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Invited Review

Nitric oxide in the stress axis

M.O. López-Figueroa, H.E.W. Day, H. Akil and S.J. Watson

Mental Health Research Institute, University of Michigan, USA

Summary. In recent years nitric oxide (NO) has emerged as a unique biological messenger. NO is a highly diffusible gas, synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). Three unique subtypes of NOS have been described, each with a specific distribution profile in the brain and periphery. NOS subtype I is present, among other areas, in the hippocampus, hypothalamus, pituitary and adrenal gland. Together these structures form the limbic-hypothalamic-pituitary-adrenal (LHPA) or stress axis, activation of which is one of the defining features of a stress response. Evidence suggests that NO may modulate the release of the stress hormones ACTH and corticosterone, and NOS activity and transcription is increased in the LHPA axis following various stressful stimuli. Furthermore, following activation of the stress axis, glucocorticoids are thought to down-regulate the transcription and activity of NOS via a feedback mechanism. Taken together, current data indicate a role for NO in the regulation of the LHPA axis, although at present this role is not well defined. It has been suggested that NO may act as a cellular communicator in plasticity and development, to facilitate the activation or the release of other neurotransmitters, to mediate immune responses, and/or as a vasodilator in the regulation of blood flow. In the following review we summarize some of the latest insights into the function of NO, with special attention to its relationship with the LHPA axis.

Key words: Nitric oxide synthase, Hippocampus, Hypothalamus, Pituitary gland, Adrenal gland, Oxytocin, Vasopressin, Steroids, NADPH, CRH, ACTH, Glucocorticoids

Introduction

For many years indirect donors of nitric oxide (NO), such as nitroglycerin, have been used to treat people suffering from heart attacks due to their potent vasodilatory effects. In the late eighties, the radical gas NO was found to be the diffusible substance causing vasodilatation, previously known as "endothelium-derived relaxing factor" (EDRF) (Ignarro et al., 1987; Palmer et al., 1987). NO has an ultra-short half-life in the millisecond range. Upon formation, NO diffuses and binds to its targets, the most common of which is the heme group of soluble guanyl cyclase (sGC). This neurotransmitter is atypical, in that it is not accumulated in vesicles, does not require membrane receptors to mediate its action and has the capacity to act retrogradely, from post- to presynaptic terminals. Since the description of NO as a member of a new class of neurotransmitter, and its designation as molecule of the year by Science in 1992, a vast literature has brought insights into its ubiquitous distribution, the enzymes responsible for its synthesis and its unique mode of action (for review see Wolf, 1997).

Offprint requests to: Manuel Oscar López-Figueroa, Ph.D., 205 Zina Pitcher Place, University of Michigan, Ann Arbor, Michigan 48109-0720 USA

Nitric oxide synthase characteristics

NO biosynthesis

Nitric oxide synthase (NOS) utilizes L-arginine and O_2 as substrates of a reaction that produces L-citrulline and the radical gas NO (Fig. 1) (Knowles et al., 1989). NOS is a homodimeric enzyme divided into an oxygenase and reductase domain (for review see Stuehr, 1997). Five electrons from NADPH are transferred in sequence to flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) located in the reductase domain (Fig. 2). L-arginine binds to the oxygenase domain of NOS and its guanidino group acts as a final acceptor of the electrons. Together with O_2 the reaction forms the intermediate N^{ω}-hydroxy-arginine. The enzyme also contains binding sites for heme, tetrahydrobiopterin (BH_4) and calmodulin (CaM), all of which are required for NOS to be completely functional. A consensus sequence for phosporylation is present, but the functional significance of this sequence has yet to be demonstrated.

Thusfar, three subtypes of NOS have been described, termed subtypes I, II and III based on their order of cloning. NOS subtypes I, II and III represent unique genes and are localized on chromosomes 12, 17 and 7

respectively of the human genome. The mRNA of NOS subtype I is encoded by 29 exons, whereas subtypes II and III are encoded by 26 exons. The amino acid sequence homology between the three subtypes is less than 59% in the same species and higher than 80% for the same subtype between species.

At this point it should be noted that in addition to this numerical classification, a descriptive nomenclature based on the enzymes' first described tissue distribution, or constitutive versus inducible expression, is frequently used. Hence, subtype I is often described as brain or neuronal NOS. Subtype II, first described in cells of the immune system, is also known as immunological or inducible NOS. Finally, subtype III is commonly known as endothelial NOS as it is normally found in the endothelium of blood vessels. Subtypes I and III were first described as constitutive enzymes, as compared with the inducible subtype II. However, the description of NOS in a number of tissues other than those first described, together with the observations that expression of "constitutive" forms of NOS may be induced, can be misleading. Therefore, we will follow the numerical nomenclature in this review.

Subtype I

Bredt and Snyder were the first to describe the 10.5 Kb mRNA, which translates into an approximately 155 Kd protein corresponding to NOS subtype I (Bredt et al., 1990, 1991). Subtype I was first described as neuronal NOS because of its localization in neurons of the central and peripheral nervous systems. However, it should be noted that NOS I has been found in non-neuronal cells, such as skeletal muscle cells (Frandsen et al., 1996). This subtype of NOS is constitutively expressed, but there is potential for regulation by transcription factors such as AP-2, TEF-1/MCBF and CREB/ATF/c-Fos (Hall et al., 1994). In addition, the expression of this enzyme has been shown to be regulated in vivo by glucocorticoids at the mRNA level (Fig. 4) (López-Figueroa et al., 1998a,b). Because the promoter region of NOS I does not contain a consensus sequence for glucocorticoids, further studies are required to clarify the exact mechanism of action. At the protein level, the activity of NOS I may be regulated by increases in intracellular calcium (Bredt and Snyder, 1990), or by binding to other proteins. NOS I was initially thought to be a cytosolic enzyme, but recently it has been shown that this enzyme can bind to membrane receptors via proteins containing PDZ domains. PDZ motifs are sequences of about 90 amino-acids involved in protein-protein interactions which allows for clustering of submembranous signalling molecules (Schepens et al., 1997). NOS I possesses a PDZ domain close to its C-terminal, which binds to the PDZ domain of PSD (postsynaptic density) and related proteins, such as PSD-95 (Brenman et al., 1996). The subsequent binding of PSD-95 to NMDA receptors permits a more efficient activation of NOS I. In addition, PSD-95 can reinforce its binding to membranes via N-terminal palmitoylation (Topinka and Bredt, 1998). In contrast to this membrane association, a NOS-PDZ interaction with CAPON C-terminus (carboxy-terminal PDZ ligand of nNOS) may compete with PSD-95 for binding (Jaffrey et al., 1998), thus re-locating NOS activity in the cytosol. A further mechanism by which NOS activity may be regulated is via a novel "protein inhibitor of nNOS" (PIN), which has been shown to interact and inhibit NOS dimerization (Jaffrey and Snyder, 1996).

Recently, splice variants of NOS I have been described, for example in skeletal muscle (Silvagno et al., 1996). In addition, knockout mice lacking exon 2 still express at least two other variants, known as NOSß and NOSγ, which exhibit specificity in their tissue and temporal expression (Eliasson et al., 1997). Furthermore, alternative promoter regions for NOS I have been described (for review see Brenman et al., 1997). Hence, there appears to be potential for regulation of NOS I at many levels.

Subtype II

This highly active enzyme is normally located in the

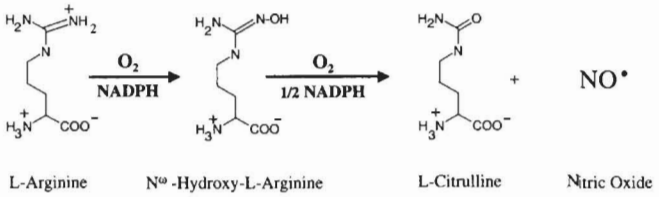


Fig. 1. Simplified schematic to illustrate the formation of the free radical nitric oxide by NOS.

