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NADPH-diaphorase activity in the paraventricular nucleus of the hypothalamus: distribution, coexistence and functional implications

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ABSTRACT

In mammals neurons of the hypothalamic paraventricular nucleus exhibit intense and selective staining (mainly magnocellular component) for NADPH-diaphorase. Several coexistences of this enzyme with neurobiological substances and functional implications have been reported. Additionally, different experimental conditions such as salt loading, food deprivation, stress, lactation, hypophysectomy and hormonal treatments promote changes in the expression of the enzyme in the paraventricular nucleus. In the present paper we analyze the information available about the distribution of NADPH-diaphorase activity in the paraventricular nucleus, the coexistence with different bioactive molecules and its possible functional roles. We conclude that 1.- There is a widespread expression of NADPH-diaphorase activity in the three different types of neurons located in the paraventricular nucleus (magno-, parvo- and mediocellular ones). However, the highest concentration of the neurons showing this activity is situated at the level of the magnocellular component. 2.- These NADPH-diaphorase neurons co-express neuropeptides and other neuroactive substances, although there is not a general correspondence with a specific one suggesting that NADPH-diaphorase is not related to general mechanisms involving these substances. 3.- NADPH-diaphorase activity in the paraventricular nucleus can be modulated by different hormones and experimental conditions. 4.- Based upon the foregoing, nitric oxide producing neurons in the paraventricular nucleus are an important cellular population of this nucleus involved in multiple mechanisms, especially neuroendocrine functions. However, the exact role of this messenger molecule in the paraventricular nucleus is not totally known.

Key words: Nitric oxide synthase - Coexistence - Magnocellular - Parvocellular - Mediocellular.

1. *Paraventricular nucleus. General considerations.*

Over the last decades the hypothalamic paraventricular nucleus (PVN) has been the focus of interest as an excellent model system in which to study hypothalamic integrative mechanisms. This nucleus plays a major role in the central control of various important neuroendocrine, homeostatic and autonomic functions (Silverman and Zimmerman, 1983; Swanson and Sawchenko, 1983; Swanson et al, 1986; Kiss, 1988; Armstrong, 1995), including, among others, the control of pituitary-adrenocortical activity, body fluid homeostasis, milk ejection reflex, thyroid hormone secretion, food intake, pineal melatonin synthesis (Swanson et al., 1986; Kiss, 1988; Armstrong, 1995). Moreover, among the different hypothalamic neuronal groups, PVN is the only one containing populations of neurons that control the anterior and posterior pituitary secretions.

PVN is a very complex nuclear formation that in the rat occupies less than a third of a mm^3 of tissue on either side of the third ventricle. It is composed by a heterogeneous population of about 15,000 neurons (Kiss et al., 1983; Kiss et al., 1991), which contain a large diversity of neurotransmitters or neuropeptides (Swanson et al., 1986; Palkovits, 1988; Meister et al., 1990; Armstrong, 1995).

The cytoarchitectural complexity of this nucleus has been reflected in an important number of papers showing different subdivisions, which displayed no substantive differences. In fact, the presence of three defined cellular types (magnocellular, parvocellular and mediocellular)

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anatomically compartmentalized according not only to the cell size and the location into the nucleus, but also the neurochemical characteristics, cell packing density and connections is currently accepted (see among others, Armstrong et al., 1980; Swanson and Kuypers, 1980; Sawchenko and Swanson., 1982; Kiss et al., 1983; Swanson and Sawchenko, 1983; Swanson et al., 1986; Sánchez et al., 1990; Kiss et al., 1991; Armstrong, 1995).

Although several neuroactive substances are predominantly located in specific subdivisions of the nucleus, they may be present in various numbers of others. On the basis of these cytoarchitectural criteria, it is possible to distinguish three functional zones in the nucleus: a parvocellular area (medial) which projects to the median eminence, a mediocellular area (dorsal and posterior) which projects to medullary and spinal cord autonomic centers and a magnocellular area which projects to the posterior pituitary. However, especially at the level of the parvo- and mediocellular areas, neurons with intermixed projections exist (Kiss et al., 1991; Armstrong, 1995).

