g* for 20 min, and the supernatant was used as the sample. The enzyme activity was determined by the method of Waymire et al. [10], with minor modifications [11]. The procedure involves measuring $^{14}CO_2$ released from [1-^{14}C]tyrosine (sp. act. 52 mCi/mmol; Dupont/NEN, Boston, MA) after hydroxylation by endogenous TH and subsequent decarboxylation by addition of a crude extract of dihydroxyphenylalanine decarboxylase (DDC) obtained from pig kidneys. The assay was performed with 1 mM 6-methyltetrahydrobiopterin (Sigma Chemical Co.) used as a cofactor for TH. Enzymatic activity was expressed as pmol CO_2 formed/mg protein/15 min.

Measurement of DβH Activity

DβH activity was measured in hypothalamic tissue homogenized in 10 volumes of 0.1 M acetate buffer (pH 5.7)/0.2%Triton X-100. The enzyme activity was determined as previously described [12, 13]. The procedure involves measuring the [^{3}H]octopamine formed by the hydroxylation of [^{3}H]tyramine (sp. act. 18.2 Ci/mmol, Dupont/NEN) by DβH. We used 5 μM $CuSO_4$ and 50 μM hydroxy-mercuribenzoate to inhibit endogenous inhibitor of the enzyme present in the tissue homogenate. Enzymatic activity was expressed as fmol octopamine formed/mg protein/h.

Measurement of DβH by the Immunodot Technique

In addition to the role of DβH in transforming DA to NE in noradrenergic neurons, DβH is a constitutive protein of the membrane of NE-storing vesicles [14]. Changes in the amount of the protein represent changes in the number of storage vesicles of NE and hence in the availability of NE to be released under stimulatory conditions. To measure the amount of DβH, samples were homogenized in 10 volumes of PBS containing 10 μM of phenylmethyl-sulfonyl-fluoride (Sigma Chemical Co.) as protease inhibitor. Samples were centrifuged at 3500 × *g* for 10 min, the pellet was discarded, and the supernatant was assayed. We used a rabbit antibody against DβH from the adrenal medulla (Eugene Tech. Int. Inc., Ridgefield Park, NJ). To determine the amount of DβH, samples containing 50 μg of protein were vacuum-filtered through 0.45-μm nitrocellulose paper filters, and the adsorbed samples were allowed to interact for 30 min with a 1:1000 dilution of the primary antibody and were then processed with an immunostaining ABC kit (Pierce Laboratories Inc., Rockford, IL). The final product was determined by the development of green color, with ABTS (2,2′-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) used as substrate. Because of the water solubility of the product it was directly eluted from the nitrocellulose, and the absorbance of the solution was read. Results are presented as absorbance units per milligram of protein.

Measurement of Tissue NE, DA, and Protein Contents

The hypothalamic tissue (AH or MH) was homogenized in 10 volumes of 0.2 M perchloric acid. The suspensions were centrifuged at 15 000 × *g* for 10 min, and NE and DA present in the supernatant was determined by the radioen-

