Nitric Oxide as Key Mediator of Neuron-to-Neuron and Endothelia-to-Glia Communication Involved in the Neuroendocrine Control of Reproduction

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Key Words

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Abstract

Nitric oxide (NO) is a peculiar chemical transmitter that freely diffuses through aqueous and lipid environments and plays a role in major aspects of brain function. Within the hypothalamus, NO exerts critical effects upon the gonadotropin-releasing hormone (GnRH) network to maintain fertility. Here, we review recent evidence that NO regulates major aspects of the GnRH neuron physiology. Far more active than once thought, NO powerfully controls GnRH neuronal activity, GnRH release and structural plasticity at the neurohemal junction. In the preoptic region, neuronal nitric oxide synthase (nNOS) activity is tightly regulated by estrogens and is found to be maximal at the proestrus stage. Natural fluctuations of estrogens control both the differential coupling of this Ca^{2+} -activated enzyme to glutamate N-methyl-D-aspartic acid receptor channels and phosphorylation-mediated nNOS activation. Furthermore, NO endogenously produced by neurons expressing nNOS acutely and directly suppresses spontaneous firing in GnRH neurons, which sug-

gests that neuronal NO may serve as a synchronizing switch within the preoptic region. At the median eminence, NO is spontaneously released from an endothelial source and follows a pulsatile and cyclic pattern of secretion. Importantly, GnRH release appears to be causally related to endothelial NO release. NO is also highly involved in mediating the dialogue set in motion between vascular endothelial cells and tanycytes that control the direct access of GnRH neurons to the pituitary portal blood during the estrous cycle. Altogether, these data raise the intriguing possibility that the neuroendocrine brain uses NO to coordinate both GnRH neuronal activity and GnRH release at key stages of reproductive physiology.

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Introduction

As the final common pathway for the central control of reproduction, gonadotropin-releasing hormone (GnRH) neurons are regulated by multiple neuronal networks and interactions with non-neuronal cells that intermingle in a complex array of transsynaptic inputs [1–3] and paracrine communication [4, 5], subjected to the modulatory influence of gonadal steroids that prompts

both chemical [6, 7] and structural plasticity [8, 9]. In rodents, the cell bodies of GnRH neuroendocrine neurons are mostly located in the preoptic region. Neuroendocrine GnRH neurons send their neuronal processes to the median eminence of the hypothalamus, where GnRH is released into the pituitary portal vessels. Upon reaching the anterior pituitary, GnRH elicits the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn stimulate gametogenesis and gonadal hormone secretion.

The present article will review recent findings that have unveiled some of the regulatory mechanisms involving neuronal and non-neuronal sources of nitric oxide (NO) in the control of GnRH neuron function. NO is indeed a very dynamic chemical transmitter that is well positioned to play a crucial role in regulating both the activity and secretion of GnRH neurons.

Nitric Oxide: Production, Synthesizing Enzymes and Downstream Targets

Originally described as the endothelial-derived relaxing factor [10–12], NO is a highly labile gaseous messenger molecule that is generated as a by-product from the conversion of L-arginine to L-citrulline. The formation of NO requires the enzyme nitric oxide synthase (NOS), in which there are three isoforms: two constitutive (i) neural-type NOS I (nNOS) and (ii) endothelial-type III (eNOS), and one inducible NOS II (iNOS) isoform [13]. All three isoforms generate NO by oxidizing a guanidino nitrogen group from L-arginine utilizing nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor [13, 14].

Mapping of the constitutive forms of NOS, through specific antibodies and NADPH-diaphorase histochemistry, show both nNOS and eNOS are expressed in the brain [15–21]. While eNOS is mainly found in the vasculature in both the periphery and the brain, it has also been documented in neurons of the hippocampus [17, 19]. The nNOS isoform is located in several populations of neuronal cells in the cerebellum, olfactory bulb, hippocampus, and is also highly expressed within neurons of the hypothalamus [15, 18, 22, 23]. While the activity of the inducible isoform is Ca^{2+} independent [24], the constitutive NOS isoforms activities are dependent on both Ca^{2+} and calmodulin [12, 14, 25, 26]. In the central nervous system, it has been well described that the activation of N-methyl-D-aspartic acid (NMDA) receptors (NMDA-R) by glutamate provides the necessary increase in intracellular cal-

