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Nitric oxide mediates sexual behavior in female rats

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ABSTRACT **Nitric oxide (NO), an active free radical formed during the conversion of arginine to citrulline by the enzyme NO synthase (NOS), mediates vasorelaxation, cytotoxicity, and neurotransmission. Neurons containing NOS (NOergic) are located in the hypothalamus. These NOergic neurons control the release of several hypothalamic peptides. Release of NO from these NOergic neurons stimulates pulsatile release of luteinizing hormone-releasing hormone (LHRH) *in vivo* and LHRH release *in vitro*. LHRH not only induces LH release, which induces ovulation, but also facilitates female sexual behavior. Sexual behavior can be induced reliably in estrogen-primed ovariectomized female rats by progesterone (P). This behavior consists of proceptive behavior to attract the male and the assumption of a clear characteristic posture, lordosis, when mounted by the male. To ascertain the role of NO in the control of sexual behavior in female rats, an inhibitor of NOS, N^G-monomethyl-L-arginine was microinjected into the third cerebral ventricle (3V) of conscious, ovariectomized, estrogen-primed rats with indwelling cannulae. N^G-Monomethyl-L-arginine (10–1000 μg) prevented P-facilitated lordosis when administered intracerebroventricularly into the 3V, 20 min prior to the 3V injection of P. N^G-Monomethyl-D-arginine, which does not inhibit NOS, did not inhibit lordosis under the same experimental conditions. Microinjection into the 3V of sodium nitroprusside (SNP), which spontaneously releases NO, facilitated lordosis in estrogen-primed rats in the absence of P. The facilitation of lordosis induced by either P or SNP was prevented by intracerebroventricular injection of hemoglobin, which binds NO. Lordosis facilitated by P or SNP was blocked by injection of LHRH antiserum into the 3V. The results are interpreted to mean that the P-facilitated lordosis response is mediated by LHRH release. Furthermore, since NO release from SNP also facilitates lordosis in the absence of P and this response could be blocked by LHRH antiserum, we conclude that P brings about the release of NO, which stimulates LHRH release that facilitates lordosis. Thus, the results indicate that NO induces LHRH release and that LHRH then plays a crucial role in mediation of sexual behavior in the female rats.**

Nitric oxide (NO) formed by the conversion of arginine to citrulline by the enzyme NO synthase (NOS) has multiple regulatory effects in a variety of tissues and systems (1, 2), including the brain (3). NOS has been demonstrated by immunocytochemistry in various neurons in the brain including the paraventricular and supraoptic nuclei of the hypothalamus (4). The physiological role of NO as a messenger in the central nervous system was first suggested by the demonstration that neonatal cerebellar cultures released a factor that had properties similar to NO (5), followed by observations of NO-forming activity in brain extracts and slices (6, 7). NO acts as a messenger in the brain by activating soluble guanylyl cyclase, which generates cGMP formation (8). Brain NOS, like the NOS in the vascular endothelium is a constitutive enzyme, in contrast to the inducible NOS, which is produced in macrophages exposed to bacterial liposaccharide or cytokines (8).

NO plays an important part in controlling the release of hypothalamic peptides (9). For example, NO acts as a signal transducer in norepinephrine (NE)-induced prostaglandin E_2 release from medial basal hypothalamic explants (10). Both NE and prostaglandin E_2 regulate the pulsatile release of luteinizing hormone-releasing hormone (LHRH) (10–13), which in turn induces pulsatile luteinizing hormone (LH) release from the gonadotropes of the anterior pituitary gland (14). *In vivo* and *in vitro* experiments using N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NOS, demonstrated NO-mediated release of pulsatile LH *in vivo* and LHRH *in vitro* (15, 16).

The hypothalamic hypophysiotropic peptide hormone, LHRH, not only releases LH from the pituitary but also induces sexual behavior (14, 17). LHRH induces lordosis after mounting by the male in estrone-primed ovariectomized (17) and estradiol benzoate-primed adrenalectomized female rats (18). These observations raise the possibility that NO might also be involved in the mediation of sexual behavior in female rats. In the present experiments, we investigated the effect(s) of agents that enhance or inhibit NO formation on the sexual behavior of female rats. The results indicate a crucial role for NO in LHRH-potentiated mating behavior as measured by the lordosis response in estrogen (E)-primed ovariectomized female rats.

