The Role of Nitric Oxide in Reproduction

(43950B)

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Other papers of this symposium describe the discovery of the various forms of nitric oxide synthase (NOS). All forms of NOS convert arginine in the presence of oxygen to equimolar quantities of nitric oxide (NO) and citrulline. The inducible form is produced in immune cells by bacterial lipopolysaccharide (LPS) or cytokines which act in the nucleus to induce mRNA to synthesize the enzyme. This enzyme is then produced in large quantities. The inducible enzyme does not require activation by calcium and calmodulin since these are intrinsic to the molecule, and consequently, it releases NO without further stimulation of the cell. This NO kills bacteria and viruses but also kills other cells in the region (1).

The next form of the enzyme discovered was the so-called endothelial NOS, which is a constitutive enzyme present in endothelium of blood vessels. It is activated by parasympathetic nerves via the release of acetylcholine, which increases the intracellular free calcium in the endothelial cells. Calcium combines with calmodulin and activates the enzyme. The NO formed diffuses to the overlying smooth muscle where it activates soluble guanylate cyclase and causes the formation of cyclic GMP which relaxes the smooth muscle (2–6).

While incubating hippocampal slices with glutamic acid, Garthwaite discovered that the medium contained endothelial-derived relaxing factor, later shown to be NO, and postulated that it was NO (7). This was proven by mass spectroscopy by Palmer *et al.* (5). Therefore, there is also a neural form of the enzyme (neural NOS). It is also a constitutive enzyme and is activated by an increase in intracellular free calcium that combines with calmodulin activating the enzyme (7–10). Bredt and Synder isolated the enzyme (11), developed antibodies against it and showed by immunocytochemistry its wide distribution in the nervous system (12). A large population of NOS-containing neurons, termed NOergic neurons, is located in the hypothalamus with the largest number of NOergic neurons in the supraoptic and paraventricular nuclei with axonal projections to the median eminence, pituitary stalk and neural lobe of the hypophysis, which has the densest staining for NOS observed in the entire nervous system. Therefore, we postulated that NO would have powerful actions within the hypothalamus. We also observed that there were cells which were positive for NOS in the anterior lobe of the pituitary (12) and hypothesized that the gas would also be of importance there.

Luteinizing Hormone–Releasing Hormone

Since this review is devoted to the actions of NO in reproduction, we will first discuss its possible role in control of luteinizing hormone–releasing hormone (LHRH) release. We have termed this the reproductive peptide because it controls mating behavior by actions in the brain and is released into the hypophyseal portal vessels, which transmit it to the anterior lobe of the pituitary. There, LHRH causes release of gonadotropins that in turn stimulate the gonads to produce sex steroids. LHRH induces ovulation in female and spermatogenesis in male mammals (13).

LHRH is found in cell bodies in the preoptic area of the brain and, in most species, in the arcuate nucleus. Both of these sets of neurons project axons to the median eminence region to release the peptide into the hypophyseal portal system of veins which transport it to the anterior lobe of the pituitary (13, 14). LHRH particularly stimulates the release of luteinizing hormone (LH), but it also to a lesser extent stimulates follicle-stimulating hormone (FSH) from the gonadotropes of the gland. The LH and FSH then circulate to the gonads, where FSH promotes follicular development and LH evokes ovulation. FSH and LH also stimulate production of gonadal steroids, in particular estrogen, in females. In the male, FSH induces spermatogenesis, whereas LH acts on the interstitial cells of the testis to cause production of testosterone. Therefore, LHRH has prime importance in regulating

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not only gonadal function, but also steroid secretion in both male and female mammals (13).

Role of NO in Control of LHRH Release—*In Vitro* Studies

We explored the possibility that NO controls the release of LHRH by incubating arcuate nucleus-median eminence (MBH) explants from male rats *in vitro*, as we have previously shown this to be an effective method of studying LHRH release. Sodium nitroprusside (NP) (100–300 μM), a spontaneous releaser of NO, increased the release of LHRH in a dose-related fashion (2; unpublished data). N^G-monoethyl-L-arginine (NMMA) is a competitive inhibitor of NOS (2). It had no effect on basal release of LHRH; but NMMA (300 μM) completely blocked norepinephrine-induced release of LHRH.