On the other hand, intermixing between different types of cells in various subdivisions have been clearly observed and many neurons of the PVN possess the ability to express multiple biologically active molecules simultaneously (Hatton, 1986; Hökfelt et al., 1986; Swanson et al., 1986; Ceccatelli et al., 1989; Meister et al., 1990; Villar et al., 1990; Alonso et al., 1992 a and b; Arévalo et al., 1993 a; Sánchez et al., 1994, among others). In addition, the chemical phenotype of these neurons can change with the demand of hormone release (Kiss et al., 1984; Swanson et al., 1986; Larsen, 1992; Armstrong, 1995). Thus, it is essential to take into account in the meaning of any particular chemical map that the existence of coexistences and the degree of the same must be considered in the context of the functional state and sex of the animal.

For the present analysis the nomenclature and nuclear boundaries proposed by Swanson and Kuypers (1980) and Kiss et al. (1991) were used. As in previous papers from our group (Arévalo et al., 1992, 1993 a; Alonso et al., 1992 b; Sánchez et al., 1994, 1996 a) and in order to facilitate putative comparisons in the chemoarchitecture of the PVN, we considered both the anterior and medial magnocellular subdivisions of Swanson and Kuypers as the one called the commissural subdivision, which includes the anterior commissural nucleus (Swanson and Kuypers, 1980). In addition, based upon the size of the neurons, the dorsal and lateral parvocellular subdivisions have been called dorsal and lateral mediocellular subdivisions (Kiss et al., 1991; Sánchez et al., 1997 a and b).

2. *NADPH-diaphorase histochemical technique*

Three methods are currently used to demonstrate nitric oxide (NO)-producing neurons in tissue sections, including the hypothalamus: immunohistochemistry with antisera raised against nitric oxide synthase (NOS) (see Yamada et al., 1996), in situ hybridization with antisense probes complementary to NOS mRNA (see Ceccatelli et al., 1996) and the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemical method: NADPH-diaphorase (ND) (Arévalo et al., 1992).

NOS catalyzes the conversion of L-arginine to L-citrulline and NO, in a stochiometric reaction. Several isoforms of NOS have been purified and molecularly cloned (Moncada and Higgs, 1993; Bredt and Snyder, 1994). The neuronal isoform and the endothelial isoform are constitutive, calcium/calmodulin dependent, enzymes which produce NO for short periods in response to receptor activation. In addition, NO may be produced by the macrophage isoform (present in some populations of glial cells) in a calcium-independent manner, obtaining larger amounts of NO for long periods.

ND-positive neurons are frequently stained in a Golgi-like way with extensive details of the dendritic arborizations and lengthy axons, providing an important anatomical information. ND is widely found throughout the Central Nervous System. The highest concentration of the enzyme is found in the cerebellum, whereas in other brain regions, e.g. cerebral cortex and hippocampus ND is expressed very selectively in only a few neurons.

Although the distribution of ND-active cells is reliable in animals studied under the same experimental conditions, several aspects must be considered. As we described previously, of special interest for the interpretation of some data at the level of the PVN is that the existence and ratio of coexpression among different messenger molecules, including ND, must be considered in the context of the functional state, sex of the animal and the methodological approach, especially the method of ND/NOS detection. Thus, the interpretation of ND activity in a given brain region must be cautious and besides its quality as an excellent neuroanatomical marker, this staining may indicate variations in the physiological conditions of a given cell.

3. *ND activity in the PVN*

The first papers to mention in the hypothalamus the presence of ND activity were published in the mid eighties (Vincent, 1986; Sagar and Ferriero, 1987). These authors described positive neurons located in the PVN (both in the magno- and parvocellular subdivisions) and in the supraoptic nucleus.

