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Role of nitric oxide in the control of luteinizing hormone-releasing hormone release *in vivo* and *in vitro*

(plasma luteinizing hormone/N^G-monomethyl-L-arginine/nitroprusside/hemoglobin/nitric oxide synthase)

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ABSTRACT **Nitric oxide (NO) synthase, the enzyme which converts arginine into citrulline plus NO, a highly active free radical, has been found in many neurons in the brain, including neurons in the hypothalamus. Our previous experiments showed that norepinephrine-induced prostaglandin E_2 release from hypothalamic explants incubated *in vitro* is mediated by NO. Since the release of luteinizing hormone-releasing hormone (LHRH) is also driven by norepinephrine and prostaglandin E_2, we hypothesized that NO might also control pulsatile release of LHRH *in vivo*, resulting in turn in pulsatile release of luteinizing hormone (LH). To ascertain the role of NO in control of pulsatile LH release *in vivo*, an inhibitor of NO synthase, N^G-monomethyl-L-arginine (NMMA), was microinjected into the third cerebral ventricle (1 mg/5 μl) of conscious castrate male rats at time 0 and 60 min later; blood samples were taken every 10 min during this period. NMMA blocked pulsatile LH release within 20 min, and plasma LH concentration declined further without pulses after the injection at 60 min. Pulsatile release of LH was not altered in diluent-injected controls. NMMA did not alter pulsatile release of follicle-stimulating hormone, which suggests that its release does not require NO. Incubation of medial basal hypothalami with norepinephrine (10 μM) induced an increase in LHRH release that was inhibited by NMMA (300 μM). NMMA alone did not alter basal LHRH release, whereas it was augmented by sodium nitroprusside (100 μM), which releases NO spontaneously. This augmentation was prevented by hemoglobin (2 μg/ml), which binds the NO released by nitroprusside. Our previous experiments showed that norepinephrine-induced release of prostaglandin E_2 is mediated by NO. Nitric oxidergic neurons were visualized in the median eminence adjacent to the LHRH terminals. The combined *in vivo* and *in vitro* results indicate that the pulsatile release of LHRH induced by norepinephrine is brought about by α_1-adrenergic activation of NO synthase. NO then induces prostaglandin E_2 release that activates exocytosis of LHRH secretory granules into the portal vessels to induce pulsatile LH release.**

Nitric oxide (NO) released from vascular endothelium by cholinergic stimulation diffuses to the adjacent vascular smooth muscle and elicits relaxation (1–4). The mechanism by which this occurs begins with the release of acetylcholine from cholinergic terminals. It combines with muscarinic cholinergic receptors on the endothelial cells and increases intracellular Ca^{2+}. The Ca^{2+} interacts with calmodulin to activate constitutive NO synthase, which then converts arginine into NO plus citrulline (1–4). Constitutive NO synthase occurs in the brain (5, 6); this enzyme has been purified and antibodies have been generated against it (7, 8). Neurons containing the constitutive NO synthase were found in various parts of the brain by immunocytochemistry (8); relatively large numbers of these neurons occur in the paraventricular and supraoptic nuclei of the hypothalamus (8).

These observations suggested a role for NO in hypothalamic function, and we have previously reported that norepinephrine-induced prostaglandin E_2 (PGE_2) release from medial hypothalamic tissue incubated *in vitro* is mediated by NO (9). Earlier reports associated this increase in PGE_2 release with the release of luteinizing hormone-releasing hormone (LHRH) (10–12). These observations raise the possibility that NO mediates norepinephrine-induced LHRH release.

Luteinizing hormone (LH) is released in a pulsatile fashion in the living animal, and this release is caused by pulsatile release of LHRH (13). Considerable evidence indicates that norepinephrine acts via α_1-adrenergic receptors to induce PGE_2 release and consequent pulsatile release of LHRH (10–12, 14). The LHRH is released into capillaries of the hypophyseal portal veins and is carried to the hypophyseal gonadotropes which release LH (14). Consequently, in the present experiments we investigated the possible role of NO in the generation of LHRH release *in vitro* and pulsatile LH release *in vivo*. The results provide compelling evidence for a crucial role for NO in the generation of pulsatile LH release.