MATERIALS AND METHODS

Ovariectomized Sprague–Dawley rats (160–180 g body weight) obtained from Sasco (Houston) were housed with a 12/12-h light/dark cycle and given food and water ad libitum. All the animals were administered hormones and tested for sex behavior. 17β-Estradiol benzoate (herein used as E; 10 μg in sesame oil) was injected subcutaneously (s.c.) followed by progesterone (P) s.c. (100 μg in sesame oil) 48 h later. Four hours after P administration, the animals were tested for sex behavior in the presence of sexually active males in a 50 × 45 × 24 cm polystyrene arena. Both proceptive (hop-darting, ear wiggling, and approaches to the male) and receptive behavior (the number of lordoses, acceptance behavior on being mounted by the male, and the number of mounts by the male) of each female rat in the presence of a male was scored and recorded. The results of all the experiments were converted to lordosis quotient (LQ), defined as a percentage of full lordosis response (perineum elevated, all four legs extended

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Abbreviations: NO, nitric oxide; NOS, NO synthase; LHRH, luteinizing hormone-releasing hormone; L-NMMA, N^G-monomethyl-L-arginine; D-NMMA, N^G-monomethyl-D-arginine; 3V, third cerebral ventricle; E, estrogen; P, progesterone; SNP, sodium nitroprusside; NE, norepinephrine; Hb, hemoglobin; NRS, normal rabbit serum; LQ, lordosis quotient; i.c.v., intracerebroventricularly.

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from the initial crouch position, and the head at an angle of 45° from the floor) divided by the number of mounts by the male. Only animals that exhibited complete lordosis after s.c. injection of steroids as described above on initial testing were used for further experiments. A week prior to further tests, an indwelling cannula was implanted into the third cerebral ventricle (3V) of animals by the technique of Antunes-Rodrigues and McCann (19). Then, the animals were again tested for sexual behavior as described above with a minor modification. E-primed animals were administered 2 μg of P (in 2 μl of sesame oil) intracerebroventricularly (i.c.v.) 48 h after E, and sexual behavior was observed for 30–150 min thereafter in the presence of a sexually active male. If the rats had not displayed lordosis by 150 min, the experiment was terminated. Animals that exhibited complete lordosis were used in the experiments.

Test Substances. L-NMMA, N^G-monomethyl-D-arginine (D-NMMA), sodium nitroprusside (SNP), hemoglobin (Hb), E, and P were obtained from Sigma. LHRH antiserum was a gift from Ayella Barnea (Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center, Dallas).

Injections i.c.v. Forty-eight hours after E priming, the test substances L-NMMA (10, 100, and 1000 μg), D-NMMA (1, 10, and 100 μg), and SNP (0.1, 1, 10, and 100 μg) were microinjected into the 3V in 2 μl of saline over 60 s. Twenty minutes after the administration of the test substance, 2 μg of P was microinjected into the 3V and sexual behavior was observed in the presence of a sexually active male until lordosis occurred. If sexual behavior had not occurred by 150 min, the experiment was terminated. Control animals received saline instead of the test compounds. In some experiments involving SNP, certain of the experimental animals did not receive P (see *Results*). In experiments where Hb (2 μg/ml of saline) was used, SNP was incubated with Hb for 5 min and, subsequently, 2 μl were injected into the 3V. In studies with LHRH antiserum, 2 μl of the antiserum (1:10 dilution in saline) was administered into the 3V of E-primed (48 h) animals 2 h prior to P and/or SNP. In these experiments, normal rabbit serum (NRS) was used as control. In all experiments, controls included animals that received vehicle alone, E alone, and E followed by P (positive controls).