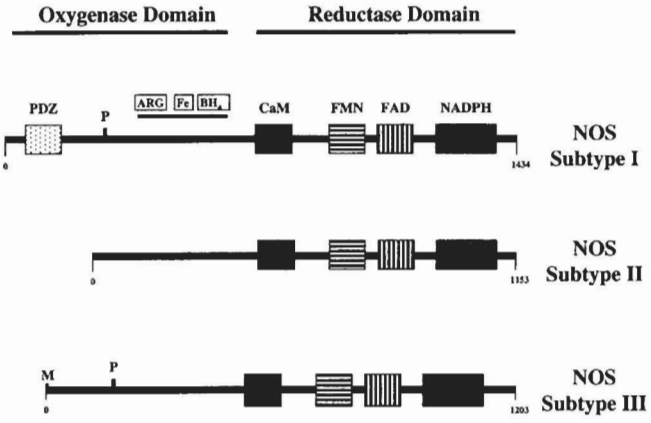


Fig. 2. Schematic representation of the amino acid sequences of NOS. The reductase domain contain binding sites for NADPH, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). Calmodulin (CaM), tetrahydrobiopterin (BH_4), L-arginine (Arg) and a catalytic heme center are present in the oxidative domain of the enzymes. NOS subtype III is myristoylated at its N-terminal site. NOS subtype I is characterized by a PDZ domain. P: phosphorylation site.

cytosol of cells related to the immune system, for example macrophages. However, it should be noted that this subtype may be found in smooth muscle cells, hepatocytes and other cell types (Bandaletova et al., 1993). NOS subtype II was originally described as inducible NOS, since under basal conditions this subtype of NOS is almost undetectable but is induced at the transcriptional level by lipopolysaccharides and cytokines (Xie et al., 1992). Of particular interest with respect to stress is the transcriptional regulation of NOS subtype II by glucocorticoids in vascular endothelial cells (Radomski et al., 1990). In addition, NOS II contains several consensus sequences for the binding of transcription factors such as AP-1, interferon gamma (IFN-γ), as well as response elements upstream from "TATA box" for LPS. Once the 4.4 Kb mRNA is expressed, it is translated into a 130 Kd protein (Lowenstein et al., 1992). Calmodulin is tightly bound and the activity of this subtype is calcium independent.

Subtype III

The last isoform of NOS to be cloned (Lamas et al., 1992), subtype III is generally found in the endothelium of blood vessels. This subtype of NOS produces the NO that is responsible for vasodilatation via binding to the heme group of guanyl cyclase, thereby increasing cGMP production and thus smooth muscle relaxation. The N-terminal of this enzyme is myristoylated and palmitylated allowing it to bind to membranes (Pollock et al., 1992). For example, acylation targets the protein to plasmalemmal caveolae, which are microdomains implicated in signal transduction (Shaul et al., 1996). The activity of this constitutively expressed 135Kd-protein is dependent on calcium, although a transcriptional activation of its 4.8Kb mRNA by shear stress produced by the flowing blood has been demonstrated (Xiao et al., 1997). The promoter region of this gene contains a putative shear stress-response element, as well

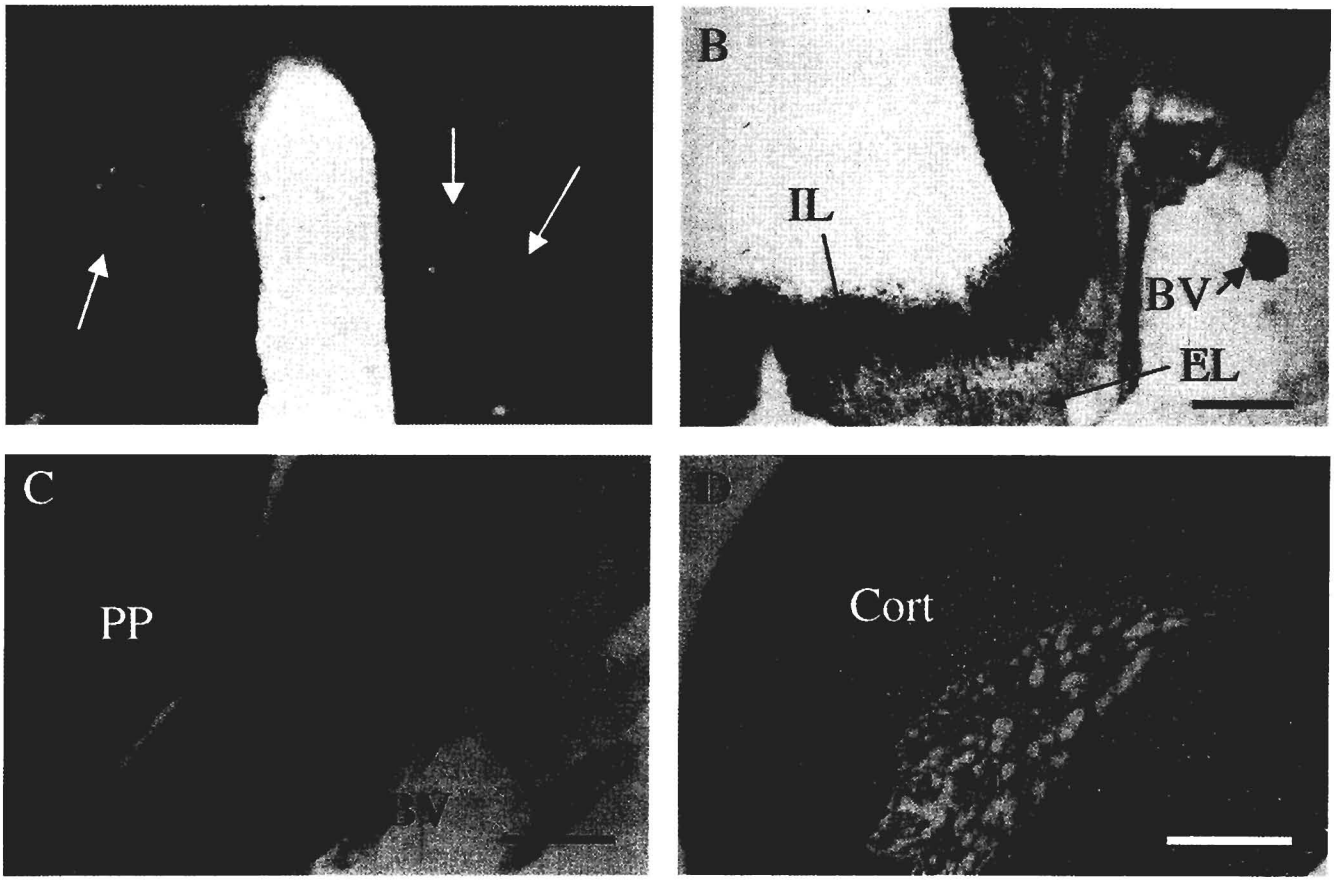
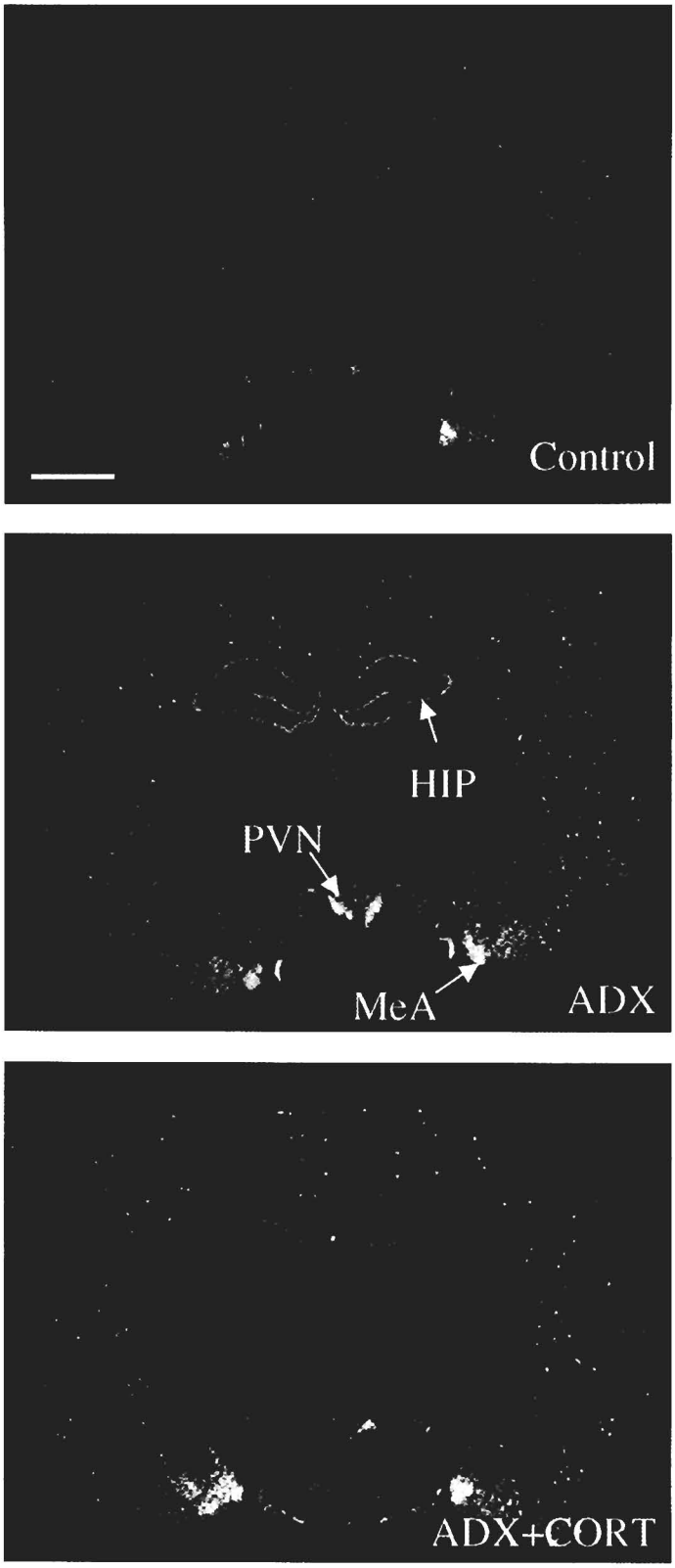