MATERIALS AND METHODS

All experiments utilized adult male rats of the Sprague–Dawley strain (Holtzmann, Madison, WI) weighing 200–250 g. The *in vivo* studies utilized castrate males with increased plasma concentrations of follicle-stimulating hormone (FSH) and LH as a result of removal of negative feedback of gonadal steroids. The rats were castrated under ether anesthesia and used for experiments 2–3 weeks later. Intact males were used for the *in vitro* experiments. All rats were kept in group cages in a light (0500 to 1900 hr) and temperature (23–25°C)-controlled room with free access to laboratory chow and water.

***In Vivo* Studies.** Six to eight days prior to experiments an indwelling cannula was implanted in the third cerebral ventricle by the technique of Antunes-Rodrigues and McCann (15) while the rats were anesthetized with tribromoethanol given intraperitoneally. One day before the experiment an indwelling catheter was introduced into the right external jugular vein and advanced to the right atrium for collection of blood samples according to the technique of Harms and Ojeda (16). Immediately after collection of the first blood

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Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; LHRH, LH-releasing hormone; NMMA, N^G-monomethyl-L-arginine; PGE_2, prostaglandin E_2.

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sample (0.3 ml), the test substances were microinjected into the third ventricle in 5 μl of 0.9% NaCl (saline). Additional (0.3 ml) blood samples were collected every 10 min for 120 min. An equal volume of saline was injected immediately after removal of each sample to restore blood volume. In certain animals N^G-monomethyl-L-arginine (NMMA) (1 mg in 5 μl of saline), an inhibitor of NO synthase, was microinjected into the third ventricle. NMMA or saline was microinjected over 60 sec. Sixty minutes after the first injection, the animals which had received NMMA received a second injection of the substance, whereas the control animals received a second injection of saline.

After centrifugation, the plasma was stored frozen at −20°C prior to radioimmunoassay for FSH and LH. LH was assayed with the NIHRP3 standard and antiserum against LH. FSH was assayed with the NIHRP2 standard and antiserum against FSH. Both assay kits were provided by the National Pituitary Agency (University of Maryland, Baltimore).

***In Vitro* Studies.** Following decapitation and removal of the brain, the medial hypothalamus was dissected by making frontal cuts just behind the optic chiasm, extending dorsally 2 mm. A horizontal cut extended from this point caudally to just behind the pituitary stalk, where another frontal cut was made. Longitudinal cuts were made 1 mm lateral to the midline bilaterally. The hypothalami were preincubated in Krebs–Ringer bicarbonate-buffered medium (pH 7.4) containing 0.1% glucose for 30 min prior to replacement with fresh medium or medium containing the substances to be tested. The incubation was continued for 30 min followed by removal of the medium and storage of samples at −20°C prior to assay for LHRH. Incubations were carried out in a Dubnoff metabolic shaker (50 cycles per min; 95% O_2/5% CO_2) (9). LHRH was measured by radioimmunoassay using highly specific LHRH antiserum kindly provided by A. Barnea (University of Texas Southwestern Medical Center, Dallas). The sensitivity of the assay was 0.2 pg per tube and the curve was linear up to 100 pg of LHRH.

Chemicals used were norepinephrine, HCl, NMMA, sodium nitroprusside, and rat hemoglobin, all from Sigma.

Immunocytochemistry of NO Synthase. Adult male Sprague–Dawley rats were anesthetized with equithesin, flushed with Tris-buffered saline (TBS, pH 7.4) via cardiac perfusion, and fixed with 4% paraformaldehyde. Brains were removed and a block of hypothalamus from the optic chiasm to the rostral border of the mammillary body was postfixed with the same fixative for 24 hr. The tissues were washed in TBS and Vibratome-sectioned at 40 μM. Free-floating sections of hypothalamus containing the median eminence were incubated in 1% H_2O_2 for 60 min to remove any inherent peroxidase activity. Sections were permeabilized with 0.4% Triton X-100 in TBS for 30 min then blocked with 4% normal goat serum (NGS) for an additional 30 min. The tissues were incubated at room temperature for 48 hr with affinity-purified antisera directed against NO synthase, provided by S. H. Snyder and T. M. Dawson (The Johns Hopkins University, Baltimore). One antiserum was used at a dilution of 1:60 and the other at 1:100. Both antisera were diluted in TBS with 0.1% Triton X-100 and 2% NGS and were absorbed at their respective dilution overnight with bovine serum albumin (BSA) at 500 μg/ml. Because BSA was used as the conjugate for the production of these antisera, it was added to eliminate possible false positive staining as a result of any residual anti-BSA activity within the serum that could crossreact with albuminoid substances in the brain (17).

