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Review article

The role of nitric oxide in the hypothalamic control of LHRH and oxytocin release, sexual behavior and aging of the LHRH and oxytocin neurons

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Abstract: Nitric oxide (NO) affects reproductive processes both at the level of the brain and reproductive tract and this review is focused on its role as an essential regulator of the hypothalamic control of reproduction. The data gathered indicate that glutamate stimulates noradrenergic neurons which subsequently activate NO-ergic cells via α_1-adrenergic receptors. The released NO diffuses into luteinizing hormone-releasing hormone (LHRH) terminals where it triggers LHRH secretion by activation of guanylyl cyclase and cyclooxygenase. The NO released by estrogen-stimulated NO-ergic ventromedial neurons plays a crucial role in the regulation of sexual behavior. Furthermore, an increased expression of inducible nitric oxide synthase in the LHRH and oxytocin neurons underlies the destructive action of NO on the aging of the hypothalamic neuroendocrine pathways. Within the hypothalamo-hypophyseal system, NO exerts an inhibitory effect in the control of oxytocin secretion. This action seems to employ an indirect mechanism by which NO may modulate the release of GABA. This review provides an overview of the role of NO in hypothalamic control of LHRH and oxytocin release, aging of the LHRH and oxytocin neurons and sexual behavior. (www.cm-uj.krakow.pl/FHC)

Key words: Nitric oxide - LHRH - Oxytocin - Sexual behavior - Hypothalamus

Introduction

Nitric oxide, an active radical synthesized by nitric oxide synthase (NOS) [61], is known to play multiple physiological roles [75-77, 123]. In female reproductive organs nitric oxide has been recognized as an important regulator of parturition, pregnancy, implantation, oviduct function and steroidogenesis. Moreover, a correlation between circulating NO and follicular development, implicates luteinizing hormone-releasing hormone (LHRH) in the regulation of NO synthesis and folliculogenesis, thereby functionally linking hypothalamic structures with ovarian NO function [106].

In the nervous system, NO acts as a messenger of interneuronal information but, unlike traditional neurotransmitters, it is not found in the synaptic vesicles [14, 36]. In nerve cells, NO is generated by Ca^{2+}/calmodulin-stimulated NOS which catalyzes the production of NO and L-citruline from L-arginine, O_2 and NADPH-derived electrons [37] (Fig. 1). The NO is not released into the synaptic space and does not act at the postsynaptic membrane, but diffuses through cell membranes to reach its targets in neighboring neurons [41]. In the target cell, NO binds to the iron of the heme moiety of hemoprotein soluble guanylyl cyclase and cyclooxygenase, thus utilizing cyclic GMP and prostanoids as second messengers [43]. Because of its unique mechanism of action NO represents a completely new class of gaseous neurotransmitters [124, 132].

The NO releasing, NO-ergic neurons [11] express three major isoforms of the NOS enzyme. Neuronal NOS (nNOS) and endothelial (eNOS), referred to as constitutive NOS, are responsible for the continuous basal release of NO and both require calcium/calmodulin for activation [42]. A third isoform is an inducible calcium-independent subtype (iNOS) whose expression is triggered by inflammatory signaling [85]. The three isoforms of NOS are products of separate genes that share 50-60% amino acid homology [81] and display sequence similarity to the carboxy-terminal end of cytochrome P-450 reductase [13]. All NOS isoforms require nicotinamide adenine dinucleotide phosphate

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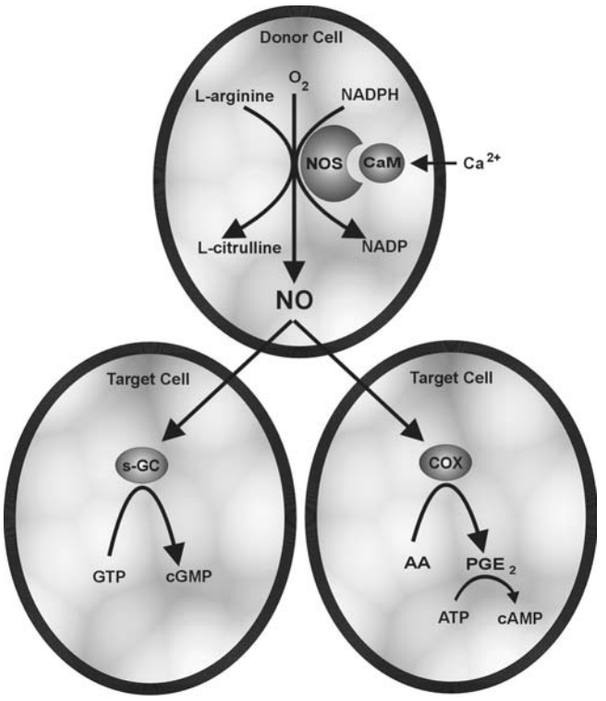