cium required for activation of nNOS, whereas other pathways that increase intracellular Ca^{2+} are much less efficient in eliciting nNOS activity [12, 14, 27]. In endothelial cells, the abundance of the principal resident coat protein of caveolae, caveolin-1, appears to be an important regulator of eNOS activity [28]. Caveolin-1 interacts with eNOS [29] and leads to its inhibition in a reversible process modulated by Ca^{2+} -calmodulin [30]. Both nNOS and eNOS must be bound to the membrane, and require further phosphorylation of 1412 and 1179 serine residues, respectively, through the PI3K/AKT signaling pathway, for full activation [31, 32]. The eNOS isoform is a dually acylated peripheral membrane protein that targets into the Golgi region and plasma membrane of endothelial cells and is, itself, compartmentalized to the membrane [31]. This stands in contrast to the nNOS isoform that requires a scaffolding protein to couple itself to the membrane [33–35]. Recent studies suggested that nNOS might also be subjected to repressive sets of posttranslational modifications in vivo, such as phosphorylation at the Ser847 site by calcium-calmodulin-dependent kinase II [32, 36, 37]. Thus, the regulation of the activity of nNOS appears to be mainly dependent on three factors: sufficient Ca^{2+} influx through activation of NMDA-R, subcellular localization via a scaffolding protein, and phosphorylation of specific serine residues. Regarding eNOS, the most important mechanism for sustaining its activity is phosphorylation on serine-1179, enabling the enzyme to function at resting Ca^{2+} cytosolic concentrations [for review, see 38] and interaction of eNOS with caveolin is used to prevent inadequate NO production under basal conditions [39].

Since NO is not stored in vesicles, following its synthesis, NO diffuses across the biological membranes to produce its effect. However, the ability of NO to exert an action is delineated by its half-life and the proximity of NO-containing cells to their target, which must be within 200 μm [40–42]. This distance indeed corresponds to the physiological sphere of influence from a single-point source of NO that emits for 1–10 s [41]. In the brain, NO is typically considered to be a retrograde neurotransmitter (i.e. increasing release of other neurotransmitters, such as GABA and glutamate from presynaptic sites); however, it also acts at postsynaptic sites [for review, see 43]. The most well-documented target or ‘receptor’ of NO is the soluble guanylate cyclase (sGC), in which the activation by NO results in formation of cyclic GMP (cGMP) to mediate intracellular effects [44, 45]. NO binds to the ferrous heme of sGC with high affinity to change the conformation of sGC and therefore dramatically increases its

catalytic activity [46]. Following treatment of NO, the synthesis of prostaglandins (PGs) has also been documented [47]. The conversion of arachidonic acid to PGs requires cyclooxygenase (COX), thus COX may also be considered as a downstream target of NO. Similar to the sGC enzyme, COX contains an iron-heme group suggesting a direct interaction between NO and COX [48, 49]. Indeed, it was demonstrated that COX is a direct target for NO, and the increase of PGs was independent of cGMP [48, 49]. Of note, the formation of PGE₂ by COX appears to play an important role in the neuroendocrine control of the reproductive axis [50, 51].

Neuronal Nitric Oxide Modulates GnRH Neuronal Activity and Secretion during the Ovarian Cycle

Since the early 1990s, NO has been known to be a regulator of LH secretion [for review, see 1, 52–54]. Indeed, several pharmacological studies suggested NO as a key modulator of GnRH secretion [55, 56], and a contributor in the onset of the preovulatory surge of GnRH/LH [57–61]. Intracerebroventricular injection of NOS antisense oligodeoxynucleotides was shown to block the LH surge in steroid-primed ovariectomized rats [60], whereas intracerebral infusion of N^G-nitro-L-arginine methyl ester (L-NAME, a NOS inhibitor) either within the preoptic

region (fig. 1) or the median eminence caused a marked disruption of rat estrous cyclicity [62, 63]. Intriguingly, in mutant mice, the first targeted disruption of the nNOS gene in exon 2 did not markedly alter fertility [64]. However, these mutant mice retained residual nNOS activity [64]. In contrast, deletion of exon 6, which harbors the catalytic domain of nNOS, was shown to cause hypogonadotropic hypogonadism and infertility in mutant mice [65].

Neuronal NO Exerts an Acute Postsynaptic Action on GnRH Neurons

Early studies using immortalized GnRH cell lines suggested that NO acts directly at the level of the cell body to either inhibit [66] or stimulate GnRH release and synchronize pulsatile GnRH secretion [55, 66–68]. In contrast to these in vitro models where GnRH-secreting cells express NO-producing enzymes [67, 69, 70], consistent mapping of the nNOS isoform within the hypothalamus demonstrated that GnRH perikarya are surrounded by nNOS neurons, but do not express nNOS themselves [22, 71–73] (fig. 1a). Due to the scattered nature of GnRH neurons across the preoptic region, the putative action of NO at the level of the GnRH cell body was difficult to assess. With the advancement in genetic techniques and the engineering of mice expressing green fluorescent protein (GFP) in GnRH neurons [74–76], GnRH neuronal activ-

