GnRH and Gonadotropin Release Is Decreased in Chronic Nitric Oxide Deficiency

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Nitric oxide synthetase (NOS), the conversion enzyme for nitric oxide (NO) is localized in the anterior pituitary of female rats, particularly in gonadotrophs and folliculo-stellate cells, suggesting that NO regulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary. The focus of this study was to determine the effect of chronic NO deficiency on the subsequent pituitary release of LH and FSH *in vitro* and the hypothalamic immunoexpression of GnRH *in vivo*. NO deficiency was induced by adding the NOS inhibitor, *N*-nitro-L-arginine (L-NNA, 0.6g/L) to the drinking water of female Wistar rats. After 8 weeks, the animals were euthanized, the pituitaries were removed, and they were incubated *in vitro*. Pituitaries were perfused for 4 hr in the presence of pulsatile gonadotropin release hormone (GnRH, 500 ng/pulse) every 30 min. *S*-Nitroso-L-acetyl penicillamine (SNAP, an NO donor, 0.1 mM) or *L*-nitro-arginе methyl ester (L-NAME, a NOS inhibitor, 0.1 mM) was added to the media and perfusate samples were collected at 10-min intervals. LH and FSH levels in the perfusate were measured by double antibody radioimmunoassays. Pituitaries from the NO-deficient rats had a significantly smaller GnRH-stimulated release of LH and FSH compared with proestrous control rats. The addition of S-NAP to the perfusate resulted in decreased LH and FSH secretion in the control group, but increased LH secretion in the NO-deficient group. The addition of L-NAME to the perfusate suppressed LH secretion from control pituitaries, but not in pituitaries from NO-deficient animals. Immunohistochemistry of brain slices demonstrated that NO-deficient rats had a large qualitative decrease of GnRH in the median eminence compared with their controls. This decrease was particularly evident in the external capillary plexus of the median eminence. We concluded that chronic NO deficiency is associated with a decreased GnRH in neurosecretory terminals in the external capillary layer of the median eminence, accompanied by a decrease in LH and FSH release from the pituitaries. [Exp Biol Med Vol. 226(7):701–706, 2001]

Key words: nitric oxide; luteinizing hormone; follicle stimulating hormone; gonadotropin

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It is well established that nitric oxide (NO), a free radical gas, is not only an endothelium-derived relaxing factor (1), but also an inter- and intracellular messenger in many biological systems such as the endocrine system (2). NO arises from the oxidation of one of the guanidino nitrogens of arginine by NO synthase (NOS), yielding NO and citrulline. NO has emerged as an important regulator of a variety of endocrine and neurohormonal events such as vasorelaxation, neurotransmission, and immunocytotoxicity (2). There are also several lines of evidence linking NO to the control of reproduction due to its ability to regulate luteinizing hormone (LH) releasing hormone (RH) secretion from the hypothalamus. One such study by Rettori *et al.* (3) demonstrated that incubation of the arcuate nucleus-median eminence explants with the NO donor, sodium nitroprusside, increased LHRH release in a dose-dependent manner.

The localization of NOS to neurons in the brain and periphery is highly selective (4). Studies have suggested that the localization of NOS to neuronal projections in the neurohypophysis and adrenal medulla may reflect a role for NO in regulating pituitary hormone release (5). This possibility is supported by finding that NO (6) stimulates catecholamine release in adrenal medullary preparations.

In the present study, we evaluated the effect of chronic NO deficiency on GnRH-induced levels of LH and FSH from pituitary gonadotropins. Furthermore, we correlated these *in vitro* changes with *in vivo* studies in the hypothalamic centers and the external capillary plexus of the median eminence as revealed by immunohistochemistry. These studies were an attempt to establish the acute and chronic influences of NO and its role in pituitary secretion.

Material and Methods

Animals. Adult female Wistar rats (225–250 g; 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, one-half of the rats were placed on drinking water containing the NOS inhibitor *N*-nitro-L-arginine for 8 weeks (L-NNA, 0.6 g/L, Sigma Chemical Company, St. Louis, MO). The remaining rats were left

untreated during this time period. Vaginal smears were obtained daily, and systolic blood pressure measurements were made weekly on restrained rats by the tail cuff method using a standard physiological recorder (Narco Bio-System, Austin, TX). NO deficiency was defined by vaginal smears demonstrating predominantly cornified cells and secondly, by a systolic blood pressure of 150 mmHg for 6 consecutive weeks.

