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The role of nitric oxide in reproduction

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Abstract

Nitric oxide (NO) plays a crucial role in reproduction at every level in the organism. In the brain, it activates the release of luteinizing hormone-releasing hormone (LHRH). The axons of the LHRH neurons project to the mating centers in the brain stem and by afferent pathways evoke the lordosis reflex in female rats. In males, there is activation of NOergic terminals that release NO in the corpora cavernosa penis to induce erection by generation of cyclic guanosine monophosphate (cGMP). NO also activates the release of LHRH which reaches the pituitary and activates the release of gonadotropins by activating neural NO synthase (nNOS) in the pituitary gland. In the gonad, NO plays an important role in inducing ovulation and in causing luteolysis, whereas in the reproductive tract, it relaxes uterine muscle via cGMP and constricts it via prostaglandins (PG).

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Introduction

It is already apparent that nitric oxide (NO) plays a crucial role in reproduction at all levels from the brain to the gonads and to the accessory sex organs. In this review, we will concentrate on the role of NO in control of hypothalamic-pituitary function, and in sexual behavior, and only briefly describe its effects on the reproductive organs themselves.

Hypothalamic control of gonadotropin secretion

The control of gonadotropin secretion is extremely complex as revealed by the research of the past 35 years since the discovery of luteinizing hormone-releasing hormone (LHRH) (1), now commonly called gonadotropin-releasing hormone (GnRH) (2). This was the second of the hypothalamic-releasing hormones to be characterized and was then shown to have an effect on follicle stimulating hormone (FSH) release, albeit smaller than that on LH release. For this reason, it was renamed GnRH (2,3). Overwhelming evidence indicates that there must be a separate FSH-releasing factor (FSHRF) since pulsatile release of LH and FSH can be dissociated. In the castrate male rat, roughly half of the FSH pulses occur in the absence of LH pulses and only a small fraction of the pulses of both gonadotropins are coincident. LHRH antisera or antagonists can suppress

release of LH without altering FSH release (4). LH but not FSH pulses can be suppressed by alcohol (5), delta-9-tetrahydrocannabinol and cytokines, such as interleukin-1 alpha (IL-1α) (6). In addition, a number of peptides inhibit LH but not FSH release and a few stimulate FSH without affecting LH (4,7).

The hypothalamic areas controlling LH and FSH are separable. Stimulation in the dorsal anterior hypothalamic area can cause selective FSH release, whereas lesions in this area selectively suppress the pulses of FSH and not LH (8). In contrast, stimulations or lesions in the medial preoptic region can augment or suppress LH release without affecting FSH release. Electrical stimulation in the preoptic region releases only LH, whereas lesions in this area inhibit LH release without inhibiting FSH release. The medial preoptic area contains the perikarya of the LHRH neurons. The axons of these neurons project from the preoptic region to the anterior and mid-portions of the median eminence. Extracts of the anterior mid-median eminence contain LH-releasing activity commensurate with the content of immunoassayable LHRH, whereas extracts of the caudal median eminence and organum vasculosum lamina terminalis contain more FSH-releasing activity than can be accounted for by the content of LHRH (4).

Lesions confined to the rostral and mid-median eminence can selectively inhibit pulsatile LH release without altering FSH pulsations, whereas lesions which destroy the caudal and mid-median eminence can selectively block FSH pulses in castrated male rats (4,9,10). Therefore, it appears that the putative FSHRF is produced in neurons with perikarya in the dorsal anterior hypothalamic area with axons which project to the mid- and caudal median eminence to control FSH release selectively.

We, followed by several other groups, reported FSH-releasing activity in the stalk-median eminence. The activity was purified and separated from the LH-releasing activity in 1965 as measured by *in vivo* bioassays (11). Later, using radioimmunoassay for identification of FSH and LH release, it was reported that these activities could not be separated; however, it has now been clearly shown that the FSH-releasing activity can be separated from bioactive and radioimmunoassayable LHRH by gel filtration through Sephadex G-25 on the same type of column as used in the earlier research. FSH- and LH-releasing activity were assayed by the increase in plasma FSH and LH, respectively, in ovariectomized, estrogen-progesterone blocked rats. The separation was confirmed by radioimmunoassay of the LHRH in the fractions (11). The separation of the two activities was also demonstrable by assay of FSH and LH released from hemipituitaries incubated *in vitro* (12). In both assay systems, FSHRF emerged from the column just before elution of LHRH.

