Effects of gonadal hormones on central nitric oxide producing systems.

EFFECTS OF GONADAL HORMONES ON CENTRAL NITRIC OXIDE PRODUCING SYSTEMS.

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Abbreviations (anatomical nomenclature according to Franklin and Paxinos, 1997).

5-HT: serotonin
AR: androgen receptor
ARC: arcuate nucleus
ARO: aromatase
BAOT: bed nucleus of the accessory olfactory tract
BST: bed nucleus of the stria terminalis
cGMP: cyclic guanosine-monophosphate
DA: dopamine
E_2: estradiol
ERalpha: estrogen receptor alpha
ERbeta: estrogen receptor beta
GnRH: gonadotrophin hormone-releasing hormone
-IR: -immunoreactive
L-NAME: L-nitro-arginine methyl ester
MeA: medial amygdala
MeAV: medial amygdala, anteroventral subdivision
MPA: medial preoptic area
MPOM: medial preoptic nucleus, medial pars
NADPH: nicotinamide adenine dinucleotide phosphate
nNOS: neuronal nitric oxide synthase
NO: nitric oxide
PVN: paraventricular nucleus
T: testosterone
VMH: ventromedial nucleus

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Abstract

Nitric oxide (NO)-containing neurons are widely distributed within the central nervous system, including regions involved in the control of reproduction and sexual behavior. The expression of neuronal NO-synthase (nNOS) is influenced by testosterone in male rat, and by estrogens in female. Moreover, nNOS may co-localize with gonadal hormones' receptors. Gonadal hormones may influence nNOS expression in adulhood as well as during the development. In fact, in mice knockout for estrogen receptor alpha, the nNOS-expressing population is deeply reduced in specific regions. In physiological conditions, the female in mammalian species is exposed to short-term changes of gonadal hormones levels (estrous cycle). Our recent studies, performed in the rat vomeronasal system and in mouse hypothalamic and limbic systems reveal that, in rodents, the expression of nNOS-producing elements within regions relevant for the control of sexual behavior are under the control of gonadal hormones. The expression of nNOS may vary according to the rapid variations of hormonal levels that take place during the estrous cycle. This seems in accordance with the hypothesis that gonadal hormone activation of NO-cGMP pathway is important for lordosis behavior, as well as that this system is activated during mating behavior. Finally, comparative data available for other vertebrates suggest that class-specific and species-specific differences occur in the nNOS system of hypothalamus and limbic structures. Therefore, particular caution is needed to generalize data obtained from studies in rodents.

Keywords: sex steroids, NADPH-diaphorase, BAOT, amygdala, hypothalamus, estrous cycle

Nitric oxide (NO) is an inorganic free radical gas (˙N=O), whose synthesis from L-arginine requires an enzyme known as NO synthase (NOS), and contributes to the formation of citrulline. Besides these substrates, NO synthesis requires also coenzymes (as reduced nicotinamide adenine dinucleotide phosphate, NADPH), cofactors, the presence of calmodulin (Knowles et al., 1989) (**Fig.1A**), as well as the co-operation of the superoxide dismutase (Schmidt et al., 1996). NO is believed to be a neuronal messenger (Vincent, 1994), whose action takes place primarily by inducing an increase of soluble cyclic guanosine monophosphate (cGMP) in target cells (Miki et al., 1977). Molecular cloning and the study of immunological properties suggested that there are at least three isoforms of NOS that have been purified and characterized from nervous tissue, macrophages, and endothelial cells (Alderton et al., 2001). All these isoforms are present within the brain in different cellular compartments (**Fig.1B**), but the neuronal isoform (nNOS) is largely predominant (Bredt et al., 1990).

The neuronal nitric oxide synthase.

NO is an unusual neuronal messenger molecule that revolutionized our conceptions of how neurons communicate (Dawson and Snyder, 1994). It was first recognized as a neuronal messenger molecule when it was demonstrated that glutamate, acting on the *N*-methyl-D-aspartate receptor in cultures of cerebellar granule cells, releases a factor with properties resembling NO. The neuronal NO-forming enzyme (nNOS) is activated by increases in intracellular calcium, which subsequently binds to calmodulin to activate the enzyme. Due to the importance of NADPH as H+ donor for the functioning of nNOS, it was quickly understood that the old histochemical reaction for NADPH-diaphorase (Thomas and Pearse, 1964) could be utilized to detect nNOS positive neurons (Bredt et al., 1991). Subsequent investigations have shown that NOS catalytic activity accounts for NADPH diaphorase staining (for a review see Dawson and Dawson, 1996).

Neuroanatomical distribution

NO-producing neurons have been localized, with both histochemical and immunohistochemical methods, in several parts of the mammalian and non-

mammalian central nervous system including the olfactory system, the cerebral cortex, the diencephalon, the brainstem, the cerebellum and the spinal cord (Vincent and Kimura, 1992, Brüning et al., 1994, Egberongbe et al., 1994, Holmqvist et al., 1994, Panzica et al., 1994, Rodrigo et al., 1994, Brüning and Mayer, 1996, Gotti et al., 2005). Based on the present literature, it appears that the nNOS system in the brain does not overlap completely with any other known neurotransmitter system, even though several studies demonstrated co-localization with cholinacetyltransferase, serotonin (5-HT), and numerous neuropeptides (see for reviews: Panzica et al., 1998, Prast and Philippu, 2001).

In particular, nNOS-immunoreactive (nNOS-IR) neurons and fibers were described in several hypothalamic and limbic nuclei of rodents (Bhat et al., 1996, Hadeishi and Wood, 1996, Ng et al., 1999). Many of these nuclei are implicated in the control of reproduction: e.g., medial preoptic nucleus (MPOM) (**Fig.1C, D**), paraventricular nucleus (PVN) (**Fig. 1E, F**), supraoptic nucleus, arcuate nucleus (ARC), ventromedial nucleus (VMH) (**Fig.1G, E**), bed nucleus of the stria terminalis (BST), and amygdaloid complex (**Fig.1G, F**). The nNOS-IR elements are generally weakly or moderately stained small neurons, with a large supply of positive fibers. In the PVN, positive neurons are mainly situated in the ventral part, in the medial parvicellular portion, and around the fornix (**Fig.1F**). A dense network of fibers covers the VMH, whereas the weakly stained neurons are localized in its more lateral subdivision (**Fig1H**).