Following incubation in primary antiserum, the tissues were washed in TBS with 1% NGS and incubated for 24 hr at room temperature with biotinylated goat anti-rabbit immunoglobulin serum (Vector Laboratories) diluted 1:200 in TBS with 1.5% NGS. The sections were then washed in TBS with 1% NGS, washed in TBS, and incubated in ABC reagent (Vector Elite) for 45 min. Tissues were washed in TBS containing 3,3′-diaminobenzidine (0.5 mg/ml; Sigma) and 0.01% H_2O_2 for 2 min. After immunocytochemistry, all sections were washed and mounted for observation at the light microscopic level.

To ensure specificity of immunostaining, some sections were incubated in primary antiserum absorbed 24 hr before with BSA (as above), as well as purified NO synthase (200 μg/ml; also a gift of S. H. Snyder). Additionally, for some sections, TBS was substituted for the primary antiserum.

Statistics. Pulses were analyzed according to the method of Cahoreau *et al.* (18). Areas under the plasma hormone curves were calculated by trapezoidal integration. The results were analyzed by one-way analysis of variance with repeated measures and significance of differences was determined by the Student–Newman–Keuls test. The differences between the means of two groups were calculated by Student's *t* test.

RESULTS

***In Vivo* Experiments.** Microinjection of saline into the third cerebral ventricle did not alter mean plasma LH concentrations or pulsatile LH release during the experiment (2 hr) (Fig. 1). In striking contrast, the intraventricular injection of NMMA caused a rapid cessation of pulsatile LH release, so that no pulses were seen 30–50 min postinjection (Fig. 1). However, in most animals plasma LH began to increase slightly by 60–70 min postinjection. The second injection of NMMA at 60 min induced a further decline in plasma LH and there were no LH pulses 80–120 min after the initial injection. The height of the remaining LH pulses (8.9 ± 1.2 ng/ml, mean ± SEM) was similar to controls (11.6 ± 2.3 ng/ml). The area under the plasma LH curve was not decreased significantly below that of the control animals in the first 60 min after injection of NMMA (Fig. 2), because of the pattern just described. However, it was significantly reduced during the 60-min period after the second injection of NMMA.

In contrast to the suppressive effect of NMMA on pulsatile LH release and mean plasma levels, pulsatile release of FSH was unaffected and the FSH pulses were frequently asynchronous with those of LH (Fig. 3). The area under the plasma FSH curve was not affected (data not shown).

***In Vitro* Studies.** In the first experiment, the effects of increasing NO concentration with sodium nitroprusside, which spontaneously releases NO, and the effect of hemo-

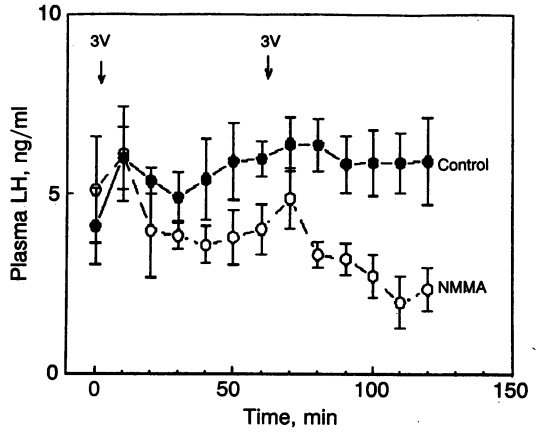

FIG. 1. Effect on the release of LH of microinjection of NMMA (1 mg in 5 μl of saline) into the third ventricle (3V) of conscious castrate male rats. An equal volume of saline was injected into the 3V of the control rats. NMMA or saline was injected a second time at 60 min. There were six rats per group. Values in this and subsequent figures are means ± SEM.