Pituitary Removal and Perfusion. On the day of the experiment, rats (control group $n = 6$ and NO-deficient group $n = 6$) were euthanized by decapitation. The anterior pituitary was removed immediately and the posterior pituitary was discarded. Each anterior lobe was placed in alpha-modified minimal essential medium (α-MEM, Sigma) 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 apparatus (Endotronics, Minneapolis, MN); this perfusion apparatus consisted of 12 500-μl chambers. Each chamber was perfused at 100 μl/min with α-MEM only or one of the following treatments: α-MEM + *S*-nitroso-L-penicillamine (SNAP, 0.1 mM) and α-MEM + *L*-nitro-arginine methyl ester (L-NAME, 0.1 mM). The pituitaries were perfused for 4 hr at 37°C with 10-sec GnRH pulses (500 ng per pulse) given every 30 min in a humidified atmosphere of 95% oxygen and 5% carbon dioxide. The perfusate was collected at 10-min intervals directly following the initial placement of the pituitary into the chamber. After 240 min, the pituitaries were weighed and the culture media were frozen (–20°C) until radioimmunoassay (RIA).

RIA. LH and FSH levels were measured using double antibody RIAs at 10-min intervals using reagents supplied by National Pituitary Program of NIH (FSH-RP-1 and LH-RP-1 were the standards used). All samples and standards were assayed in duplicate. The levels of LH and FSH from a given sample of pituitary were summed across the 4-hr period.

Immunohistochemistry. Each rat (control group $n = 5$, NO-deficient $n = 5$) was anesthetized with chloral hydrate (400 mg/kg body wt, intraperitoneally). The rat was perfused transcardially with 50 cc of 0.1 M PBS (pH 7.5) to flush the blood from the vascular system, followed by 200 cc of 4% paraformaldehyde in 0.1 M sodium phosphate-buffered solution (PBS, pH 7.5). One hour following the completion of the 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 normal and NO-deficient rat brains were processed at the same time to 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 IgG (1:200, Vector Laboratories) at room temperature for 1 hr. Sections were then immersed in a avidin-biotin complex (ABC, Vector Elite Kit; amplifications solutions made according to Vector's instructions in the kit) for 30 min and stained for 10 min with 3,3 diaminobenzidine and H_2O_2 solution. Each slice was placed on a slide containing glycerol/PBS solution and the slide was then cover-slipped. Control sections, omitting the primary antibody, were included in every experiment.

Statistics. Statistical analysis of the LH and FSH release data was performed using two-way ANOVA followed by Fisher's pair-wise comparison test.

Results

Starting in the first week of L-NNA treatment, the systolic blood pressures were significantly higher in all of the animals treated with L-NNA in their drinking water compared with the controls (Fig. 1). Systolic blood pressure in these animals increased to approximately 40 mmHg above the control and they were maintained throughout the experiment.

An analysis of the vaginal smears indicated that all of the L-NNA-treated animals were in constant estrus.

LH and FSH Release. GnRH-stimulated LH release by pituitaries from proestrous control animals perfused with α-MEM medium is characterized by a progressive increasing secretory response for 4 hr (Fig. 2A). The addition of the NO donor S-NAP to the perfusion media significantly decreased the GnRH-stimulated LH release when compared with pituitaries perfused with α-MEM (control) media (Fig. 2A). Also, the addition of L-NAME, the NOS inhibitor, to the perfusion media inhibited GnRH-stimulated LH release (Fig. 2A). The average LH secretion by the pituitaries in the three above conditions can be observed in Figure 2A (bar