Fig. 1. Within the preoptic region, NO release modulates GnRH neuronal activity and is required for estrous cyclicity. **a** The relationship of NO-producing neurons stained with NADPH-diaphorase to GnRH neurons in GnRH-GFP transgenic mice. NOS and neuronal NADPH diaphorase are identical in brain and peripheral tissues [20, 21]. Arrows show close relationships between NADPH-diaphorase-containing neurons (dark precipitate) and GnRH-GFP neurons (green). Scale bar: 40 μ m. **b** Whole-cell patch-clamp recordings show that L-arginine (L-Arg, the precursor of NO) alters spontaneous firing in GnRH neurons (top panel). Application of L-NAME, a NOS inhibitor, prevented L-Arg-mediated inhibition of spontaneous discharges in L-Arg-responsive GnRH neurons (bottom panel) [73]. **c** Microscopic visualization of nNOS catalytic activity using immunofluorescence to localize L-citrulline, a by-product of NO synthesis. Representative illustration of nNOS-expressing neurons (red) and L-citrulline detection (green) by fluorescent immunocytochemistry in coronal brain sections from diestrous (Di 16h) and proestrous (Pro 16h) rats. V3 = Third ventricle; oc = optic chiasm. Right panels: high magnifications of the areas delineated by rectangles. Arrowheads indicate double-labeled neurons expressing nNOS and L-citrulline. Arrows indicate nNOS single-labeled neurons. Low magnification scale bar: 210 μ m; high magnification scale bar: 60 μ m. **d** Top panel: representative profiles of spontaneous NO secretion from preoptic region explants at

two different stages of the estrous cycle in the female rat. Differential current measured by the self-referencing probe, converted to flux (vertical axis), increased after the addition of single preoptic explants in survival medium at the time indicated by arrow. NO effluxes recorded from each explant during the 90-min data acquisition phase were averaged and plotted on the bar graph. Comparisons between stages of the estrous cycle indicated that preoptic explants produced significantly more NO on the afternoon of proestrus than on the afternoon of diestrus (* $p = 0.014$). Bottom panel: representative response of a proestrous rat preoptic explant to vehicle (veh, arrowhead) or to 1 mM L-NAME (arrow). Comparisons between treatments indicated that L-NAME, but not vehicle, significantly reduced NO production by the preoptic region during proestrus (* $p = 0.017$). The transient drops in current upon substance application (arrowhead, arrow) are artifacts. **e** Disruption of estrous cyclicity in young adult rats by the pharmacological blockade of NO synthesis targeted to the preoptic region of the hypothalamus. L-NAME (5 mM) or vehicle (NaCl 0.9%) delivered via a stereotaxically implanted stainless steel cannula connected to a subcutaneously placed osmotic pump delivering its content at a rate of 0.5 μ l/h for 7 days. Infusion starts at P0 (arrow pointing up) and ends 7 days later (arrow pointing down), when the pump content is exhausted. Di = Diestrus; Pro = proestrus; Es = estrus [adapted from 62, 73, with permission].

ity can now be readily examined in brain slice preparations. Using patch-clamp recordings from GnRH-GFP mouse brain slices, our laboratory has provided the first electrophysiological evidence that NO is a direct modulator of native GnRH neuron excitability [73]. Both endogenous and exogenous sources of NO were shown to cause acute inhibition of spontaneous firing in GnRH neurons [73] (fig. 1b). Importantly, L-arginine (the natural sub-

strate for NOS-mediated NO production) inhibitory effects on GnRH bursting activity were abolished when brain slices were incubated with L-NAME, a broad-spectrum NOS inhibitor (fig. 1b), or (4S)-N-(4-amino-5-[aminoalkyl]aminopentyl)-N'-nitroguanidines (AAANG), a selective inhibitor of nNOS [73, 77]. The ability of NO to inhibit GnRH neuron firing and induce GnRH neuron hyperpolarization is retained during syn-

aptic uncoupling with a medium containing low- Ca^{2+} and high- Mg^{2+} to block Ca^{2+} -dependent presynaptic release, and TTX to block voltage-gated sodium channels. This indicates a direct action of NO at a postsynaptic site to change membrane properties in GnRH neurons [73]. Notably, NO appears to require the sGC-cGMP signaling cascade to modulate neuronal excitability in GnRH neurons. The presence of a sGC antagonist blocked the inhibition of firing promoted by NO in GnRH neurons, while an exogenous cGMP analog mimicked the action of NO [73]. By revealing that NO is a direct modulator of GnRH neuronal activity, these data introduce the intriguing possibility that the neuronal release of this highly diffusible gaseous neurotransmitter with a short half-life (<1 s) may be a key mechanism used by the neuroendocrine brain to both modulate bursting firing patterns, and set into phase the bursting activity of GnRH neurons (fig. 1). Because NO production is key to the occurrence of the preovulatory surge of GnRH [57–61], it is tempting to speculate that NO may serve as a transitory switch between pulse and peak release of GnRH in proestrus [78–80]. Such a switch could be operated by estrogens during the onset of the preovulatory surge [7] and might require the intervention of additional neuronal circuits, including kisspeptin neurons, as discussed below.