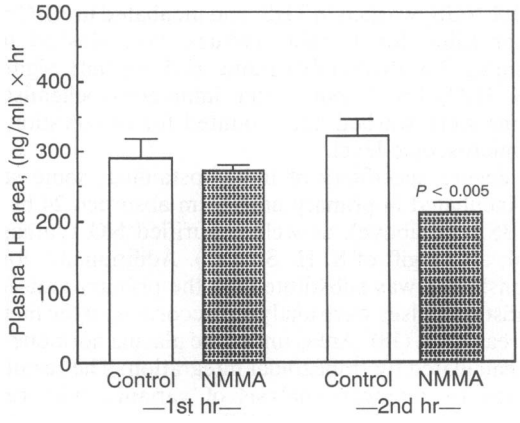

FIG. 2. Area under the curve of plasma LH after third-ventricular injection of NMMA or saline in the rats whose plasma LH concentrations are illustrated in Fig. 1.

globin, a scavenger of NO, were evaluated. Nitroprusside (100 μM) significantly increased LHRH release. Hemoglobin (2 μg/ml) had no effect on control release of LHRH but completely abolished the effect of nitroprusside (Fig. 4).

In further experiments, NMMA (300 μM) had no effect on control release of LHRH; however, it completely blocked the stimulatory effect of norepinephrine (10 μM) on LHRH release (Fig. 5).

Localization of Nitric Oxidergic Neurons. As reported previously (8), we observed NO synthase immunoreactivity in the supraoptic and paraventricular regions of the hypothalamus. Additionally, we noted a few positive cells in the lateral hypothalamic area, as well as numerous cells scattered within the amygdaloid nucleus. Importantly, by using a greater concentration of antiserum and longer incubation times than in previous studies, we now report NO synthase immunoreactivity in cells within the median eminence. These cells were few in number and were not present in every section; however, when present, they were most frequently observed scattered in the dorsal and dorsolateral areas of the internal layer of the median eminence, just ventral to the floor of the third ventricle (Fig. 6). These NO synthase-containing cells were observed with both antisera employed, and in each case, positive staining in all regions of the hypothalamus, including the median eminence, was eliminated by prior absorption of the antiserum with purified NO synthase.

DISCUSSION

There is substantial evidence that the pulsatile release of LH is brought about by pulsatile release of LHRH that is driven

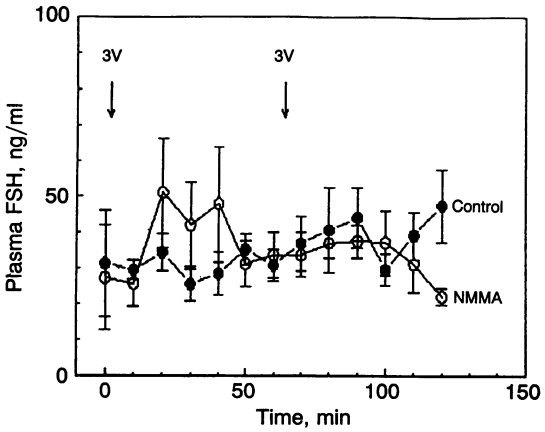

FIG. 3. Effect of microinjection of NMMA into the third ventricle (3V) of conscious castrate male rats on the release of FSH. These are the same rats as in Fig. 1.

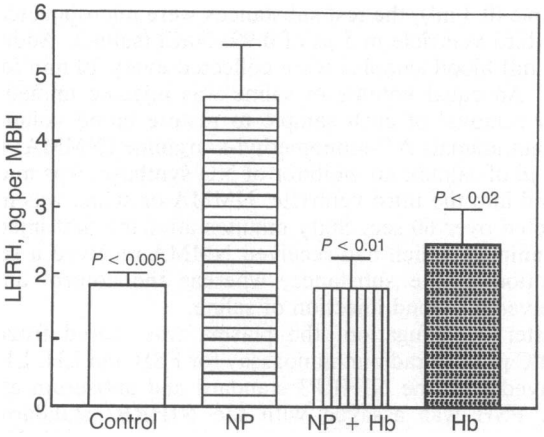

FIG. 4. Effect of sodium nitroprusside (NP, 100 μM) or hemoglobin (Hb, 2 μg/ml) on release of LHRH from the median basal hypothalamus (MBH). There were six to eight MBH tubes per group. *P* values are versus the NP group.

in turn by pulsatile release of norepinephrine; the latter acts on α_1-adrenergic receptors to induce LHRH release from terminals of LHRH-secreting neurons in juxtaposition to hypophyseal portal capillaries in the median eminence (15). The LHRH that is released diffuses into hypophyseal portal vessels and is transported to the pituitary, where it activates the release of LH from the gonadotropes (15). Since microinjection of the NO synthase inhibitor NMMA into the third cerebral ventricle decreased plasma LH and abolished LH pulses, the data provide strong evidence that NO is essential to generation of pulsatile LH release *in vivo*. NMMA did not alter pulsatile FSH release, which suggests that the influence of NO is specific for LHRH release and that hypothalamic control over FSH is different from that over LH (19). Considerable evidence indicates that control of FSH may be mediated by another hypothalamic peptide, FSH-releasing factor, which has yet to be isolated (20).