Fig. 3. Localization of NOS in the HPA axis. **A.** Coronal section through the hypothalamus of a rat brain processed for NOS subtype I immunohistochemistry. The highest activity is observed in the magnocellular cells (PVNm) of the paraventricular nucleus (PVN). Scattered parvocellular cells (PVNp) are positive for NOS. Scale bar: 200 μm. **B.** Presence of NADPH-d positive nerve fibers in the internal layer (IL) of the median eminence. The external layer (EL) exhibits very little NADPH-d activity. Blood vessels (BV) in the proximity of the medial eminence also express NADPH-d activity. Scale bar: 200 μm. **C.** Section of a pituitary gland reacted for NADPH-d activity. The posterior part (PP) is strongly stained and signal is also present in gonadotrophs and folliculo-stellate cells of the anterior pituitary (AP). The intermediate lobe (I) is devoid of staining. The endothelium of crossing blood vessels is also stained. Scale bar: 50 μm. **D.** In the adrenal gland, the cortex (Cort) and a few scattered cells are observed in the medulla (M). Scale bar: 500 μm.

as AP-1 and AP-2 binding sites, and the human promoter is characterized by the absence of a "TATA box" sequence (Marsden et al., 1993).



NOS and the LHPA axis

LHPA axis overview

The stress response is the result of a complex neural and endocrine interaction, and the precise mechanisms that elicit these processes are still enigmatic. Central to the endocrine stress response is the paraventricular nucleus of the hypothalamus (PVN). Activation of the parvocellular cells of this nucleus, leads to the release of a variety of neuropeptides, including corticotropin-releasing hormone (CRH). These peptides access cells of the anterior pituitary via the median eminence and portal blood system. The subsequent stimulation of corticotrophs results in the release of adrenocorticotropin (ACTH) into the general circulation, which in turn leads to the de novo synthesis and release of glucocorticoids (corticosterone in rat and cortisol in human) from the adrenal cortex. Whilst the PVN is thought to be the critical nucleus in eliciting a stress response, many other brain regions are known to modulate the activity of the PVN, and therefore the stress response. For example, the amygdala, bed nucleus of the stria terminalis (BST), hippocampus and prefrontal cortex, represent some of the structures which are believed to modulate the LHPA axis. Indeed, the hippocampus is thought to be one of the primary sites for glucocorticoid negative feedback, which is critical for the termination of a stress response. For this reason, the hippocampus may be included in the term limbic-hypothalamic-pituitary-adrenal (LHPA) axis.

Methods for studying NO

Because of the difficulties encountered in measuring the highly reactive NO, most of the studies described to date are based on the localization of its synthesizing enzyme NOS. Three main techniques have been used to localize NOS. The NADPH-diaphorase histochemical technique is based on the fact that all NOS enzymes contain a binding site for NADPH and therefore exhibit diaphoretic activity. However, because other enzymes, for example cytochrome-P450, also posses diaphoretic activity, the reaction is not necessarily specific for NOS, and results from this technique have been viewed with a certain degree of skepticism. A second frequently used method is immunohistochemistry, whereby specific

Fig. 4. Photomicrographs showing the effect of glucocorticoids on NOS subtype I mRNA expression in the rat brain. **A.** In situ hybridization in a section from a sham operated animal. **B.** The signal is increased one week after bilateral adrenalectomy in the hippocampus (Hip) and PVN. **C.** In an adrenalectomized animal treated with corticosteroids, the levels of NOS subtype I mRNA return to normal. Medial amygdala (MeA). Scale bar: 2 mm.

antibodies are raised against the different isoforms of NOS, allowing visualization of the proteins at the light or electron microscopy level. Finally, the cloning of three isoforms of NOS has allowed the utilization of cDNA or cRNA probes to determine the expression of their respective mRNAs. In addition, other indirect techniques have been used to measure NO production, including assays for L-citruline, cGMP, nitrites and nitrates.

Localization of NOS in the LHPA axis

Studies on the localization of NOS in the hippocampus have been controversial. Initially, it was suggested that NOS was only present in interneurons of the hippocampus (Bredt et al., 1990; Vincent and Kimura, 1992; Valtschanoff et al., 1993; Vincent, 1994). Subsequent studies described the presence of NADPH-d activity (Vaid et al., 1996) and NOS subtype I immunoreactivity (Wendland et al., 1994) both in interneurons and CA1 pyramidal cells. In addition, in situ hybridization has been used to demonstrate the presence of NOS subtype I mRNA in the CA1, CA3 and dentate gyrus (DG) of the hippocampus (Fig. 4) (Endoh et al., 1994; López-Figueroa et al., 1998a,b). Although NOS subtype III immunoreactivity (Dinerman et al., 1994) has been reported within pyramidal cells of the hippocampus, more recent evidence suggest that this labeling was not specific (Stanarius et al., 1997; López-Figueroa et al., 1998a).