Previous experiments showed that prostaglandin E_2 (PGE_2) is required for LHRH release both *in vivo* and *in vitro* (15–17). Consequently, we evaluated the effect of NO on PGE_2 release and found that NP would increase PGE_2 release as determined by radioimmunoassay (RIA) (18) and would also highly significantly augment the incorporation of C^{14} arachidonate into labeled prostanoids, particularly PGE_2 but also $PGF_{1\alpha}$, 6-keto $PGF_{1\alpha}$, but not thromboxane (19). We concluded that NO must activate cyclooxygenase since it was able to convert labeled arachidonate into prostanoids. Needleman's group (personal communication, 1994) has shown that NO activates purified type I and type II cyclooxygenase, which is consistent with our findings. This NO-induced activation of cyclooxygenase with consequent increased PGE_2 release was thought to induce LHRH release.

Additional experiments revealed a critical role for norepinephrine in the release of LHRH both *in vivo* and *in vitro* by α_1 adrenergic receptors (15–17). Norepinephrine similarly increased PGE_2 release as well as LHRH release into the medium as determined by RIA (15). Conversely, NMMA blocked the release of PGE_2 and LHRH induced by norepinephrine (15). Therefore, we hypothesized that NE activates NOS and that the NO released activates LHRH release via PGE_2 release. NO activates the LHRH terminals in the median eminence by activating cyclooxygenase.

For release of PGE_2 to occur, phospholipase A_2 (PLA_2) must also be activated, so that membrane phospholipids can be converted into arachidonate, the substrate of cyclooxygenase. We postulated that this was via an increase in intracellular free calcium which then activated PLA_2. Since the principal mechanism of action of NO in other tissues is via activation of soluble guanylate cyclase with the generation of cyclic guanosine monophosphate (cGMP) (4, 6), we wondered whether the cGMP generated by NO could be responsible for elevating intracellular free calcium. Indeed, NP, the releaser of NO, increased the release of cGMP into the medium. Furthermore, cGMP ($10^- M$) activated LHRH release (19). Most previous studies have indicated that cGMP lowers intracellular free calcium. This is the mechanism by which it relaxes vascular smooth muscle; however, recent experiments by Muallem's group (20) in isolated pancreatic acinar cells demonstrated that low concentrations of cGMP increased intracellular free calcium, whereas higher concentrations lowered it.

Therefore, we hypothesize that the concentration of cGMP generated by the NO released from NOergic neurons by norepinephrine elevates intracellular free calcium, thereby activating PLA_2. This causes generation of arachidonate which is converted into PGE_2 and other arachidonate metabolites by activated cyclooxygenase. Cyclooxygenase is directly activated by NO, probably by altering the conformation of the heme group of cyclooxygenase just as it alters the heme group of soluble guanylate cyclase to activate that enzyme (4).

LHRH, as is the case with other hormones, is not released continuously but instead is released in pulses with a frequency that depends on the species and the hormonal milieu. In the rat, pulses occur in general every 20–30 min. We postulate that the pulsatile release of LHRH is caused by release of norepinephrine from noradrenergic terminals in the MBH which acts on α_1 receptors on the NOergic neurons to increase intracellular free calcium, which combines with calmodulin and activates NOS. The activated enzyme converts arginine into equimolar quantities of citrulline and NO, and the latter diffuses to the LHRH terminal to activate it as just described (19). We have further evidence that this is the pathway, since norepinephrine can activate NOS in MBH explants as measured by conversion of C^{14}-labeled arginine to C^{14} citrulline, which is relatively stable in contrast to NO which decays with a half-time of 5–10 sec. The labeled citrulline can be readily measured (19).

Indeed, by a modification of the method of Bredt and Snyder (9), we have shown that there is activity of the enzyme even in the absence of norepinephrine which can be inhibited by the inhibitor of NOS, nitroarginine methyl ester (NAME). This reduces the formation of labeled citrulline by about 50% (19). Since these same inhibitors do not reduce the basal LHRH release, it appears that the activity of the enzyme under these so-called basal conditions, although present, generates insufficient NO to stimulate the LHRH terminals. Incubation of the explant with norepinephrine dramatically increased the production of labeled citrulline, and this effect was blocked by prazosine, a specific α_1 blocker (19). Previous experiments have shown that phentolamine, an α blocker blocks the release of LHRH induced by norepinephrine. There is

no need for an α_1 receptor on the LHRH terminal because phentolamine does not affect the response of the terminals to NP. This is a convenient test which we have developed to assess the responsiveness of the LHRH terminals to NO released from NP (21) (Fig. 1).