RESULTS

Effect of NOS Inhibitors on Sexual Behavior. Injection of L-NMMA, a competitive inhibitor of NOS, into the 3V of E-primed female rats 20 min prior to the administration of P inhibited P-facilitated lordosis (Fig. 1*A*) at all the doses (1000–10 μg) examined. In contrast, saline-injected controls exhibited excellent sexual behavior after P administration. L-NMMA had no effect on the behavior of rats primed with E but not given P (data not shown). Similar effects on sexual behavior were observed on i.c.v. administration of another NOS inhibitor, N^G-nitro-L-arginine methyl ester (data not shown). However, it was much less potent than L-NMMA, requiring a minimum effective dose of 100 μg.

Effects of D-NMMA on Sexual Behavior. D-NMMA, which unlike L-NMMA and N^G-nitro-L-arginine methyl ester, does not inhibit NOS, was ineffective in inhibiting sex behavior at the doses (100–1 μg) used (Fig. 1*B*). Sexual behavior of rats treated with D-NMMA was equivalent to that of the controls that received saline instead of D-NMMA.

Effects of SNP on Lordosis. The influence of increasing concentrations of SNP, which spontaneously releases NO, was investigated by administering SNP i.c.v. in E-primed rats. The lowest dose of SNP (0.1 μg) only stimulated lordosis in few rats, but higher doses (1 and 10 μg), when microinjected into the 3V of E-primed rats, were able to facilitate receptive behavior in the absence of P (Fig. 2*A*). At the highest dose used (100 μg), the rats became lethargic and refused to accept the males and to exhibit lordosis response. However, within 60–90 min, they became hyperactive and started running in the cages. SNP did not alter the lordosis response in the presence of P (data not shown). Proceptive behavior in the presence of SNP did not parallel that observed in the presence of P. Though some hop-darting was observed, no ear wiggling was seen after SNP treatment. The effect of Hb, a scavenger of NO, on SNP-facilitated lordosis was evaluated. Hb (2 μg/ml) suppressed the lordosis response facilitated by either SNP or P in E-primed animals (Fig. 2*B*).

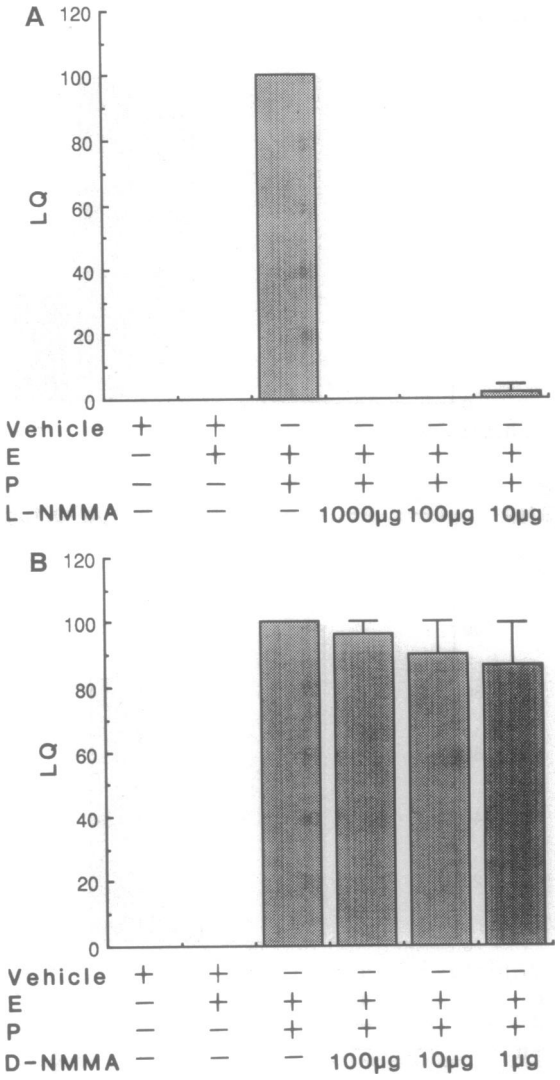

FIG. 1. (*A*) Effect of administration of L-NMMA (1000–10 μg/2 μl) i.c.v. on the lordosis response of ovariectomized E-primed (s.c.) female rats. Microinjection of L-NMMA was followed 20 min later by injection of P into the 3V. Lordosis response of the female rats in the presence of sexually active male rats was observed and expressed as LQ. Control rats received vehicle alone or E followed by vehicle in the place of L-NMMA. There were 12 rats per group. Values in this and subsequent figures are the mean ± SEM. (*B*) Dose–response data of D-NMMA (1000–1 μg/2 μl) on P-facilitated sexual behavior of ovariectomized E-primed female rats in the presence of male rats. The microinjections of the compound D-NMMA and P, the schedule of administration, and the controls were similar to those described for *A*.