Summy-Long and collaborators demonstrated for the first time that NADPH-d activity was present in magnocellular neurons of the PVN and in the posterior pituitary (Summy-Long et al., 1984). Later, an extensive bibliography confirmed and further described NADPH-d activity (Arévalo et al., 1992; Sánchez et al., 1994; Siaud et al., 1994; Vanhalato and Soinila, 1995) and NOS immunoreactivity (Villar et al., 1994; Bhat et al., 1995) in the PVN. NOS appears to be most abundant in the magnocellular division of the PVN, but scattered cells exhibiting moderate staining are observed in the anterior and medial parvocellular subdivisions, as well as in the dorsomedial cap and posterior regions (Fig. 3A). In the ventral area, large to medium cells are strongly stained. A comparable pattern of mRNA for NOS I is observed using the technique of in situ hybridization (Fig. 4) (Calzà et al., 1993; Ceccatelli and Eriksson, 1993; Grossman et al., 1994; Kadowaki et al., 1994; Villar et al., 1994 Ceccatelli et al., 1996; López-Figueroa et al., 1998a,b). Colocalization studies have demonstrated that NOS is present in approximately 33% of vasopressin (Calka and Block, 1993b; Torres et al., 1993; Sánchez et al., 1994; Hatakeyama et al., 1996) and 70% of oxytocin cells, in the magnocellular division (Torres et al., 1993; Miyagawa et al., 1994; Sánchez et al., 1994; Hatakeyama et al., 1996; Yamada et al., 1996). Within the parvocellular division, depending upon the experimental conditions, it is estimated that 5 to 15% of CRH positive neurons colocalize with NOS (Torres et al., 1993; Siaud et al., 1994). Other neuropeptides such as enkephalin (Yamada et al., 1996), calretinin (Arévalo et al., 1993), PACAP (Okamura et al., 1994), calbindin (Alonso et al., 1992a) and somatostatin (Alonso et al., 1992b) have been colocalized with NOS in various subregions of the PVN. Also, a small number of angiotensin-(1-7) cells were observed to colocalize with NADPH-d positive cells in the PVN (Calka and Block, 1993a). Furthermore, NOS positive neurons have been colocalized with NMDA R1 in the PVN (Bhat et al., 1995), suggesting that the intracellular increases in calcium following direct activation of glutamate receptors of the hypothalamus may have a direct effect on NOS activity. NOS I is the most abundant subtype within the PVN (Bhat et al., 1996; Ceccatelli et al., 1996 López-Figueroa et al., 1998b). NOS subtype II is not present in the hypothalamus under basal conditions (López-Figueroa et al., 1998b), but is induced in the PVN following intraperitoneal administration of LPS (Wong et al., 1996). Within neurons of the PVN, NOS III is not expressed (López-Figueroa et al., 1998b), but NOS III mRNA has been described in endothelial cells of hypophyseal blood vessels in the vicinity of the median eminence (Fig. 3B) (Ceccatelli et al., 1996). The internal zone of the median eminence is enriched with NOS positive fibers corresponding to magnocellular hypothalamic neurons projecting to the posterior pituitary. In contrast, the external zone of the median eminence, the area which corresponds to the parvocellular projection to the anterior pituitary exhibits very few NOS/NADPH-d positive fibers (Fig. 3B).

The majority of NADPH-d positive cells of the PVN project to the pituitary (Vanhalato and Soinila, 1995), while approximately 4% of NADPH-d positive cells project to the spinal cord (Hatakeyama et al., 1996). In the pituitary gland, the posterior lobe presents NADPH-positive nerve terminals and fibers (Sagar and Ferriero, 1987; Bredt et al., 1990). Pituicytes present low levels of NADPH-d activity, and there is almost no signal in the intermediate lobe, with the exception of some epithelial cells and extensions between the melanotrophes (Vanhatalo and Soinila, 1995). By contrast, NOS is present in gonadotrophs and folliculo-stellate cells of the anterior pituitary, as demonstrated by immunohistochemistry and in situ hybridization (Ceccatelli et al., 1993). To date, there has been no demonstration of NOS within the corticotrophs.

NOS immunoreactivity and NADPH-d activity have been demonstrated in the adrenal gland (Fig. 3D) (Bredt et al., 1990; Dun et al., 1993). In particular, the adrenal cortex expresses high levels of NOS, whilst scattered ganglion cell bodies are stained in the adrenal medulla (Tsuchiya et al., 1996)

In addition to the presence of NOS in the LHPA axis, NOS has been described in many areas of the CNS such as the cortex, basal ganglia, hypothalamus, thalamus, amygdala (Fig. 4), cerebellum and brainstem, as well as in passing fibers or terminals in peripheral organs such as the pineal gland (Bredt et al., 1990;

Vincent and Kimura, 1992; Vincent, 1994; López-Figueroa and Møller, 1996). Many of these areas have potential to influence the stress response, via direct or indirect projections. For example, NOS subtype I is highly expressed in the medial nucleus of the amygdala, and a nitrergic innervation from the amygdala to the PVN suggests an involvement of NO in the neuroendocrine regulation of the PVN (Tanaka et al., 1997).

Physiological roles of NO in the stress system

As previously described, NO is a unique intercellular messenger, and during development NOS shows an interesting pattern of expression, which is suggestive of a role as signaling molecule (Bredt and Snyder, 1994). In rats, as soon as embryonic day 15, NOS activity is observed in the PVN (Terada et al., 1996), increasing until postnatal day 14 when it reaches adult levels (Fig. 5) (Torres et al., 1993; Terada et al., 1996). It is thought that NO, particularly because of its capacity to act retrogradely, could play an important role in plasticity processes that take place during development and continue during the adulthood. For instance, NO produced in CA1 pyramidal cells of the hippocampus acts on the presynaptic terminals of Schaeffer collaterals, potentiating the secretion of glutamate and other neurotransmitters, partially mediating the plasticity process of long-term potentiation (LTP) (Zorumski and Izumi, 1993).

NO has been implicated in the regulation of secretion of neurotransmitters from nerve terminals. At the level of the pituitary gland NO has been shown to have both inhibitory and facilitatory effects on the secretion of hormones, such as growth hormone (GH) and luteinizing hormone (LH) (Kato, 1992; Ceccatelli et al., 1993). Although, NOS containing cells are not highly colocalized with the direct effectors of the stress axis, the unique characteristic of NO as a diffusible neurotransmitter suggests its potential action on the secretion of neurotransmitters from adjacent nerve terminals. In addition, the demonstration of the regulation of hypothalamic cGMP by NO (Bhat et al., 1996) suggests an important role in secretion.