All neuronal types of the nucleus (magno-, parvo- and mediocellular) show positive labelling (Sánchez et al., 1997 a and b). In the magnocellular subdivisions, most of the stained neurons are located in the posterior part of the nucleus, forming a dense cluster of ND-active neurons (Alonso et al., 1992 a and b; Arévalo et al., 1992 and 1993 a; Calka and Block 1993 a and b; Torres et al., 1993; Sánchez et al., 1994; Amir., 1995; Vanthalo and Soinila., 1995; Yamada et al., 1996) (Figs. 1 and 2). A few stained cells are present in the commissural subdivision (Fig. 4) (Arévalo et al., 1992).

In the parvocellular subdivisions, the neurons are predominantly situated close to the wall of the third ventricle, in the anterior part of the periventricular subdivision (Arévalo et al., 1992). In the latter, the number of stained neurons is higher in the dorsal part of the ventricle than in the ventral one. Positive neurons are also situated in the anterior and medial parvocellular subdivisions (Figs. 1 and 2). However, the number of these neurons is very scarce, especially in the medial subdivision (Fig. 2) (Arévalo et al., 1992). In the dorsal and lateral mediocellular subdivisions, some labelled neurons are present. In the dorsal one, they form a small cluster located close to the dorsal part of the third ventricle (Fig. 1). In the lateral subdivision ND-stained neurons are also present (Fig. 3) (Arévalo et al., 1992).

On considering the ontogeny of ND in the PVN, Torres et al. (1993) have reported the emergence of ND expression in the postnatal rat. ND is present as a ring of fibers around small bipolar neurons at P1 of life. Progressively, the staining appears in the perikarya, predominantly located in magnocellular neurons. NOS-positive neurons achieve their maturity about the time of weaning.

4. *Coexistence of ND with neuropeptides and other neuroactive substances in the PVN.*

Although a general coexistence has not been found, it has been shown that specific groups of ND-active neurons colocalize neurotransmitters, neuropeptides and others neuroactive substances in different brain regions. Several partial coexistences of ND have been found in the PVN. Most of these coexistences have been studied in magnocellular neurons since the ND-activity is preferentially located in this neuronal type. The area-specific patterns of coexpression and segregation with other molecules found in these studies provide a clue to the cellular mechanisms underlying the ND selective staining.

At present, in this nucleus colocalizations of ND with vasopressin, oxytocin, somatostatin, corticotropin-releasing factor, angiotensin, calbindin D-28K, calretinin, enkephalin, dynorphin, cholecystokinin, pituitary adenylate cyclase-activating polypeptide (PACAP-38), galanin message-associated peptide, acetylcholinesterase are known. Finally, there are additional examples of coexistence of ND/NOS and other messenger molecules such as substance P in the rest of hypothalamic nuclei (Yamada et al., 1996) that are not present in the PVN and as a consequence are not a focus of the present analysis.

4.1. ND-Vasopressin. Several studies from different groups have shown partial coexistence of this neuropeptide and ND in the PVN (Calka and Block, 1993 a; Torres et al., 1993; Sánchez et al., 1994). In general terms, all these studies described that neurons showing both markers were preferentially located at the level of the magnocellular subdivisions (Fig. 5). In the parvocellular subdivisions only a few double labelled cells were present (Calka and Block, 1993 a). However, these authors specify that the double labelled neurons found in the parvocellular subdivisions showed morphological characteristics consistent with magnocellular neurons. Nevertheless, the presence of coexistence of ND and the antidiuretic hormone in non-magnocellular neurons of the PVN has been clearly described (Sánchez et al., 1996 a) (Fig. 5).

With regard to the number of cells showing coexistence, there is general agreement that the degree of coexistence is very low. In fact, Torres et al. (1993) found that the degree of coexistence was 3.6% and Sánchez et al. (1994) also reported a similar degree of coexistence in magnocellular subdivisions, although the same was slightly higher (10% in the magnocellular commissural subdivision and 5% in the magnocellular posterior subdivision).