Fig. 1. Schematic diagram of the role of NO in transcellular signal transduction. NOS increases its activity in response to intracellular Ca^{2+} influx, which stimulates, via calmodulin (CaM), the NOS enzyme. NOS catalyses the conversion of O_2 and L-arginine to NO and L-citrulline. Activation of NOS requires nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor. NO diffuses to NO-responsive target cell where it binds to a heme moiety of soluble guanylyl cyclase (sGC) which, following activation, catalyses cyclic GMP (cGMP) formation. Possible NO target may be heme moiety of another hemoprotein, mainly, cyclooxygenase (COX) which, following activation, converts arachidonic acid (AA) into prostaglandin E_2 (PGE_2). Thus, PGE_2 activates adenylate cyclase causing an increase in cAMP. Since both cGMP and cAMP are second messengers, they can affect multiple enzymatic pathways in target neurons.

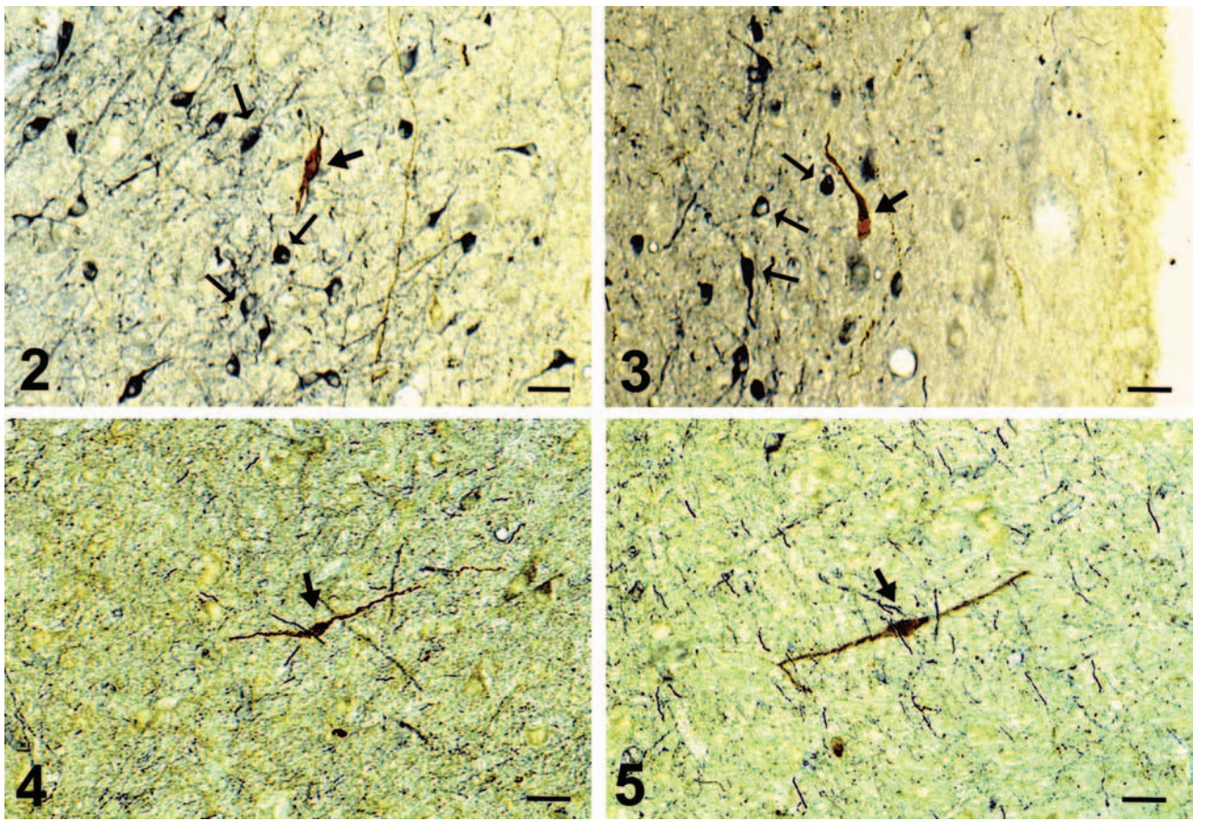
(NADPH) as an electron donor, as well as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (THB) for efficient generation of NO [68]. All three isoforms of the enzyme express enzymatic activity of NADPH-diaphorase which is used as a histochemical marker for NOS [29, 51, 130].