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Nitric oxide, neuroendocrine functions and behaviors

The localization of nNOS in diverse cell types, belonging to a variety of neuronal systems, suggests a widespread role in neuromodulation for the free radical NO. The effects on blood vessel tone and neuronal function form the basis for the important role that NO has on neuroendocrine function and behavior. This messenger molecule appears to be involved in a variety of physiological activities, such as long-term potentiation, neuroprotection, neural degeneration, and the regulation of peptidergic secretion (for reviews see: Dawson and Snyder, 1994, Dawson and Dawson, 1996). In particular, nitric oxide plays a crucial role in reproduction at various level in the organism. In the brain, it activates the release of gonadotrophin hormone-releasing

hormone (GnRH) enabling the lordosis reflex to be elicited by stimuli from the male. In the periphery, NO via cGMP induces erection in males and ovulation in females (for reviews see: McCann et al., 1999, McCann et al., 2003).

Finally, NO influences several motivated behaviors including aggressive, ingestive, and sexual behaviors. Learning and memory are also influenced (for reviews see: Nelson et al., 1997, Nelson and Chiavegatto, 2001)

Nitric oxide and reproductive behaviors

Several studies have suggested complex relations among NO producing elements and sexual behavior. In females, the NO/cGMP/protein kinase G pathway is involved in the lordosis induced by progesterone and some of its ring A-reduced metabolites (Gonzalez-Flores and Etgen, 2004). Moreover, treatment with competitive inhibitors of nNOS attenuates progesterone-induced lordosis, whereas administration of a NO donor facilitates sexual behavior. This last effect is blocked by the administration of a GnRH antiserum. Since progesterone-facilitated lordosis is mediated by GnRH release, these results indicate that NO induces GnRH release that then plays a role in the control of female sexual behavior (Mani et al., 1994). In male rats, the treatment with L-arginine, the natural nNOS substrate, facilitates male sexual behavior (Benelli et al., 1995, Sato et al., 1998), whereas administration of nNOS inhibitors reduces the number of mounts and prevents ejaculations (Benelli et al., 1995, Sato et al., 1998). In addition, disruption of the nNOS gene in knock out mice results in an inappropriate sexual behavior of males (Nelson et al., 1995).

Other studies suggest the involvement of NO in the maternal behavior, in particular the participation of NO in the aggressive behavior that lactating females express against intruders during this period. It has been demonstrated that disruption of the nNOS gene in mice produces important deficits in maternal aggression (Gammie and Nelson, 1999). Moreover, intracerebroventricular administration of the NO inhibitor L-nitro-arginine methyl ester (L-NAME) during postpartum not only attenuates maternal aggression, but also disrupts the expression of maternal behavior, eliminating pup retrieval (Popeski and Woodside, 2004).

In summary, these studies suggest that NO facilitates the expression of sexual behavior in males and females and also that this neurotransmitter has a relevant role in aggression in lactating females.

Direct or indirect control of NO on sexual behavior

Is the effect of NO on the reproductive behaviors the results of its direct control or is mediated by some other neurotransmitter system? Some reports seems to indicate that, in rats, the involvement of NO in the control of reproductive behavior is modulated by gonadal hormones and probably mediated by interactions with other neurotransmitter systems such as dopamine (DA) in males (for reviews see: Hull et al., 1997, Hull et al., 1999, Hull et al., 2002) and noradrenaline in females (Chu and Etgen, 1997, Lagoda et al., 2004). In the male rat, testosterone (T) acts by increasing nNOS immunoreactivity (Du and Hull, 1999), NO, in turn, stimulates the release of DA in the medial preoptic area (MPA) (Lorrain and Hull, 1993, Lorrain et al., 1996, Dominguez et al., 2004). The increased DA release enhances responsiveness to stimuli from an estrous female and increases the probability, rate, and efficiency of copulation (Lorrain et al., 1996). It was not known, however, which metabolite(s) of T regulate(s) DA and/or nNOS in the MPA of male rats. The results of a recent study indicate that estradiol (E_2) up-regulates nNOS-IR in the MPA and it maintains tissue content of DA at levels similar to those in T-treated rats. Dihydrotestosterone did not influence nNOS-IR, while attenuating the effect of castration on tissue DA content (Putnam et al., 2005).

In the females rat, it has been recently demonstrated that microinjection of L-NAME, an NO synthesis inhibitor, into the MPA blocked copulation in naive rats and impaired copulation in sexually experienced males (Lagoda et al., 2004). Moreover the NO-cGMP system may mediate the facilitator effect of α1–adrenoreceptors on lordosis behavior in female rats (Chu and Etgen, 1997).

In summary, these studies suggest that NO might facilitates the expression of rodents' sexual behavior in both sexes, probably through its action on specific neurotransmitter systems.

Neuronal nitric oxide synthase and gonadal hormones

The distribution of nNOS in several brain regions of mammals overlaps that of gonadal hormones' receptors. Regions like the BST, the amygdala, the preoptic region, the mediobasal hypothalamus, or the magnocellular nuclei are characterized by the presence of two types of estrogen receptors (ERalpha and ERbeta) (Shughrue and Merchenthaler, 2001, Merchenthaler et al., 2004), of androgen receptors (AR),

(Simerly et al., 1990) and of progesterone receptors (PR), (Lauber et al., 1991). Only a few studies have, however, detailed the co-expression of nNOS (or NADPH-diaphorase activity) with these receptors.

In particular, in the female guinea pig, quantitative analysis showed that approximately 16% of the nNOS-IR cells in the rostral preoptic area and 55% of nNOS-IR cells in the ventrolateral nucleus displayed PR immunoreactivity (Warembourg et al., 1999), a similar distribution was observed also in the ewe (Dufourny and Skinner, 2002).