Finally, concerning vasotocin (the nonapeptide produced in the hypothalamo-hypophysial system of non-mammalian vertebrates and closely related to the mammalian vasopressin –see Sánchez et al., 1991–), in the Japanese quail the coexistence of ND and vasotocin has also been evaluated (Sánchez et al., 1996 b). No colocalization has been found in any hypothalamic area, in contrast with the above-mentioned results in rodents. This fact clearly indicates a high interspecies variability in the distribution (Panzica et al., 1994) and coexistences with bioactive molecules (Sánchez et al., 1996 b) of ND activity in the hypothalamus.

4.2. ND-Oxytocin. At present different studies have shown the coexistence of ND and oxytocin (Torres et al., 1993; Miyagawa et al., 1994; Sánchez et al., 1994; Yamada et al., 1996). There is agreement about the location and type of these neurons displaying both markers (anterior part of the nucleus in magnocellular neurons) (Fig. 4).However, some discrepancies in the ratio of coexistence have been noticed. In fact, Sán-

chez et al. (1994) found in the different subdivisions of the nucleus a ratio of colocalization never higher than 13%, with the lowest value in the posterior part of the nucleus, whereas Torres et al. (1993) found that 36% of the neurons expressing NOS also displayed oxytocin. Miyagawa et al (1994) reported that most oxytocin neurons express ND and, finally Yamada et al. (1996) showed that about 70% of the oxytocin neurons coexpress NOS.

4.3. ND-corticotropin-releasing factor. At the level of the parvocellular component, the presence of neurons coexpressing ND and corticotropin-releasing factor has been demonstrated (Torres et al., 1993; Siaud et al., 1994; Yamada et al., 1996). In addition, according to Yamada et al. (1996) some magnocellular neurons also colocalize both markers. The degree of coexistence in the parvocellular neurons varies from 4.5% (Torres et al., 1993) to 15% (Siaud et al., 1994).

4.4. ND-angiotensin. Calka and Block (1993b) reported a very limited coexistence of ND and angiotensin. According to these authors, a few double labelled (ND and angiotensin) neurons were present in the magnocellular subdivisions. No parvocellular neurons showed this coexistence.

4.5. ND-somatostatin. Colocalization of ND and somatostatin has been exclusively found in the periventricular subdivision (Alonso et al., 1992 a; Yamada et al., 1996). Only a neuronal subpopulation represented by a few neurons expressed both markers (3.39%). The double-labelled cells were predominantly located in the ventral part of the subdivision.

4.6. ND-Calbindin D-28 K. An important number of neurons expressing ND and CaBP-D28K simultaneously has been recognized at the level of the different magnocellular subdivisions in which this calcium-binding protein is strongly expressed (Alonso et al., 1992 b; Sánchez et al., 1992). In the anterior magnocellular subdivision colocalization rises up to 21.17% whereas in the posterior magnocellular subdivision the degree of coexistence was slightly lower: 17.35%.

4.7. ND-calretinin. Infrequent cellular coexistence of ND and calretinin has been reported by Arévalo et al. (1993 a) (Fig. 7). In fact, the degree of coexistence was never higher than 1% in the different subdivisions. In order to explain this low ratio of coexpression it is important to bear in mind that calretinin is mainly located in the parvocellular and mediocellular components (Arévalo et al., 1993 b) of the PVN whereas ND activity is preferentially situated in the magnocellular component.

4.8. ND-enkephalin. Yamada et al. (1996) observed a high colocalization between NOS and enkephalin in the medial parvocellular subdivision and in less degree in the magnocellular component. These authors showed that this coexpression is the most frequent combination in many parts of the hypothalamus although Murakami (1994) reported that only 6-9% of enkephalinergic neurons in the PVN expressed ND activity.

4.9. ND-dynorphin B. Murakami (1994) showed that about 37%-84% dynorphin β-immunoreactive neurons of the parvocellular and magnocellular components of the PVN colocalized ND-activity.