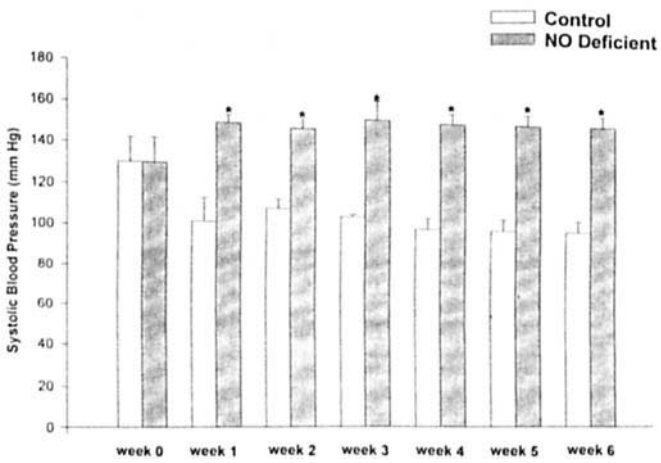

Figure 1. Systolic blood pressure of control and NO-deficient rat. Results are expressed as means ± SEM. *$P < 0.05$ versus control. (Control, $n = 6$ and NO-deficient, $n = 6$).

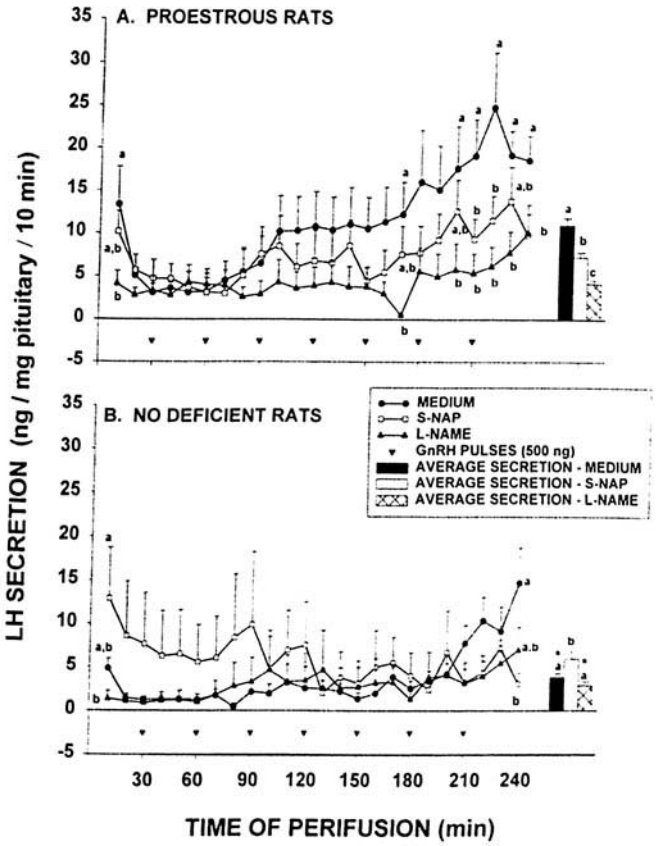

Figure 2. (A and B) GnRH-stimulated LH secretion in the presence or absence of S-NAP or L-NAME at 10-min intervals (expressed as nanograms per milligram of pituitary per 10 min) by pituitaries from proestrous (controls; A) and NO-deficient (B) rats *in vitro*. The 4-hr average GnRH-stimulated LH release from control and NO-deficient rat pituitaries is shown in the bar graph of A and B. $^*P < 0.05$ proestrous versus NO-deficient by one-way ANOVA. (a, b, and c) At each time point with different letters are significantly different ($P < 0.05$, ANOVA, Fishers LSD). Arrowhead indicates the addition of GnRH.