In the search for FSHRF, we first believed that it might be an analogue of LHRH and we had many such analogues synthesized and tested the forms of GnRH that are known to exist in lower species. We had not tested lamprey (l) GnRH-III, but when we realized that antiserum which cross-reacted with l-GnRH-III and l-GnRH-I immunostained neural fibers in the arcuate nucleus that proceeded to the median eminence of human brain, it occurred to us that l-GnRH-III could be the FSHRF since l-GnRH-I had little activity to release either LH or FSH. Indeed, l-GnRH-III was a potent FSH-releasing factor with little or no LH-releasing activity both *in vitro* when incubated with hemipituitaries and *in vivo* when injected into ovariectomized, estrogen progesterone-blocked rats. The lowest dose tested in that preparation (10 ρmol) produced a highly significant increase in plasma FSH with no rise in LH. Immunocytochemistry indicated that this peptide is present in the rat hypothalamus in the region which had previously been shown to control FSH release. There-

fore, it is either FSHRF or a very closely related peptide (12).

FSH and LH themselves have intrahypothalamic actions to alter gonadotropin secretion which are probably of physiologic significance (13). Growth factors are also involved (14). Gonadal steroids play an important role in controlling LHRH release and pituitary responsiveness to the peptide (2-4). In the male, the influence is inhibitory by androgens at both the hypothalamic and pituitary level, whereas in females there is a biphasic effect of estrogen to first suppress the release of LHRH and the pituitary responsiveness to it, and then, after a delay to stimulate the release of LHRH and to augment the responsiveness of the pituitary to the peptide. Furthermore, there is a self-priming action of LHRH to further augment the responsiveness of the gonadotropes to the peptide when the gland is under the influence of estrogen (2,3,7).

The pulse frequency and amplitude of pulses of LH are also altered by gonadal steroids and this plays an important role in the control of the menstrual cycle and in the induction of puberty (2-4). In turn, pulsatile release of LH and FSH is under the control of a host of classical transmitters and peptides as further discussed below.

Role of NO in control of LHRH release

NO is formed in the body by NO synthase (NOS), an enzyme which converts arginine in the presence of oxygen and several cofactors into equimolar quantities of citrulline and NO. There are 3 isoforms of the enzyme. One of these, neural (n) NOS, is found in the cerebellum and various regions of the cerebral cortex and also in various ganglion cells of the autonomic nervous system. Large numbers of nNOS-containing neurons, termed NOergic neurons, were also found in the hypothalamus, particularly in the paraventricular and supraoptic nuclei with axons projecting to the median eminence and neural lobe which also contains large amounts of nNOS. These findings indicated that the enzyme is synthesized at all levels of the neuron from perikaryon to axon terminals (15).

Because of this distribution in the hypothalamus in regions which contain peptidergic neurons that control pituitary hormone secretion, we decided to determine the role of this soluble gas in hypothalamic-pituitary function. The approach used was to use sodium nitroprusside (NP) that spontaneously liberates NO to see if this altered the release of various hypothalamic transmitters. Hemoglobin, which scavenges NO by a reaction with the heme group on the molecule and inhibitors of NOS, such as N^G-monomethyl-L-arginine (NMMA), a competitive inhibitor of NOS, were used to determine the effects of decreased NO. Two types of studies were performed. In the first set of experiments, medial basal hypothalamic (MBH) explants were preincubated *in vitro* and then exposed to neurotransmitters which modify the release of the various hypothalamic peptides in the presence or absence of inhibitors of the release of NO. The response to NO itself, provided by sodium NP, was also evaluated. Anterior pituitaries were incubated similarly *in vitro* and the effect of these compounds that increase or decrease the release of NO into the tissue on the release of pituitary hormones was examined.