In male and female rats, ERalpha co-localize with NADPH-diaphorase elements in the VMH (Okamura et al., 1994b), whereas AR co-localize with nNOS-IR cells in the premammilary nucleus of the male rats (Yokosuka and Hayashi, 1996, Yokosuka et al., 1997). A recent study (Sato et al., 2005) detailed in a greater detail the co-localization of ERalpha and AR with nNOS-IR cells in male rat preoptic and anterior hypothalamic regions. In particular, in the anteroventral periventricular nucleus ERalpha co-localize with 77% and AR with 60% of nNOS cells, in the MPOM ERalpha co-localize with the 53% and AR with 52% of nNOS cells.

In male mouse, ERalpha co-localize with 90% of nNOS cells in the MPA and 50% of nNOS cells in the medial amygdala (MeA), whereas a more limited number of nNOS cells co-localize in the BST (16%) or in the PVN (10%) (Scordalakes et al., 2002). AR co-localize with a more limited number of nNOS cells in the same nuclei (e.g.: 20% in MPA, 6% in BST, 20% in MeA, 10% in PVN) (Scordalakes et al., 2002).

These results indicate that in all the investigated mammalian species a relationship between gonadal steroids' receptors and nNOS or NADPH-diaphorase is present. However, species-specific differences have been detected in the proportion of the colocalization.

The regionally specific distribution of nNOS-IR elements and their co-existence with gonadal hormones' receptors suggest the existence of significant neuroendocrine relationships. Therefore, several studies investigated the role played by gonadal hormones in the regulation of the nNOS system. In mammals, sex steroids control the expression of nNOS in the preoptic-hypothalamic region. In the male, castration decreases the number of nNOS-IR neurons in the rat (Du and Hull, 1999) and the hamster (Hadeishi and Wood, 1996) MPA. In the female, E_2 increases, the NADPH–diaphorase staining in the guinea pig ventrolateral nucleus (Warembourg et al., 1999) and in the rat PVN (Sanchez et al., 1998), and MPA (Okamura et al., 1994a). It

increases also the nNOS mRNA in the ventrolateral subdivision of the rat VMH (Ceccatelli et al., 1996). However, effects of hormonal manipulations are not univocal; in fact other studies demonstrated either no effects of castration or an increase of mRNA for nNOS in the hypothalamus of male rat (Shi et al., 1998, Singh et al., 2000). A recent study evidenced an E_2-induced decrease, mediated by the ERbeta, in the number of nNOS-positive neurons in rat hypothalamic slices cultures of PVN (Gingerich and Krukoff, 2005).

These discrepancies in the effects of gonadal hormones on the nNOS could be due to a combination of several factors: differences between species, methodology used, parameter studied or even a regional specificity. Changes in mRNA content (Shi et al., 1998, Singh et al., 2000) may not be directly related to changes in immunoreactive material (Du et al., 1998), as was previously demonstrated for other peptidergic systems (Miller et al., 1992). In addition, the presence of NOS-IR cells is not always reflecting the same amount of NADPH-diaphorase positive elements as recently demonstrated for the mouse basal forebrain (Gotti et al., 2004). Differential expression of protein inhibitor of nNOS (Jaffrey and Snyder, 1996) should also be considered in the future to better clarify these relationships.

Estrogen receptors seem to be important also for the differentiation of the limbic-hypothalamic nNOS system. A first study on the distribution of nNOS in mice knockout for ERalpha (ER□KO) demonstrated significant changes in the limbic-hypothalamic region, when compared to that observed in wild-type mice (Panzica et al., 2000). However, what we have observed was a nucleus-specific decrease rather than a total disappearance of the system. In particular, a significant decrease in NOS-IR cell number has been observed in PVN and ARC, as well as a significant decrease in the density of NOS immunostained fibres in MPA. Other regions that are important targets for estrogens in females, as the VMH, do not show significant differences. In the BST we have, on the contrary, observed an increase in the cell number at the more caudal level of the nucleus. To confirm the important role of estrogens, we observed a significant decrease of nNOS-IR elements in the MPA of aromatase knockout mice (ArKO) (Sica et al., 2002). A moderate decrease in immunoreactivity was also detected in the PVN and VMH.

By using a double mutant mouse in which males lacked functional ERalpha, AR, or both, Scordalakes et al. (2002) investigated the roles of these steroid receptors in

nNOS-IR cell numbers and immunoreactive area staining under T and E_2 treatments. Their data demonstrated that functional ERalpha is correlated with more nNOS-IR cells under T treatment and more immunoreactive area staining in the MPA under both T and E_2 treatments. However, the presence of ERalpha decreases nNOS-IR cell number in the BNST under E_2 treatment. AR has action on posterior ventral region of MeA and both receptors show action on PVN. In summary, these data suggest that ERalpha and AR interact to regulate nNOS in male and female brain in a site-specific manner.

Sex differences and effect of estrous cycle.

In general, all the reported studies were based on medium or long treatments with gonadal hormones (from one to several weeks). Obviously, this is a not physiological condition in adult laboratory rodents, where, in the female, the levels of circulating ovarian hormones change in a very short period (the total duration of estrous cycle is 4-5 days). Based on the presumption that gonadal hormones may influence the expression of nNOS, we wondered if short-term changes might influence the number of nNOS-IR elements and if this fact could also influence the demonstration of sexual dimorphism for this system.

In a study performed in mice (Martini et al., 2004) we considered four hypothalamic and limbic nuclei that are involved in the control of sexual behavior and targets for gonadal hormones: MPA, BST, ARC, VMH (Nishizuka, 1978, Leedy et al., 1980, García-Segura et al., 1988, Claro et al., 1995, Kato and Sakuma, 2000). In some of these nuclei (e.g.: MPA, ARC) we observed statistically significant changes in the population of nNOS-IR elements throughout the estrous cycle, whereas, in the other two nuclei (e.g. BST and VMH) we have not detected any statistically significant variation (see **Fig. 2a-d**). Changes in the number of nNOS-IR cells in MPA and ARC do not follow the same pattern. In MPA, the highest number of positive neurons was detected during estrus, whereas in proestrus and diestrus we have the lower values. In ARC, the highest number of nNOS-IR cells was detected in proestrus, this value is significantly different from metestrus and diestrus.

Sex differences were statistically significant only in the BST, with females showing more nNOS-IR cells than males. For the MPA and ARC, in which nNOS-IR varied with the estrous cycle, significant sex differences depended on the phase of the

estrous cycle. Therefore, this indicates the presence of a sexual diergism (i.e. functional sex difference, Rhodes and Rubin, 1999) rather than a real dimorphism.