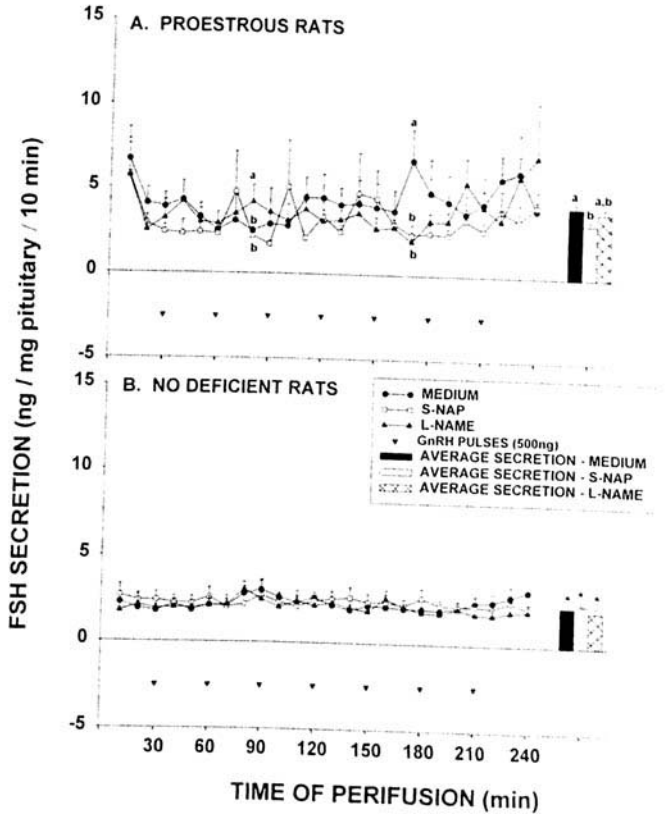

Figure 3. (A and B) GnRH-stimulated FSH secretion in the presence or absence of S-NAP or L-NAME at 10-min intervals (expressed as nanograms per milligram of pituitary per 10 min) by pituitaries from proestrous (controls; A) and NO-deficient (B) rats *in vitro*. The 4-hr average GnRH-stimulated FSH release from control and NO-deficient rat pituitaries is shown in the bar graph (A and B). $^*P < 0.05$ proestrous versus NO-deficient by one-way ANOVA. (a, b, and c) At each time point with different letters are significantly different ($P < 0.05$; ANOVA, Fishers LSD). Arrow indicates the addition of GnRH.

graph). The average secretory response confirms inhibition of GnRH-stimulated LH secretion by both the NO donor S-NAP and NOS inhibitor L-NAME.

The GnRH-stimulated LH secretion by pituitaries from NO-deficient rats perfused with control media had a significantly lower average secretion when compared with pituitaries from proestrus control rats (Fig. 2, A and B). The addition of S-NAP to the media significantly increased the average GnRH-stimulated LH secretion by pituitaries from NO-deficient rats, while the addition of L-NAME did not significantly alter the already suppressed secretion (Fig. 2B). The effect of S-NAP occurred early in the perfusion period.

When GnRH-stimulated FSH secretion by the same pituitaries was determined it, was observed that FSH was secreted continuously over the perfusion period in a more or less uniform pattern (Fig. 3A). The addition of S-NAP to the perfusion media significantly decreased the average FSH secretion, while the addition of L-NAME did not significantly alter the average secretory pattern or amount of secretion, but did decrease FSH at selected time points (Fig. 3A). GnRH-stimulated FSH secretion by pituitaries from NO-deficient rats was significantly lower and less variable over the perfusion period when compared with secretion by pituitaries from proestrous rats (Fig. 3B). The addition of S-NAP or L-NAME did not significantly effect GnRH-stimulated FSH secretion by NO-deficient pituitaries (Fig. 3B).

Immunohistochemistry. In normal rats, the omission of the primary antibody resulted in lack of immunostaining both at caudal (Fig. 4A) and more rostral (Fig. 4D) median eminence, as well as adjacent hypothalamic levels. Similarly, at higher magnification, immunostaining was not observed either in the fiber layer or the external capillary plexus of the median eminence (Fig. 4B) or in the subependymal layer and subjacent neuropil at the level of the arcuate nucleus (Fig. 4C). In contrast, intense immunostaining for GnRH occurred in all of the above mentioned areas at the level of the infundibular recess, in sections incubated with the primary antibody (Fig. 5, arrowheads). The most intensely stained area was shaped as a crescent just lateral and ventral to the angle of the recess (Fig. 5A, arrow). At higher power, many stained nerve fibers were observed coursing in the subependymal layer of the arcuate nucleus