Previous histochemical and immunocytochemical studies revealed populations of NO-ergic neurons in various brain structures [12, 105, 132, 133]. Quantitative biochemical analysis has indicated the hypothalamus, after the cerebellum, as the second concentration site for nNOS activity in the brain [35]. In direct support of this notion are observations [15, 18, 105, 133, 139] showing numerous populations of hypothalamic, NO-ergic neurons particularly in supraoptic, paraventricular and circular nuclei, and also in the preoptic area, ventromedial and arcuate nuclei. The staining techniques have revealed NOS activity in the neuronal perikarya and processes, indicating that NO produced in the neuron may be released by the entire cell surface, including the neurosecretory terminals of the hypothalamic neurosecretory pathways [71]. Indeed, occurrence of the NO synthesizing neurons throughout the hypothalamic regions involved in neuroendocrine regulation of gonadotropin secretion, sexual behavior and parturition coupled with its high permeability range up to 300 μm [37], enables NO-ergic cells to affect multiple hypothalamic systems. This review will address the significance of nitric oxide as a modulator of hypothalamic reproductive functions, focusing mainly on its effect upon LHRH and oxytocin release and sexual behavior.

Effect of nitric oxide on LHRH release

Recent immunocytochemical studies identifying NOS, as well as histochemical visualization of NADPH-d have revealed numerous populations of NO-generating neurons throughout the hypothalami of different species including rat [15-17, 105, 133], mouse [84], guinea pig [134], cat [74], monkey [110], pig [18] and human [31, 109]. Within the hypothalamus, prominent NOS stainings were reported for neurons of the preoptic area which is a well-documented production site for LHRH [24, 60, 62, 119, 120, 137, 138, 143]. Nuclei of the preoptic area together with arcuate/median eminence (ARC/ME) complex constitute the hormonal sex center [32]. Interestingly, LHRH and both NOS mRNAs [44, 52] as well as NOS proteins [18, 48] were shown to be expressed in separate populations of preoptic neurons. The exceptionally high activity of NOS in the population of preoptic neurons localized in the direct vicinity of the LHRH hypothalamic system (Figs. 2-5) indicates a capacity for NO-ergic control over the LHRH production and release [18, 44, 48]. Indeed, *in vivo* application of NOS inhibitors resulted in the suppression of pulsatile and steroid-induced LHRH release [9, 100]. These observations are consistent with *in vitro* studies on dissected arcuate/median eminence complex and with immortalized GT-1 LHRH-producing cells, showing an inhibitory action of the NOS inhibitors on LHRH secretion [8, 79, 103], confirmed currently by study of Karanth *et al.* [56-58]. Moreover, sodium nitroprusside, a spontaneous NO donor, has been shown to increase LHRH release from the ARC/ME complex and from cultured GT-1 cell line [79, 103] indicating a key function of NO in the modulation of LHRH secretion.