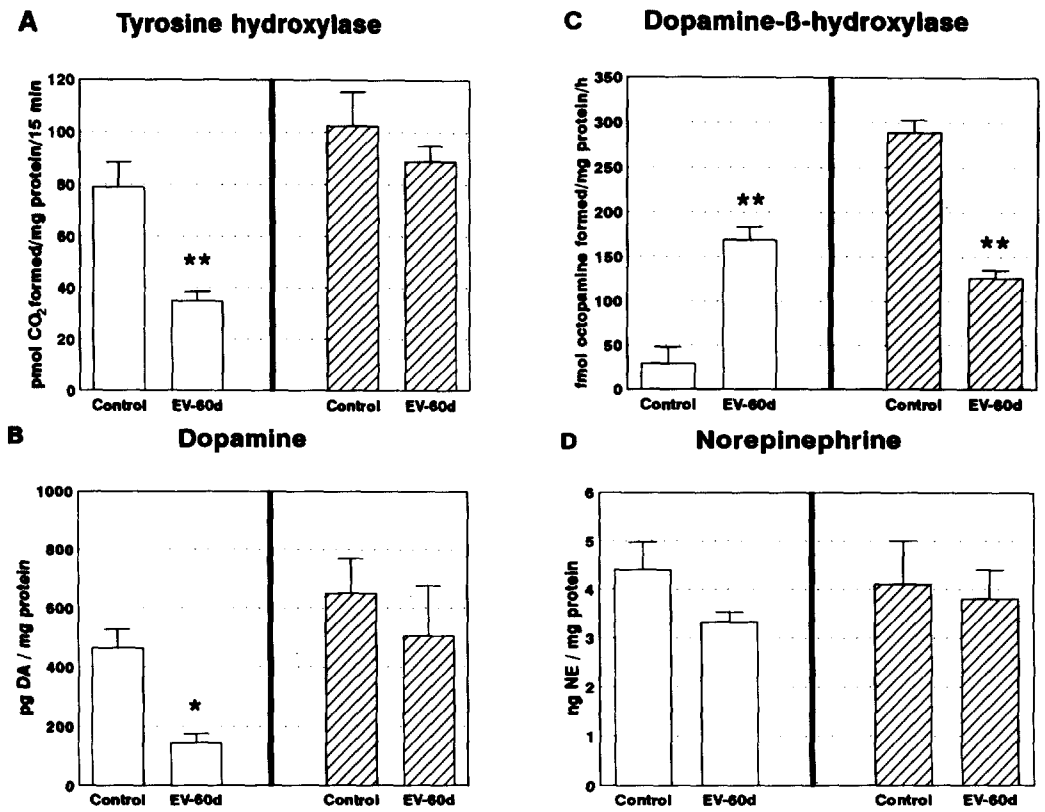

FIG. 1. Effect of EV administration on TH (A) and DβH (C) activities and DA (B) and NE (D) content in the AH (open bars) and MH (hatched bars). Controls were age-matched rats killed during estrus. Results correspond to mean ± SEM of 3–5 animals for each experimental point. * $p < 0.05$ vs. control. ** $p < 0.01$ vs. control.

zymatic method of Saller and Zigmond [15], as previously described [16]. Acid-insoluble material was dissolved in 1 M NaOH, and its protein content was determined by the method of Lowry et al. [17], with BSA (fraction V) used as standard.

Release of Hypothalamic NE In Vitro

The procedure employed was modified from our previous studies [16, 18]. Hypothalamic blocks from control or PCO rats were preincubated for 20 min in Krebs-bicarbonate buffer (pH 7.4) gassed with 95% O_2:5% CO_2 and then incubated for 30 min at 37°C with 2 μCi of [^{3}H]NE (sp. act. 40.1 Ci/mmol, Dupont/NEN). After the tissue was washed in Krebs bicarbonate buffer (6 washes of 10 min each) to remove nonincorporated radioactivity, the hypothalamic tissue was placed in a multiwell plate with 24 flat-bottom wells containing 1.5 ml of buffer per well. Tissues were maintained for 1 min in each well. After 3 passages, depolarization was effected by removal of the tissue to another well where a train of monophasic electrical pulses (80 V, 10 Hz, 2 msec/pulse for 1 min) was delivered via a parallel set of platinum electrodes and generated by a Grass S-4 stimulator. After stimulation, tissues were washed 3 times (1 min each). In some experiments, after this first stimulation period, the hypothalamic tissues were washed for 10 min and subjected to the same protocol of passages through the multiwell plate and a second train of pulses under the same conditions as in the first stimulation period, or in the presence of 1 μM morphine (morphine-HCl, Sigma Chemical Co.), for study of the effect of an opioid agonist on the release of NE. The entire stimulation protocol was simultaneously repeated for AH and MH from one control and one PCO rat in parallel superfusion chambers.

At the end of the experiment, the hypothalamic tissues were homogenized in 0.4 N perchloric acid, and the [^{3}H]catecholamines remaining in the tissue were determined by scintillation counting; the radioactivity incorporated by the tissue and the radioactivity released during stimulation were then calculated. The latter, which represents [^{3}H]NE overflow from hypothalamic nerve terminals, was expressed as fractional release, i.e., as percentage of the total radioactivity present in the tissue [16, 18]. The total amount of NE released under stimulation (net release) was calculated as the area (A) under the stimulation minus the spontaneous release. To compare the effect of morphine on the release, we calculated the ratio A_2/A_1, which represents the ratio of the net release obtained in the presence (A_2) of morphine and the net release in the absence (A_1) of the drug. This calculation allows quantification of the effect of morphine independent of change on the release of NE due to treatment.