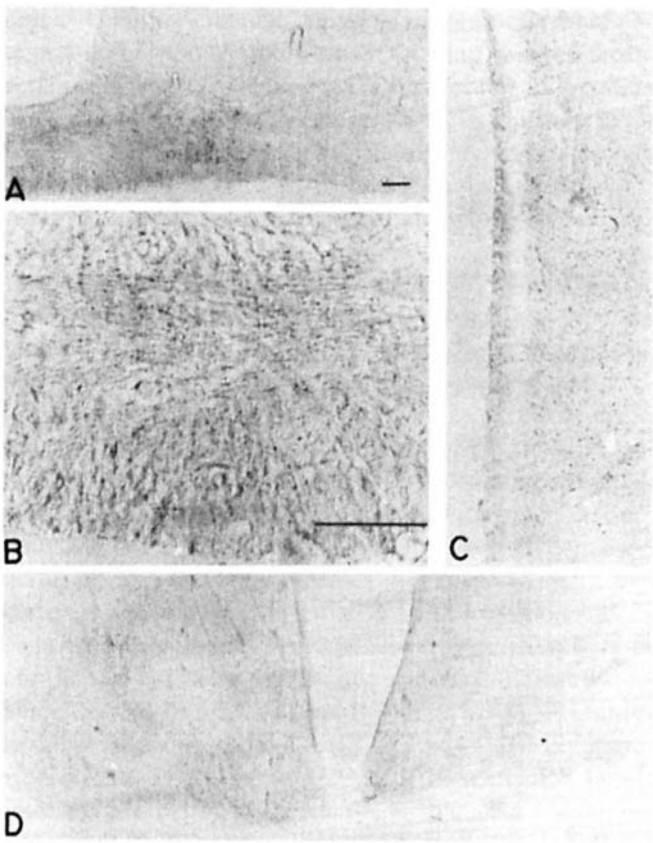

Figure 4. (A–D) Control immunostained coronal sections through hypothalamus of normal tissue by omission of primary antibody. (A) Rostral medial eminence. Part of the third ventricle is shown at the upper left. ×10 objective. (B) Higher magnification of the median eminence showing part of the fiber layer and external capillary plexus. ×40 objective. (C) Ependyma and subjacent neuropil at the level of the arcuate nucleus. ×40 objective. (D) Caudal median eminence and adjacent arcuate nuclei showing no staining. ×40 objective.

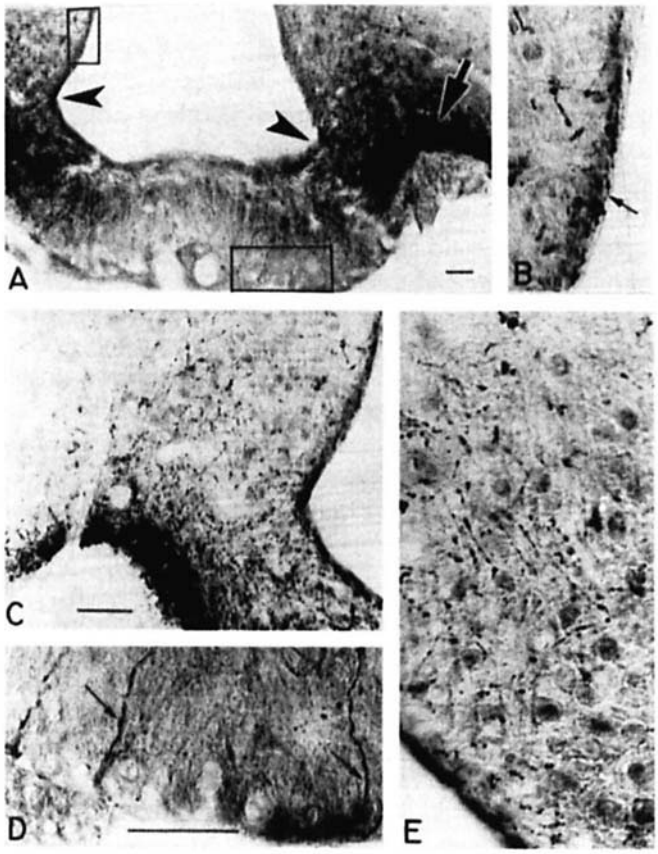

Figure 5. (A–E) Normal hypothalamus at the level of the caudal median eminence stained for GnRH. (A) Low power magnification of the median eminence at the level of the lateral recesses (arrowheads). Note the intense staining of the lateral fiber tract (arrow) and the adjacent arcuate nucleus. Upper and lower rectangles are reproduced in B and D, respectively. ×10 objective. (B) Higher power of area delimited by upper rectangle in A. Single immunostained fibers (arrow) course in the ependymal layer close to the ventricular surface. ×40 objective. (C) Region of the arcuate nucleus and lateral fiber tract with numerous labeled fibers. ×20 objective. (D) External capillary plexus layer of the median eminence delimited by lower rectangle in A. Note that single fibers (arrows) oppose the perimeter of the capillaries. ×40 objective. (E) Region of the arcuate nucleus with stained fibers radiating ventrolaterally. Some nerve cell bodies appear to be faintly labeled. ×40 objective.