In order to determine if the results *in vitro* also held *in vivo*, substances were microinjected into the third ventricle (3V) of the brain of conscious, freely moving animals to determine the effect on pituitary hormone release (6).

Our most extensive studies were carried out with regard to the release of LHRH. Not only does LHRH act after its secretion into the hypophyseal-portal vessels to stimulate LH and to a lesser extent FSH release, but it also induces mating behavior in female rats and penile erection in male rats by hypotha-

lamic action. Our experiments showed that release of NO from sodium NP *in vitro* promoted LHRH release and that the action was blocked by hemoglobin, a scavenger of NO. NP also caused an increased release of prostaglandin E_2 (PGE_2) from the tissue, which in previous experiments was shown to play an important role in release of LHRH. Furthermore, it caused the biosynthesis and release of prostanoids from ^{14}C arachidonic acid. The effect was most pronounced for PGE_2, but there also was release of lipoxygenase products that have been shown to play a role in LHRH release. Inhibitors of cyclooxygenase, the enzyme responsible for prostanoid synthesis such as indomethacin and salicylic acid blocked the release of LHRH induced by norepinephrine (NE), providing further evidence for the role of NO in the control of LHRH release via the activation of cyclooxygenase-1 (6,16,17). The action is probably mediated by the association of NO with the heme group of cyclooxygenase thus altering its conformation. The action on lipoxygenase is similar; although it contains ferrous iron, the actual presence of heme in lipoxygenase has yet to be demonstrated (6,16,17).

The previously accepted pathway for the physiologic action of NO is by activation of soluble guanylate cyclase by interaction of NO with the heme group of this enzyme, thereby causing conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP), which mediates the effects on smooth muscle by decreasing the intracellular $[Ca^{2+}]$. On the other hand, Muallem's group (18) has shown in incubated pancreatic acinar cells that cGMP has a biphasic effect on intracellular $[Ca^{2+}]$, increasing it at low concentration and lowering it at higher concentrations. We postulate that the NO released from the NOergic neurons, near the LHRH neuronal terminals, increases the intracellular free calcium required to activate phospholypase A_2 (PLA_2). PLA_2 causes the conversion of membrane phospholipids in the LHRH terminal to arachidonate, which then can be converted to PGE_2 via the activated cyclooxygenase. The released PGE_2 activates adenyl cyclase causing an increase in cAMP release, which activates protein kinase-A, leading to exocytosis of LHRH secretory granules into the hypophyseal portal capillaries for transmission to the anterior pituitary gland (19).

NE has been shown to be a powerful releaser of LHRH. In the present experiments, we showed it acted by activation of the NOergic neurons since the activation of these neurons and the release of LHRH could be blocked by a competitive inhibitor of NOS, NMMA. NE acts to stimulate the release of NO from the NOergic neurons by α_1 adrenergic receptors since its action can be blocked by phentolamine, an α receptor blocker, and prazosine, an α_1 receptor blocker. Activation of the α_1 receptors is postulated to increase intracellular $[Ca^{2+}]$ that combines with calmodulin to activate NOS leading to generation of NO.

We measured the effect of NE on the content of NOS in the MBH explants at the end of the experiments by homogenizing the tissue and adding ^{14}C arginine and measuring its conversion to citrulline on incubation of the homogenate. Since arginine is converted to equimolar quantities of NO and citrulline, measurement of citrulline production provides a convenient estimate of the activity of the enzyme. The NO disappears rapidly making its measurement very difficult. NE caused an increase in the apparent content of the enzyme. That we were actually measuring enzyme content was confirmed, because incubation of the homogenate with L-nitroarginine methyl ester, another inhibitor of NOS, caused a drastic decline in the conversion of arginine to citrulline. We further confirmed that we actually had increased the content of enzyme by isolating the enzyme according to the method of Bredt and Snyder (20) and then measuring the conversion of labeled arginine to citrul-

line. The conversion was significantly increased by NE (19).

