Chronic Nitric Oxide Deficiency Is Associated with Altered Leutinizing Hormone and Follicle-Stimulating Hormone Release in Ovariectomized Rats[1]

MARIA J. BARNES,* KAREN LAPANOWSKI,* JOSE A. RAFOLS,† DAVID M. LAWSON,* AND JOSEPH C. DUNBAR[2,*]

*Departments of *Physiology and †Anatomy, Wayne State University School of Medicine, Detroit, Michigan 48201*

Nitric oxide (NO) synthase (NOS) has been found in the gonadotrophs and folliculo-stellate cells of the anterior pituitary. Previous observations from our laboratory suggest that NO may play a role in regulating gonadotropin secretion. Because estrogen secretion by the ovary can influence gonadotropin secretion, we investigated the hypothesis that chronic *in vivo* NO deficiency has a direct estrogen-independent effect on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion. Chronic NO deficiency was induced by adding an NOS inhibitor, N-nitro-L-arginine (L-NNA, 0.6 g/l) to the drinking water of ovariectomized (OVX) rats. The control OVX rats were untreated. After 6–8 weeks, the animals were sacrificed, and the pituitaries were removed and perfused continuously for 4 hr in the presence of pulsatile gonadotropin-releasing hormone (GnRH, 500 ng/pulse) every 30 min. *S*-Nitroso-L-acetyl penicillamine (SNAP, an NO donor, 0.1 m*M*) or L-nitro-arginine methyl ester (L-NAME, an NOS inhibitor, 0.1 m*M*) was added to the media and perfusate samples were collected at 10-min intervals. GnRH-stimulated LH and FSH levels were significantly lower in pituitaries from OVX/NO-deficient pituitaries compared with pituitaries from the OVX control group. The addition of SNAP significantly decreased LH and FSH secretion by pituitaries from OVX control animals, but significantly increased their secretion by pituitaries from the OVX/NO-deficient animals. L-NAME also suppressed LH and FSH secretion by pituitaries from the OVX control animals and stimulated their release by pituitaries from the NO-deficient/OVX animals. Immunohistochemistry of frontal sections through the hypothalamus demonstrated that OVX/NO deficiency is associated with increased GnRH in the median eminence. We conclude that NO has a chronic stimulatory effect on LH and FSH release and the subsequent altered secretory responsiveness to NO agonist or antagonist is the result of chronic NO suppression. Exp Biol Med 227: 817–822, 2002

Key words: LH; FSH; nitric oxide; ovariectomy; GnRH.

Nitric oxide (NO) has been demonstrated to have a major involvement in the hypothalamic control of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion. Studies also support the hypothesis that the ovary is a major timer of the estrous cycle and that estradiol controls gonadotropin secretion by acting directly on the pituitary gland (1–3). The intermittent basal release of gonadotropin-releasing hormone (GnRH) by the hypothalamus is believed to play a permissive role, and is a necessary component of this control system. Other studies have supported the hypothesis that estrogen acts on the pituitary to increase GnRH receptors (4) and on the hypothalamus to produce a preovulatory surge of GnRH (5). Studies by Rebar *et al.* (6) on the interaction of estrogen and gonadotropins demonstrated that the infusion of estradiol resulted in increasing circulating levels of LH in normal cycling women during the late follicular phase of the menstrual cycle.

A number of reports have indicated that an important transcriptional target of estrogen is the NO synthase (NOS) gene (1, 2, 7). Estrogen stimulates the constitutive synthesis of NO in numerous tissues, including blood vessels, heart, uterus, and skeletal muscle (8). Studies have also shown that both pregnancy and estrogen therapy enhance neuronal and endothelial NOS expression, whereas the inducible NOS isoform is unaffected (8). Additional evidence demonstrate that a region of the gene for endothelial NOS contains transcription factor-binding sites for estrogen (9). Previous

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[2] To whom requests for reprints should be addressed at Dr. Joseph C. Dunbar, Department of Physiology, 5374 Scott Hall, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201-1928. E-mail: jdunbar@med.wayne.edu

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studies from our laboratory demonstrated that NO deficiency is associated with chronically elevated levels of estrogen and a decrease in GnRH secretion (10, 11).