The *in vitro* experiments provide convincing evidence for a role of NO in the control of norepinephrine-induced LHRH release: the generation of NO by nitroprusside enhanced LHRH release, and the enhancement was abolished by a scavenger of NO, hemoglobin. Because hemoglobin had no effect on the basal release of LHRH, this suggests that this unstimulated release does not require NO. Norepinephrine-induced LHRH release was completely blocked by NMMA, which again failed to alter control release. Thus, NO release is essential for norepinephrine-induced LHRH release.

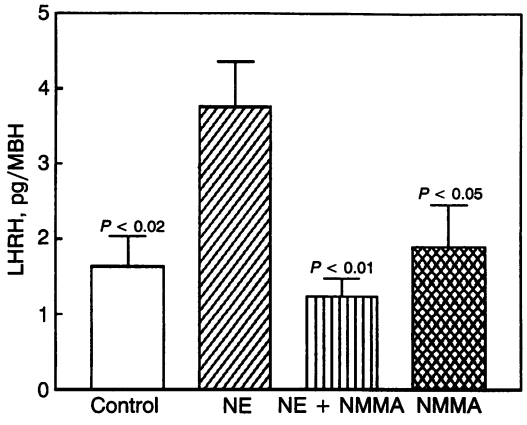

FIG. 5. Effect of NMMA (300 μM) on norepinephrine (NE, 10 μM)-stimulated LHRH release from the median basal hypothalamus (MBH). There were six to eight MBH tubes per group. *P* values are versus the NE group.

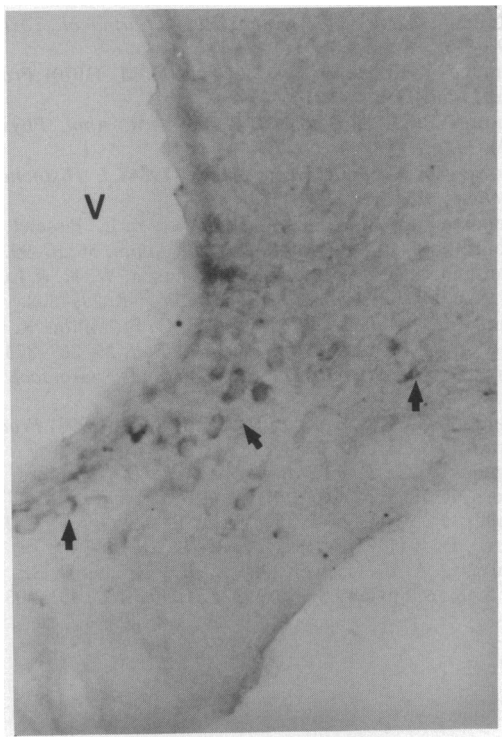

FIG. 6. Section of rat hypothalamus demonstrating a few NO synthase-positive cells (arrows) in the median eminence region. V, third ventricle. (×900.)