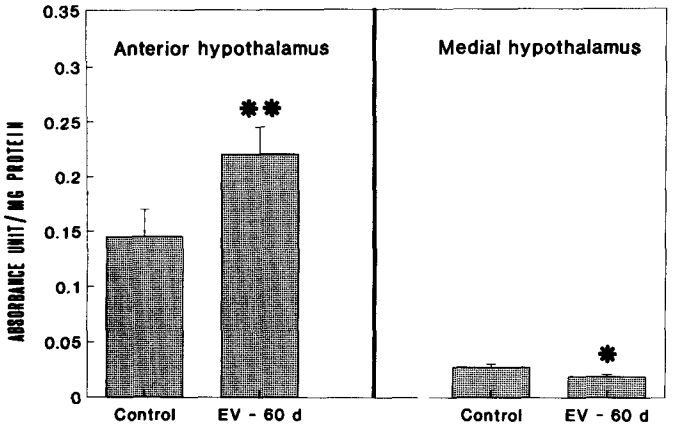

FIG. 2. Effect of EV administration on amount of DβH in AH (right panel) and MH (left panel) assayed by immunodot technique. Results are presented as absorbance unit/mg of protein and correspond to mean ± SEM of 5 animals in each group. * $p < 0.05$ vs. control. ** $p < 0.01$ vs. control.

Statistics

Differences between two group means were analyzed with Student's *t*-test. When percentages were compared, the data were normalized by means of an arc-sine transformation before statistical evaluation. Comparisons between several groups were performed by use of a one-way analysis of variance, followed by the Student-Newman-Keuls multiple comparison test for unequal replications [19].

RESULTS

Effect of EV on the Biosynthesis and Content of Hypothalamic Catecholamines

A decrease in TH activity ($p < 0.01$) was observed in the AH from rats with PCO (Fig. 1A, left panel). This reduction in AH-TH activity was accompanied by a comparable decrease in the content of DA in the same tissue (Fig. 1B, left panel). In contrast, no changes in TH activity and DA content was observed in the MH (Fig. 1, A and B, right panels). A clear increase in the activity of DβH was found in the AH (Fig. 1C, left panel), but the increase in DβH was not followed by a comparable increase in the content of NE (Fig. 1D, left panel). Interestingly, the DβH activity in the MH was lower ($p < 0.01$) in PCO rats (Fig. 1C), whereas the MH-NE contents were similar in PCO and control rats (Fig. 1D). Consistent with increased DβH activity in the AH (Fig. 1C, left panel) and decreased DβH activity in the MH (Fig. 1C, right panel), similar results were obtained with immunodot analysis, which showed an increase in the amount of DβH protein in the AH and a decreased amount in the MH (Fig. 2).

Effect of EV on [^{3}H]NE Release from the AH and MH

The effect of EV administration on the induced release of NE is presented in Figure 3. After 60 days of EV administration, a moderate increase in the electrically induced release of NE ($p < 0.05$) from the AH was seen. No difference in the induced release of NE from the MH of the same rats was observed. No changes in the spontaneous release of NE from either the AH or MH was found.