Glutamic acid (GA), acting at least in part through n-methyl-d-aspartate (NMDA) receptors, also plays a physiologically significant role in controlling the release of LHRH. Therefore, we determined where GA fit into the picture. It also acted via NO to stimulate LHRH release, but we showed that the effect of GA could be completely inhibited by the α-receptor blocker phentolamine. Consequently, we concluded that GA acted by stimulation of the noradrenergic terminals in the MBH to release NE which then initiated NO release and stimulation of LHRH release (21).

Oxytocin has actions within the brain to promote mating behavior in the female and penile erection in the male rat. Since LHRH mediates mating behavior, we hypothesized that oxytocin would stimulate LHRH release that, after secretion into the hypophyseal portal vessels, mediates LH release from the pituitary. Consequently, we incubated MBH explants and demonstrated that oxytocin (0.1 to 100 nM) induced LHRH release via NE stimulation of nNOS. Therefore, oxytocin may be very important as a stimulator of LHRH release. Furthermore, NO acted by negative feedback to block oxytocin release (22).

One of the few receptors to be identified on LHRH neurons is the gamma amino butyric acid a ($GABA_a$) receptor. Consequently, we evaluated the role of GABA in LHRH release and the participation of NO in this. The data showed that GABA blocked the response of the LHRH neurons to NP that acts directly on the LHRH terminals. We concluded that GABA suppressed LHRH release by blocking the response of LHRH neuronal terminals to NO. Additional experiments showed that NO stimulated the release of GABA, thereby providing an inhibitory feed-forward pathway to inhibit the pulsatile release of LHRH initiated by NE. As NE stimulated the release of NO, this would stimulate the release of GABA, which would then block the response of the LHRH neuron to the NO released by NE (23).

Other studies indicated that NO would suppress the release of dopamine and NE. We have already described the ability of NE to stimulate LHRH release and the fact that dopamine also acts as a stimulatory transmitter in the pathway. Therefore, there is an ultra short-loop negative feedback mechanism to terminate the pulsatile release of LHRH since the NO released by NE would diffuse to the noradrenergic terminals and inhibit the release of NE, thereby terminating the pulse of NE, LHRH and finally LH (24).

We further examined the possibility that other products from this system might have inhibitory actions. Indeed, we found that as we added increasing amounts of NP, we obtained a bell-shaped dose-response curve of the release of LHRH, such that the release increased with increasing concentrations of NP up to a maximum at about 600 μM and then declined with higher concentrations. When the effect of NP on NOS content at the end of the experiment was measured, we found that high concentrations of NP lowered the NOS content. Furthermore, NP could directly decrease NOS content when incubated with MBH homogenates, results that indicate a direct inhibitory effect on NOS probably by interaction of NO with the heme group on the enzyme. Thus, when large quantities of NO are released, as could occur following induction of iNOS in the brain during infections, the release of NO would be decreased by an inhibitory action on the enzyme at these high concentrations. Furthermore, high concentrations of cGMP released by NO also acted in the explants or even in the homogenates to suppress the activation of NOS. This pathway could also be active in the presence of high concentrations of NO, such as would occur in infection by induction of inducible NOS by bacterial or viral products (19).

Effect of cytokines (IL-1 and granulocyte macrophage colony-stimulating factor) on the NOergic control of LHRH release

The cytokines so far tested, for example IL-1 (25) and granulocyte macrophage colony-stimulating factor (GMCSF) (26), act within the hypothalamus to suppress the release of LHRH as revealed in both *in vivo* and *in vitro* studies. We have examined the mechanism of this effect and found that for IL-1, it occurs by inhibition of cyclooxygenase as shown by the fact that there is blockage of the conversion of labeled arachidonate to prostanoids, particularly PGE_2, and the release of PGE_2 induced by NE is also blocked (25).