Previous reports indicated that noradrenaline (Fig. 6), and to a lesser extent dopamine can stimulate hypothalamic LHRH release [88, 89, 118]. In this context it is interesting that preoptic noradrenergic neurons may coexpress NOS (Figs. 7, 8). Recent studies have shown



Figs. 2, 3. Double labeling of LHRH/NADPH-d of the porcine medial preoptic nucleus. The analysis revealed two separate populations of neighboring nerve cells expressing NADPH-d histochemical activity (blue) (small arrows) or LHRH-immunoreactivity (brown) (large arrow). Bar = 30 μm. **Fig. 4.** Double labeling of LHRH/NADPH-d in the porcine medial preoptic nucleus. Arrow indicates point of possible contact between differentially stained LHRH-immunoreactive cell (brown) and NADPH-d-positive fiber (blue). Bar = 30 μm. **Fig. 5.** Double labeling of LHRH/NADPH-d in the porcine lateral preoptic nucleus. Arrow indicates point of the possible contact between the NADPH-d-positive fiber (blue) and LHRH-IR neuron (brown). Bar = 30 μm.

that adrenergic stimulation of LHRH release involves activation of the adrenergic receptor on NO-ergic neurons [20, 100, 116]. Noradrenaline exerts its effect via the α_1-adrenergic receptor which stimulates the release of NO from NO-ergic neurons. The NO diffuses to the adjacent LHRH neurons causing an increase in the free intracellular calcium required for the activation of phospholipase A_2. It is believed that phospholipase A_2 converts membrane phospholipids in the LHRH terminals to arachidonate, which can then be processed by activated cyclooxygenase into PGE_2. The PGE_2-dependent activation of adenylate cyclase causes cAMP release, which in turn activates the protein kinase-A leading to exocytosis of LHRH secretory granules from neurosecretory terminals [20, 69, 70, 100].

LHRH reaches gonadotrophs of the anterior pituitary gland via hypophyseal portal vessels, thereby mediating the LH release, which in turn stimulates steroid secretion from the ovary and induces ovulation [40]. Although there is no doubt that ovarian steroids affect the secretory activity of the hypothalamic LHRH neurons, paradoxically those neurons do not contain estrogen receptor (ER) [50, 64, 118, 128, 136]. It has been shown that NO-ergic neurons embracing the preoptic LHRH cells express estrogen receptor (Fig. 9) [18, 87] and treatment with estradiol benzoate increased NOS expression in these cells [87], indicating the role of NO as a transducer of estrogenic information for LHRH neurons. The separate cellular expression of NOS/ER *versus* LHRH in preoptic neurons does not seem to be crucial, since NO produced at a single point source should be able to act within an area of 0.3 mm in diameter [37]. It has been well documented that ER-expressing preoptic neurons may contain many active substances known to affect LHRH release such as neurotensin [50], galanin [7], natriuretic peptide [135], GABA [34], CGRP [49]. It remains to be elucidated whether the preoptic ER/NO-ergic neurons may produce parallel to NO additional modulators controling the secretory function of LHRH neurons or the activity of the NO-ergic system itself. Such versatility in the histochemical signaling of ER/NO-ergic neurons would strengthen the position of NO