Collectively, these previous studies suggest that estrogen is the major cause of increased LH pulse amplitude and frequency seen in normal individuals. In the present study, we reinvestigated the hypothesis that chronic *in vivo* NO deficiency had a direct effect on LH and FSH secretion in female rats and that NO deficiency regulatory effects are estrogen independent.

Methods

Animals. Adult female Wistar rats (225–250g; Harlan Sprague-Dawley, Indianapolis, IN) were group housed in a temperature-controlled room with a 12:12-hr light:dark cycle and were allowed rat chow and water *ad libitum*. On the third day after arrival, all rats were ovariectomized (OVX) under xylazine (4 mg/kg) and ketamine (80 mg/kg) anesthesia, and one-half of the rats were immediately placed on drinking water containing the NOS inhibitor N-nitro-L-arginine (L-NNA, 0.6g/L). Blood pressures were obtained daily using the tail cuff method to monitor the NO deficiency. The index for NO deficiency was when the animals achieved a blood pressure of greater than 150 for more than 5 days, as previously described (11).

On the day of the experiment, rats (OVX [controls] $n = 6$ and NO-deficient/OVX animals $n = 6$) were sacrificed by decapitation. The pituitary was removed immediately and the posterior pituitary was discarded. Each anterior lobe was placed in α-modified minimal essential medium (α-MEM; Sigma Chemical Company, St. Louis, MO) containing 0.1% bovine serum albumin but without phenol red. Once in the medium, each pituitary was cut into four sections, and two sections were placed in each chamber of the perfusion system (Endotronics, Minneapolis, MN). Two of these systems provided 12 500-μl chambers. Each chamber was perfused at 100 μl/min with α-MEM only or α-MEM containing one of the following treatments: S-nitroso-L-penicillamine (SNAP, 0.1 m*M*) or L-nitro-arginine methyl ester (L-NAME, 0.1 m*M*). The pituitaries were perfused in a humidified atmosphere of 95% oxygen and 5% carbon dioxide for 4 hr at 37°C with 10-sec GnRH pulses (500 ng per pulse) given every 30 min. The perfusate was collected at 10-min intervals starting directly after the initial placement of the pituitary into the chamber. After the incubation, the pituitary sections were removed and weighed. The culture media was collected and frozen (–20°C) for subsequent radioimmunoassay (RIA) determination. Replicate experiments were performed using all experimental groups.

LH and FSH levels were measured using double antibody RIAs using reagents supplied by National Pituitary Program of the national Institutes of health (FSH-RP-1 and LH-RP-1 were the standards used). All samples and standards were assayed in duplicate. The secretion of LH and FSH from a given pituitary were summed from samples across the 4-hr period.

Immunohistochemistry. Rats (OVX group $n = 5$, NO deficient/OVX $n = 5$) were anesthetized with chloral hydrate (404 mg/kg body weight, i.p.). After NO deficiency was attained with L-NNA in the drinking water, each rat was perfused transcardially with 50 ml of 0.1 *M* PBS (pH 7.5) to flush the blood from the vascular system, followed by 200 ml of 4% paraformaldehyde in 0.1 *M* sodium phosphate-buffered solution (PBS; pH 7.5). One hour following the completion of perfusion, the brain was carefully removed and stored in the perfusion fixative at 4°C for a minimum of 24 hr. The brain was sectioned coronally using a vibratome, and the 50-μm slices were stored at 4°C until immunohistochemistry procedures were performed. A profile of 10 sections from both OVX and NO-deficient/OVX rat brains were processed at the same time in parallel to prevent confounding processing effects and ensure uniformity of immunostaining. Brain sections were washed three times in 0.1 *M* PBS (pH 7.5), treated with 3% hydrogen peroxide to quench endogenous peroxidases, washed three times again with PBS, and then treated with 0.1% Triton X 100 for 15 min. The sections were incubated in blocking solution (Vector Laboratories, Burlingame, CA) for 30 min and were then incubated with a rabbit anti-GnRH Serum (1:500; Phoenix Pharmaceutical, Mountain View, CA) at 4°C overnight. The following morning, the sections were treated with a secondary antibody consisting of diluted biotinylated goat anti-rabbit immunoglobulin (Ig) G (1:200, Vector Laboratories) at room temperature for 1 hr. Sections were then immersed in a solution of avidin-biotin complex (ABC, Vector Elite Kit; amplifications solutions made according to Vector's instructions in the kit) for 30 min and were stained for 10 min with 3,3 diaminobenzidine and hydrogen peroxide solution. Each slice was placed on a slide containing glycerol/PBS solution, a coverslip was applied, and the slides were photographed and the staining was evaluated visually. Control sections, omitting the primary antibody, were included in every experiment.