Moretto *et al.* (22) have also concluded that NO releases LHRH with arcuate median eminence explants, and also from the GT1 LHRH tumoral cell line. In fact, perifusion of the GT1 cells yielded pulsatile LHRH release with a frequency similar to that of the intact rat (23). Furthermore, the GT1 LHRH tumor cells appear to express receptors to many transmitters including adrenergic receptors, glutamergic receptors, and LHRH receptors (24, 25). In tissue taken from living animals, it has not been possible to demonstrate either NOS, or adrenergic, glutamergic, or estrogen receptors on LHRH neurons. Therefore, we feel that even though this GT1 cell system presents an interesting model of the *in vivo* system, it is not a true model. In fact, we postulate that in the absence of input from other neurons and even glial elements, these isolated LHRH cells generate NOS and various receptor types on their cell surface which are not present normally. These allow the isolated cells to manifest activity that is surprisingly reminiscent of that which occurs in the living animal.

In Vivo Studies

To determine if our *in vitro* studies had relevance to the living animal, we evaluated the role of NO in control of pulsatile LH and FSH release *in vivo* in castrated male rats bearing indwelling third ventricular (3V) cannulae and jugular catheters, which extended to the right atrium for convenient blood sampling in these conscious, freely moving animals. Indeed, intraventricular injection of NMMA (1 mg in 2 μl 0.9% NaCl), but not the saline diluent, after a delay, caused a cessation of LH but not FSH pulses, indicating that NO was required for pulsatile LHRH release but presumably not for pulsatile release of the putative FSH-releasing factor which controls the separate release of FSH (21, 26).

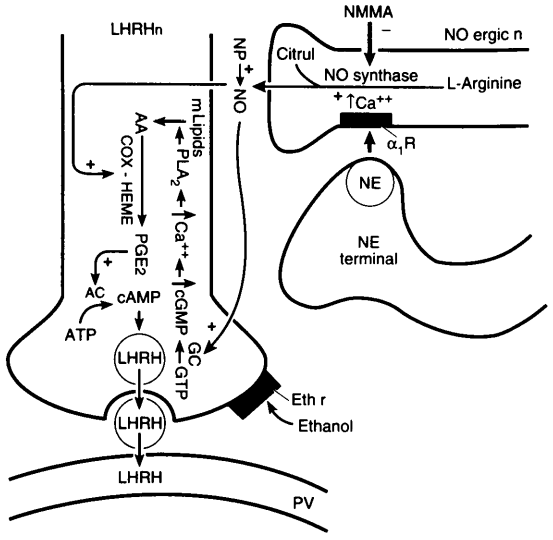

Figure 1. Diagram of the proposed mechanism of release of LHRH and the effect of ethanol (ETOH) thereon. For explanation see text. NMMA, N^G-monomethyl-L-arginine; LHRHn, LHRH neuron; NOn, nitricoxidergic neuron; NE terminal, norepinephrine terminal; α_1r, α_1 receptor; Citrul, citrulline; NP, nitroprusside; NO, nitric oxide; Hb, hemoglobin; GTP, guanosine triphosphate; GC, guanylate cyclase; cGMP, cyclic guanosine monophosphate; PLA_2, phospholipase A_2; m Lipids, membrane phospholipids; AA, arachidonic acid; COX - HEME, cyclooxygenase$_1$; PGE_2, prostaglandin E_2. (Reproduced from Canteros *et al.* Proc Natl Acad Sci USA **92**:3417–3434, 1995, with permission.)

Role of NO in the Stimulation of LHRH Release by Glutamate

Glutamate plays a crucial role in control of LHRH release by actions both in the median eminence arcuate region and in the medial preoptic area (27). In both cases, the effect is stimulatory and there is no doubt that glutamate plays a physiologically significant role not only in the pulsatile release of LH thought to be mediated primarily in the median eminence arcuate region, but also in the preovulatory type release mediated through the preoptic region, which is responsible for not only for the preovulatory discharge of LH, but also for the induction of puberty (27).

In confirmation of earlier work, glutamic acid (10 m*M*) induced a highly significant release of LHRH from MBH explants. Hemoglobin (20 μ*M*/ml), a scavenger of NO, incubated together with glutamic acid completely blocked its affect. Furthermore, NMMA (300 μ*M*), a competitive inhibitor of NOS, had a highly significant inhibitory effect on the action of glutamic acid, but again neither NMMA nor hemoglobin altered basal LHRH release, indicating that NO is not involved in this release. As previously demonstrated, NP highly significantly increased LHRH release (28).