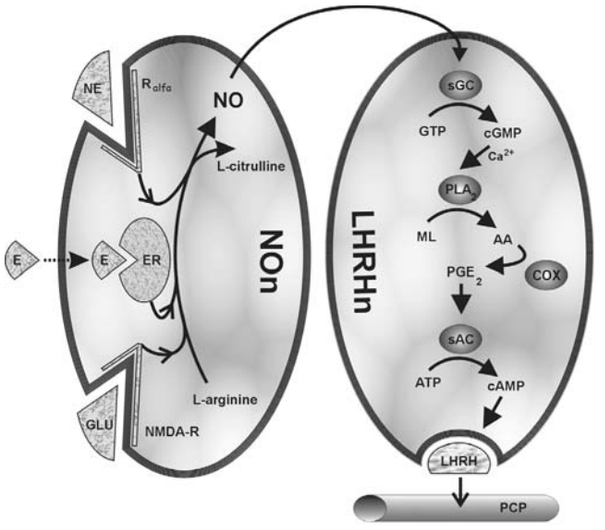


Fig. 6. Schematic diagram showing the role of NO in the control of LHRH release. NO stimulates the release of luteinizing hormone-releasing hormone (LHRH) in response to norepinephrine (NE), estrogen (E) and glutamic acid (GLU). NO released from vicinal NO-ergic neuron diffuses to NO-responsive LHRH neurosecretory neuron causing sGC-catalyzed conversion of GTP into cGMP. The increased cGMP accompanied by elevated Ca^{2+} activates phospholipase A_2 (PLA_2) to provide arachidonic acid (AA) from hydrolysis of membrane phospholipids. COX then causes the conversion of arachidonate into PGE_2. PGE_2 activates adenylate cyclase leading to an increase in cAMP and subsequent activation of protein kinase A, which induces exocytosis of LHRH into the primary capillary plexus (PCP) of the median eminence. NOn, NO-ergic neuron; LHRHn, LHRH-ergic neuron; R_{alfa}, α_1 adrenergic receptor; ER, estrogen receptor; NMDA-R, N-methyl-D-aspartate receptor; sGC, soluble guanylyl cyclase; ML, membrane phospholipids; COX, cyclooxygenase; sAC, soluble adenylate cyclase.

as a mediator of the steroidogenic control of LHRH release.

The role of NO in modulating LHRH-induced gonadotropin secretion depends also on oxytocin. In human, [26] the administration of oxytocin did not affect the gonadotropin responses to LHRH. In contrast, NOS inhibitor N,G-nitro-L-arginine methyl ester (L-NAME) substantionally reduced both luteinizing hormone (LH) and follicle stimulating hormone (FSH) release induced by LHRH. When L-NAME was applied in the presence of oxytocin, the LH and FSH responses to LHRH were similar to those observed after the administration of LHRH alone. These results indicate oxytocin capacity to abolish L-NAME inhibitory action on LHRH-induced LH and FSH release. The exact mechanism of this NO restoring action of oxytocin in the control of gonadotropin secretion induced by LHRH has to be confronted with the fact that NO itself affects oxytocin release (see last chapter).

Glutamate is another possible candidate for NO-ergic control of LHRH secretion. Stimulation of the NMDA receptor increased LHRH release [10] whose action was shown to be mediated by NO [66], probably due to expression of the NMDA receptor in NOS-containing hypothalamic cells [6]. Suppression of the glutamate-stimulated LHRH release by phentolamine, an α_1-adrenoreceptor blocker, also suggests that glutamatergic control of the LHRH release is mediated by adrenergic neurons [55]. The available evidence suggests that NO may exert a bidirectional action, in part mediating the adrenergic stimulatory effects on LHRH release through the PGE_2 pathway [102]. On the other hand, NO released as a consequence of adrenergic stimulation may suppress noradrenaline release, constituting an ultra-short feedback loop restraining the LHRH release [115].

Role of nitric oxide in sexual behavior

The ventromedial nucleus regarded as a hypothalamic center controlling sexual behavior [91, 92] contains both nitric oxide synthase [15, 18, 133, 140] and estrogen receptors [90]. In the ventrolateral aspect of the nucleus, the estrogen receptors have been found to be expressed in numerous NADPH-d-positive neurons (Fig. 10) [18, 97] indicating a potential role of NO in sexual behavior. The biological significance of such colocalization is demonstrated by an increased expression of NADPH-d [88] as well as both nNOS mRNA and protein following estrogen stimulation of ovariectomized rats [22] and by increase in the number of NADPH-d cells following estradiol treatment in the ovariectomized ewes [30]. This suggests that estrogen may directly regulate the neuronal expression of NOS in the ventromedial nucleus. Consequently, an increase in nNOS may result in elevated NO production and is potentially relevant to the facilitation of lordosis behavior [97].

To confirm the role of NO in female sexual behavior, Mani *et al.* [67] applied an intracerebroventricular (ICV) injection of NOS inhibitor in ovariectomized, estrogen primed rats. N^G-monomethyl-L-arginine prevented progesterone-facilitated lordosis, whereas the ICV microinjection of sodium nitroprusside, a spontaneous NO donor, facilitated lordosis in estrogen-primed rats in the absence of progesterone. Concurrently, the nitric oxide-cGMP-protein kinase G pathway has been involved in the facilitation of progesterone-induced lordosis and proceptivity behavior in estrogen-primed rats [39]. The NO-ergic neurons could affect sexual behavior through their action on LHRH neurons [79, 100] since LHRH facilitates the display of lordosis behavior in the estrogen-primed female rat [93]. In line with this contention, NO mediates the stimulatory action of norepinephrine [100], glutamate [66], oxytocin [99] and leptin [141] on LHRH secretion. Taken together, the NO-cGMP physiological pathway, with NO as a key intercellular messenger, is especially suited as a convergent mechanism for control of reproductive functions by various neurotransmitters and hormones [70].