Statistics. Statistical analysis of the LH and FSH release data was performed using one-way analysis of variance (ANOVA).

Results

LH and FSH Release. GnRH-stimulated release of FSH and LH summed over the 4-hr perfusion period was significantly lower in pituitaries from OVX/ NO-deficient animals when compared with FSH and LH released by pituitaries from OVX rats (Fig. 1).

The addition of the NO donor SNAP to the perfusion media significantly ($P < 0.05$) decreased the GnRH-stimulated LH and FSH release by pituitaries from OVX animals. However, SNAP increased significantly ($P < 0.05$) both LH and FSH release by pituitaries from OVX/NO-deficient rats (Figs. 2 and 3).

The induction of NO deficiency by the addition of L-NAME to the media significantly ($P < 0.05$) decreased both LH and FSH release by pituitaries from OVX rats (Figs. 4

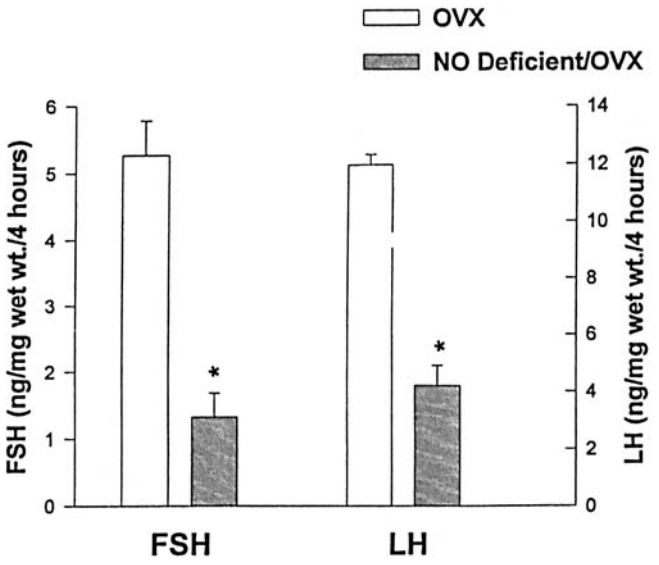

Figure 1. GnRH stimulated LH and FSH release from OVX and NO-deficient/OVX rat pituitaries *in vitro*. Values are the means ± SEM. *$P < 0.0001$ vs OVX.