Glutamic acid has been previously shown to be a potent releaser of norepinephrine in the brain. Navarro *et al.* (29) have just reported that glutamic acid and other excitatory amino acids induced norepinephrine release from MBH explants. Therefore, we hypothesized that the action of glutamic acid to release LHRH was mediated by stimulation of noradrenergic terminals in the MBH. Indeed, phentolamine completely blocked the release of LHRH induced by glutamic acid (30), but as indicated earlier, phentolamine did not alter the release of LHRH induced by NP (30). Therefore, we believe that our results establish the concept that glutamate acts in the basal hypothalamus by activating the noradrenergic terminals, which in turn stimulate the NOergic neurons by α_1 receptors. These activate LHRH terminals by releasing NO. There is no need to postulate the presence of either glutamergic receptors or noradrenergic receptors on the LHRH terminal (19, 30) (Fig. 2).

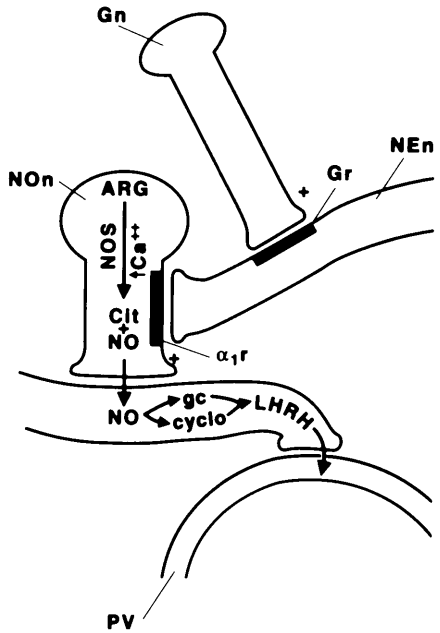

Figure 2. Schematic diagram indicating the proposed pathway of glutamic acid stimulation of LHRH release. For explanation see text. (Reproduced from Kamat *et al.* Brain Res Bull **37**:233–235, 1995, with permission).

This research needs to be expanded with *in vivo* studies and extended to the possible role of noradrenaline in the stimulatory effect of glutamate on LHRH release in the rostral preoptic region involved in preovulatory control of LH release. In that regard, Kalras' group presented evidence that inhibitors of NOS can inhibit excitatory amino acid stimulation of LHRH release *in vivo* (31).

What Is the Role of Glutamate in Pulsatile LHRH Release?

Clearly, it is involved since blockade of its receptors interrupts it and it acts by stimulation of noradrenergic terminals which in turn activate the release of NO from the NOergic neurons.

Since lesions of the locus ceruleus, a site of noradrenergic neurons whose axons project to the basal hyothalamus, block pulsatile LH release in the castrate male rat and also block the preovulatory surge of LH (Franci J *et al.*, unpublished), we believe that the pulses may actually be generated by pulsatile discharge from brain stem noradrenergic neurons. This does not agree with the accepted concept of generation of pulses by the pulse generator located in the arcuate region (32). There is no doubt that there is a marked increase in multiple unit activity of neurons in this region, which is coincident with the LHRH pulses. If indeed the pulses are originating in the brain stem from locus ceruleus and other noradrenergic neurons, there should the pulsatile activity there coincident with that in the arcuate median eminence region. To our knowledge, this has not yet been demonstrated. Alternatively, there may be tonic firing from the locus region which is required. In that case, perhaps activation of local glutamate neurons which stimulate the noradrenergic terminals may be the generator of the pulse. Further research is required to answer these questions.

Even this complex scenario of control of LHRH release is an over simplification since a number of other neurotransmitters and neural modulators are involved in pulsatile LHRH release, among them neuropeptide Y (NPY). A variety of other transmitters are almost certainly involved such as dopamine and various other neuropeptides (13).

Role of Gamma Amino Butyric Acid in LHRH Release via NO

Gamma amino butyric acid (GABA) plays a dual role in LHRH control in female rats: it inhibits LHRH by action on LHRH neurons in the medial preoptic area and it has a stimulatory effect on LHRH release in the arcuate nucleus-median eminence region (33). It also has dual effects on LHRH release in males (33). Therefore, we hypothesized that NO might also control the release of GABA from the MBH *in vitro*.