4.10. ND-cholecystokinin. The most frequent colocalization between both substances was observed in the posterior magnocellular subdivision followed by the medial parvocellular part (Yamada et al., 1996). The percentage of NOS neurons showing cholecystokinin was 24.5%.

4.11. ND-pituitary adenylate cyclase-activating polypeptide. Okamura et al. (1994a), by means of a combination of pituitary adenylate cyclase-activating polypeptide (PACAP-38) immunocytochemistry and ND-histochemistry, reported that virtually all PACAP-38-immunoreactive neurons in the PVN displayed ND activity.

4.12. ND-Acetylcholinesterase. Partial coexistence between both markers has been preferentially described in magnocellular neurons (Fig. 8) whereas in the parvocellular ones only some double labeled neurons were detected, mainly located in the periventricular and medial subdivisions (Crespo et al., 1998).

ND positive (blue), different bioactive molecules (brown) and coexistences (dark brown or black) labelled neurons in the PVN.

Figs 1 to 3.– ND-active neurons in the PVN. 1: Note the preferential location of neurons in the magnocellular posterior subdivision. A group of positive neurons is situated at the level of the dorsal mediocellular subdivision (arrowheads). In the medial and periventricular parvocellular subdivisions some scattered neurons can be seen (arrows). 40 x. **2:** Typical magnocellular neurons of the posterior subdivision. 100 x. **3:** Some ND-active neurons can be seen in the lateral mediocellular subdivision. 100 x.

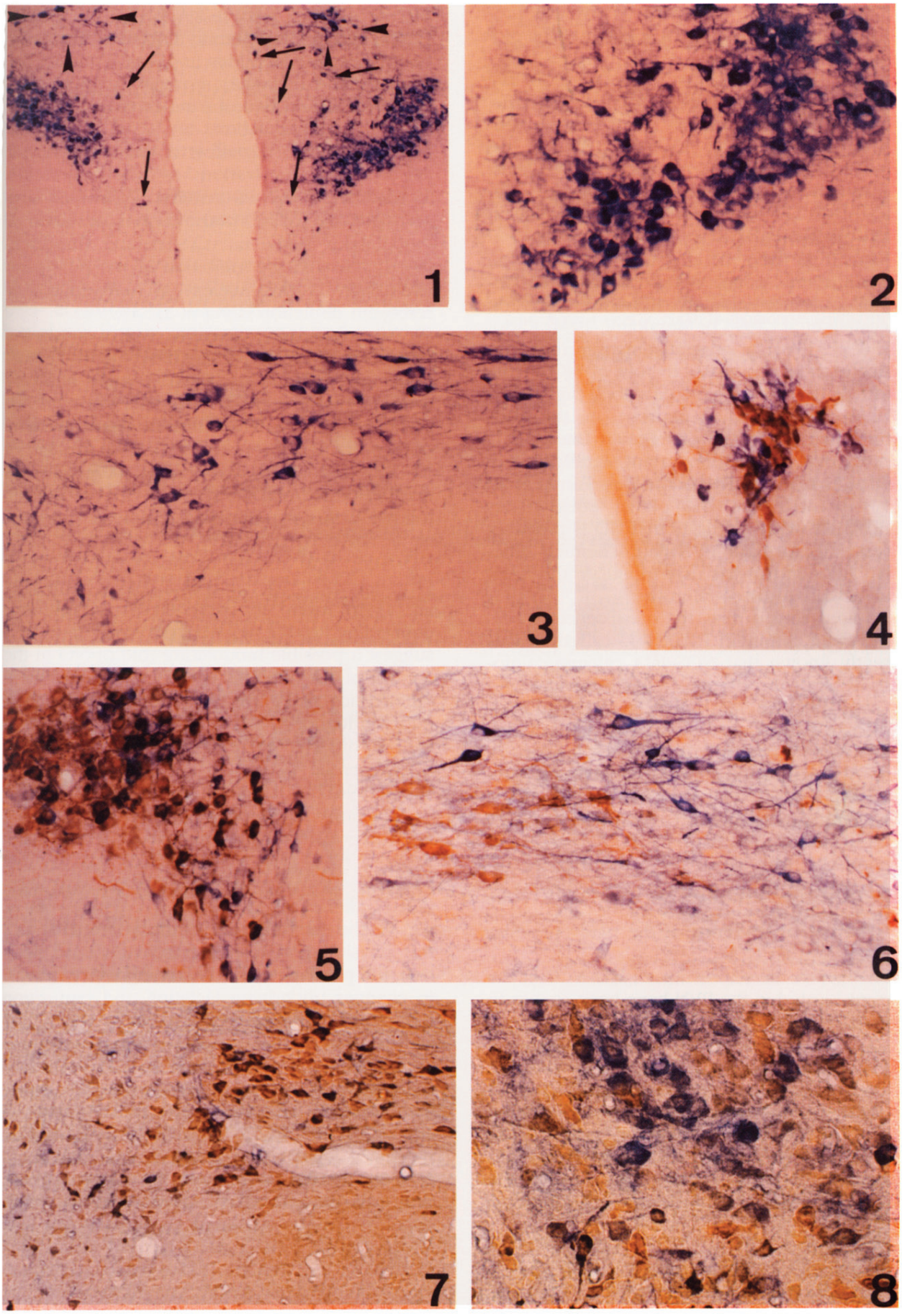
Fig. 4.– ND and oxytocin-labelled neurons in the commissural subdivision. 100 x.