Nitric oxide and aging of LHRH and oxytocin systems

While the involvement of NO in hypothalamic regulation of LHRH and oxytocin release is becoming accepted, the putative role of NO as a potential proapoptotic factor for LHRH and oxytocin neurons has not been extensively studied until recently. Vernet *et al.* [131] suggested that increased expression of iNOS may lead to neurotoxicity, which can be involved in impaired pulsatile LHRH secretion, as well as acts as a possible inducer of age-associated neuronal loss. Recent findings of aging-related iNOS induction in LHRH and oxytocinergic neurons [33] support the view that iNOS expression is associated with the previously observed decrease in the number of LHRH [45, 46] and oxytocin [5] cells. This suggests an additional, possibly destructive action of NO on the hypothalamic neuroendocrine pathways.

The endogenous factors that induce iNOS expression in aging LHRH and oxytocinergic hypothalamic neurons are unknown. Nevertheless, indirect observations seem to indicate cytokines as potential regulators of the age-related iNOS induction. Earlier studies revealed that TNF-γ in the cerebrospinal fluid and peripheral circulation and IL-1β and interferon-γ were increased in monocytes by aging [19, 80, 122]. Cytokines were found to be synthesized in the hypothalamus [125]. Accordingly, observation that the exogenous administration of interleukin 1-α can block the nitrergic control of LHRH release both *in vivo* and *in vitro* [101] through iNOS induction, additionally implicates cytokines in aging-related control of iNOS expression in the hypothalamic neurons.

Mechanism of action of nitric oxide on the hypothalamic oxytocin release

A number of studies have reported expression of NOS in magnocellular neurons of the hypothalamic neurosecretory system including supraoptic and paraventricular nuclei as well as neurohypophysis [12, 18, 29, 105, 112, 133]. It was also noted that, in addition to NOS, the hypothalamic magnocellular neurons coexpress oxytocin [73] implying a role for NO in parturition and lactation.

Indeed, there is a growing evidence that NO functions as a local modulator of magnocellular neuronal activity, since late pregnancy and parturition causes down-regulation of the endogenous NOS in magnocellular neurons and hypophysis [89].

Additional evidence substantiating the modulatory role which NO plays in hypothalamic magnocellular neurons comes from functional studies. Application of the NOS inhibitor L-NAME, revealed that NO exerted an inhibitory role in the control of oxytocin secretion in the rat [54] and human [25]. This effect correlates with data from electrophysiological studies where sodium nitroprusside (NO donor) and L-arginine (NO precursor) inhibited supraoptic neurons *in vivo* [126], whereas L-NAME and hemoglobin (NO scavenger) stimulated them *in vitro* [65]. An inhibitory action of NO upon magnocellular neurons seems to employ an indirect mechanism by which NO may modulate the release of other neurotransmitters in the brain [96]. The importance of NO-dependent neurotransmitter release in the brain has been especially well established with regard to GABA [21, 47, 86, 114]. A detailed morphological study revealed that GABA-ergic synapses constitute nearly 40% of the total synaptic connections of the supraoptic neurons [129], thus providing morphological evidence for a key position of GABA as a modulator of oxytocinergic neurons [78, 98]. Stern and Ludwig [127] recently showed that sodium nitroprusside and L-arginine increased the frequency and amplitude of $GABA_A$ miniature inhibitory postsynaptic currents (mIPSCs) in oxytocin cells. This supports the notion that NO-ergic inhibition of neuronal excitability in the oxytocin neurons relies on the pre- and postsynaptic potentiation of GABA-ergic synaptic activity in the supraoptic neurons. Alternatively, the stimulatory effect of NO on GABA-ergic, supraoptic and paraventricular [142] neurons, may reflect one of its regulatory actions, since in the hippocampus GABA release is biphasically dependent on NO concentration. Low concentration range around basal NO levels inhibits GABA outflow, while on the contrary, high concentrations of NO enhance GABA release [38].