First, we evaluated the effect of GABA on the release of LHRH from MBH explants from male rats and discovered that this inhibitory neurotransmitter inhibited the release of LHRH, and that this inhibition is mediated by NO since the inhibitory effect was prevented by hemoglobin, a scavenger of NO, or by NMMA.

Second, we evaluated the effect of NO on the release of GABA. Our results demonstrated that NP doubled the release of GABA from MBH explants and that the release was largely blocked by hemoglobin, a scavenger of NO. Therefore, it is clear that NO stimulates the release of GABA from this tissue. Under the conditions of incubation used, effective concentrations of NO were not released spontaneously since hemoglobin, which would remove the NO produced, had no effect on GABA release. Furthermore, when NMMA in a concentration previously found to inhibit NOS (26) was incubated with the tissues, there was no inhibition of GABA release. That NO was being generated, even if not in effective concentrations, was evidenced by the formation of C^{14} citrulline from labeled arginine and the partial blockade of its production by NAME as described above.

As expected, elevation of extracellular K^+ concentration drastically stimulated the release of GABA from the tissue. This release was augmented by NO as revealed by the further dramatic increase in release induced by NP. Evidence that NO plays a significant role in high K^+-induced release of GABA was the finding that inhibition of NOS by NMMA reduced high K^+-induced release of the inhibitory transmitter.

High K^+-induced release of transmitters and poly-

peptides is thought to be due to opening of voltage-dependent Ca^{++} channels as a result of K^+-induced depolarization of cell membranes (34). Presumably, NO acts to further increase intracellular Ca^{++} in the presence or absence of increased extracellular K^+ to promote additional exocytosis of GABA. The accepted mechanism of action of NO in most tissues is via activation of soluble guanylate cyclase leading to generation of cyclic GMP which activates protein kinase G (1–6). Indeed, monobutyryl cGMP (10^{-3}–10^{-2} *M*) significantly increased basal and high K^+-evoked GABA release and content, which supports the hypothesis that NO stimulates GABA release and synthesis via NO activation of soluble guanylate cyclase (35). The released cGMP in turn can cause an increase in intracellular Ca^{++} (20). This mechanism appears to operate also in NO-induced release of the hypothalamic peptides LHRH (19), growth hormone–releasing hormone (36), prolactin-releasing factors (37), and somatostatin (38).

Since GABA inhibited the release of LHRH and its effect was blocked by the inhibitor of NOS, NMMA, it is apparent that NO is involved not only in the stimulation of LHRH release by norepinephrine shown earlier (26), but the inhibition of LHRH release induced by GABA (Fig. 3). In the intact animal, we visualize that norepinephrine acting via α_1 receptors stimulates NOergic neurons to release NO, which directly stimulates the LHRH terminals to release LHRH. After passage down the portal vessels LHRH stimulates the release of LH from the gonadotropes. However, at the same time the NO released would also stimulate release of GABA, which would act directly on the LHRH terminals to inhibit the release of LHRH, thereby terminating the response to norepinephrine and thereby the pulse of LHRH and LH. We postulate that the response of the LHRH terminals to the NO released is terminated only when the concentration of GABA rises to a threshold level after a pulse of LHRH has been released (Fig. 3).

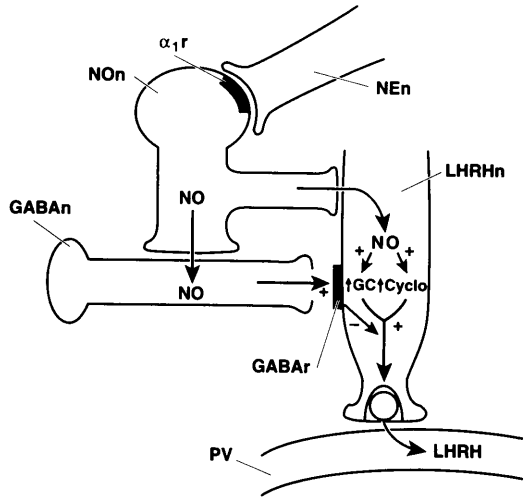

Figure 3. Schematic diagram of the role of GABA in the control of LHRH release. For description see text. NEn, norepinephrine terminal; NOn, NOergic neuron; GABAn, GABAergic neuron; NO, nitric oxide; α_1r, α_1 adrenergic receptor; GABAr, GABA receptor; LHRHn, LHRH terminal; PV, portal vessel. (Reproduced from Seilicovich *et al.* Proc Natl Acad Sci USA **92**:3421–3424, 1995, with permission.)