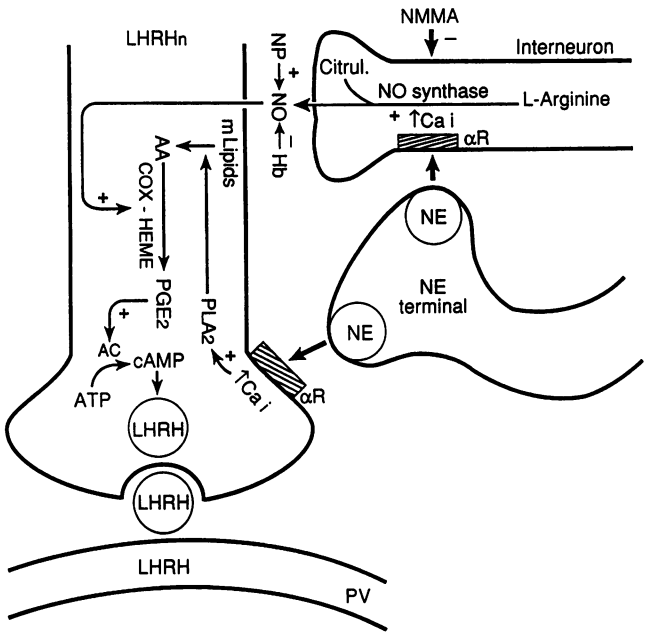

FIG. 7. Schematic diagram of hypothetical interactions of norepinephrine (NE) terminals with NOergic interneuron and LHRH terminals to generate NO, PGE_2, cyclic AMP, and LHRH release. For detailed description, please see *Discussion*. Abbreviations: n (suffix), neuron; mLipids, membrane phospholipids; AA, arachidonic acid; COX-HEME, cyclooxygenase; AC, adenylate cyclase; ATP, adenosine triphosphate; NP, nitroprusside; Hb, hemoglobin; citrul., citrulline; Cai, internal Ca^{2+}; αR, α_1-adrenergic receptor; PLA2, phospholipase A_2; PV, portal vessel; +, stimulation or increase; −, inhibition or decrease.

Our previous experiments have demonstrated that norepinephrine-induced PGE_2 release is mediated by NO (9). Because norepinephrine-induced PGE_2 release was implicated in the release of LHRH by prior experiments from our laboratory and others (10–12), it seemed logical that NO might also mediate LHRH release. This hypothesis was substantiated in the present experiments.

The mechanism by which norepinephrine operates to control LHRH release is conceptualized to involve three neurons (Fig. 7). A norepinephrine terminal synapses with a NO-releasing interneuron (NOergic neuron) and makes an axo-axonal synapse on an axon of a LHRH neuron which terminates on a portal capillary. The norepinephrine neuron perhaps arises from the locus coeruleus; lesions of the locus coeruleus can block the preovulatory and pulsatile release of LH (J. Franci and S.M.M., unpublished data). We hypothesize that the norepinephrine terminal synapses on the NOergic neuron, and on adjacent LHRH terminals, via α_1-adrenergic receptors. The activation of each of these receptors leads to conversion of inositol phosphates into inositol trisphosphate. Inositol trisphosphate activates protein kinase C, which liberates Ca^{2+} from intracellular stores. In the NOergic neuron, the increased intracellular Ca^{2+} interacts with calmodulin and activates the constitutive NO synthase in this cell, which leads to generation of NO plus citrulline (21). The NO diffuses across to the axon of the LHRH neuron and activates cyclooxygenase by interaction with the heme group on the enzyme (9). We have demonstrated herein the presence of NOergic neurons in proximity to the LHRH neuronal axons. The increased Ca^{2+} generated by the α_1-adrenergic receptor combination with norepinephrine on the LHRH axon activates phospholipase A_2 to provide arachidonate from hydrolysis of membrane phospholipids. Arachidonate is then converted by activated cyclooxygenase to PGE_2, which activates adenylate cyclase, leading to an increase in cyclic AMP and thus to activation of protein kinase A, which induces exocytosis of LHRH secretory granules.

We believe that NO acts on the cyclooxygenase enzyme rather than by its conventional mechanism—i.e., activation of the soluble guanylate cyclase (21)—since the postulated increase in intracellular Ca^{2+} in the LHRH terminal should inhibit guanylate cyclase. That the cyclooxygenase enzyme is activated is indicated by the ability of indomethacin, an inhibitor of cyclooxygenase, to block the PGE_2 and LHRH release induced by norepinephrine (10).

We have recently obtained evidence for participation of NO in other pathways within the hypothalamus. For example, NO appears to mediate via activation of cyclooxygenase the cholinergic and the interleukin-2-induced stimulation of corticotropin-releasing factor release which is mediated by cholinergic neurons within the hypothalamus (22). On the other hand, growth hormone-releasing factor-induced somatostatin release via NO appears to be induced by activation of the guanylate cyclase pathway (23).

LHRH is the most important reproductive peptide known; it plays a role not only in the release of LH from the pituitary but also in the induction of sexual behavior (14, 24). It follows that NO might also be involved in mediation of sexual behavior. Furthermore, NOergic neurons arising from the anococcygeal region of the spinal cord innervate the bulbocavernosa muscles of the penis. The release of NO causes relaxation of their smooth muscle, allowing them to be filled with blood and thus inducing the resultant penile erection (25, 26). Therefore, it appears that NO is extremely important to reproduction at many levels in the organism.

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