There is evidence for the involvement of NO in the regulation of hypothalamic blood flow (Ceccatelli et al., 1992). In addition, the median eminence is a highly vascularized region, and the endothelium of those blood

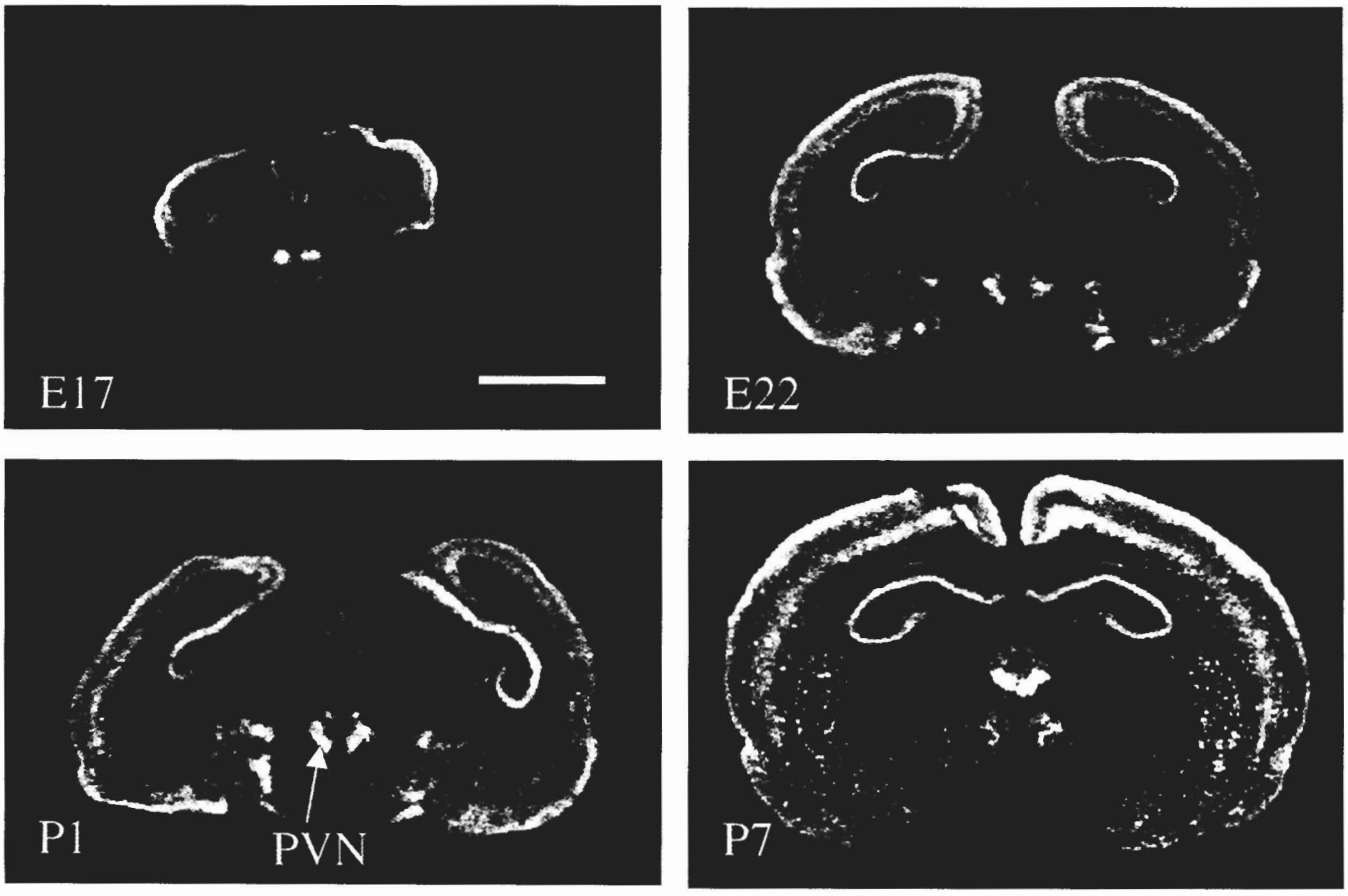


Fig. 5. Photomicrographs showing the distribution of NOS subtype I mRNA during development, through embryonic (E) day 17 to postnatal (P) day 7. Scale bar: 2 mm.

vessels constitutively express NOS subtype III (Ceccatelli et al., 1996). The NO produced by these cell types is important not only for maintaining the blood flow or vascular tone, but also for permitting the flow of the released secretagogs. Also, nitrergic nerve fibers innervating the blood vessels might contribute to additional control. In this regard, the use of several NOS inhibitors suggested that NOS III is involved in the ACTH response to administration of IL-1ß in rats (Turnbull and Rivier, 1996).

NO produced by NOS subtype II may mediate neurotoxicity when produced in high amounts (Dawson and Dawson, 1996). However, there are no indications of toxic effects in the stress axis of immune stimulated rats (Kutamura et al., 1996; Wong et al., 1996).

Differential regulation of the stress axis

We can define stress as any physiological or physical alterations resulting in an interaction between neuronal and endocrine systems which ultimately results in the production of glucocorticoids. Many different classes of stimuli, which utilize a wide variety of neuronal pathways, result in a stress response (Herman and Cullinan, 1997). For example, salt loading, hypoxia or an immune stress, affect physiological homeostasis by a relatively direct activation of the PVN, without intervention of limbic areas. Other stressors, such as restraint, fear or novel environment, involve differing degrees of previous experience, which requires limbic processing without affecting the physiological homeostasis. Despite the relatively low abundance of NOS in many key structures of the LHPA axis as described above, the involvement of nitric oxide following a number of different stressors has been demonstrated. Current evidence suggests a complex role for NO in the modulation of the central stress response, which may in part reflect the differing mechanisms of stress regulation.

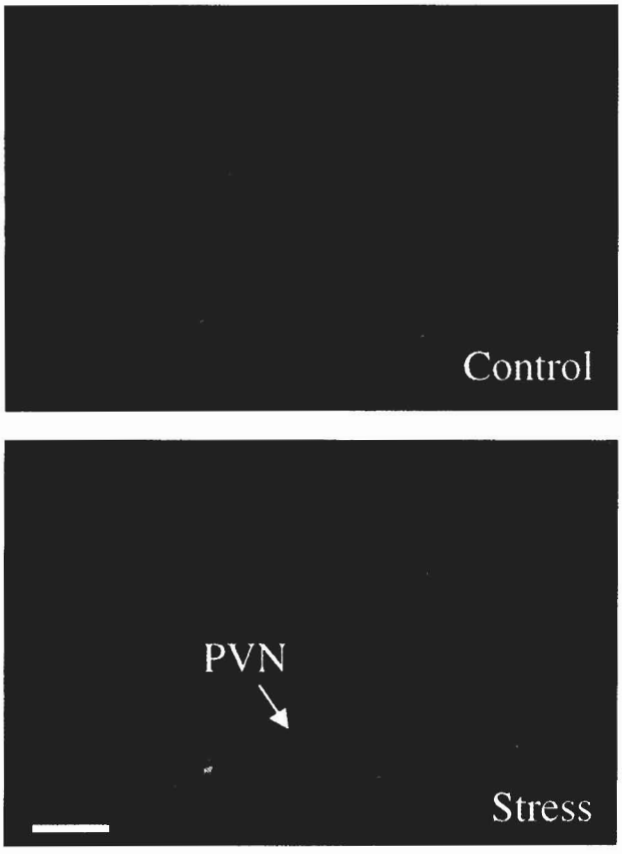


Fig. 6. Localization of NOS subtype I mRNA in control and 24 hours following 20 minutes of forced swim stress. Note the increase in mRNA in the PVN of the stressed animal. Scale bar: 2 mm.