A principal mechanism of action is by suppression of the LHRH release induced by NO donors such as NP (25). We first believed that there were IL-1 and GMCSF receptors on the LHRH neuron which blocked the response of the neuron to NO. However, since we had also shown that GABA blocks the response to NP and earlier work had shown that GABA receptors are present on the LHRH neurons, we evaluated the possibility that the action of cytokines could be mediated by stimulation of GABAergic neurons in the MBH. Indeed, in the case of GMCSF, its inhibitory action on LHRH release can be partially reversed by the $GABA_a$ receptor blocker, bicuculline, which also blocks the inhibitory action of GABA, itself, on the response of the LHRH terminals to NO. Furthermore, GMCSF reduced the NOS content measured by the citrulline method, suggesting that there were GMCSF receptors of the NOergic neurons that inhibited them leading to decreased synthesis of NOS. Therefore, we believe that the inhibitory action of cytokines on LHRH release not only is mediated by stimulation of GABA neurons but also by inhibition of NOergic neurons (26).

Role of NO in mating behavior

LHRH controls lordosis behavior in the female rat and is also involved in mediating male sex behavior. Studies *in vivo* have shown that NO stimulates the release of LHRH that induces sex behavior. This behavior can be stimulated by 3V injection of NP and is blocked by inhibitors of NOS. Apparently, there are two LHRH neuronal systems: one, with axons terminating on the hypophyseal portal vessels, the other with axons terminating on neurons which mediate sex behavior (27). NO is also involved in inducing penile erection by the release of NO from NOergic neurons innervating the corpora cavernosa of the penis. The role of NO in sex behavior in both sexes has led us to change the name of NO to the sexual gas (15).

Effect of NO in the release of other hypothalamic peptides

NO appears to act similarly by stimulating the release of corticotropin-releasing hormone (CRH) (28), growth hormone-releasing hormone (GHRH) (29), somatostatin (30), but not FSHRF (6) since this is not affected by inhibitors of NOS or by donors of NO. On the other hand, the release of vasopressin and oxytocin release is also suppressed by NO (22).

Action of NO to control release of anterior pituitary hormones

NOS is localized in certain pituitary cells, principally the folliculostellate cells, which are modified glial cells that bear a resemblance to macrophages, and also in the LH gonadotropes as revealed by immunocytochemistry. When pituitaries are incubated *in vitro*, most pituitary hormones are secreted only in small quantities. The exception to this rule is prolactin, which is secreted in large amounts because of removal of inhibitory hypothalamic control by dopa-

mine (31). In the case of pituitary hormones that are secreted at low levels because of lost stimulatory hypothalamic input, NO donors increase hormone release, for example, in the case of LH and GH. In contrast, in the case of prolactin, which is released in large amounts, NO donors suppress the release of the hormone and inhibitors of NOS usually enhance the release, indicating that the gland can further increase the release of prolactin *in vitro*.

Dopamine, the most important prolactin-inhibiting factor, acts on dopamine type 2 receptors (D_2 receptors) in the gland. The dramatic inhibitory action of dopamine can be prevented by D_2 receptor blockers and also is prevented by incubation in the presence of inhibitors of NOS. Therefore, we conclude that the primary inhibitory action of dopamine is mediated by the stimulation of D_2 receptors on the NOS-containing cells in the pituitary gland, with the resultant release of NO which diffuses to the lactotropes and activates guanylate cyclase, causing the release of cGMP that mediates the inhibition of prolactin secretion. Consistent with this hypothesis is the fact that NO donors suppress prolactin release and the addition of cyclic GMP can also lower the release of the hormone from incubated pituitaries (31).

The LH-releasing action of LHRH has long been known to be caused by an increase in intracellular free calcium and this is also true for FSH (2,3,32). Since NOS has been localized to gonadotropes, it occurred to us that NO might play a role in control of gonadotropin secretion. Indeed, in early work, we found that cGMP, but not cAMP, would activate both FSH and LH release (33). We have now shown that blockade of NO formation by the use of the inhibitor of NOS, NMMA, inhibits the release of FSH and LH induced by LHRH. Presumably, NO activates guanylyl cyclase (GC) causing the release of cGMP, which, as already indicated, can release both gonadotropins, presumably by acting on protein kinase-G (34).

Certain cytokines can also affect gonadotropin secretion by the pituitary including GMCSF (Kimura M and McCann SM, unpublished data), which increases LH release from the gland. It has not yet been determined whether these effects are also mediated via NO.