In the rat, variations in expression of nNOS, or associated NADPH-diaphorase activity, during the estrous cycle were studied in two structures belonging to the vomeronasal system: the bed nucleus of the accessory olfactory tract (BAOT) and the anteroventral subdivision of medial amygdala (MeAV) (Collado et al., 2003, Carrillo et al., 2004). These two nuclei are implicated in the control of reproductive behaviors (Masco and Carrer, 1984, Del Cerro et al., 1991, Izquierdo et al., 1992, Kondo, 1992, Dominguez and Hull, 2001, Sheehan et al., 2001). In both structures two types of positive neurons were identified: intensely- and medium-stained elements. The sensitivity to E_2 of these two subpopulations of NO producing cells may vary depending on the investigated nucleus. In the BAOT, there was a greater density of medium-stained cells in estrous females then in males or diestrous females **(Fig. 2E**) (Collado et al., 2003). However, in the MeAV nucleus the intensely stained cells were the most sensitive group. Estrous females had significantly more NADPH-diaphorase-positive cells than did male and diestrous female (**Fig. 2F**) (Carrillo et al., 2004). These hormone-dependent fluctuations in NADPH-diaphorase activity suggest that distinct subpopulations in the BAOT-medial amygdala pathway might regulate the expression of reproductive behaviors in which these two structures are involved. As was the case of MPA and ARC in mice, a functional diergism occurs also in rat BAOT and MeAV.

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Comparative studies

The study of the distribution and characteristics of nNOS or NADPH-diaphorase positive neurons has been performed mostly in mammalian species. However, several papers described the presence and distribution of this system also in the brain of other vertebrates, such as fishes, amphibians, reptiles, and birds (for a review see Panzica et al., 1998). The general pattern of distribution of nNOS or NADPH-diaphorase positive neurons is similar in all vertebrates, with some noteworthy variation. Hence, populations of positive neurons were observed in the basal ganglia and in the so-called mesopontine system of all vertebrates, whereas neuronal populations located in the diencephalon or in higher telencephalic centers showed a high degree of variations

of the distribution according to the investigated species (for discussions see Panzica et al., 1998).

In birds a series of investigations, partly done in our laboratories, have elucidated that differences are even more marked when considering the extent of co-localization with neurotransmitters or neuropeptides (Panzica et al., 1996, Sanchez et al., 1996, Panzica and Garzino, 1997). No sex dimorphism was observed in the hypothalamus of Japanese quail (G.C. Panzica and C. Dermon, unpublished results). In a recent study (Balthazart et al., 2003), we investigated the anatomical relationships between aromatase (ARO) and nNOS-containing neurons. Major groups of nNOS-immunoreactive/ NADPH-positive neurons were adjacent to the main ARO-IR cell groups, such as the MPOM, the BST and the VMH. However, examination of adjacent sections indicated that there is very little overlap between the NOS-IR and ARO-IR cell populations. This notion got further support by double-labeled sections where no double-labeled cells could be identified. In sections stained simultaneously by histochemistry for NADPH and immunohistochemistry for ARO, many NADPH-positive fibers and punctate structures were closely associated with ARO-IR perikarya. Taken together, these data indicate that nNOS is not or very rarely co-localized with ARO, but that nNOS elements (cell bodies, processes, presumptive synaptic terminals) are closely associated with ARO-IR cells, suggesting that they might rise inputs modulating the expression or the activity of ARO in the quail brain. Further studies should clarify these suggested relationships.

Following this study as well as the mammalian model, we investigated if, in male quail, gonadal hormones may regulate the nNOS system (Martini et al., 2005). We have therefore studied the nNOS system in adult male quails that were either intact, gonadectomized or treated with Silastic implants of T. The study was performed in regions where both nNOS-IR neurons and gonadal hormones' receptors are present (Panzica et al., 1994, Gahr, 2001), namely BST, VMH, area ventralis tegmentalis, and substantia grisea centralis. Quantitative analyses revealed no significant effect of gonadectomy or of exogenous T on the number of nNOS-IR cells within these nuclei. In conclusion, these data indicate that the hypothalamic nNOS system of male quail is not under the control of T. This suggests that the role played in mammals by NO in the control of male sexual behavior, as well as other functions such as osmoregulation or regulation of cerebral blood flow (Nilsson and Soderstrom, 1997), may not be considered a general feature of all vertebrates or may be exerted by other mechanism

than those hypothesized for mammals.

Conclusions

The studies reported in this short review indicate that, in mammals, gonadal hormones directly influence the expression of nNOS in a wide population of hypothalamic and limbic neurons. The effects of sex steroids have been chiefly demonstrated after medium- or long-term treatments, but they are also very significant in physiological situations, as during the estrous cycle. Changes are not similar in the whole system, but they vary in a very specific way according to each nucleus that has been investigated. It is difficult to differentiate true sexually dimorphic distributions of nNOS-IR elements from sexually diergic ones. Only for those nNOS populations that are insensitive to the estrous cycle (e.g.: VMH or BST), it is relatively easy to test whether their distribution is dimorphic. However, short-term effects may differ from long-term ones. For example, in female rat, the VMH is strongly stimulated by the long-term administration of estrogens (Ceccatelli et al., 1996), whereas, in mice, we have not detected any significant changes during the estrous cycle (Martini et al., 2004). At the moment no clear conclusion can be drawn on this topic, in particular, studies on genetically altered mice cannot fully clarify among organizational versus activational effects. Therefore, additional studies are needed following the strategies recently indicated for studying sex differences in the brain (Becker et al., 2005).

In addition, data that are available so far in other vertebrate models are even more disperse. As we have reported for the Japanese quail, one of the more studied avian species for the control of sexual behavior, they are apparently in strong contradiction to the physiological implications of nNOS in the control of reproduction that have been suggested for mammals (Martini et al., 2005). Therefore, the comparative studies are not clarifying our knowledge, but, on the contrary, point to species-specific or class-specific differences, mainly targeted to the hypothalamic and limbic system.

References

Alderton WK, Cooper CE, Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. Biochem J 357: 593-615.