Effect of LHRH Antiserum on SNP-Facilitated Sexual Behavior. Administration of LHRH antiserum i.c.v. 60 min prior to the injection of P inhibited lordosis in E-primed animals (Fig. 3*A*). In contrast, the proceptive and receptive

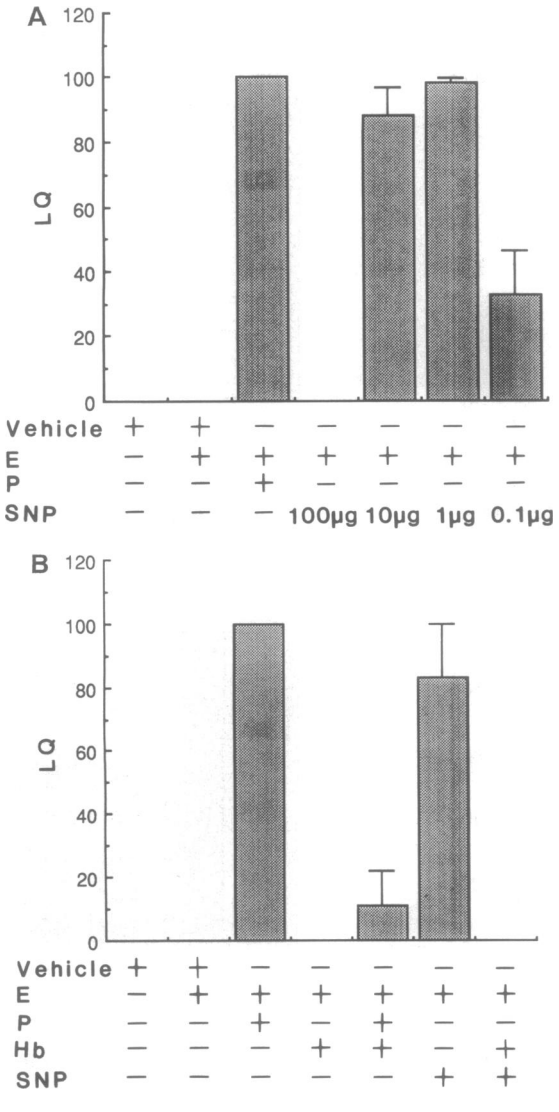

FIG. 2. (*A*) Effect of microinjection of SNP (100–0.1 μg/2 μl) into the 3V of ovariectomized E-primed female rats on the lordosis response in the absence of P. The controls were similar to those described for Fig. 1*A*. (*B*) Effect of Hb (2 μg/ml) on P- and SNP (1 μg)-facilitated lordosis response in female rats. Hb was incubated with SNP for 5 min prior to the microinjections. In cases where Hb effects on the P-facilitated lordosis response were evaluated, Hb was administered directly into the 3V, prior to the administration of P. The other criteria were similar to that described for Fig. 1*A*.