Potential role of the adipocyte hormone, leptin, in reproduction

The hypothesis that leptin may play an important role in reproduction stems from several findings. First, the Ob/Ob mouse, lacking the leptin gene, is infertile and has atrophic reproductive organs (35). Gonadotropin secretion is impaired and very sensitive to negative feedback by gonadal steroids as is the case for prepubertal animals (36). It has now been shown that treatment with leptin can recover the reproductive system in the Ob/Ob mouse by leading to growth and function of the reproductive organs and fertility (37) via secretion of gonadotropins (38).

The critical weight hypothesis for the development of puberty states that puberty occurs when body fat stores have reached a certain level (39). This hypothesis in its original form does not hold since if animals are underfed, puberty is delayed but with access to food, rapid weight gain leads to onset of puberty at weights well below the critical weight under normal nutritional conditions (40). We hypothesized that during this period of refeeding or at the time of the critical weight in the normally fed animal, there is increased release of leptin into the blood stream from the adipocytes and that this acts on the hypothalamus to stimulate the release of LHRH with the resultant induction of puberty. Indeed, leptin has recently been shown to induce puberty (41).

Therefore, we initiated studies on its possible effect on hypothalamic-pituitary function. We expected that it would also be active in adult rats and therefore studied its

effect on the release of FSH and LH from hemipituitaries, and also its possible action to release LHRH from MBH explants *in vitro*. To determine if it was active *in vivo*, we used a model that we employed to evaluate stimulatory effects of peptides on LH release, namely the ovariectomized, estrogen-primed rat. Since our supply of leptin was limited, we began by microinjecting it into the 3V in conscious animals bearing implanted third ventricular cannulae, and also catheters in the external jugular vein extending to the right atrium, so that we could draw blood samples before and after the injection of leptin and measure the effect on plasma FSH and LH (42).

Effect of leptin on LH release

We found that, under our conditions, leptin had a bell-shaped dose-response curve to release LH from anterior pituitaries incubated *in vitro*. There was no consistent stimulation of LH release with a high concentration of 10 µM. The effects became significant with 100 nM and remained on a plateau through 10 pM with the reduced release at a concentration of 1.0 pM that was no longer statistically significant. The release was not significantly less than that achieved with 40 nM LHRH. Under these conditions, there was no additional release of LH when 100 nM leptin was incubated together with 40 nM LHRH. In some experiments, there was an additive effect when leptin was incubated with LHRH; however, this effect was not always observed. The data indicate that leptin was only slightly less effective than LHRH itself to release LH.

Effect of leptin on FSH release

In the incubates from these same glands, we also measured FSH release and found that it showed a pattern similar to that of LH, except that the sensitivity for FSH release was much less than that for LH. The minimal effective dose for FSH was 1.0 nM, whereas it was 10 pM for LH. The responses were roughly of the same magnitude at the effective concentrations as obtained with LH and the responses were clearly equivalent to those observed with 4 nM LHRH. The combination of LHRH with leptin at a concentration which was less than required to give an effect, gave a clear additive effect.

Effect of leptin on prolactin release

The results with prolactin differed from those with FSH and LH in that the maximal response (a 4.5-fold increase) was obtained with 10 µM, the highest concentration tested and prolactin release declined with lower concentrations, such that there was no longer a significant effect with 10 nM leptin.

Effect of leptin on LHRH release

There was no significant effect of leptin in a concentration range of 1 pM to 1 µM on LHRH release during the first 30 min of incubation; however, during the second 30 min, the highest concentration, 1 µM, produced a borderline significant decrease in LHRH release followed by a tendency to increase with lower concentrations and a significant, plateaued increase with 1 to 100 pM, with the lowest concentrations tested. The overall statistical significance obtained by combining the results with both effective doses was $P<0.01$.