Balthazart J, Panzica GC, Krohmer RW (2003) Anatomical relationships between aromatase-immunoreactive neurons and nitric oxide synthase as evidenced by NOS immunohistochemistry or NADPH diaphorase histochemistry in the quail forebrain. J Chem Neuroanat 25: 39-51.

Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadee W, Steiner M, Taylor J, Young E (2005) Strategies and Methods for Research on Sex Differences in Brain and Behavior. Endocrinology 146: 1650-1673.

Benelli A, Bertolini A, Poggioli R, Cavazzuti E, Calzà L, Giardino L, Arletti R (1995) Nitric oxide is involved in male sexual behavior of rats. Eur J Pharmacol 294: 505-510.

Bhat G, Mahesh VB, Aguan K, Brann DW (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 DS, Glatt CE, Hwang PM, Fotuhi M, Dawson TM, Snyder SH (1991) Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 7: 615-624.

Bredt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 347: 768-770.

Brüning G, Mayer B (1996) Localization of nitric oxide synthase in the brain of the frog, Xenopus laevis. Brain Res 741: 331-343.

Brüning G, Wiese S, Mayer B (1994) Nitric oxide synthase in the brain of the turtle Pseudemys scripta elegans. J Comp Neurol 348: 183-206.

Carrillo B, Pinos H, Panzica GC, Guillamon A, Collado P (2004) Nitrergic expression during estrous phase and morphologic sexual dimorphism in the medial amygdala anteroventral subdivision in rat. Horm Behav 46: 85-86.

Ceccatelli S, Grandison L, Scott REM, Pfaff DW, Kow LM (1996) Estradiol regulation of nitric oxide synthase mRNAs in rat hypothalamus. Neuroendocrinology 64: 357-363.

Chu HP, Etgen AM (1997) A Potential Role of Cyclic GMP in the Regulation of Lordosis Behavior of Female Rats. Horm Behav 32: 125–132.

Claro F, Segovia S, Guilamón A, Del Abril A (1995) Lesions in the medial posterior region of the BST impair sexual behavior in sexually experienced and inexperienced male rats. Brain Res Bull 36: 1-10.

Collado P, Guillamon A, Pinos H, Perez-Izquierdo MA, Garcıa-Falgueras A, Carrillo B, Rodrıguez C, Panzica GC (2003) NADPH-diaphorase activity increases during estrous phase in the bed nucleus of the accessory olfactory tract in the female rat. Brain Res 983: 223–229.

Dawson TM, Dawson VL (1996) Nitric-oxide synthase: Role as a transmitter mediator in the brain and endocrine system. Annu Rev Med 47: 219-227.

Dawson TM, Snyder SH (1994) Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J Neurosci 14: 5147-5159.

Del Cerro MC, Izquierdo MA, Collado P, Segovia S, Guillamon A (1991) Bilateral lesions of the bed nucleus of the accessory olfactory tract facilitate maternal behavior in virgin female rats. Physiol Behav 50: 67-71.

Dominguez JM, Hull EM (2001) Stimulation of the medial amygdala enhances medial preoptic dopamine release: implications for male rat sexual behavior. Brain Res 917: 225-229.

Dominguez JM, Muschamp JW, Schmich JM, Hull EM (2004) Nitric oxide mediates glutamate-evoked dopamine release in the medial preoptic area. Neuroscience 125: 203-210.

Du J, Hull EM (1999) Effects of testosterone on neuronal nitric oxide synthase and tyrosine hydroxylase. Brain Res 836: 90-98.

Du J, Lorrain DS, Hull EM (1998) Castration decreases extracellular, but increases intracellular, dopamine in medial preoptic area of male rats. Brain Res 782: 11-17.

Dufourny L, Skinner DC (2002) Influence of Estradiol on NADPH Diaphorase/Neuronal Nitric Oxide Synthase Activity and Colocalization with Progesterone or Type II Glucocorticoid Receptors in Ovine Hypothalamus. Biol Reprod 67: 829-836.

Egberongbe YI, Gentleman SM, Falkai P, Bogerts B, Polak JM, Roberts GW (1994) The distribution of nitric oxide synthase immunoreactivity in the human brain. Neuroscience 59: 561-578.

Franklin KBJ, Paxinos G (1997) The mouse brain in stereotaxic coordinates. New York: Academic Press.

Gahr M (2001) Distribution of Sex Steroid Hormone Receptors in the Avian Brain: Functional Implications for Neural Sex Differences and Sexual Behaviors. Microsc Res Tech 55: 1–11.

Gammie SC, Nelson RJ (1999) Maternal aggression is reduced in neuronal nitric oxide synthase-deficient mice. J Neurosci 19: 8027-8035.

García-Segura LM, Hernandez P, Olmos G, Tranque P, Naftolin F (1988) Neuronal membrane remodelling during the oestrus cycle: a freeze-fracture study in the arcuate nucleus of the rat hypothalamus. J Neurocytol 17: 377-383.

Gingerich S, Krukoff TL (2005) Estrogen modulates eNOS and nNOS expression via an ERβ-dependent mechanism in hypothalamic slice cultures. Endocrinology 146: 2933-2941.

Gonzalez-Flores O, Etgen AM (2004) The nitric oxide pathway participates in estrous behavior induced by progesterone and some of its ring A-reduced metabolites. Horm Behav 45: 50-57.

Gotti S, Chiavegatto S, Sica M, Viglietti-Panzica C, Nelson RJ, Panzica G (2004) Alteration of NO-producing system in the basal forebrain and hypothalamus of Ts65Dn mice: an immunohistochemical and histochemical study of a murine model for Down syndrome. Neurobiol Dis 16: 563-571.

Gotti S, Sica M, Viglietti Panzica C, Panzica GC (2005) The distribution of nitric oxide sythase immunoreactivity in the mouse brain. Microsc Res Tech *in press*.

Hadeishi Y, Wood RI (1996) Nitric Oxide Synthase in Mating Behavior Circuitry of Male Syrian Hamster Brain. J Neurobiol 30: 480-492.

Holmqvist BI, Östholm T, Alm P, Ekström P (1994) Nitric oxide synthase in the brain of a teleost. Neurosci Lett 171: 205-208.