Estrogens Modulate Neuronal Nitric Oxide Signaling within the Preoptic Region

Should NO act to synchronize GnRH neuronal cell activity, a critical question remains: Does the release of NO/nNOS activity fluctuate in parallel with gonadal hormones across the estrous cycle? Amperometric measurements performed with a NO-specific probe on rat hypothalamus explants showed that NO production varies during the estrous cycle in the preoptic region and that the amplitude of NO effluxes is significantly higher in proestrus (when estrogen levels are highest) than in diestrus [62] (fig. 1d). Concurrently, the microscopic visualization of NOS catalytic activity using immunohistofluorescence to localize L-citrulline, which is formed stoichiometrically with NO [12] showed that the proportion of nNOS neurons producing NO is significantly higher in proestrus than in diestrus II in the preoptic region (average citrulline/nNOS co-expression, Di 16h $78 \pm 5\%$, Pro 16h $90 \pm 2\%$; t test, $p = 0.049$, $n = 6$ per stage of the estrous cycle; fig. 1c) [62].

Intriguingly, in contrast with previous studies suggesting that estrogen could modulate hypothalamic nNOS gene expression [58, 81–84], these changes in NO secretion seen during the estrous cycle are not associated with

changes in nNOS protein synthesis but rather with changes in nNOS activity [62, 85]. Estradiol induces the formation of a NMDA-R-nNOS complex in neurons of the preoptic region [62, 85], thus enhancing NO secretion [86] by coupling nNOS to its main stimulatory calcium influx pathway [12, 14] (box). This differential coupling of nNOS with NMDA-Rs during the estrous cycle was shown to involve the scaffolding protein postsynaptic density-95 (PSD-95) [62, 86] (fig. 2) and to require estrogen receptor (ER) activity [86] (box). PSD-95 knockdown via the administration of antisense PSD-95 oligodeoxynucleotides strikingly impaired ovarian cyclicity [62] (fig. 2) and nNOS activity in preoptic neurons both in vitro [86] and in vivo [62]. Importantly, inhibition of estradiol-induced NO release by selective NMDA-R blockers demonstrated that estrogen actually promotes the coupling of glutamatergic fluxes for NO production in preoptic neurons [86]. The evidence that NO-producing neurons in the hypothalamus could be targets of glutamate was demonstrated by neuroanatomical studies showing that virtually all preoptic nNOS neurons, which are also known to express ER- α [87, 88], express NMDA receptors [62, 71]. Interestingly, other studies have shown that most NMDA-R-expressing neurons of the preoptic region also contain ER- α [89], which can be visualized in cell nuclei, perikaryal cytoplasm, and dendrites [90, 91]. In parallel to promoting changes in protein-protein interactions, natural fluctuations of estrogens across the ovarian cycle were also recently shown to regulate the state of activation of nNOS through changes in nNOS stimulatory phosphorylation levels in the preoptic region [85]. Phosphorylation of nNOS at Ser1412 was shown to be maximal on the afternoon of proestrus and this phosphorylation-activated nNOS isoform was seen to physically interact with the PSD-95/NMDA-R complex at the plasma membrane [85] (box). Correspondingly, in physiologically relevant environmental conditions associated with low LH release, such as fasting, the phosphorylation of nNOS is decreased [92]. In vitro experiments performed in primary culture of hypothalamic neurons showed that estradiol promotes phosphorylation of nNOS at Ser1412 via a Src/PI3K/Akt-dependent pathway [93]. The above findings suggest a direct action of estrogens on nNOS neurons of the preoptic region. This would couple NO production to glutamate fluxes that exert a well-known stimulatory influence on GnRH secretion during their positive feedback on the hypothalamus [94, 95]. The resulting production of NO may then act on GnRH neuron cell bodies to synchronize their activity and adjust their firing behavior in a meaningful manner to enable peak release of GnRH.

Box: Schematic representation of the possible estradiol-mediated changes in protein-protein interactions involved in the control of nNOS activity in the preoptic region of the hypothalamus during the ovarian cycle. nNOS activity is primarily regulated by increases in the local intracellular $[\text{Ca}^{2+}]$ (*), which activates nNOS through calmodulin (CaM) binding [14]. The physical interaction of nNOS with NMDA-Rs involves the PSD-95 scaffolding protein and the assembly of a ternary complex [34]. Only Ca^{2+} influx through the NMDA-R promotes an efficient NO production [12, 14]. In parallel, nNOS is also subjected to posttranscriptional modifications (such as phosphorylation) that modulates its catalytic activity [32, 142–144]. Natural fluctuations of estrogen levels across the ovarian cycle, i.e. low in diestrus and high in proestrus, regulate the activation state of nNOS by modulating its coupling with NR2B-containing NMDA-Rs by the PSD-95 scaffolding protein. In turn, this regulation, that could be mediated by estrogen-dependent ER activation [86], results in the phosphorylation