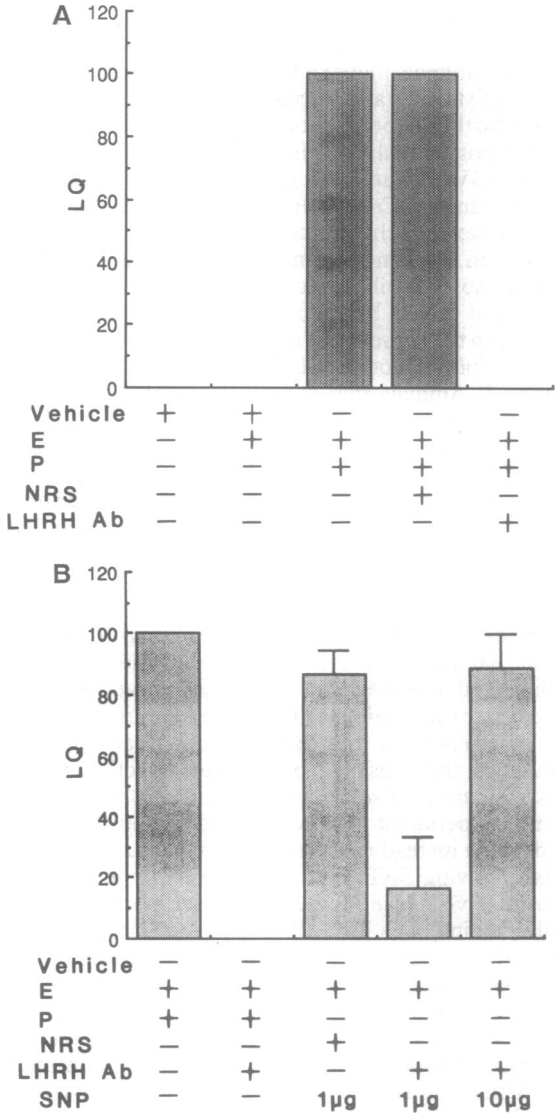

FIG. 3. (*A*) Effect of LHRH antiserum (Ab) on P-facilitated sexual behavior of ovariectomized E-primed female rats. NRS was used in lieu of the antiserum as a control. P was administered 2 h after the microinjection of the LHRH antiserum or NRS into the 3V. (*B*) Effect of LHRH antiserum on SNP-facilitated lordosis response in ovariectomized E-primed female rats. The antiserum or NRS was administered 2 h prior to the microinjection of SNP into the 3V. Sexual behavior was observed 30–75 min after the administration of SNP or P. LHRH antiserum blocked the lordosis response facilitated by 1 μg of SNP. However, this blockade could be overcome by a higher dose of SNP (10 μg).

behavior was not blocked in control animals that received 3V injections of NRS. In further experiments, the sexual behavior of E-primed rats induced by a facilitatory dose (1 μg) of SNP was also blocked by LHRH antiserum (Fig. 3*B*). This suppressive effect of LHRH antiserum could be overcome by increasing the dose of SNP by an order of magnitude.

DISCUSSION

Injection of the NOS inhibitors, L-NMMA and N^G-nitro-L-arginine methyl ester, into the 3V of E-primed rats completely blocked the lordosis response after the i.c.v. administration of P, and NO released into the 3V by the breakdown of SNP mimicked the response to P in the E-primed rat. The data provide strong evidence that NO is capable of inducing sexual behavior. This conclusion was further supported by the fact that Hb, a scavenger of NO, could block the lordosis response induced by either P or SNP.

In all the experiments involving SNP, the effects on proceptive behavior were not as distinct as seen in the presence of P. While some hop-darting type of solicitous behavior was evident, ear wiggling was not seen. The reason that SNP had little effect on proceptive behavior is not apparent. Perhaps this behavior is not dependent on NO-induced LHRH release since in earlier experiments injection of LHRH induced lordosis but had little effect on proceptive behavior (17).

Previous evidence indicates that release of NO from NO-ergic neurons is critical to the release of LHRH, which then diffuses to the hypophyseal portal vessels (14, 15). The portal transport of LHRH to the pituitary stimulates LH release from the gonadotropes. Furthermore, previous studies showed not only that LHRH could induce lordosis in response to sexual stimulation in the E-primed rat (17, 18, 20–23) but also that LHRH antiserum could block lordosis

facilitated by P (24, 25). The LHRH antiserum that we tested also blocked P-facilitated lordosis response and lordosis induced by the release of NO by SNP microinjected into the 3V.