Hull EM, Du J, Lorrain DS, Matuszewich L (1997) Testosterone, preoptic dopamine, and copulation in male rats. Brain Res Bull 44: 327-333.

Hull EM, Lorrain DS, Du J, Matuszewich L, Lumley LA, Putnam SK, Moses J (1999) Hormone-neurotransmitter interactions in the control of sexual behavior. Behav Brain Res 105: 105-116.

Hull EM, Meisel RL, Sachs BD (2002) Male Sexual Behavior. In: Hormones, Brain and Behavior, Pfaff, D. (ed) vol.1, pp. 1-134. Elsevier Science.

Izquierdo MAP, Collado P, Segovia S, Guillamon A, Del Cerro MCR (1992) Maternal behavior induced in male rats by bilateral lesions of the bed nucleus of the accessory olfactory tract. Physiol Behav 52: 707-712.
Jaffrey SR, Snyder SH (1996) PIN: an associated protein inhibitor of neuronal nitric oxide synthase. Science 274: 774-777.
Kato A, Sakuma Y (2000) Neuronal activity in female rat preoptic area associated with sexually motivated behavior. Brain Res 862: 90-102.
Knowles RG, Palacios M, Palmer RMJ, 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 U S A 86: 5159-5162.
Kondo Y (1992) Lesions of the Medial Amygdala Produce Severe Impairment of Copulatory Behavior in Sexually Inexperienced Male Rats. Physiol Behav 51: 939-943.
Lagoda G, Muschamp JW, Vigdorchik A, Hull EM (2004) A Nitric Oxide Synthesis Inhibitor in the Medial Preoptic Area Inhibits Copulation and Stimulus Sensitization in Male Rats. Behav Neurosci 118: 1317-1323.
Lauber AH, Romano GJ, Pfaff DW (1991) Gene Expression for Estrogen and Progesterone Receptor mRNAs in Rat Brain and Possible Relations to Sexually Dimorphic Functions. J Steroid Biochem Mol Biol 40: 53-62.
Leedy MG, Vela EA, Popolow HB, Gerall AA (1980) Effect of prepuberal medial preoptic area lesions on male rat sexual behavior. Physiol Behav 24: 341-346.
Lorrain DS, Hull EM (1993) Nitric oxide increases dopamine and serotonin release in the medial preoptic area. Neuroreport 5: 87-89.
Lorrain DS, Matuszewich L, Howard RV, Du JF, Hull EM (1996) Nitric oxide promotes medial preoptic dopamine release during male rat copulation. Neuroreport 8: 31-34.
Mani SK, Allen JMC, Rettori V, McCann SM, O'Malley BW, Clark JH (1994) Nitric oxide mediates sexual behavior in female rats. Proc Natl Acad Sci U S A 91: 6468-6472.
Martini M, Sica M, Eva C, Viglietti Panzica C, Panzica GC (2004) Dimorphism and effects of estrous cycle on the nitrinergic system in mouse hypothalamus. Horm Behav 46: 95-96.
Martini M, Zito G, Viglietti-Panzica C, Panzica GC, Balthazar J (2005) Neuronal nitric oxide synthase in forebrain and mesencephalon is not affected by castration and testosterone therapy in Japanese quail. Soc Neurosc Abstr in press.
Masco DH, Carrer HF (1984) Pathways conducting amygdaloid influence on feminine sexual behavior in the rat. Behav Brain Res 11: 205-212.
McCann SM, Haens G, Mastronardi C, Walczewska A, Karanth S, Rettori V, Yu WH (2003) The role of nitric oxide (NO) in control of LHRH release that mediates gonadotropin release and sexual behavior. Curr Pharm Des 9: 381-390.
McCann SM, Mastronardi C, Walczewska A, Karanth S, Rettori V, Yu WH (1999) The role of nitric oxide in reproduction. Braz J Med Biol Res 32: 1367-1379.
Merchenthaler I, Lane MV, Numan S, Dellovade TL (2004) Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses. J Comp Neurol 473: 270-291.
Miki N, Kawabe Y, Kuriyama K (1977) Activation of cerebral guanylate cyclase by nitric oxide. Biochem Biophys Res Commun 75: 851-856.

Miller MA, De Vries GJ, Al Shamma HA, Dorsa DM (1992) Decline of vasopressin immunoreactivity and mRNA levels in the bed nucleus of the stria terminalis following castration. J Neurosci 12: 2881-2887.
Nelson RJ, Chiavegatto S (2001) Molecular basis of aggression. Trends Neurosci 24: 713-719.
Nelson RJ, Demas GE, Huang PL, Fishman MC, Dawson VL, Dawson TM, Snyder SH (1995) Behavioural abnormalities in male mice lacking neuronal nitric oxide synthase. Nature 378: 383-386.
Nelson RJ, Kriegsfeld LJ, Dawson VL, Dawson TM (1997) Effects of nitric oxide on neuroendocrine function and behavior. Front Neuroendocrinol 18: 463-491.
Ng YK, Xue YD, Wong PT (1999) Different distributions of nitric oxide synthase-containing neurons in the mouse and rat hypothalamus. Nitric Oxide 3: 383-392.
Nilsson GE, Soderstrom V (1997) Comparative aspects on nitric oxide in brain and its role as a cerebral vasodilator. Comp Biochem Physiol A 118: 949-958.
Nishizuka M (1978) Topography of the neurons responding to estrogen in the hypothalamic arcuate nucleus of immature female mice. Brain Res 152: 31-40.
Okamura H, Yokosuka M, Hayashi S (1994a) Estrogenic induction of NADPH-diaphorase activity in the preoptic neurons containing estrogen receptor immunoreactivity in the female rat. J Neuroendocrinol 6: 597-601.
Okamura H, Yokosuka M, McEwen BS, Hayashi S (1994b) Colocalization of NADPH-diaphorase and estrogen receptor immunoreactivity in the rat ventromedial hypothalamic nucleus: Stimulatory effect of estrogen on NADPH-diaphorase activity. Endocrinology 135: 1705-1708.
Panzica GC, Arevalo R, Sanchez F, Alonso JR, Aste N, Viglietti-Panzica C, Aijon J, Vazquez R (1994) Topographical distribution of reduced nicotinamide adenine dinucleotide phosphate-diaphorase in the brain of the Japanese quail. J Comp Neurol 342: 97-114.
Panzica GC, Cozzi B, Viglietti-Panzica C (1998) Colocalization studies in the avian brain provide new insights on the roles of NO in higher vertebrates. Europ J Anat 2: 63-76.
Panzica GC, Garzino A (1997) Anatomically specific colocalization of NADPH-diaphorase and choline acetyltransferase in the quail brainstem. Neurosci Lett 231: 151-154.
Panzica GC, Garzino A, Garcia Ojeda E (1996) Coexistence of NADPH-diaphorase and tyrosine hydroxylase in the mesencephalic catecholaminergic system of the Japanese quail. J Chem Neuroanat 11: 37-47.
Panzica GC, Verzè L, Viglietti-Panzica C, Rissman EF (2000) Modifications of NOS-immunoreactive system in estrogen receptor alpha knock-out male mice. Soc Neurosc Abstr 26: 1275.
Popeski N, Woodside B (2004) Central nitric oxide synthase inhibition disrupts maternal behavior in the rat. Behav Neurosci 118: 1305-1316.
Prast H, Philippu A (2001) Nitric oxide as modulator of neuronal function. Prog Neurobiol 64: 51–68.
Putnam SK, Sato S, Riolo JV, Hull EM (2005) Effects of testosterone metabolites on copulation, medial preoptic dopamine, and NOS-immunoreactivity in castrated male rats. Horm Behav 47: 513-522.
Rhodes ME, Rubin RT (1999) Functional sex differences ('sexual diergism') of central nervous system cholinergic systems, vasopressin, and hypothalamic-pituitary-