Consequently, when noradrenergic stimulation of NOergic neurons occurs, there are two effects. First, diffusion of NO to the LHRH terminal provokes release of LHRH. Second, stimulation of GABA release via NO occurs to terminate release of LHRH stimulated by NO. This inhibitory GABAergic mechanism accounts for the termination of the pulse. There may be other mechanisms as well, since it has been shown that LHRH has an autocrine action on cells that release it (39), thereby terminating LHRH release. After a delay, the next pulse would be induced by another barrage of noradrenergic firing, releasing additional norepinephrine to retrigger the NOergic neurons.

GABA has been shown to play an important role in the control of release of several hypothalamic peptides which include CRH as well as LHRH (33). GABA is colocalized with NOS in cerebral cortical neurons (35); therefore, it is possible that this relationship may hold true in the hypothalamus as well. Then, we would have a situation in which NOS releases NO which induces GABA release from the same neuron.

Mechanism of Interleukin-1 Inhibition of LHRH Release

A major cytokine released during infection is interleukin-1 (IL-1). IL-1α injected into the 3V inhibits pulsatile LH, but not FSH release (21). *In vitro* studies have shown that it can block norepinephrine-induced LHRH release from MBH explants and this occurs by blockade of the release of PGE_2. Consequently, it is clear that it has an action to suppress LHRH release. To determine the mechanism, we employed the NP test (30) mentioned above since NP appears to stimulate LHRH release directly via activation of guanylate cyclase and cyclooxygenase. Indeed, we found that IL-1α (10^{-11} *M*) completely blocked the release of LHRH induced by NP. Therefore, one site of action of IL-1 to suppress LHRH release involved in LH release and mating behavior is via direct suppression of the LHRH terminals. This is probably the mechanism of inhibition of LH secretion and loss of libido during infection which is caused by increased production of cytokines in the periphery and in the brain (21).

Mechanism of Action of Ethanol to Inhibit LHRH Release

It has previously been shown that alcohol can suppress reproductive function in humans, monkeys, and small rodents by inhibiting release of LH (40). The principal action is via suppression of the release of

LHRH, both *in vivo* and *in vitro*. Therefore, experiments were designed to determine the mechanism by which alcohol inhibits LHRH release.

Ethanol had no effect on the production of NO from MBH explants measured by the C^{14} citrulline method, and also did not modify the increased release of NO induced by norepinephrine. Therefore, it does not act at that step in our postulated pathway of LHRH control. Ethanol also failed to affect the increase in cGMP induced by NP. On the other hand, as might be expected from previous experiments indicating that LHRH release was brought about by PGE_2, NP increased the conversion of C^{14} arachidonic acid to its metabolites, particularly PGE_2 in these experiments. Ethanol completely blocked the release of LHRH induced by NP, the conversion of labeled arachidonate to PGE_2 and the increase in PGE_2 release induced by NP. Therefore, the results support the theory that norepinephrine acts to stimulate NO release from NOergic neurons. This NO diffuses to the LHRH terminals, where it activates guanylate cyclase leading to an increase in cGMP. At the same time it also activates cyclooxygenase. The increase in cGMP increases intracellular free calcium activating PLA_2 to provide arachidonic acid, the substrate for conversion by the activated cyclooxygenase to PGE_2 which then activates the release of LHRH. Since alcohol inhibits the conversion of labeled arachidonic acid to PGE_2, it must act to inhibit cyclooxygenase either directly or by blocking the increase in intracellular free calcium induced by cGMP, which is crucial for activation of PLA_2 and perhaps also cyclooxygenase (19) (Fig. 1).

Role of NO in Controlling Release of Pituitary Hormones Directly from the Adenohypophysis

NOS occurs in scattered anterior pituitary cells, but there are none in the intermediate lobe of the gland (12). In the anterior lobe, it has been localized to folliculostellate cells and gonadotropes, presumably LH gonadotropes since these cells were immunoreactive to LH antiserum (41).