Earlier evidence for the role of NO in the release of LHRH was obtained in both *in vivo* (15) and *in vitro* (16) studies. In the current study, the greatest dose of L-NMMA used in the dose–response experiments was equivalent to that used in the prior *in vivo* study of Rettori *et al.* (15), yet our experiments show that some inhibition of LHRH release was observed with L-NMMA doses 100-fold less. Both studies provide excellent support for the specificity of the action of L-NMMA on NOS since D-NMMA was ineffective. In these studies we have shown that 3V injection of SNP causes a dose-dependent induction of sexual behavior in the ovariectomized E-primed rat. At the highest dose, toxic effects were observed ≈1 h after drug administration, probably due to cyanide production. Neurons containing NOS (NOergic neurons) have been reported in the vicinity of LHRH neuronal fibers in the arcuate-median eminence region (15). Thus, our experiments lend strong support to the concept that NO released from NOergic neurons in the vicinity of the LHRH terminals induces LHRH release. At the highest dose, the animals were lethargic and did not exhibit lordosis. Since the action of β-endorphin, which releases NO (26), is similar (27), this may be the cause for their failure to exhibit lordosis. Approximately 1 h after drug administration, these rats were hyperactive, possibly due to cyanide production during breakdown of SNP.

As indicated above, LHRH neuronal terminals that end in juxtaposition to the hypophyseal portal vessels control the release of LH. Other terminals of the LHRH neurons also release the peptide into the ventromedial hypothalamus and the midbrain central gray (28). In these sites, the peptide acts as a neurotransmitter or neuromodulator to facilitate sexual behavior. Indeed, LHRH injected into the medial preoptic area, arcuate nucleus (20, 21), or the midbrain central gray area (22, 23) elicits lordosis.

Since serotonergic (29), dopaminergic (30), and noradrenergic systems (31) facilitate lordosis, the transmitters evoking LHRH release could be those released from any or all of these systems. We propose that a neuron containing one of these neurotransmitters (for example, NE) synapses via α_1 receptors with a NOergic neuron and also makes an axo-axonal synapse on an axon of a LHRH neuron terminating on a portal capillary within the hypothalamus or in the midbrain central gray (Fig. 4). These latter LHRH terminals could synapse on other neurons mediating sex behavior.

The activation of the α_1 receptors leads to the conversion of inositol phosphates into inositol triphosphate, which then liberates Ca^{2+} from intracellular stores. Increased intracellular Ca^{2+} in NOergic neurons can interact with calmodulin and activate NOS, which leads to the generation of NO and citrulline (32). NO diffuses across to the axon of the LHRH neuron and directly activates LHRH release.

In addition to LHRH, a number of other drugs can substitute for P in the activation of sexual receptivity (33). Indeed, cAMP induces sexual behavior in the E-primed rat (34), perhaps related to the previously described role of cAMP in inducing exocytosis of LHRH secretory granules. cGMP (33) can also induce sexual behavior and this could be a consequence of NO release from NOergic neurons, which could activate guanylyl cyclase in the LHRH neuron and induce release of LHRH by the usual pathway of NO action (16). Oxytocin also has been shown to induce sex behavior in the E-primed rat, and we speculate that it may act by activating the NOergic neurons, which in turn release LHRH and, consequently, induce sexual behavior. Therefore, it appears that NO is an extremely important neurochemical mediator of sexual behavior in the female rat.

Sexual behavior in the ovariectomized female rat has always been thought to be a consequence of the synergistic interaction between E and P (35, 36). However, sexual behavior in the normal rat occurs in the presence of much lower concentrations of P than are necessary to induce sexual behavior in the ovariectomized E-primed animal. In both cases there are undoubtedly several synapses distal to the LHRH synapse.

Recent studies have shown that inhibitors of NOS are capable of blocking oxytocin-induced and apomorphine-induced penile erection in the male rat by a central action since the doses were insufficient to act peripherally (37). Moreover, the potency of the compounds in preventing penile erection parallels their potency in inhibiting the activity of this enzyme in the brain (38). This suggests that NO is also involved within the brain to activate male sexual behavior. It is already well established that NOergic terminals in the corpora cavernosa of the penis mediate penile erection by local action (39). Furthermore, recent reports indicate an important role for NOergic terminals in the control of uterine smooth muscle myometrium as well (40). Thus, NO appears to play an important role in reproduction by its actions not only within the brain but also within the peripheral reproductive organs themselves.