Glutamate may be another neurotransmitter that is possibly interrelated with NO-ergic regulation of the supraoptic oxytocinergic neurons. Synaptic terminals expressing glutamate immunoreactivity account for approximately one-third of all synaptic terminals contacting supraoptic magnocellular neurons [72]. It is known that NO synthesis in neurons is stimulated by glutamate [59, 63]. NO can also regulate the release of glutamate depending on NO concentration in local tissues. Low NO levels decrease the release of glutamate, whereas higher concentration enhance neuronal glutamate [113, 117]. Binding of glutamate to the ionotropic NMDA glutamatergic receptor initiates opening of the Ca^{2+} channel. Augmentation of intracellular Ca^{2+} concentration leads to its binding to calmodulin, a cofactor for nitric oxide synthase and phospholipase A_2. A subsequent synthesis of NO and arachidonic acid may activate an intracellular messenger [94]. Once synthesized, NO can affect neuronal pathways in two ways [104].

In the first system, NO stimulates cGMP via guanylate cyclase in target cells [3] such as neurons and glia. The second pathway acts as a negative feedback regulator of NMDA receptor activity constituting a self-protection mechanism for NO-ergic neurons against

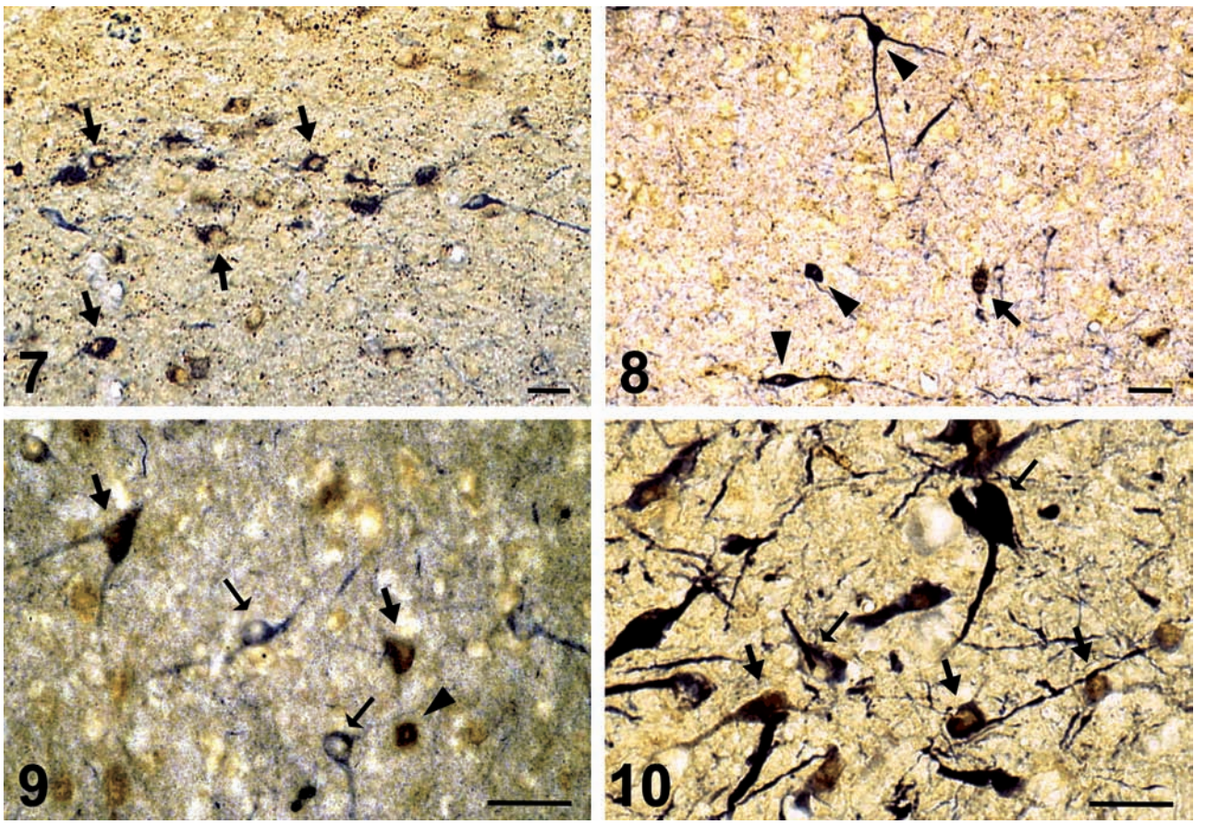


Fig. 7. The location of double labeled brown DBH-containing/blue NADPH-d-positive neurons (arrows) in the medial preoptic nucleus of the pig. Bar = 30 μm. **Fig. 8.** The lateral preoptic nucleus of the pig contains double labeled brown DBH-containing and blue NADPH-d-positive neurons (arrow) accompanied by NADPH-d-positive cells (arrowheads). Bar = 30 μm. **Fig. 9.** The NADPH-d-positive neurons (blue) of the porcine medial preoptic nucleus expressing nuclear estrogen receptors (ER) (brown) (large arrows), devoid of the receptor (small arrows) and ER-positive but NADPH-d-negative neuron (arrowhead). Bar = 30 μm. **Fig. 10.** Double labeled brown ER-expressing/blue NADPH-d-positive neurons (large arrows) adjacent to the NADPH-d-positive ER-negative cells (small arrows) in the ventro-medial nucleus of the pig. Bar = 30 μm. All micrographs are reproduced from [18], with permission of the publisher.