Effect of intraventricularly injected leptin on plasma gonadotropin concentration in ovariectomized, estrogen-primed rats

The injection of the diluent for leptin (5 µl Krebs-Ringer bicarbonate) into the 3V had no effect on pulsatile FSH or LH release, but the injection of leptin (0.5 nM) uniformly produced an increase in plasma LH with a variable time-lag ranging from 10-50

min, so that the maximal increase in LH from the starting value was highly significant ($P<0.01$) and constituted a mean increase of 60% above the initial concentration. In contrast, leptin inhibited FSH release when compared with the diluent, but the effect was delayed and occurred mostly during the second hour. Therefore, at this dose of estrogen, it appears that leptin stimulates the release of LHRH and inhibits the release of FSHRF (43).

Mechanism of action of leptin on the hypothalamic pituitary axis

Recently, we have shown that leptin exerts its action at both the hypothalamic and pituitary level by activating NOS since its effect to release LHRH, FSH and LH *in vitro* is blocked by NMMA (34).

Leptin in essence is a cytokine secreted by the adipocytes which, like the cytokines, appears to reach the brain via a transport mechanism mediated by the Ob/Ob_a receptors (44) in the choroid plexus (45). These receptors have an extensive extracellular domain, but a greatly truncated intracellular domain (44) and mediate transport of the cytokine by a saturable mechanism (46). Following uptake into the cerebrospinal fluid (CSF) through the choroid plexus, leptin is carried by the flow of CSF to the 3V, where it either diffuses into the hypothalamus through the ependymal layer lining the ventricle or combines with Ob/Ob_b (44) receptors on terminals of responsive neurons that extend to the ventricular wall.

The Ob/Ob_b receptor has a large intracellular domain which presumably mediates the action of the protein (45). These receptors are spread widely throughout the brain (45), but localized particularly in the region of the paraventricular (PVN) and arcuate nuclei (AN). Leptin activates stat 3 within 30 min after its intraventricular injection (47). Stat 3 is a protein which is important for carrying information to the cell nucleus to initiate DNA-directed mRNA synthesis. Following injection of bacterial lipopolysaccharide (LPS), stat 3 is also activated, but in this case, the delay is 90 min presumably because LPS induces IL-1ß mRNA in the same areas, namely the PVN and AN (48). IL-1ß mRNA would then cause production of IL-1ß that would activate stat 3. On entrance into the nucleus, stat 3 would activate or inhibit DNA-directed mRNA synthesis. In the case of leptin, it activates CRH mRNA in the PVN, whereas in the AN, it inhibits neuropeptide Y (NPY) mRNA resulting in increased CRH synthesis and presumably release in the PVN and decreased NPY synthesis and release in the AN (45). Presumably, combination of leptin with these transducing receptors also either increases or decreases the firing rate of that particular neuron. In the case of the AN-median eminence (ME) area, leptin may enter the median eminence by diffusion between the tanycytes or alternatively by combining with its receptors on terminals of neurons projecting to the tanycytes. Activation or inhibition of these neurons would induce LHRH release.

The complete pathway of the action of leptin on the MBH to stimulate LHRH release is not known. Arcuate neurons bearing Ob/Ob receptors may project to the ME and to the tanycyte/portal capillary junction. Leptin would either combine with its receptors on the terminals that transmit information to the cell bodies in the AN or diffuse to the AN to combine with its receptors on the perikarya of AN neurons. Since leptin decreases NPY mRNA and presumably NPY biosynthesis in NPY neurons in the AN, we postulate that leptin causes a decrease in NPY release. Since, NPY inhibited LH release in intact and castrated male rats (49), we hypothesize that NPY decreases the release of LHRH by inhibiting the noradrenergic neurons which mediate pulsatile release of LHRH. Therefore, when the release of NPY is inhibited by leptin, noradrenergic impulses are generated, which act on α_1

receptors on the NOergic neurons causing the release of NO which diffuses to the LHRH terminals and activates LHRH release by activating guanylate cyclase and cyclooxygenase$_1$ as shown in our prior experiments reviewed above. Leptin acts to activate NOS as indicated since its release of LHRH is blocked by inhibition of NOS (42). LHRH enters the portal vessels and is carried to the anterior pituitary gland where it acts to stimulate FSH and particularly LH release by combining with its receptors on the gonadotropes. The release of LH and to a lesser extent FSH is further increased by the direct action of leptin on its receptors in the pituitary gland (50).