(Fig. 5B) or, seemingly, between the ependyma (Fig. 5B, arrow). Many of the nerve fibers projected ventrolaterally through the arcuate nucleus and reached the densely stained crescent area (Fig. 5C), which represents a tightly packed bundle of GnRH-containing fibers. From the crescent, many fibers entered the median eminence from its lateral aspect, although other fibers appeared to follow a more medial route close to the subependymal layer. From the densely stained fiber layer of the median eminence, single varicose fibers radiated towards the external capillary plexus and terminated as prominent swellings adjacent to the capillaries (Fig. 5D, arrows).

Immunopositive nerve cell bodies were seen scattered in the arcuate nucleus. Fibers of some of these neurons were more strongly stained than the cytoplasm, suggesting, in general, that the staining of the cell bodies may not have been as specific as that found in nerve fibers.

In NO-deficient rats, a qualitative decrease in GnRH immunostaining was observed in the hypothalamus when compared with that of control animals. At the level of the infundibular recess, the crescent-shaped area was thinner and appeared to contain fewer stained fibers than that in control brains (compare Fig. 5A with Fig. 6A). Examination of adjacent areas at higher magnification (Fig. 6, B and C, from areas delineated by rectangles in A) revealed a contrasting pattern from the control rat. Many stained nerve fibers projected from the crescent-shaped area into the lateral aspect of the median eminence and its fiber layer (Fig. 6B). However, practically no nerve fibers were immunostained from the fiber layer into the external capillary plexus (Fig. 6C).

Discussion

Several studies suggest that NO may play a regulatory role in the release of the pituitary hormones, LH and FSH, from rat pituitaries (2, 3, 7). Additionally, NO deficiency has been implicated as a possible mechanism of pituitary dysfunction (2, 6). In the present study, chronic NO deficiency resulted in a significant decrease in the average amount of gonadotropin released by pituitaries in response

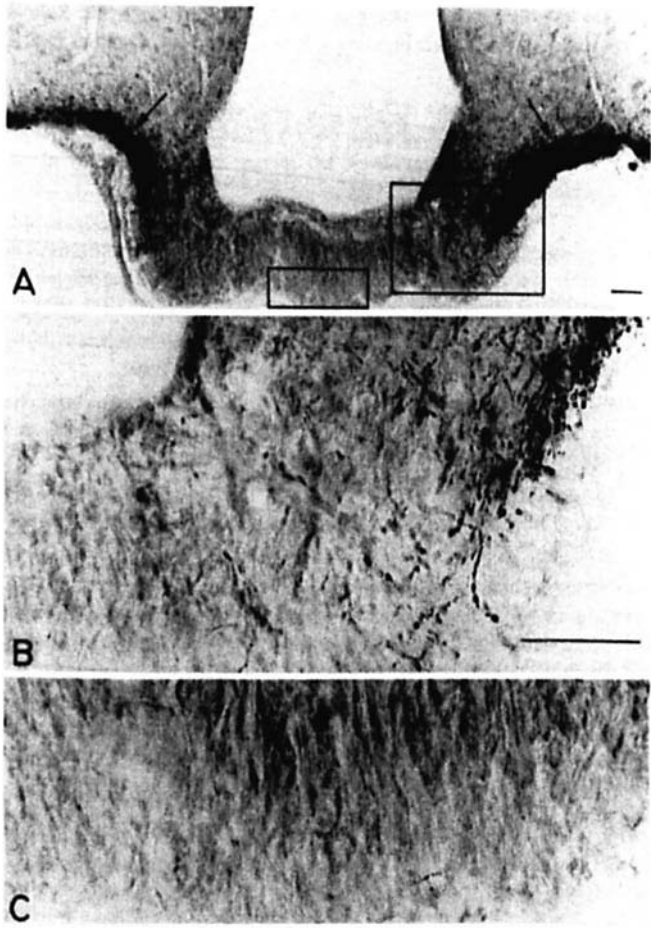

Figure 6. (A–C) Coronal sections through the caudal median eminence stained for GnRH from an NO-deficient animal. (A) Low power of the caudal median eminence and adjacent arcuate nuclei. The upper and lower rectangles are reproduced at higher magnification in B and C, respectively. Note the qualitative decrease in the size of the lateral fiber tracts (arrows) compared with that in the normal brain (Fig. 5A). ×10 objective. (B) Area delimited by upper rectangle in A. Note some labeled fibers in the lateral region of the median eminence. ×40 objective. (C) Area enclosed by lower rectangle in A. Very few labeled fibers enter in the external capillary layer and none are seen opposing the perimeter of the capillaries. ×40 objective.