overexcitation by glutamatergic stimulation. This modulatory function is based on the presence of a redox, vicinal sulfhydryl group-containing site located on NMDA receptors. The thiol groups in reduced state allow Ca^{2+} influx, whereas they inhibit intracellular Ca^{2+} current while being oxidized to disulfides [1, 121]. Via NO release, the NO-ergic magnocellular neurons in addition to affecting hypothalamic glutamatergic neurons may also directly control the redox modulatory site of its NMDA receptors and thereby down-regulate Ca^{2+} influx and their own NOS catalytic activity. Cui *et al*. [28] further supports this notion, showing that in the supraoptic nucleus the NO reduces NMDA-induced depolarization in a cGMP-independent manner. An alternative regulatory mechanism emerges, in which neuronal excitability could be modulated by NO-dependent synaptic activity. This regulation of neuronal excitation could proceed via an ultra-short feedback mechanism based on auto control of the intracellular Ca^{2+} influx in supraoptic NO-ergic/oxytocinergic neurons. A feedback NO inhibition of NOS has already been reported elsewhere [4].

Hypothalamic supraoptic magnocellular neurons coexpress both nitric oxide synthase and oxytocin [73]. It has been demonstrated that estrogens up-regulate oxytocin production in the rat [23, 27, 53]. By the end of pregnancy, oxytocin accumulation increases by 50% of its total pituitary content and it is released during parturition to promote uterine contraction [107]. On the other hand, estradiol has been shown to increase neuronal expression of NOS in paraventricular [108], preoptic and ventromedial nuclei [87, 88] following ovariectomy and estradiol replacement. The number of cells stained for NADPH-d in both supraoptic and paraventricular nuclei increased in late pregnancy and lactation, during steroid treatment that mimicked late pregnancy and after chronic central oxytocin infusion in estrogen primed rats [95]. Although one has to keep in mind that Okere and Higuchi [89] reported contrasting results, the prevailing evidence indicates that estrogenic regulation of hypothalamic magnocellular neurons results in up-regulation of oxytocin production and release [23, 27, 53]. This effect occurs in oxytocin-producing neurons that also express

NOS, inducible by ovarian steroids [73]. In this context, expression of ER in preoptic and ventromedial NO-ergic neurons of the rat and pig, implicating NO as a mediator of estrogenic regulation of gonadotropin release, suggests NO as a candidate for estrogen-dependent regulator of the oxytocin release. Although this hypothesis requires additional verification, Alves *et al*. [2] have already revealed oxytocin neurons expressing ERβ in the supraoptic nucleus. This further suggests that estrogens can directly modulate a specific oxytocin system through an ERβ-mediated mechanism.

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