One focus has been to study the relationship between the CNS, immune and endocrine systems, and the mechanisms underlying the modulation of the immune response by NO. NOS subtype II mRNA expression was demonstrated to be increased in the PVN, median eminence and pineal gland following intraperitoneal injection of lipopolysaccharide (LPS; also termed endotoxin), a component of the cell wall of Gram-negative bacteria (Wong et al., 1996). In addition, an increase in NOS I mRNA expression was observed 4 hours after i.v. LPS administration, which was preceded by an increase in CRH heteronuclear RNA and c-fos mRNA (Lee et al., 1995). About 40-50% of the NOS positive neurons were colocalized with Fos (Hatakeyama et al., 1996). Brunetti and collaborators suggest that the *in vitro* increase in CRH and ACTH secretion observed in hypothalamic and pituitary cell cultures following stimulation with interleukin is mediated by NO, but basal CRH release is not affected by NO (Brunetti et al., 1993, 1996). Moreover, McCann's group, using hypothalamic slices, has confirmed these data, and further suggest that diffusible NO is involved in the release of CRF induced by carbachol but not by norepinephrine (Karanth et al., 1993). In contrast, it has been suggested that NO has an inhibitory effect on the immune stimulated release of CRH, ACTH and corticosterone *in vivo* (Costa et al., 1993; Rivier and Shen, 1994; Lee at al., 1995; Turnbull and Rivier, 1996). The contradictory results might be explained by methodological differences, since in cell cultures or hypothalamic slides the tissue is isolated from other neuronal or hormonal influences present in the intact rat. The mechanisms whereby NO acts to regulate the stress response to a systemic immune challenge are not clear. However, because ACTH secretion is observed 30 min following systemic LPS stimulation, it has been suggested that there is a prior action at the median eminence activating peptidergic nerve terminals (Lee et al., 1995; Rivier, 1995). Although IL-1ß also stimulates the stress axis when injected directly into the brain, this effect does not appear to involve NO (Rivier and Shen, 1994; Wong et al., 1996).

For other stressors, including salt loading (Kadowaki et al., 1994; Villar et al., 1994), water or food deprivation (Ueta et al., 1995; O'Shea et al., 1996) or lactation (Ceccatelli and Eriksson, 1993), NOS activity and expression has also been shown to be increased in

the PVN. The increase in NOS mRNA following stress may be a mechanism to maintain a high production of NO which could then act to modulate the release of other neurotransmitters. Following immobilization stress an increase in NOS mRNA and protein expression has been demonstrated in the ventral medial parvocellular subdivision of the PVN (Calzà et al., 1993; Hatakeyama et al., 1996; Kishimoto et al., 1996; Krukoff and Khalili, 1997). This increase was observed more quickly in older rats as compared to young ones (Calzà et al., 1993). The same type of stressor also increased the expression of NOS in the adrenal cortex (Tsuchiya et al., 1996, 1997). In contrast to that observed following immune challenge, inhibition of NOS activity by L-NAME attenuated Fos expression in the PVN following immobilization stress (Amir et al., 1997), and furthermore blunted the ACTH response to noxious stimuli such as electroshocks and water avoidance (Rivier, 1994). The opposing effects of L-arginine antagonists on ACTH secretion, following immobilization or noxious stress versus immune stress, suggests that the differential activation of the stress axis may define the role that NO plays. In keeping with this idea, differential activation by stress of NOS containing cells in different nuclei of the brain has been demonstrated by Krukoff and Khalili (1997), such that the net effect of the activated circuitry may have different consequences, depending on the stressor.

To summarize, the use of specific inhibitors and the stress-induced regulation of NOS in areas critical for LHPA axis activation, suggests that NO plays a physiological role in the LHPA axis response to a wide variety of stressors. Although the exact mechanisms whereby NO modulates the LHPA axis remain unknown, NO appears to be an important and complex modulator of the stress response.

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References

Alonso J.R., Sánchez F., Arévalo R., Carretero J., Aijón J. and Vázquez R. (1992a). CaBP D-28k and NADPH-diaphorase coexistence in the magnocellular neurosecretory nuclei. Neuroreport 3, 249-252.

Alonso J.R., Sánchez F., Arévalo R., Carretero J., Vázquez R. and Aijón J. (1992b). Partial coexistence of NADPH-diaphorase and somatostatin in the rat hypothalamic paraventricular nucleus. Neurosci. Lett. 148, 101-104.

Amir S., Rackover M. and Funk D. (1997). Blockers of nitric oxide synthase inhibit stress activation of c-fos expression in neurons of the hypothalamic paraventricular nucleus in the rat. Neuroscience 77, 623-627.

Arévalo R., Sánchez F., Alonso J.R., Carretero J., Vázquez R. and Aijón J. (1992). NADPH-diaphorase activity in the hypothalamic magnocellular neurosecretory nuclei of the rat. Brain Res. Bull. 28, 599-603.

Arévalo R., Sánchez F., Alonso J.R., Rubio M., Aijón J. and Vázquez R. (1993). Infrequent cellular coexistence of NADPH-diaphorase and calretinin in the neurosecretory nuclei and adjacent areas of the rat hypothalamus. J. Chem. Neuroanat. 6, 335-341.

Bandaletova T., Brouet I., Bartsch H., Sugimura T., Esumi H. and Ohshima H. (1993). Immunohistochemical localization of an inducible form of nitric oxide synthase in various organs of rats treated with *Propionibacterium acnes* and lipopolysaccharide. APMIS 101, 330-336.

Bhat G.K., Mahesh V.B., Lamar C.A., Ping L., Aguan K. and Brann D.W. (1995). Histochemical localization of nitric oxide neurons in the hypothalamus: association with gonadotropin-releasing hormone neurons and co-localization with N-methyl-D-aspartate receptors. Neuroendocrinology 62, 187-197.

Bhat G., Mahesh V.B., Aguan K. and Brann D.W. (1996). Evidence that brain nitric oxide synthase is the major nitric oxide synthase isoform in the hypothalamus of the adult female rat and that nitric oxide potently regulates hypothalamic cGMP levels. Neuroendocrinology 64, 93-102.

Bredt D.S. and Snyder S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. USA 87, 682-685.

Bredt D.S. and Snyder S.H. (1994). Transient nitric oxide synthase neurons in embryonic cerebral cortical plate, sensory ganglia, and olfactory epithelium. Neuron 13, 301-313.

Bredt D.S., Hwang P.M. and Snyder S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347, 768-770.

Bredt D.S., Hwang P.M., Glatt C.E., Lowenstein C., Reed R.R. and Snyder S.H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 351, 714-718.

Brenman J.E., Chao D.S., Gee S.H., McGee A.W., Craven S.E., Santillano D.R., Wu Z., Huang F., Xia H., Peters M.F., Froehner S.C. and Bredt D.S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell 84, 757-767.

Brenman J.E., Xia H., Chao D.S., Black S.M. and Bredt D.S. (1997). Regulation of neuronal nitric oxide synthase through alternative transcripts. Dev. Neurosci. 19, 224-231.

Brunetti L., Preziosi P., Ragazzoni E. and Vacca M. (1993). Involvement of nitric oxide in basal and interleukin-1 beta-induced CRH and ACTH release in vitro. Life Sci. 53, L219-222.

Brunetti L., Volpe A.R., Ragazzoni E., Preziosi P. and Vacca M. (1996). Interleukin-1 beta specifically stimulates nitric oxide production in the hypothalamus. Life Sci. 58, L373-377.

Calka J. and Block C.H. (1993a). Angiotensin-(1-7) and nitric oxide synthase in the hypothalamo-neurohypophysial system. Brain Res. Bull. 30, 677-685.

Calka J. and Block C.H. (1993b). Relationship of vasopressin with NADPH-diaphorase in the hypothalamo-neurohypophysial system. Brain Res. Bull. 32, 207-210.

Calzà L., Giardino L. and Ceccatelli S. (1993). NOS mRNA in the paraventricular nucleus of young and old rats after immobilization stress. Neuroreport 4, 627-630.

Ceccatelli S. and Eriksson M. (1993). The effect of lactation on nitric oxide synthase gene expression. Brain Res. 625, 177-179.