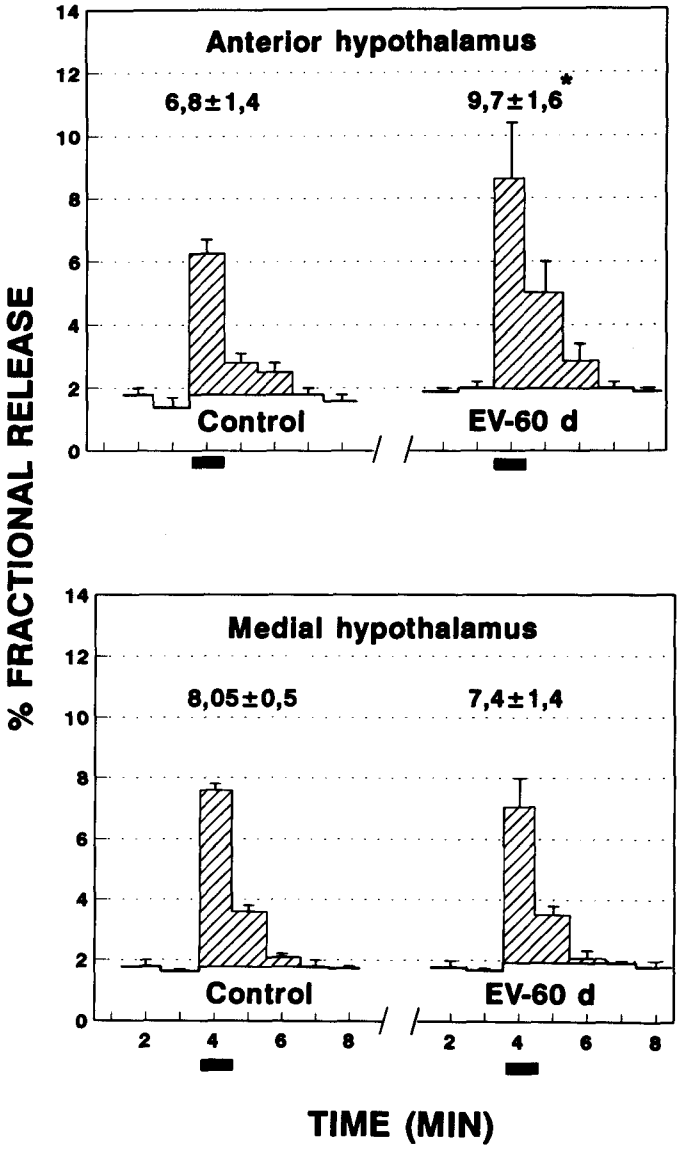

FIG. 3. Effect of EV administration on induced release of [^{3}H]NE] from AH (upper panel) and MH (lower panel). Blocks from AH and MH from control and 60-day EV-treated rats were preincubated with [^{3}H]NE and then stimulated with a train of 600 pulses at 10 Hz, 2 msec-length, and 80 V for 1 min (black bar). Numbers on top correspond to net release (shaded area) for control and EV-treated rats. Results are presented as percentage of fractional release and represent mean ± SEM of 4 individual experiments in each group with exception of AH from EV-60d that corresponds to 6 animals. *$p < 0.05$ vs. control.

After long-term (5 mo) treatment with EV, a clear increase in the net release of NE ($p < 0.05$) from the AH was shown in treated rats vs. age-matched controls (Fig. 4, upper panel). On the other hand, the induced release of NE from the MH was significantly decreased ($p < 0.01$) after long-term EV treatment (Fig. 4, lower panel).