of nNOS, an effect known to increase nNOS enzymatic activity [31, 32]. Estrogens may induce nNOS phosphorylation through the activation of the Src/PI3K/Akt pathway [93]. The mechanism by which estrogen promotes nNOS anchoring to the PSD-95/NMDA-R complex remains unknown. Estrogen exhibits widespread effects throughout the brain and its actions are intensively investigated, but very few studies have considered the relationship between estrogen and PSD-95. In vitro estrogen upregulates the synthesis of PSD-95 via the Akt/protein kinase B pathway [145] and alters the shape of hippocampal dendritic spine [146] where PSD-95 is present in high amounts. Perhaps the nNOS association with PSD-95 is enhanced at synaptic sites in response to dynamic events at the postsynaptic density. One plausible mechanism for this would be that the estrogen signalling mediates the coalescence of cytoskeleton-tethered nNOS [147] to PSD-95 through spine formation, which would require remodeling of the actin cytoskeleton [148] [adapted from 85, with permission].

A Role for NO in Kisspeptin Signaling?

ER α -expressing neurons of the anteroventral periventricular nucleus (AVPV), the median preoptic nucleus (MnPO) and the periventricular hypothalamic nucleus (Pe), containing a significant subset of hypothalamic nNOS neurons [23, 62, 73, 84], are critical for estrogen-positive feedback to the GnRH neurons [96, 97]. While NO trans-

mission was originally postulated as one of the signals mediating gonadal steroid-induced and preovulatory GnRH/LH surge [57, 60, 61], the emergence for the key role of *Kiss1*-expressing neurons in the AVPV has eclipsed this notion [98–100]. Even though mutant studies unequivocally revealed the fundamental role of kisspeptin signaling in the central control of mammalian reproduc-

Fig. 2. The estrous cycle promotes changes in nNOS coupling to glutamate NMDA-R channels and requires the scaffolding protein PSD-95 in the preoptic region. **a** Co-immunoprecipitation experiments show that NMDA-R NR2B subunit is highly physically associated with nNOS on the afternoon of proestrus (top panel). The bottom panel shows that differential NR2B/nNOS-complex formation involves changes in physical association of PSD-95/nNOS. **b–d** Central administration of an antisense PSD-95 ODN results in estrous cycle disruption in sexually mature rats. **b** Confocal microscopy images showing that AS-PSD-95 FITC-coupled ODNs directly target NR2B-expressing nNOS neurons (arrowhead) in vivo when stereotactically infused into the preoptic region. **c** Confocal microscopy images illustrating a GnRH-immunoreactive neuron (red) surrounded by cells that incorporated AS-PSD-95 FITC-coupled ODNs (green, arrowheads)

in the preoptic region. **d** Representative estrous cycle profiles showing disruption of estrous cyclicity in young adult rats by the infusion of AS-PSD-95 into the hypothalamic preoptic region. Infusion starts at day 0 (arrow pointing up), and ends 7 days later (arrow pointing down), when the pump content is exhausted. Di = Diestrus; Pro = proestrus; Es = estrus; SE-PSD-95, sense PSD-95-ODNs. **e** Selective decrease of PSD-95 expression and its association with NR2B in hypothalamic neurons treated with the antisense oligodeoxynucleotide to PSD-95 (PSD-95 ODN) in vitro. Cells were treated with the antisense ODN (5 μ M) or a sense sequence for 7 days, then protein extracts were immunoprecipitated with antibodies to PSD-95 and sequentially immunoblotted with NR2B and PSD-95 antibodies. The supernatant resulting from immunoprecipitation was subjected to straight Western blotting for actin [adapted from 62, with permission].

Fig. 3. In the median eminence of the hypothalamus, endothelial NO secretion may represent one of the synchronizing cues that by coordinating GnRH release from GnRH neuroendocrine terminals that are distributed over 2 mm within the median eminence allows the occurrence of functionally meaningful episodes of GnRH secretion. **a** Photomicrograph showing GnRH axonal fibers in the external zone of the median eminence (green fluorescence, arrows) in close apposition to eNOS-immunoreactive portal vasculature (red fluorescence, arrowheads). 3V = Third ven-

tricle. The dotted lines outline the third ventricle. Scale bar: 75 μ m. **b** Real-time amperometric measurement of spontaneous NO release from median eminence explants at different stages of the rat estrous cycle. DiII = Diestrus II; PRO = proestrus; E = estrus. **c** On the afternoon of proestrus, the preovulatory GnRH/NO release is blocked with L-NIO, an NOS inhibitor selective for eNOS at 0.5 μ M. * and ^a = Significantly different from treated samples, $p = 0.05$. AUC = Area under the curve during a 30-min period [adapted from 54, 114, with permission].

tion [101–105], recent studies demonstrated that intracerebroventricular injection of NMDA, the selective agonist of glutamate NMDA-Rs, induces nNOS neuron activation and LH release in kisspeptin and kisspeptin receptor knockout mice [106]. These findings suggest that glutamate-stimulated NO production triggers GnRH neuronal function in a kisspeptin-independent manner. Interestingly, preliminary data show that kisspeptin- and nNOS-containing neurons morphologically interact in the preoptic region [107]. Thus, it is possible that in addition to act both directly on GnRH neurons and via actions on synaptic afferents [108–110], kisspeptin neurons may also talk to nNOS neurons and promote NO production that could serve as an intermediary synchronizing ‘switch’. This interesting possibility awaits further study.