Ceccatelli S., Lundberg J.M., Fahrenkrug J., Bredt D.S., Snyder S.H. and Hökfelt T. (1992). Evidence for involvement of nitric oxide in the regulation of hypothalamic portal blood flow. Neuroscience 51, 769-772.

Ceccatelli S., Hulting A.L., Zhang X., Gustafsson L., Villar M. and Hökfelt T. (1993). Nitric oxide synthase in the rat anterior pituitary

gland and the role of nitric oxide in regulation of luteinizing hormone secretion. Proc. Natl. Acad. Sci. USA 90, 11292-11296.

Ceccatelli S., Grandison L., Scott R.E., Pfaff D.W. and Kow L.M. (1996). Estradiol regulation of nitric oxide synthase mRNAs in rat hypothalamus. Neuroendocrinology 64, 357-363.

Costa A., Trainer P., Besser M. and Grossman A. (1993). Nitric oxide modulates the release of corticotropin-releasing hormone from the rat hypothalamus in vitro. Brain Res 605, 187-192.

Dawson V.L. and Dawson T.M. (1996). Nitric oxide neurotoxicity. J. Chem. Neuroanat. 10, 179-190.

Dinerman J.L., Dawson T.M., Schell M.J., Snowman A. and Snyder S.H. (1994). Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. Proc. Natl. Acad. Sci. USA 91, 4214-4218.

Dun N.J., Dun S.L., Wu S.Y. and Forstermann U. (1993). Nitric oxide synthase immunoreactivity in rat superior cervical ganglia and adrenal glands. Neurosci. Lett. 158, 51-54.

Eliasson M.J., Blackshaw S., Schell M.J. and Snyder S.H. (1997). Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. Proc. Natl. Acad. Sci. USA 94, 3396-3401.

Endoh M., Maiese K. and Wagner J.A. (1994). Expression of the neural form of nitric oxide synthase by CA1 hippocampal neurons and other central nervous system neurons. Neuroscience 63, 679-689.

Frandsen U., Lopez-Figueroa M. and Hellsten Y. (1996). Localization of nitric oxide synthase in human skeletal muscle. Biochem. Biophys. Res. Commun. 227, 88-93.

Grossman A.B., Rossmanith W.G., Kabigting E.B., Cadd G., Clifton D. and Steiner R.A. (1994). The distribution of hypothalamic nitric oxide synthase mRNA in relation to gonadotrophin-releasing hormone neurons. J. Endocrinol. 140, R5-8.

Hall A.V., Antoniou H., Wang Y., Cheung A.H., Arbus A.M., Olson S.L., Lu W.C., Kau C.L. and Marsden P.A. (1994). Structural organization of the human neuronal nitric oxide synthase gene (NOS1). J. Biol. Chem. 269, 33082-33090.

Hatakeyama S., Kawai Y., Ueyama T. and Senba E. (1996). Nitric oxide synthase-containing magnocellular neurons of the rat hypothalamus synthesize oxytocin and vasopressin and express Fos following stress stimuli. J. Chem. Neuroanat. 11, 243-256.

Herman J.P. and Cullinan W.E. (1997). Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends Neurosci. 20, 78-84.

Ignarro L.J., Buga G.M., Wood K.S., Byrns R.E. and Chaudhuri G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. USA 84, 9265-9269.

Jaffrey S.R., Snowman A.M., Eliasson M.J., Cohen N.A. and Snyder S.H. (1998). CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. Neuron 20, 115-124.

Jaffrey S.R. and Snyder S.H. (1996). PIN: an associated protein inhibitor of neuronal nitric oxide synthase. Science 274, 774-777.

Kadowaki K., Kishimoto J., Leng G. and Emson P.C. (1994). Up-regulation of nitric oxide synthase (NOS) gene expression together with NOS activity in the rat hypothalamo-hypophysial system after chronic salt loading: evidence of a neuromodulatory role of nitric oxide in arginine vasopressin and oxytocin secretion. Endocrinology 134, 1011-1017.

Karanth S., Lyson K. and McCann S.M. (1993). Role of nitric oxide in interleukin 2-induced corticotropin-releasing factor release from incubated hypothalami. Proc. Natl. Acad. Sci USA 90, 3383-3387.

Kato M. (1992). Involvement of nitric oxide in growth hormone (GH)-releasing hormone-induced GH secretion in rat pituitary cells. Endocrinology 131, 2133-2138.

Kishimoto J., Tsuchiya T. and Emson P. (1996). Immobilization-induced stress activates neuronal nitric oxide synthase (nNOS) mRNA and protein in hypothalamic-pituitary-adrenal axis in rats. Brain Res. 720, 159-170.

Kitamura Y., Takahashi H., Matsuoka Y., Tooyama I., Kimura H., Nomura Y. and Taniguchi T. (1996). In vivo induction of inducible nitric oxide synthase by microinjection with interferon-γ and lipopolysaccharide in rat hippocampus. Glia 18, 233-243.

Knowles R.G., Palacios M., Palmer R.M.J. and Moncada S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. Proc. Natl. Acad. Sci. USA 86, 5159-5162.

Krukoff T.L. and Khalili P. (1997). Stress-induced activation of nitric oxide-producing neurons in the rat brain. J. Comp. Neurol. 377, 509-519.

Lamas S., Marsden P.A., Li G.K., Tempst P. and Michel T. (1992). Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc. Natl. Acad. Sci. USA 89, 6348-6352.

Lee S., Barbanel G. and Rivier C. (1995). Systemic endotoxin increases steady-state gene expression of hypothalamic nitric oxide synthase: comparison with corticotropin- releasing factor and vasopressin gene transcripts. Brain Res. 705, 136-148.

López-Figueroa M.O. and Møller M. (1996). Nitric oxide synthase in the pineal gland. Histol. Histopathol. 11, 1089-1100.

López-Figueroa M.O., Itoi K. and Watson S.J. (1998a). The biology of nitric oxide. Part 6. Regulation of nitric oxide synthase mRNA expression in the rat brain by glucocorticoids. Moncada S., Toda N., Maeda H. and Higgs E.A. (eds). Portland. London. (in press).

López-Figueroa M.O., Itoi K. and Watson S.J. (1998b). Regulation of nitric oxide synthase mRNA expression in the rat hippocampus by glucocorticoids. Neuroscience (in press).

Lowenstein C.J., Glatt C.S., Bredt D.S. and Snyder S.H. (1992). Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. Proc. Natl. Acad. Sci. USA 89, 6711-6715.

Marsden P.A., Heng H.H., Scherer S.W., Stewart R.J., Hall A.V., Shi X.M., Tsui L.C. and Schappert K.T. (1993). Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. J. Biol. Chem. 268, 17478-17488.

Miyagawa A., Okamura H. and Ibata Y. (1994). Coexistence of oxytocin and NADPH-diaphorase in magnocellular neurons of the para-ventricular and the supraoptic nuclei of the rat hypothalamus. Neurosci. Lett. 171, 13-16.

Okamura H., Miyagawa A., Takagi H., Esumi H., Yanaihara N. and Ibata Y. (1994). Co-existence of PACAP and nitric oxide synthase in the rat hypothalamus. Neuroreport 5, 1177-1180.

O'Shea R.D. and Gundlach A.L. (1996). Food or water deprivation modulate nitric oxide synthase (NOS) activity and gene expression in rat hypothalamic neurones: correlation with neurosecretory activity? J. Neuroendocrinol. 8, 417-425.

Palmer R.M., Ferrige A.G. and Moncada S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327, 524-526.

Pollock J.S., Klinghofer V., Forstermann U. and Murad F. (1992). Endothelial nitric oxide synthase is myristylated. FEBS Lett. 309, 402-404.