The most important effect of NO at the pituitary level appears to be in controlling the release of prolactin, a hormone important in reproduction and required for lactation. When hemipituitaries were incubated in the presence of NP for 30 min, all of the doses tested significantly suppressed prolactin release. When the incubation was continued for 60 min, the 50 μM concentration no longer suppressed prolactin release significantly; however, 100 and 500 μM concentrations equally suppressed release by 25%–30%. This suppression was completely blocked by the scavenger of NO, hemoglobin. Analogs of arginine, such as NMMA, increased prolactin release by approximately 30%. Since in other tissues most of the actions of NO are mediated by activation of soluble guanylate cyclase with the formation of cyclic GMP, we evaluated the effects of cyclic GMP on prolactin release. A concentration of 10^{-2} *M* cGMP produced approximately a 40% reduction in prolactin release. Dopamine (10^{-7} *M*), an inhibitor of prolactin release, reduced prolactin release, and this inhibitory action was significantly blocked by either hemoglobin (20 μg/ml) or NMMA and was completely blocked by NAME (1 m*M*) (42).

In contrast to these results with prolactin, when LH was measured in the same media in which the effect of NP was tested on prolactin release, there was no effect of NP, hemoglobin, or the combination of NP plus hemoglobin on LH release. Therefore, in contrast to its inhibitory action on prolactin release, NO had no effect of basal LH release; however, it blocked LHRH-induced LH release (41), and therefore when NO is released by pituitary cells—for example, after stimulation of NOS synthesis by LPS, it may blunt LHRH-induced LH release.

Immunocytochemical studies by Hökfelt's group have shown that NOS is present in the folliculostellate cells and in the gonadotropes of the pituitary gland (41). We conclude that NO produced by either of these cell types diffuses to the lactotropes where it inhibits prolactin release and that NO mediates the prolactin-inhibiting activity of dopamine (42).

Role of NO in Sex Behavior

Previous research has indicated not only that LHRH causes the release of LH and to a lesser extent FSH into the portal vessels to trigger gonadotropin secretion, consequent production of gonadal steroids and ovulation, but also that it plays a crucial role in induction of mating behavior in all vertebrate forms so far examined (43, 44). Therefore, we evaluated the role of NO in mating behavior in ovariectomized, estrogen plus progesterone–treated rats. If these animals were only given estrogen, but not progesterone, they could be induced to mate within 30 min by progesterone injected intraventricularly into the 3V cannula. That this mating is induced via the release of LHRH from neurons within the brain has been shown, antisera directed against LHRH having blocked progesterone-induced mating (45).

Mating behavior is judged by lordosis, a characteristic arching of the back with elevation of the rump of the female rat to allow intromission by the male. We found that NMMA injected in the same dose that caused cessation of LH release, also blocked estrogen plus progesterone–induced mating. The dose could be reduced 100-fold with maintenance of this blockade. Furthermore, D-NMMA, which is inactive in inhibiting NOS, had no effect. Another inhibitor of NOS, NAME, was also capable of blocking progesterone-induced mating (46).

To determine if NO would induce mating, NP, a

releaser of NO, was injected into the 3V cannula and indeed it induced highly significant lordosis responses in doses ranging from 1 to 10 μg. That this was due to NO-induced LHRH release was shown, antisera against LHRH having blocked NP-induced mating. This blockade could be overcome by higher doses of NP, suggesting that the dose of antiserum was insufficient to inactivate completely the increased LHRH that was released (46).

Previous studies demonstrated that not only do LHRH axons project to the median eminence for release of the peptide into the portal vessels, but also some LHRH neurons synapse on neurons, which induce lordosis by activation of the lordosis reflex. These neurons, termed "lordosis" neurons, are located either in the ventromedial nuclear area of the hypothalamus or in the midbrain central gray (44). Thus, NO released from NOergic neurons activates the release of LHRH, which activates LH release on the one hand and mating behavior on the other (Fig. 4). Indeed, the central nervous system (CNS) role of NO is not limited to female mating behavior since inhibitors of NOS injected intraventricularly can also block erection in male rats (47).

Furthermore, NOergic terminals deriving from the pelvic plexus terminate in the corpora cavernosa penis of males. There, the NO activates soluble guanylate cyclase in the smooth muscle cells, generating cGMP which causes relaxation of penile smooth muscle. This allows erection to occur as blood enters via the penile arteries (48).

NO is also involved in the control of uterine smooth muscle via NOergic terminals on the uterine smooth muscle (49), and it has recently been shown to induce ovulation in rats (50).