Fig. 5.– High concentration of neurons showing ND activity and vasopressin-labelled neurons in the posterior magnocellular subdivision. Dark brown and black neurons express coexistence. 100 x

Fig. 6.– ND and oxytocin-labelled neurons in the lateral mediocellular subdivision. 100 x.

Fig. 7.– ND and calretinin-labelled neurons in the lateral mediocellular subdivision. 100 x.

Fig. 8.– ND and AChE-labelled neurons in the posterior magnocellular subdivision. 100 x.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5. *Specific possible functions of ND/NOS neurons in the PVN.*

NO is one of the neuroactive chemicals produced in the PVN in magno-, parvo and mediocellular neurons. Since it is a highly labile molecule, the presence of NO depends on the presence and activation of its synthetic enzyme, NOS. As we have previously indicated, the neuronal isoform of NOS is labeled by the ND histochemical technique (Hope et al., 1991). Thus, NOS activity as measured by histochemical staining for ND has become a useful tool to localize NO-producing cells.

Apart from the general role in the regulation of hypothalamic portal blood flow and the control of posterior pituitary hormone secretion (Ceccatelli et al., 1993 b; Murakami, 1994; Kadowaki et al., 1994), at present some information is available about the possible role of NO in the hypothalamus. As we have described previously, a wide population of neurons in the PVN (mainly magnocellular) express an intense ND staining, indicating an important significance of NO in this nucleus. By contrast, as Torres et al. (1993) pointed out, the partial coexistences found in all studies indicate that NO is probably not essential for basic PVN neurochemical functions, although this gas may modulate the functional activity of these neurons.

On analyzing, at first, the coexistence of ND-activity with calbindin D28K and calretinin it is currently known that NOS is a calcium/calmodulin-dependent enzyme (see Garthwaite and Boulton, 1995) whereas calbindin D-28k and calretinin are two calcium-binding proteins which are assumed to control intracellular levels of calcium. Although a relationship between the presence of NOS in a given nucleus or cell and a better handling of calcium could be expected, the available data indicate that both systems are independent, with degrees of colocalization ranging from 17.35% to 21.17% for calbindin (Alonso et al., 1992b) and 0% to 0.7% for calretinin in the different subdivisions of the paraventricular nucleus (Arévalo et al., 1993 a).