to GnRH. The effect of the subsequent addition of a NOS inhibitor or a NO donor to the perfusion media supports previous studies that demonstrated that the addition or removal of NO may act to decrease the amount of LH and FSH release from pituitaries (8, 9).

In addition to demonstrating the effect of chronic NO deficiency on GnRH-induced LH and FSH release, we also demonstrated that acute suppression of NO by a NO antagonist (L-NAME) in proestrous rats or the increase of NO using the NO donor S-NAP would decrease LH release. In contrast to pituitaries from proestrous rats, LH release from pituitaries of NO-deficient animals was significantly increased by the addition of the NO donor S-NAP; however, the NO inhibitor L-NAME had no significant effect. These results suggest that the role of NO in gonadotropin release at the level of the pituitary is complex. One possibility is that NO may have two actions at the pituitary level, one stimulatory (10–12) and one inhibitory, which are induced by different levels of NO. Ceccatelli *et al.* (13) reported that sodium nitroprusside, another NO donor, suppressed GnRH-stimulated LH release from pituitaries of male rats and Chatterjee *et al.* (7) showed that L-NAME enhanced GnRH-induced LH release from pituitaries of ovariectomized rats. Chronic NO deficiency was also associated with a decrease in the GnRH-induced FSH secretion. This supports the current and previous observations that chronic NO deficiency is characterized by constant estrus and increased plasma estrogen, which suppresses gonadotropin secretion (14). The addition of S-NAP decreased FSH secretion from pituitaries of proestrous rats, but L-NAME was without effect. The pituitaries from the NO-deficient rat did not respond to S-NAP or L-NAME. We would like to suggest that the dose-response to NO follows a bell-shaped or U-shaped dose-response curve (7–9, 13). The proestrous rats are acutely exposed to a high plasma estrogen, whereas the NO-deficient animals usually had estrous smears, indicating chronic exposure to fairly high estrogen levels. These different conditions could shift the dose-response curve to SNAP, resulting in an inhibitory effect in proestrus and a stimulatory effect in constant estrus. The fact that inhibition of NOS in proestrus rats lowered LH indicates that NO is stimulating in that condition, as well as in constant estrus. Further studies must be done to determine the exact pathway in which NO acts to increase or decrease gonadotropin levels, but the above explanation might help clarify the contradictory reports in the literature.

Our immunohistochemistry studies add to our understanding of NO-mediated gonadotropin secretion. Studies have demonstrated that the heaviest concentrations of the GnRH fibers are adjacent to or within the ependyma of the third ventricle and in the median eminence (15). Our studies were able to verify this; however, our studies also clearly revealed that this pathway is disrupted when there is a deficiency of NO. In NO-deficient rats, practically no GnRH-containing nerve fibers were observed projecting from the fiber layer into the external capillary plexus, suggesting either that the terminal portions of the fibers had retracted from their normal position around the capillary wall to the fiber layer or that massive depletion of the peptide from the distal portions of the neurosecretory fibers and axon terminals had occurred. Moreover, since decrease immunostaining was found in the intrahypothalamic course of the neurosecretory fibers, it appears that there may be a decrease in production and transport of the peptide following NO depletion. These results help support the present secretory studies and other studies that demonstrated that NO plays a role in the regulation of GnRH secretion (16–18).

We conclude that the acute increase or decrease in NO is associated with decreasing gonadotropin secretion. We also conclude that chronic NO deficiency is associated with a decrease GnRH release from neurosecretory terminals in the external capillary layer of the median eminence, which subsequently leads to a significant decrease in the amount of LH- and FSH-stimulated release from the pituitary. Our

combined *in vivo* and *in vitro* results clearly show that the release of LH and FSH is not only under the control of GnRH, but also under the control of NO.

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