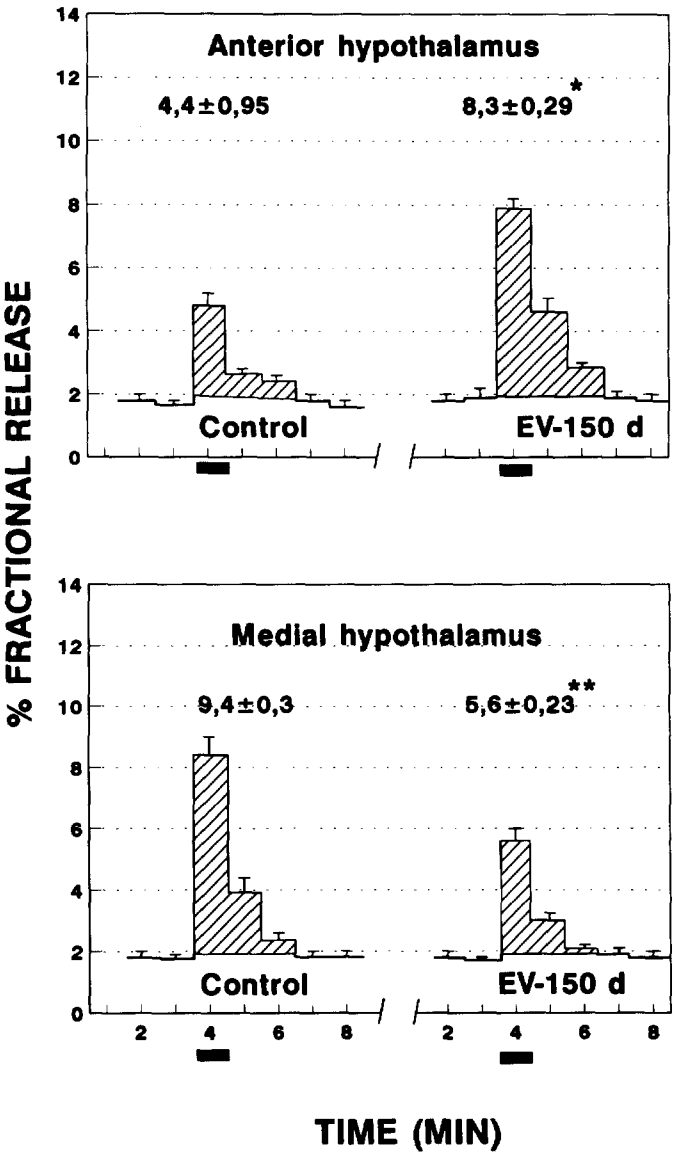

FIG. 4. Long-term effect (150 days) of EV administration on induced release of [^{3}H]NE from AH (upper panel) and MH (lower panel). Stimulation conditions were same as in Figure 3. Numbers on top correspond to net release (shaded area) for control and EV-treated rats. Results are presented as percentage of fractional release and represent mean ± SEM of 4 individual experiments in each group. * = $p < 0.05$ vs. control. ** = $p < 0.01$ vs. control.

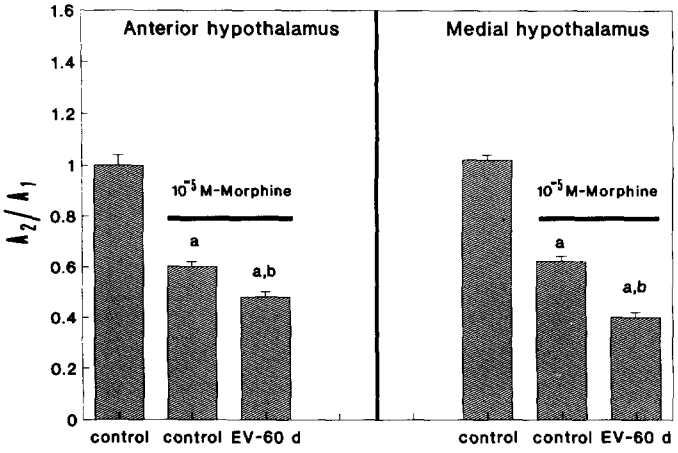

FIG. 5. Effect of morphine on induced release of [^{3}H]NE from AH (left panel) and MH (right panel) from control and EV-treated rats (dashed bars). To standardize differences in release of different groups, results are expressed as ratio A_2/A_1 for net release obtained from first stimulation in absence of morphine (A_1) and second stimulation in presence of morphine (A_2). Black bar represents ratio between 2 successive stimulations without morphine during second stimulation. Results represent mean ± SEM of 3 individual experiments. a = $p < 0.05$ vs. control ratio without morphine. b = $p < 0.05$ vs. control ratio in presence of morphine.

Effect of Morphine on [^{3}H]NE Release from the AH and MH of Control and EV-Treated Rats

The presence of 10^{-5} M-morphine in the superfusion buffer reduced the induced release of NE from the AH and MH of controls (Fig. 5). In EV-treated rats compared to controls, morphine induced a further reduction in NE release. The ratio between the net release obtained during the second (in the presence of morphine, A_2) and the first stimulation (in the absence of morphine, A_1) was significantly lower ($p < 0.05$) in EV rats than the same ratio in controls (Fig. 5). Morphine alone could not modify the spontaneous release of NE from EV-treated animals (data not shown) or from controls (0.83 ± 0.14% in AH without morphine vs. 0.78 ± 0.12% with morphine; 0.88 ± 0.20% in MH without morphine vs. 0.72 ± 0.21% with morphine; n = 3 for MH and AH).

DISCUSSION

The results of this study show that the electrically induced NE release from the AH was increased during the PCO condition induced by EV administration and was decreased in the MH of the same rats.

Synthesis, storage and release of NE are the principal processes that participate in the turnover of neurotransmitters at catecholaminergic synapses [20]. The decrease in TH activity detected in the AH from the EV-treated rats was accompanied by a decrease in DA, but not in NE, contents. The decrease in TH activity and DA content found in the AH could represent intracellular changes in the periventricular dopaminergic cells that innervate the MPOA and AH. These neurons could be damaged by EV in the same way as described for the β-endorphin neurons of the arcuate nucleus [5]. However, it is well documented that the bulk of catecholaminergic innervation of the AH is represented by noradrenergic neurons [21, 22]. Almost all of the noradrenergic neurons of the hypothalamus are derived from the lateral tegmental NE system. This system, composed of multiple cell groups in the brain stem (A_1, A_2, A_5, and A_7), projects into the MPOA (principally groups A_1 and A_2) and the adjoining anterior hypothalamic area, but not into the suprachiasmatic nucleus, arcuate nucleus, and median em-

inence [7]. Thus, the decrease in TH activity and DA content may correspond to changes occurring in noradrenergic neurons. These changes were not translated into a significant decrease in NE content, probably because of a compensatory increase in DβH activity, an observation that was partially confirmed in this study by immunodot assay (see Fig. 2). The direct relation found between DβH activity and the amount of protein determined by immunodot assay suggests that the change in the activity of DβH represents a change in the amount of enzyme molecules. Because DβH is a constituent protein of the NE storage vesicles [14], an increase in DβH activity may represent an increase in the number of NE-storage vesicles. This change in the number of NE vesicles could represent changes in the availability of NE to be released under stimulation.