The data so far presented indicate that NO could be called the sexual gas, because it is involved at all levels in reproduction from the brain to the peripheral organs, and in general it facilitates reproduction. Not only is it involved in the areas that we have described already, but also there is evidence for the presence of NOS in every locus in the male reproductive system, and it undoubtedly will be shown to play a role in testicular epididymal and vas deferens function (51) (Table I).

NO is probably a very primitive transmitter in view of its simple structure, and one could guess that it was even utilized by unicellular organisms at the dawn of life. LHRH evolved even in unicellular organisms and the yeast mating factor is a decapeptide with 60% homology to mammalian LHRH and with 1/10,000 the activity to induce LH release from rat pituitaries as the mammalian peptide (13). Therefore, it is probable that even in yeast NO might play a role in the release of the yeast-mating factor. Further studies are needed to explore the evolutionary aspects of NO synthesis and its function in primitive organisms.

Clinical Implications

If means can be found to deliver NO locally—for example, to the penis, these methods should be valuable in treatment of impotence. The problem with NO as with so many other agents involved in the control of cell function, such as prostaglandins, is the ubiquitous distribution of NOS and the ability of compounds that release NOS to circulate in the blood and generate NO

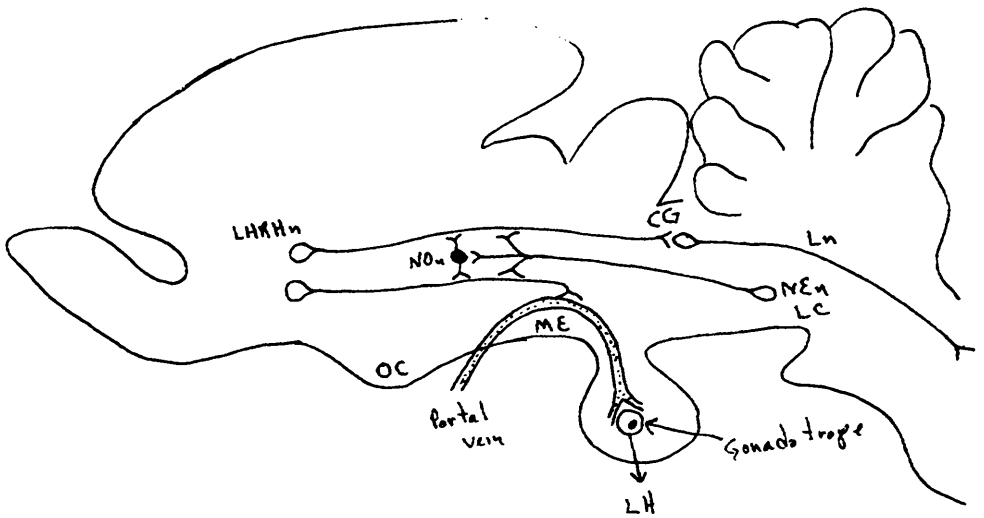

Figure 4. Schematic diagram for the dual role of NO in the induction of LH release and lordosis response. The figure indicates hypothetical interactions of norepinephrinergic neuron (NEn) terminals with NOergic (NOn) interneurons and LHRH neuron (LHRHn) terminals to generate NO, release of LHRH, secretion of LH from the pituitary, and lordosis. CG, midbrain central gray; LC, locus coeruleus; Ln, lordosis-inducing neuron; ME, median eminence; OC, optic chiasm. (Reproduced from Mani *et al.* Proc Natl Acad Sci USA **91**:6468–6472, 1994, with permission.)

Table I. Role of NO in Reproduction in the Rat

	Male	Female
Hypothalamus	Erection via LHRH	Lordosis via LHRH
	LH release via LHRH	LH release via LHRH
Pituitary	Inhibits response to LHRH	
Gonad	Present	Induces ovulation
Reproductive tract	Present	Relaxes uterine muscle via cGMP
Penis	Erection	Contracts uterine muscle via PGE_2

in distant sites. Thus, if one were to apply NP or nitroglycerine locally, it would be taken up by the circulation where it could produce coronary vasodilatation and vasodilatation of peripheral vessels. This could lead to fainting and dilatation of cerebral vessels which, in turn, could provide headaches at awkward times. These side effects may limit the utility of NO donors in treatment of sexual dysfunction. Similarly, inhibitors of the enzyme such as NMMA would also have a widespread distribution of action, such that they would do more than prevent penile erection.

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