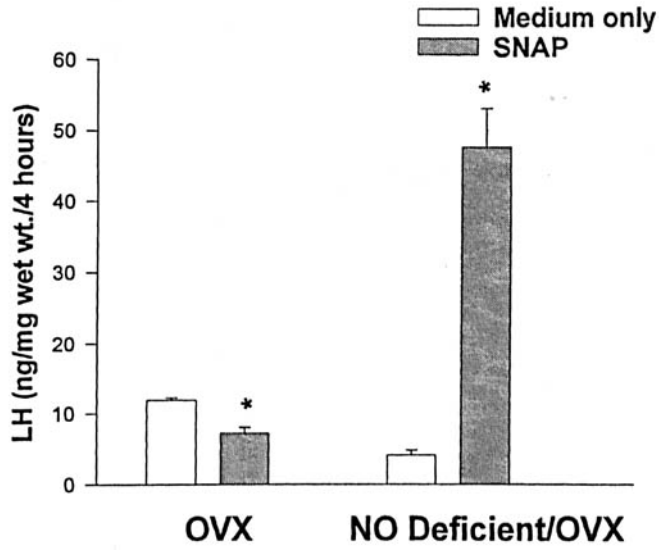

Figure 2. GnRH stimulated LH release in response to NO stimulation with SNAP from OVX and NO-deficient/OVX rat pituitaries *in vitro*. Values are the means ± SEM. *$P < 0.02$ vs medium only.

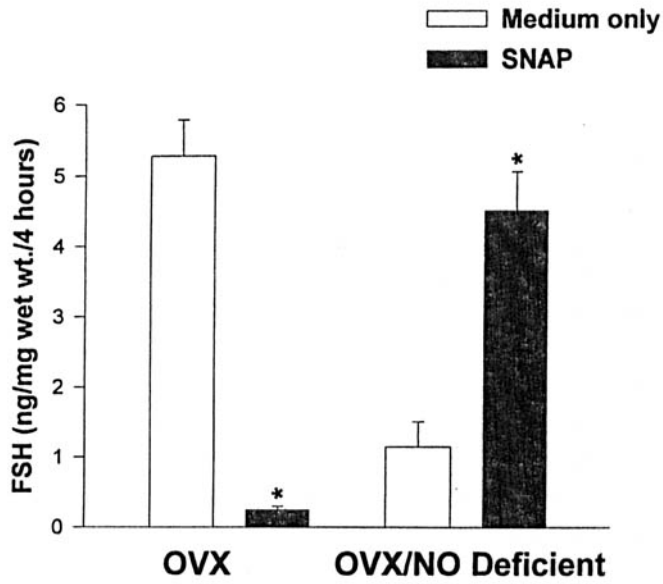

Figure 3. GnRH stimulated FSH release in response to NO stimulation with SNAP from OVX and NO-deficient/OVX rat pituitaries *in vitro*. Values are the means ± SEM. *$P < 0.0001$ vs medium only.

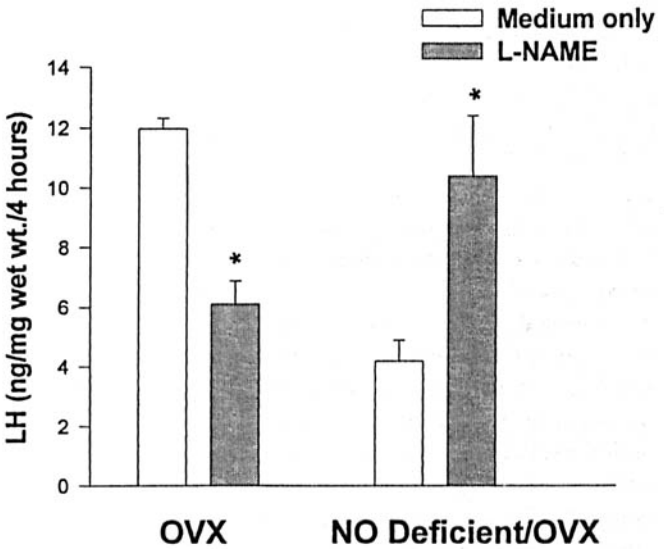

Figure 4. GnRH stimulated LH release in response to NO inhibition with L-NAME from OVX and NO-deficient/OVX rat pituitaries *in vitro*. Values are the means ± SEM. *$P < 0.0001$ vs medium only.

and 5). However, the further reduction of NO in pituitaries of OVX/NO-deficient rats by the addition of L-NAME to the media resulted in a significant ($P < 0.05$) increase in LH and FSH release (Figs. 4 and 5).