Previous studies from our laboratory have presented evidence that the changes induced by gonadal steroids in the activity of noradrenergic sympathetic neurons closely correspond to changes in DβH, but not always to TH activity, because long-term effects on NE biosynthesis are compensated for by an increase in the storage capacity of neurotransmitter and hence by the availability of NE to be released [11, 12, 23]. In the present study, a similar behavior for these enzymes was visualized in the central nervous system and under the effect of estradiol. Results from this study further confirm that the increase in DβH activity (see Figs. 1 and 2) may be responsible for an increase in the induced release of NE in the AH of rats treated with EV for 2 and 5 mo.

The decrease in the release of NE found in the MH after 5 mo of EV administration suggests that probably longer exposure to higher levels of estradiol not only produces a degeneration of the opioid neurons [5] but also can affect the catecholaminergic neurons from the medial basal hypothalamus. This last assumption is based on the possibility that some of the NE released from medial hypothalamic blocks could represent NE incorporated to dopaminergic neurons and released under stimulation. Further experiments are necessary to solve this issue.

The principal neuronal process affected in the AH and MH from rats with PCO appears to be the release of NE from a small, easily releasable pool [24]. This is suggested by the finding that the increase in NE release occurs without detection of changes in NE content. The increase in DβH activity and in the induced release of NE found in the AH could represent a specific effect on the noradrenergic neurons from the A_1 and A_2 groups of the lateral tegmental NE system. Double-staining techniques to localize GnRH and NE cells and fibers in the rat showed that NE fibers synapse with GnRH perikarya in the MPOA [25, 26]. It is interesting to note that this is the system involved in the control of GnRH release and finely regulated by opioid neurons originating from the arcuate nucleus [27, 28]. Opioid neurons can also regulate catecholamines release from the medial basal hypothalamus [29]. Thus in our EV-treated rats (Fig. 5) the increased sensitivity of electrically induced release of NE from the AH and MH to morphine suggests that μ-opioid bindings sites in these regions are increased after EV treatment, an observation in agreement with that previously demonstrated by Desjardins et al. [6]. That morphine has a regulatory role in NE-induced release and does not act as a depolarizing agent per se was demonstrated by the lack of effect of morphine on the spontaneous release of NE.

In summary, our results suggest that after the PCO condition has been established in the rat and the nerve-dependent hypersecretion of steroid from the ovary is fully operative, changes in the activity of the noradrenergic nerves of the hypothalamus appear. These changes include an increase in the response of noradrenergic nerves from the AH to stimulation and a decrease in the response from the MH. Because of the marked decrease in the number of β-endorphin neurons reported during this condition [5] and the lack of effect of opioid peptides as depolarizing agents found in this study, it is probable that noradrenergic neurons lack the negative control naturally exerted by opioid peptides [6, 27], contributing to the changes in catecholaminergic activity found in this work.

Although the PCO condition induced by EV administration to rats does not exactly reproduce the human syndrome, it shares many of the characteristics of the human PCO condition [1]. It is interesting to note that our observation on the changes in the neuronal activity of NE neurons correlates with much evidence obtained from human patients with PCO that strongly suggests an increased hypothalamic noradrenergic tone and a decreased dopaminergic activity [30, 31]. A sizably body of evidence has accumulated in support of the concept that hypothalamic function is compromised in EV-induced PCO [1, 6, 32–34]. The changes in noradrenergic activity described in this work could participate, through the modification of GnRH release, in modifications of the basal and pulsatile LH secretion described in PCO [32, 35]. The change in noradrenergic input to the hypothalamus was established after a long period of EV administration, i.e., after a primary hyperactivation of noradrenergic neurons innervating the ovary has developed [2], and opens the possibility that the increase in nerve activity of the ovary and its coupling to the increased production of steroids could participate as one of the first components of the neuroendocrinopathologic process underlying EV-induced PCO manifestation at the hypothalamic level.

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