Endothelial Nitric Oxide Controls GnRH Release at the Neurohemal Junction

Neuroendocrine GnRH neurons project to the median eminence of the hypothalamus, where they release their decapeptide into the pituitary portal blood vessels for delivery to the anterior pituitary. The median eminence,

which lies ventrally to the third ventricle in the tuberal region of the hypothalamus, is increasingly recognized as a key site for GnRH release regulation [5, 9, 111, 112]. Modulation of GnRH release by NO within the median eminence was suggested by early studies performed by McCann and colleagues. They demonstrated that NO stimulates release of GnRH from explants of the medial basal hypothalamus, containing the median eminence [for review, see 53]. Neuroanatomical studies showed that the two types of constitutive NOS were expressed within the median eminence [18, 22, 54, 113]. Notably, nNOS expression is restricted to axons of the magnocellular neurons that travel in the internal zone to end in the neurohypophysis, and are distinctly segregated from GnRH axon terminals [22]. In contrast, eNOS is abundantly expressed by vascular endothelial cells in the external zone lying only a few micrometers away from GnRH nerve terminals [54] (fig. 3a). The source of median eminence NO production involved in the regulation of GnRH release was identified with the development of amperometric methods, allowing for real-time measurements of NO release [114]. These studies demonstrated that NO was spontaneously released in a pulsatile manner, and that NO secretion was positively correlated to GnRH release

[114] (fig. 3b). Notably, the efflux of NO from the median eminence occurs at pulse rate (32 ± 1 min) [114] strikingly similar to the pulse rate of GnRH secretion from rat median eminence explants (33 ± 8 min) [115, 116]. Critically, the treatment of a selective eNOS inhibitor (N^5 -(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO)) to median eminence explants obtained from rats on the afternoon of proestrus blunted both NO and GnRH release [114], thus demonstrating these two events were causally linked (fig. 3c). These results together with the selective expression of eNOS in the capillary zone of the median eminence (fig. 3a) suggest that the major source of NO regulating GnRH release within the median eminence during the estrous cycle is endothelial in origin. The key role of median eminence NO in the control of estrous cyclicity was demonstrated by the chronic infusion of L-NAME, a NO synthesis blocker, within the median eminence that reversibly altered the ovarian cycle [63].

Estrogens Modulate Endothelial Nitric Oxide Signaling within the Median Eminence

Amperometric analysis showed a variation of the amplitude of median eminence NO across the estrous cycle reaching maximal values on the day of proestrus [114] alongside the increase in GnRH pulse amplitude known to occur in vivo [80]. Estrous cycle effects on NO effluxes are mimicked by estradiol treatment in ovariectomized rats [114], suggesting that estrogens drive the increase in the amplitude of NO release at the onset of the preovulatory surge. This long-term stimulatory effect of estrogens appears to involve upregulation of *Nos2* gene expression [117, 118] and downregulation of caveolin-1 protein synthesis [117], which is a well-known specific endogenous inhibitor of eNOS activity [119, 120]. Interestingly, estrogens were also shown to have an acute stimulatory effect on median eminence NO/GnRH release that might be mediated through an ER-dependent non-genomic signaling pathway regulating eNOS activity [121] by promoting changes in intracellular calcium concentrations in endothelial cells [122].

Endothelial Nitric Oxide May Be a Key Mediator of Neuronal-Glial Plasticity in the GnRH System during the Estrous Cycle

Within the median eminence, GnRH axon terminals are intimately associated with cell processes belonging to specialized unciliated ependymoglial cells named tanycytes. Tanycyte cell bodies are tied at their apex by tight junctions [123] and line the floor of the third ventricle. They send long fine processes that eventually contact the