Immunohistochemistry. At the level of the median eminence and adjacent arcuate nucleus, many GnRH-labeled nerve fibers were observed coursing in the neuropil of the arcuate nucleus, fiber layer of the median eminence, and beneath the ependyma lining the third ventricle of the OVX animals (Fig. 6A). A high density of GnRH fibers were present in the external capillary plexus of the median eminence (Fig. 6B) and in the ventral GnRH pathway, just lateral to the median eminence and dorsal to the pial surface (Fig. 6A, arrow and C). In contrast, a different pattern of GnRH immunostaining was found in hypothalami from OVX/NO-deficient rats. In this case, a more intense GnRH immunostaining was detected in the regions described above. Thus, the ventral GnRH tract (Fig. 7A, arrow), subependymal layer (Fig. 7B), and external capillary plexus of the median eminence (Fig. 7C) were all well demarcated by the intensity of the staining. Large, GnRH-positive varicosities surrounded sectioned profiles of blood vessels in the median eminence and the ventral pathway (Fig. 7, C and D). Varicosities of single nerve fibers in the arcuate neuropil were also intensely stained (Fig. 7E).

DISCUSSION

In this study, we have clearly shown that NO modulates GnRH-mediated gonadotropin secretion. We also have

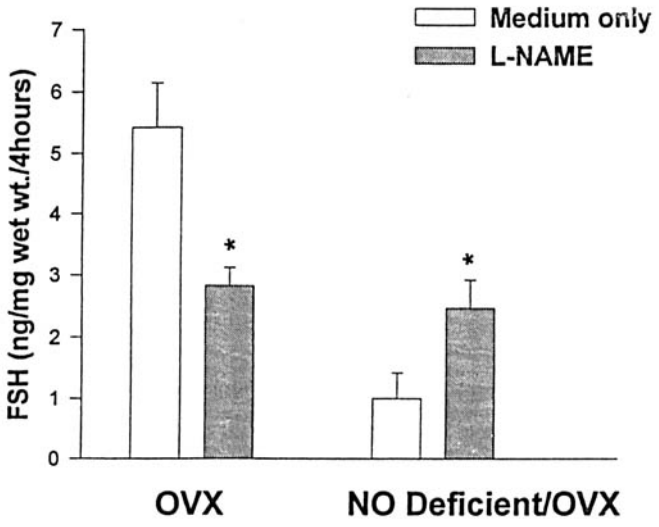

Figure 5. GnRH stimulated FSH release in response to NO inhibition with L-NAME from OVX and NO-deficient/OVX rat pituitaries *in vitro*. Values are the means ± SEM. $^*P < 0.05$ vs medium only.

demonstrated that the regulatory role of NO in LH and FSH release appears to be independent of estrogen. The suggestion that endogenous NO enhances LH and FSH release was substantiated by the observation that chronic NO deficiency resulted in a suppressed LH and FSH release in response to GnRH when compared with that from untreated OVX rats (10, 11). This observation in OVX rats is similar to the effect of chronic NO deficiency on LH and FSH levels in normal rats (10). This also suggests that the effect of NO on LH and FSH is not dependent or secondary to the presence of ovarian estrogen (10, 11).

The addition of an NO donor SNAP to the media inhibited the GnRH-stimulated LH and FSH production in OVX rats. This was also consistent with our previous observation in normal rats (10). However, when chronic NO deficiency was created in OVX animals, the subsequent addition of a NO donor now enhanced GnRH-stimulated gonadotropin release and confirmed our studies and those of others that demonstrated that the addition of NO can act to both increase or decrease the amount of LH and FSH release from the pituitaries (10, 12–16).