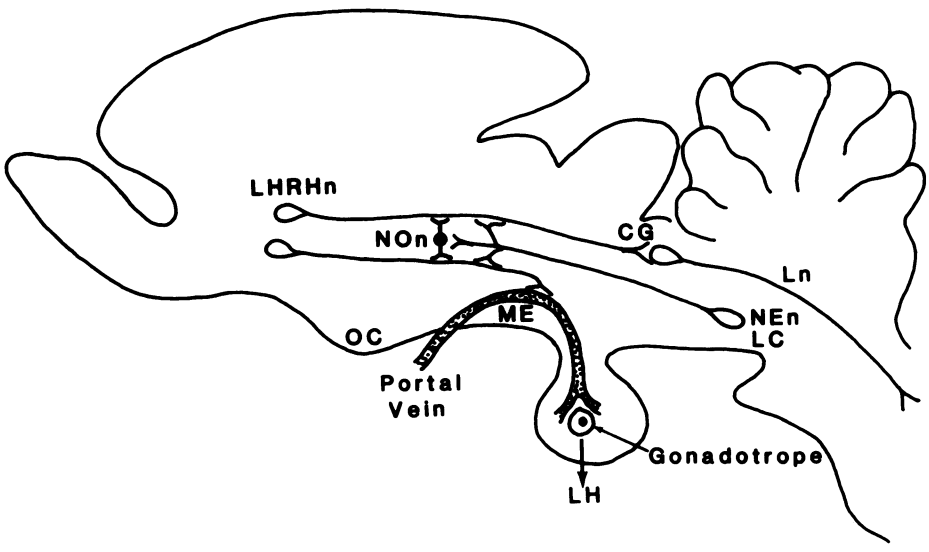

FIG. 4. Schematic diagram for the dual role of NO in the induction of LH release and lordosis response. The figure indicates hypothetical interactions of norepinephrinergic neuron (NEn) terminals with NOergic (NOn) interneurons and LHRH neuron (LHRHn) terminals to generate NO, release of LHRH, secretion of LH from the pituitary, and lordosis. CG, midbrain central gray; LC, locus coeruleus; Ln, lordosis-inducing neuron; ME, median eminence; OC, optic chiasm.

A question arises as to how the stimulatory effects of NO and LHRH relate to the well known effect of P on female sexual behavior in rats. An obvious possibility is that P, like NO, induces LHRH, which in turn induces sexual behavior (lordosis) (41, 42). Our results show that an antibody to LHRH blocks the lordotic response to P and are consistent with LHRH acting as a downstream mediator of both NO- and P-induced responses.

Recent evidence, however, suggests another alternative. For example, the stimulatory effect of P on lordosis can be blocked by antiprogestins and by direct administration into the central nervous system of antisense oligonucleotides against P receptor (PR) mRNA (43, 44). Thus, functional PR appears to be an obligate requirement for this steroid-induced behavior. It has been reported that LHRH may activate PR via a ligand-independent mechanism (45, 46). Interestingly, four distinctly different agents (LHRH, forskolin, 8-Br-cAMP, and P) can augment LHRH-stimulated LH secretion in rats and all responses are blocked by the antiprogestin RU486, implying that a common pathway is affected. As suggested by Waring and Turgeon (47), the simplest model is that RU486 acts to block PR and that the nonsteroid augmentations occur via a phosphorylation cascade, leading to ligand-independent activation of the PR. Thus PR could be a final common denominator in all of these responses and an obligate requirement for both P-mediated and nonsteroid-induced sex behavior in female rats. This alternative raises a prediction that RU486 should inhibit lordosis initiated by NO as well as by P. We recently carried out such experiments (unpublished data) and have found that RU486 prevents both NO- and P-induced lordosis.

A final, and perhaps most likely, scenario exists that both the NO- and steroid-initiated pathways are operative and that communication exists between them to an extent that they are mutually reinforcing. Future experiments will no doubt distinguish the validity of these possibilities.

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