adrenal axis activity in mammals: a selective review. Brain Res Rev 30: 135-152.

Rodrigo J, Springall DR, Uttenthal O, Bentura ML, Abadia-Molina F, Riveros-Moreno V, Martinez-Murillo R, Polak JM, Moncada S (1994) Localization of nitric oxide synthase in the adult rat brain. Philos Trans R Soc Lond B Biol Sci 345: 175-221.

Sanchez F, Alonso JR, Arevalo R, Bruning G, Panzica GC (1996) Absence of coexistence between NADPH-diaphorase and antidiuretic hormone in the hypothalamus of two galliforms: Japanese quail (*Coturnix japonica*) and chicken (*Gallus domesticus*). Neurosci Lett 216: 155-158.

Sanchez F, Martinez ME, Rubio M, Carretero J, Moreno MN, Vázquez R (1998) Reduced nicotinamide adenine dinucleotide phosphate diaphorase activity in the paraventricular nucleus of the rat hypothalamus is modulated by estradiol. Neurosci Lett 253: 75-78.

Sato S, Braham CS, Putnam SK, Hull EM (2005) Neuronal nitric oxide synthase and gonadal steroid interaction in the MPOA of male rats: co-localization and testosterone-induced restoration of copulation and nNOS-immunoreactivity. Brain Res 1043: 205-213.

Sato Y, Horita H, Kurohata T, Adachi H, Tsukamoto T (1998) Effect of the nitric oxide level in the medial preoptic area on male copulatory behavior in rats. Am J Physiol 274: R243-R247.

Schmidt HH, Hofmann H, Schindler U, Shutenko ZS, Cunningham DD, Feelisch M (1996) No .NO from NO synthase. Proc Natl Acad Sci U S A 93: 14492-14497.

Scordalakes EM, Shetty SJ, Rissman EF (2002) Roles of estrogen receptor alpha and androgen receptor in the regulation of neuronal nitric oxide synthase. J Comp Neurol 453: 336-344.

Sheehan T, Paul M, Amaral E, Numan MJ, Numan M (2001) Evidence that the medial amygdala projects to the anterior/ventromedial hypothalamic nuclei to inhibit maternal behavior in rats. Neuroscience 106: 341-356.

Shi Q, LaPaglia N, Emanuele NV, Emanuele MA (1998) Castration differentially regulates nitric oxide synthase in the hypothalamus and pituitary. Endocr Res 24: 29-54.

Shughrue PJ, Merchenthaler I (2001) Distribution of estrogen receptor beta immunoreactivity in the rat central nervous system. J Comp Neurol 436: 64-81.

Sica M, Plumari L, Honda S, Harada N, Absil P, Viglietti Panzica C, Balthazart J, Panzica GC (2002) Changes in the neuronal nitric oxide synthase immunoreactive system in male mice lacking a functional aromatase gene. Horm Behav 41: 490.

Simerly RB, Chang C, Muramatsu M, Swanson LW (1990) Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 294: 76-95.

Singh R, Pervin S, Shryne J, Gorski R, Chaudhuri G (2000) Castration increases and androgens decrease nitric oxide synthase activity in the brain: Physiologic implications. Proc Natl Acad Sci U S A 97: 3672-3677.

Thomas E, Pearse AGE (1964) The solitary active cells. Histochemical demonstration of damage resistant nerve cells with a TPN-diaphorase reaction. Acta Neuropathol (Berl) 3: 238-249.

Vincent SR (1994) Nitric oxide: a radical neurotransmitter in the central nervous system. Prog Neurobiol 42: 129-160.
Vincent SR, Kimura H (1992) Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience 46: 755-784.
Warembourg M, Leroy D, Jolivet A (1999) Nitric oxide synthase in the guinea pig preoptic area and hypothalamus: Distribution, effect of estrogen, and colocalization with progesterone receptor. J Comp Neurol 407: 207-227.
Yokosuka M, Hayashi S (1996) Colocalization of neuronal nitric oxide synthase and androgen receptor immunoreactivity in the premammillary nucleus in rats. Neurosci Res 26: 309-314.
Yokosuka M, Prins GS, Hayashi S (1997) Co-localization of androgen receptor and nitric oxide synthase in the ventral premamillary nucleus of the newborn rat: an immunohistochemical study. Dev Brain Res 99: 226-233.

Legends to the figures.

Figure 1.

A – Diagram illustrating the synthesis of nitric oxide (NO) through the action of nitric oxide synthase (NOS). **B** – Cellular localization of different types of NOS within the central nervous system. E – endothelial cell; M – microglia; A – astrocyte; N – neuron.