In addition, we observed that both LH and FSH release from pituitaries of OVX animals were significantly decreased by the NOS synthetase inhibitor L-NAME. This observation was also consistent with our previous study in animals with intact ovaries (10). We also demonstrate that L-NAME significantly enhanced the GnRH-stimulated gonadotropin release in the OVX/NO-deficient rats. In contrast to our previous study in NO-deficient rats where LH and FSH were not significantly stimulated by L-NAME, both LH and FSH responses mediated by L-NAME were increased (10). This suggests that ovarian estrogen may play a role in mediating the response to L-NAME in NO-deficient animals.

LH and FSH were inhibited or stimulated by SNAP or L-NAME in OVX or NO-deficient OVX animals, respectively. This suggests that in the pituitary, there may be a common NO mechanism impacting both LH and FSH secretory regulation. This regulatory system influenced both gonadotropins in the same fashion, especially in the absence of estrogen. These results also suggest that the role of NO in gonadotropin release is complex (14–16). We do not have an excellent explanation for the different responses to the NO donor, SNAP, and NOS antagonist, L-NAME, in the different animal models. However, a possible explanation for these results is that NO may be acting in a pathway that is biphasic. We suggest that the endogenous level of NO may determine the sensitivity of GnRH-stimulated gonadotropin released by the anterior pituitary. When the NO content is replete, a decrease or increase in NO will inhibit secretion. On the other hand, when the levels are low, as in the OVX-NO-deficient model, either a further increase or decrease will stimulate the system.

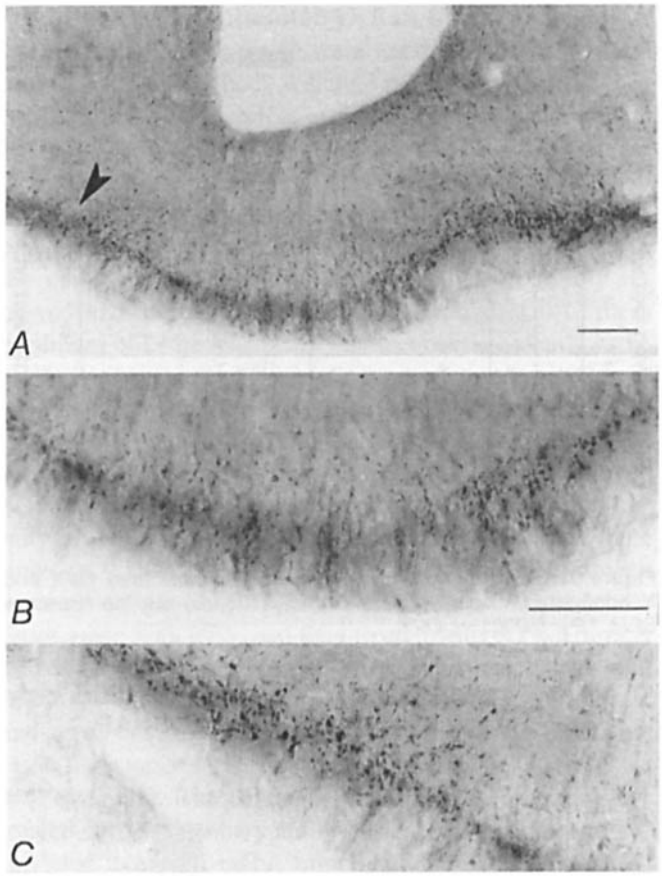

Figure 6. (A–C) Frontal sections through the hypothalamus immunostained for GnRH from the OVX control rats. (A) Low-power section of the median eminence and adjacent arcuate nucleus. Arrow points at ventral pathway reproduced at higher power in C. Scale equals ×100 μm. (B) Region of external capillary plexus. GnRH-immunostained varicose fibers terminate in the region of the capillary plexus. (C) Area of the ventral pathway from A. Magnification is the same as in B.