floor of the brain via end-feet processes where the pituitary portal vessels reside [124]. These tanycytic end-feet processes have been found not only to wrap the GnRH nerve terminals [125–128], possibly providing a diffusion barrier, but also display a high degree of structural plasticity across the estrous cycle in rats [129, 130] and seasonal breeding periods in quails [131]. During the estrous cycle, under basal conditions, e.g. in diestrus, the GnRH nerve terminals are completely enclosed by tanycyte end-feet [129, 130]. In proestrus, following activation of the reproductive axis, presumably by increasing levels of gonadal steroids [125], end-feet processes are retracted, thus allowing GnRH neurons to directly contact the pericapillary space [130]. These data argue for the importance of tanycyte structural rearrangement in delivering peak levels of GnRH to the pituitary during the preovulatory surge. The intriguing possibility that endothelial NO could be involved, at least in part, in the control of this plastic phenomenon arises from recent studies using immunopanning methods to purify vascular endothelial cells of the median eminence and culture them with isolated tanycytes [63, 132]. These studies showed that median eminence endothelial cells were capable of promoting acute actin cytoskeleton reorganization in tanycytes (within 30 min of co-culture) via the release of NO. Inhibition of NO production by preincubating vascular endothelial cells with L-NAME [63], or infection of the median eminence endothelial cells with an adenovirus expressing a dominant negative form of eNOS (DN-eNOS) [132], prevented endothelial cell-promoted actin cytoskeleton remodeling in tanycytes. Conversely, tanycyte treatment with sodium nitroprusside (SNP), a NO donor, at concentrations releasing physiological doses of NO, mimicked the co-culture effects [63, 132]. Downstream effectors of endothelial NO-mediated plasticity in tanycytes were shown to be both sGC- and COX-dependent [63]. Notably, estradiol was shown to enhance endothelial-to-tanycyte communication by causing endothelial cell-promoted acute retraction of tanycytic processes in an endothelial NO-dependent manner [132]. These effects are indeed abolished when the DN-eNOS is expressed in endothelial cells (fig. 4a) and partially mimicked when endogenous production of NO is triggered by L-arginine in non-estradiol-treated co-cultures [132]. Moreover, treatment of diestrous median eminence explants with L-arginine also caused tanycytic processes surrounding GnRH nerve terminals to undergo acute retraction (within 30 min), enabling GnRH neurons to form direct neurohemal junctions, as demonstrated by electron microscopy [63] (fig. 4b).

Fig. 4. NO mediates the vascular endothelial cell-induced plasticity in tanycytes of the median eminence. **a** eNOS activity is required for endothelial cell to promote actin cytoskeleton remodeling and cell plasticity in tanycytes. Tanycytes (Tan) cultured with endothelial cells of the median eminence (ECME) for 30 min exhibit bundles of actin filaments forming parallel stress fibers (arrows, control panel) running throughout the length of the cells, but do not exhibit cortical actin (little arrowhead). Estradiol treatment (5 nM, 48 h) promotes dramatic cytoplasm and process retraction in tanycytes co-cultured with ECME for 30 min (long arrowhead, E₂ panel). Infection of endothelial cells with an adenoviral vector expressing DN-eNOS, which abolishes eNOS activity by heterodimerizing with native eNOS [149], prevents them from promoting such plastic changes in tanycytes cultured in the presence of estradiol (E₂+DN-eNOS panel). Tanycytes co-cultured with DN-eNOS-expressing ECMEs displayed cortical actin (arrowheads). Scale bar: 10 μm. **b** Activation of en-

dogenous NO secretion in median eminence explants induces ultrastructural changes that allows GnRH nerve terminals (green, big arrowhead) to form direct neurovascular junctions in isolated median eminence explants. Median eminence explants were incubated for 30 min in the presence or absence of the NO precursor, L-arginine (L-Arg, 500 mM). Under basal unstimulated conditions (control), the GnRH axonal endings were separated from the pericapillary space (p.s.) (delineated by the parenchymatous basal lamina, white arrow). Most of the nerve endings were wrapped by a single tanycytic end-foot (Tan.). Retraction of tanycytic processes and formation of neurovascular junctions by GnRH nerve terminals that directly contact the pericapillary observed in situ (white arrow) were detected upon treatment with L-Arg. Few tanycytic processes remained around GnRH nerve terminals that had direct access to the pericapillary space (black arrows, L-Arg panel). Scale bar: 1 μm [from 63, 132, with permission].

Fig. 5. Schematic representation of endothelial-glial interactions involved in the control of GnRH neurosecretion in the median eminence. Endothelial-neuronal interactions at the level of the median eminence involves the production of NO by endothelial cells of fenestrated capillaries of the portal blood vessels. Upon its secretion, NO diffuses from its source, where it not only stimulates the release of GnRH from the neighboring GnRH neuroendocrine terminals (1') [114] but also promotes their access to the bloodstream by inducing cytoarchitectural changes in tanycytic end-feet (1–3) [63]. Downstream effectors of endothelial NO-mediated plasticity in tanycytes were shown to be both sGC and COX (1). Estrogens are likely to be the key humoral factors involved in the orchestration of the endothelia-to-glia communication that allows GnRH neurons to directly contact the pituitary portal blood vessels on the day of proestrus [132]. Estrogen treatment upregulates COX expression while leaving unchanged the expression of sGC. In addition, COX products and PGE₂ in particular (2) promote acute remodeling of actin cytoskeleton in tanycytes and cause cytoplasm retraction within tanycytic processes and end-feet (3). In parallel, estrogens stimulate eNOS expression in median eminence endothelial cells. Both tanycytes and endothelial cells were shown to express ERs in vitro [132] and in vivo [133] [adapted from 9, 150, with permission].