C, E, G – Drawings illustrating the distribution of nNOS-IR cells in mouse hypothalamus (redrawn from Gotti et al., 2005). **D, F, H, I** – Microphotographs showing nNOS positive elements in mouse hypothalamus and limbic system. **3V**, third ventricle; **aca**, anterior commissure, anterior; **AM**, anteromedial thalamic nucleus; **ARC**, arcuate hypothalamic nucleus; **BST**, bed nucleus of the stria terminalis; **D3V**, dorsal third ventricle; **DM**, dorsomedial hypothalamic nucleus; **f**, fornix; **HDB**, nucleus of the horizontal limb of the diagonal band; **ic**, internal capsule; **LA**, lateroanterior hypothalamic nucleus; **LH**, lateral hypothalamic area; **LSV**, lateral septal nucleus, ventral part; **MePV**, medial amygdaloid nucleus, posteroventral part; **MPOM**, medial preoptic nucleus; **opt**, optical tract; **ox**, optic chiasm; **PaLM**, paraventricular hypothalamic nucleus lateral, magnocellular part; **PaMM**, paraventricular hypothal nucleus medial, magnocellular part; **PaMP**, paraventricular hypothalamic nucleus medial, parvicellular part; **PaV**, paraventricular hypothalamic nucleus, ventral part; **SCh**, suprachiasmatic nucleus; **SO**, supraoptic nucleus; **VMH**, ventromedial hypothalamic nucleus; **VMPO**, ventromedial preoptic nucleus.

Figure 2. Changes in the density of nNOS-IR cells in female mice (A-D) and female rats (E-F) and comparison with males. Histograms represent means and standard errors. The maximum mean value for each nucleus was put equal to 100. **ARC**, arcuate hypothalamic nucleus; **BAOT**, bed nucleus of the accessory olfactory tract; **BST**, bed nucleus of the stria terminalis; **MeAV**, medial amygdaloid nucleus, anteroventral part; **MPA**, medial preoptic area; **VMH**, ventromedial hypothalamic nucleus; M – male; PR – proestrus; ES – estrus; ME – metestrus; DI – diestrus. *statistically different from estrous females. Δ statistically different from proestrous females. ●males statistically different from proestrous females.
Data from Collado et al. (2003), Carrillo et al. (2004), Martini et al. (2004).

Figure 1

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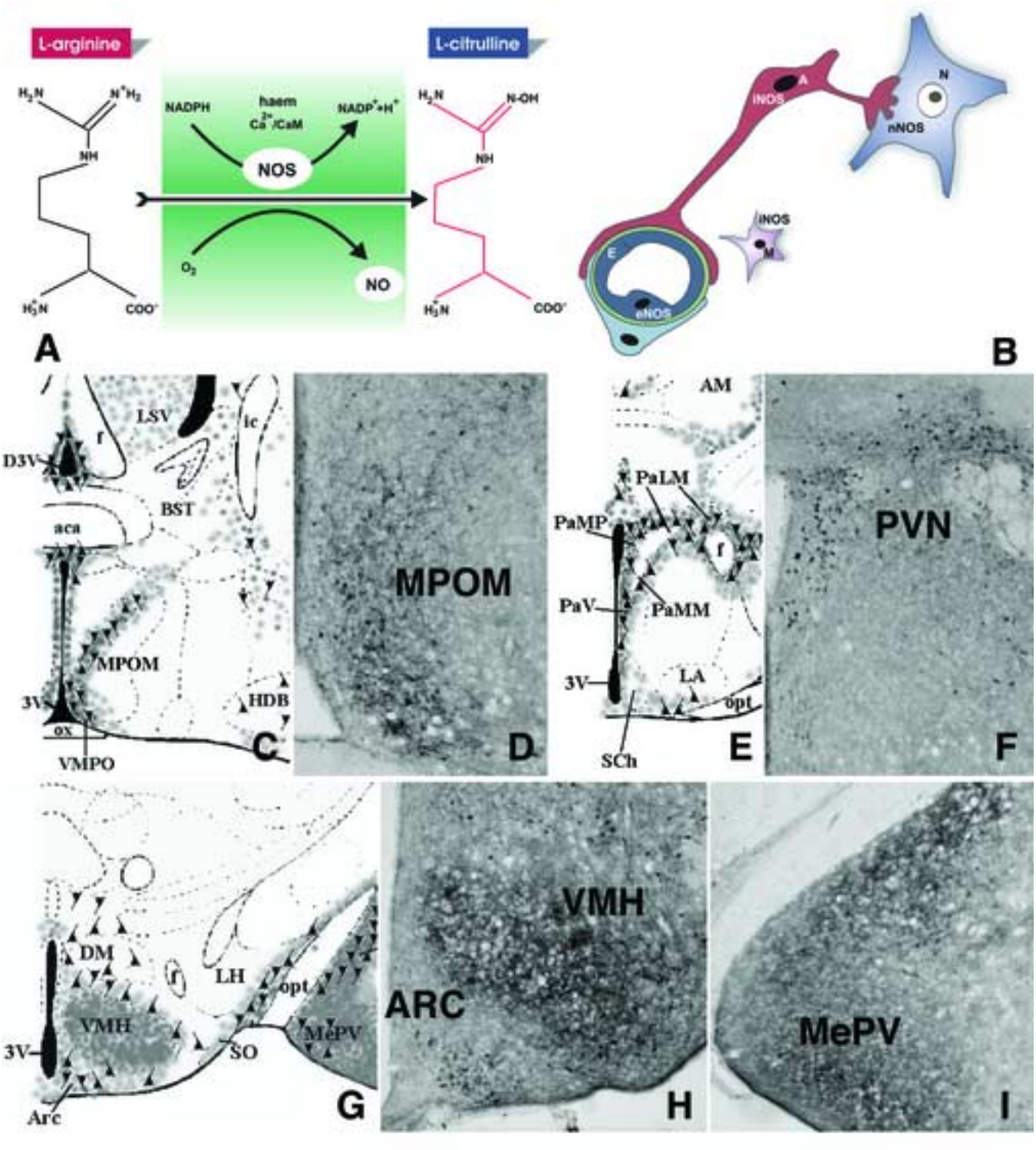

Figure 2

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