Further supporting our observation are evidence from the immunohistochemistry studies. The pattern of GnRH immunostaining in hypothalamic sections from OVX control rats are similar to that previously described by our laboratory and others (10, 17, 18). Furthermore, in OVX/NO-

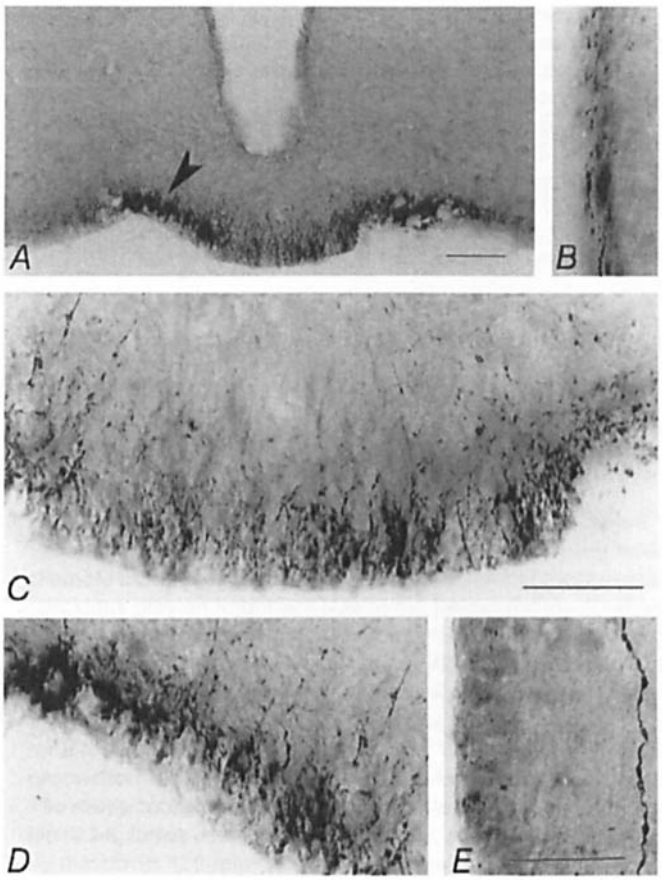

Figure 7. (A–E) Frontal sections through the hypothalamus immunostained for GnRH from OVX/NO-deficient rats. (A) Low-power median eminence and adjacent arcuate nucleus. Arrow points at ventral pathway reproduced at higher power in D. (B) GnRH-stained fibers in the ependymal layer that lines the wall of the third ventricle. (C) Region of the external capillary plexus. Generally GnRH immunostaining appears more than in the NO-deficient pretreated animal (B). Magnification is the same as in B. (D) Ventral pathway shows more immunostained fibers than that illustrated in C. (E) Single varicose fiber coursing through the arcuate nucleus.

deficient rats, the immunohistochemistry of GnRH staining appeared more intense compared with that of OVX rats. As such, in the OVX/NO-deficient condition, the intensity of the GnRH fiber tracts coursing in the hypothalamus, as well as that of neurosecretory terminals in the median eminence was increased. This would suggest either an increased endogenous GnRH synthesis and release that lead to down-regulation or decreased sensitivity of gonadotrophs. It could also suggest inhibition of release with increased accumulation of the neuropeptide in the cytoplasm of neurosecretory fibers and terminals. The apparent increase in size of single varicosities along single GnRH fibers and around capillaries of the median eminence of OVX/NO-deficient rats would also favor the notion of decreased release. Nevertheless, the increase in GnRH did not appear to enhance the subsequent release of LH or FSH in the OVX/NO-deficient rats even though they clearly had the potential for release as illustrated by SNAP and L-NAME stimulation.

We conclude that NO plays an important role in LH and FSH release and that this response is independent of the action of estrogen. The further addition of both an NO antagonist or agonist inhibits gonadotropin secretion in OVX animals and paradoxically stimulated GnRH-stimulated gonadotropin release in OVX/NO-deficient rats. Chronic NO deficiency in OVX rats acts to increase the content or release of GnRH from neurosecretory terminal in the median eminence. This study demonstrates that GnRH-stimulated gonadotropin release is decreased in OVX/NO-deficient animals and also suggests that the NO regulatory system regulates both gonadotropin in a similar fashion that is independent of estrogen.

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