Estradiol appears to sensitize tanycytes to NO since subeffective doses of SNP induced cell retraction in estradiol-treated single tanycyte cultures [132]. Both tanycytes and endothelial cells were shown to express ERs in vitro [132] and in vivo [133]. Estradiol, on top of promoting eNOS expression in endothelial cells [117, 132], also up-regulates COX-1 and COX-2 expression in tanycytes, while leaving sGC expression unchanged [132]. Together with data showing that isolated tanycytes secrete PGE₂ both under control and stimulated conditions [134], and that PGE₂ stimulates acute cellular retraction in simple tanycyte cultures [132], these findings support the conclusion that the acute estradiol-induced tanycyte retraction mediated by endothelial NO depends mainly on the activation of COX pathways in tanycytes. Direct evidence for the ability of COX products to control neuronal-glia plasticity at the neurohemal junction was obtained from experiments in which PGE₂ was applied directly to median eminence explants at concentrations known to stimulate GnRH release [135–137], and structural remodeling was observed in a matter of minutes. PGE₂ treatment caused the advancement of GnRH neurosecretory terminals towards the pericapillary space, a phenomenon that probably results from the retraction of tanycyte end-feet [132]. Intriguingly, PGE₂ failed to promote direct neurovascular contacts between GnRH axons and the vascular wall. This is in contrast to the aforementioned effects of L-arginine treatment, and it suggests that additional downstream signaling pathways, such as those involving cGMP, are required for NO to fully exert its effects on neuronal-glia plasticity (fig. 5). The recent demonstration of an involvement of NO-cGMP signaling in axonal elongation and/or growth cone orientation [63, 138–140] supports this interpretation.

Results obtained in adult rats in vivo corroborate the role of PGE₂ in the mechanism that controls neuronal-glia plasticity at the neurohemal junction for GnRH neurons. Intracerebral infusion of the same COX antagonist that blocks NO-mediated actin cytoskeleton remodeling in primary cultures (indomethacin) [63] into the median eminence resulted in a marked disruption of the ovarian cycle [132], which requires the coordinated delivery of GnRH into the hypothalamo-hypophyseal portal system. Animals treated with the COX inhibitor spent most of their time in either the diestrus or the estrus phase [132], when GnRH release is low [141] and GnRH neuroendocrine terminals are enclosed by tanycyte end-feet [129, 130]. These results highlight the physiological importance of PGs in the cell-cell communication processes regulating GnRH release and they are in full agree-

ment with pioneering data obtained over 25 years ago demonstrating that PGE₂ synthesis increases within the hypothalamic region containing the median eminence during the onset of the first preovulatory surge at puberty [50], as well as with the findings that inhibitors of COX activities alter the onset of the steroid-induced LH surge in ovariectomized female rats [51].

Taken together, these findings provide strong evidence that endothelial NO synthesis in median eminence vascular endothelial cells is a crucial target for the action of estrogen on the structural remodeling that takes place at the projection site of GnRH neurons and on GnRH neurosecretion during the estrous cycle. Estrogen may induce endothelial cells to produce NO and tanycytes to make COX in the median eminence. Endothelial NO diffuses into neural tissue and acts on tanycytes to stimulate PGE₂ production, which in turn acts with additional factors that remain unidentified to promote the acute retraction of tanycyte end-feet that allows GnRH nerve terminals to form direct neurohemal junctions (fig. 5).

Conclusion

As evidenced by the findings presented in this review, a fair amount of progress has been achieved in the last 20 years towards understanding the contributions and importance of NO in GnRH neuroendocrine regulation. Evidence has accumulated in the literature to support a role of NO at both the preoptic region and the median eminence of the hypothalamus to regulate the reproductive function. While in the preoptic region neuronal NO may serve to control the bursting activity of GnRH neurons, in the median eminence, endothelial NO may represent one of the synchronizing cues that coordinate GnRH release from neuroendocrine terminals, and thus

provides a unique regulatory mechanism that may be required for appropriate delivery of GnRH to the pituitary during the estrous cycle. In turn, NO-producing enzymes and NO receptors are subjected to regulation by estrogens, thus completing the feedback loop.

Although much has been accomplished, significant gaps still remain in our knowledge. For instance, while there is good knowledge and understanding with respect to the role of NMDA-Rs in the control of nNOS activity in the preoptic region during the estrous cycle, considerably less is known concerning the regulation of eNOS activity within the median eminence. Furthermore, the interaction of NO with hypothalamic neuropeptides key to reproductive function, such as kisspeptin, requires clarification. These gaps in our knowledge will undoubtedly be closed as the field continues to move forward. While work still remains, the body of evidence accumulated to date suggests that NO is a chemical transmitter coordinating neuronal-glial-endothelial interactions in the hypothalamus and functions as a critical central cue coordinating GnRH neurosecretion.

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