Radomski M.W., Palmer R.M. and Moncada S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc. Natl. Acad. Sci. USA 87, 10043-10047.

Rivier C. (1994). Endogenous nitric oxide participates in the activation of the hypothalamic-pituitary-adrenal axis by noxious stimuli. Endocrine J. 2, 367-373.

Rivier C. (1995). Blockade of nitric oxide formation augments adrenocorticotropin released by blood-borne interleukin-1 beta: role of vasopressin, prostaglandins, and alpha 1-adrenergic receptors. Endocrinology 136, 3597-3603.

Rivier C. and Shen G.H. (1994). In the rat, endogenous nitric oxide modulates the response of the hypothalamic-pituitary-adrenal axis to interleukin-1 beta, vasopressin, and oxytocin. J. Neurosci. 14, 1985-1993.

Sagar S.M. and Ferriero D.M. (1987). NADPH diaphorase activity in the posterior pituitary: relation to neuronal function. Brain Res. 400, 348-352.

Sánchez F., Alonso J.R., Arévalo R., Blanco E., Aijón J. and Vázquez R. (1994). Coexistence of NADPH-diaphorase with vasopressin and oxytocin in the hypothalamic magnocellular neurosecretory nuclei of the rat. Cell Tissue Res. 276, 31-34.

Schepens J., Cuppen E., Wieringa B. and Hendriks W. (1997). The neuronal nitric oxide synthase PDZ motif binds to -G(D,E)XV* carboxyterminal sequences. FEBS Lett. 409, 53-56.

Shaul P.W., Smart E.J., Robinson L.J., German Z., Yuhanna I.S., Ying Y., Anderson R.G. and Michel T. (1996). Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. J. Biol. Chem. 271, 6518-6522.

Siaud P., Mekaouche M., Ixart G., Balmefrezol M., Givalois L., Barbanel G. and Assenmacher I. (1994). A subpopulation of corticotropin-releasing hormone neurosecretory cells in the paraventricular nucleus of the hypothalamus also contain NADPH- diaphorase. Neurosci. Lett. 170, 51-54.

Silvagno F., Xia H. and Bredt D.S. (1996). Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. J. Biol. Chem. 271, 11204-11208.

Stanarius A., Topel I., Schulz S., Noack H. and Wolf G. (1997). Immunocytochemistry of endothelial nitric oxide synthase in the rat brain: a light and electron microscopical study using the tyramide signal amplification technique. Acta Histochem. 99, 411-429.

Stuehr D.J. (1997). Structure-function aspects in the nitric oxide synthases. Annu. Rev. Pharmacol. Toxicol. 37, 339-359.

Summy-Long J.Y., Salisbury R., Marietta M.P., Hartman R.D. and Weisz J. (1984). Pathways of hydrogen utilization from NADPH generated by glucose-6-phosphate dehydrogenase in circumventricular organs and the hypothalamo-neurohypophysial system: a cytochemical study. Brain Res. 294, 23-35.

Tanaka M., Ikeda T., Hayashi S., Iijima N., Amaya F., Hisa Y. and Ibata Y. (1997). Nitrergic neurons in the medial amygdala project to the hypothalamic paraventricular nucleus of the rat. Brain Res. 777, 13-21.

Terada H., Nagai T., Kimura H., Kitahama K. and Okada S. (1996). Distribution of nitric oxide synthase-immunoreactive neurons in fetal rat brains at embryonic day 15 and day 19. J. Chem. Neuroanat. 10, 273-278.

Topinka J.R. and Bredt D.S. (1998). N-terminal palmitoylation of PSD-95 regulates association with cell membranes and interaction with K^+ channel Kv1.4. Neuron 20, 125-134.

Torres G., Lee S. and Rivier C. (1993). Ontogeny of the rat hypothalamic nitric-oxide synthase and colocalization with neuropeptides. Mol. Cell Neurosci. 4, 155-163.

Tsuchiya T., Kishimoto J. and Nakayama Y. (1996). Marked increases in neuronal nitric oxide synthase (nNOS) mRNA and NADPH-diaphorase histostaining in adrenal cortex after immobilization stress in rats. Psychoneuroendocrinology 21, 287-293.

Tsuchiya T., Kishimoto J., Koyama J. and Ozawa T. (1997). Modulatory effect of L-NAME, a specific nitric oxide synthase (NOS) inhibitor, on stress-induced changes in plasma adrenocorticotropic hormone (ACTH) and corticosterone levels in rats: physiological significance of stress-induced NOS activation in hypothalamic-pituitary-adrenal axis. Brain Res. 776, 68-74.

Turnbull A.V. and Rivier C. (1996). Corticotropin-releasing factor, vasopressin, and prostaglandins mediate, and nitric oxide restrains, the hypothalamic-pituitary-adrenal response to acute local inflammation in the rat. Endocrinology 137, 455-463.

Ueta Y., Levy A., Chowdrey H.S. and Lightman S.L. (1995). Inhibition of hypothalamic nitric oxide synthase gene expression in the rat paraventricular nucleus by food deprivation is independent of serotonin depletion. J. Neuroendocrinol. 7, 861-865.

Vaid R.R., Yee B.K., Rawlins J.N. and Totterdell S. (1996). NADPH-diaphorase reactive pyramidal neurons in Ammon's horn and the subiculum of the rat hippocampal formation. Brain Res. 733, 31-40.

Valtschanoff J.G., Weinberg R.J., Kharazia V.N., Nakane M. and Schmidt H.H. (1993). Neurons in rat hippocampus that synthesize nitric oxide. J. Comp. Neurol. 331, 111-121.

Vanhatalo S. and Soinila S. (1995). Nitric oxide synthase in the hypothalamo-pituitary pathways. J. Chem. Neuroanat. 8, 165-173.

Villar M.J., Ceccatelli S., Rönnqvist M. and Hökfelt T. (1994). Nitric oxide synthase increases in hypothalamic magnocellular neurons after salt loading in the rat. An immunohistochemical and in situ hybridization study. Brain Res. 644, 273-281.

Vincent S.R. (1994). Nitric oxide: a radical neurotransmitter in the central nervous system. Prog. Neurobiol. 42, 129-160.

Vincent S.R. and Kimura H. (1992). Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience 46, 755-784.

Wendland B., Schweizer F.E., Ryan T.A., Nakane M., Murad F., Scheller R.H. and Tsien R.W. (1994). Existence of nitric oxide synthase in rat hippocampal pyramidal cells. Proc. Natl. Acad. Sci. USA 91, 2151-2155.

Wolf G. (1997). Nitric oxide and nitric oxide synthase: biology, pathology, localization. Histol. Histopathol. 12, 251-261.

Wong M.L., Rettori V., Al-Shekhlee A., Bongiorno P.B., Canteros G., McCann S.M., Gold P.W. and Licinio J. (1996). Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. Nature Med. 2, 581-584.

Xiao Z., Zhang Z. and Diamond S.L. (1997). Shear stress induction of the endothelial nitric oxide synthase gene is calcium-dependent but not calcium-activated. J. Cell. Physiol. 171, 205-211.

Xie Q.W., Cho H.J., Calaycay J., Mumford R.A., Swiderek K.M., Lee T.D., Ding A., Troso T. and Nathan C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256, 225-228.

Yamada K., Emson P. and Hökfelt T. (1996). Immunohistochemical mapping of nitric oxide synthase in the rat hypothalamus and colocalization with neuropeptides. J. Chem. Neuroanat. 10, 295-316.

Zorumski C.F. and Izumi Y. (1993). Nitric oxide and hippocampal synaptic plasticity. Biochem. Pharmacol. 46, 777-785.