We hypothesize that leptin may be a critical factor in induction of puberty as the animal nears the so-called critical weight. Either metabolic signals reaching the adipocytes, or signals related to their content of fat cause the release of leptin which increases LHRH and gonadotropin release, thereby initiating puberty and finally ovulation and onset of menstrual cycles. In the male, the system would work similarly; however, there is no preovulatory LH surge brought about by the positive feedback of estradiol. Sensitivity to leptin is undoubtedly under steroid control and we are actively working to elucidate this problem.

During fasting, the leptin signal is removed and LH pulsatility and reproductive function decline quite rapidly. In women with anorexia nervosa, this causes a reversion to the prepubertal state which can be reversed by feeding. Thus, leptin would have a powerful influence on reproduction throughout the reproductive life-span of the individual. The consequences to gonadotropin secretion of overproduction of leptin, as has already been demonstrated in human obesity, are not clear. There are often reproductive abnormalities in these circumstances and whether they are due to excess leptin production or other factors, remains to be determined (51).

In conclusion, it is now clear that leptin plays an important role in control of reproduction by acting on the hypothalamus and pituitary. It may also act in the gonads since its receptors have been demonstrated there.

Role of NO in accessory sex organs

As indicated earlier, NO activates LHRH neurons projecting to the brain stem. These neurons produce the lordosis reflex in female rats, and penile erection in the male as shown by Ignarro (see 52 for references). These brain stem neurons activate NOergic neurons in the pelvic plexus that innervate the accessory sex organs, including the penis in males and the vagina in females. In the penis, these terminals release NO that activates soluble guanylate cyclase in the smooth muscle of the corpora cavernosa, generating cGMP that causes relaxation of penile smooth muscle as shown by Ignarro. This allows erection to occur as blood enters via the penile arteries (52). Indeed, nitroglycerin applied to the surface of the penis or NP injected into the penis will induce erection in man and rat (McCann SM, unpublished data); however, the NO produced reaches the general circulation and can produce undesirable side effects such as headache and decline in blood pressure. These side effects have been circumvented to a degree by the development of Viagra, a phosphodiesterase inhibitor that more or less selectively inhibits the phosphodiesterase found in the corpora cavernosa of the penis. A comparable organ in the female is the vagina that has a weak smooth muscle constrictor at its external orifice. The vaginal constrictor relaxes and the vaginal mucosa secretes vaginal fluid on sexual arousal in women. NO secreted from the NOergic pelvic neuronal terminals in the vaginal wall may cause vaginal relaxation and secretion via liberation of cGMP. Indeed, arousal in women also appears to be enhanced by Viagra probably by delaying the breakdown of the cGMP produced.

Table 1 - Role of NO in rat reproduction.

LHRH, Luteinizing hormone-releasing hormone; FSHRF, follicle stimulating hormone-releasing factor; cGMP, cyclic guanosine monophosphate; PG, prostaglandin. Present indicates that NOS has been found, but the function of NO has not been determined.

	Male	Female
Hypothalamus	Erection via LHRH LH release via LHRH	Lordosis via LHRH LH release via LHRH
Pituitary	Mediates response to LHRH and FSHRF	Mediates response to LHRH and FSHRF
Gonad	Present	Induces ovulation; causes luteolysis
Reproductive tract	Present	Relaxes uterine muscle via cGMP; constricts it via PG's
Penis and vagina	Erection	Possibly relaxes vaginal smooth muscle and causes vaginal secretion

NO is also involved in the control of uterine smooth muscle via NOergic terminals on the uterine smooth muscle cells (53), and it has been shown to induce ovulation in rats (54), and luteal regression in rats (55) and cows (Hansel WF, unpublished data).

NO appears to be present in all parts of the male reproductive system as well and will undoubtedly be shown to play a role in testicular, epididymal and vas deferens function (56) (Table 1). Lastly, NOS is present in spermatazoa and induces sperm motility (57). Indeed, in view of its multifaceted role to promote reproduction at all levels in the organism, it has been aptly named the sexual gas (15).

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