Changes in the expression of ND/NOS in the neurons located in the PVN have been shown to occur after a number of experimental conditions. Hypophysectomy produces a transient increase in NOS in the magnocellular component of the PVN when compared to normal animals (Villar et al., 1994 a). The maximal increase in staining was observed between 5 and 7 days and by 14 days NOS was back to normal levels. These changes in expression when the axons of the cells are transected may indicate a role of NO in neurosecretory neurons after injury.

Dehydration increases the NADPH-activity in the neural lobe (Sagar and Ferriero, 1987) and it is currently well known that salt-loading and dehydration promote changes in the expression of the enzyme at the level of the PVN (Villar et al., 1994 b; Kadowaki et al., 1994; Blázquez et al, 1995). Additionally, the coexistence of ND with angiotensin and vasopressin (Calka and Block., 1993 a and b; Torres et al., 1993; Sánchez et al., 1994), two nonapeptides implicated in the control of fluid balance, strongly suggests that ND/NOS stained neurons are in close relationship with osmoregulation.

In addition to the effect of water absence in the diet, food deprivation has been also shown to modulate ND/NOS activity in the PVN suggesting a role for NO in the central regulation of food intake in the rat (O'Shea and Gundlach, 1996), although the inhibition of the NOS gene expression in this nucleus is independent of serotonin depletion (Ueta et al., 1995 a).

On considering a possible role of NO in the regulation of the hypothalamic-pituitary adrenal axis and stress, immobilization stress activates the NOS neurons of the PVN (Calzà et al., 1993; Kishimoto et al., 1996). Bilateral adrenalectomy does not affect the histochemical expression of ND activity and only a very slight increase in the number of neurons showing ND can be detected following this experimental condition (Sánchez et al., 1996 b). However, the coexpression of ND/NOS and corticotropin-releasing factor (Torres et al., 1993; Siaud et al., 1994; Yamada et al., 1996) found in parvocellular subdivisions connecting to the adenohypophysis may also suggest a possible modulating role of NO in the control of the adenohypophyseal activity involving corticotropin hormone. In addition, an inhibitory influence of NO on cytokine-induced release of corticotropin releasing factor, ACTH and corticosterone (Costa et al., 1993; Rivier and Shen, 1994) has been reported.

Lactation increases both ND-activity and NOS gene expression in this nucleus (Ceccatelli and Erikson, 1993). Since it is well established that PVN plays a major role in regulating milk ejection (Wakerley et al., 1988), a possible role of NO in the hypothalamic-pituitary regulation of this physiological response is evident. In addition, the participation of NO along with oxytocin in changes underlying lactation and parturition has been also proposed (Torres et al., 1993) since NO regulates cGMP levels in the uterus (Schmidt et al., 1992). Moreover, NO also inhibits the release of prolactin (González et al., 1996).

Nowadays, it is perfectly established that the expression of ND/NOS in the ventromedial nucleus is regulated by estradiol (Okamura et al., 1994 b; Ceccatelli et al., 1996). In agreement with this, it has recently been demonstrated that ovariectomy and/or treatment with estradiol also affects the expression of ND activity in the PVN (Sánchez et al., 1997 b). In this sense, it was previously known that in male rats castration and/or treatment with testosterone also affects ND

expression in the PVN (Sánchez et al., 1997 a). Additionally, concerning the hypothalamo-hypophysial gonadal axis it is well known that NO mediates sexual behavior in male/female rats via gonadotropin-releasing hormone (Ceccatelli et al., 1993 a; Mani et al., 1994; Benelli et al., 1995) although in the hypothalamus there is an absence of coexistence between ND/NOS and this peptide hormone (see Rubio et al., 1996). The presence of a new receptor for estradiol (β) in the hypothalamus opens new possibilities for the action of estrogens upon the ND-neurons of the PVN (Katzenellenbogen, 1997; Kuiper et al., 1997).

Very little is known about the possible effects of thyroid status upon the ND/NOS expression. Ueta et al. (1995 b) showed that induced hypothyroidism in male rats produced a highly significant reduction in NOS gene transcripts in the PVN. The addition of T3 to the diet completely prevented this reduction. On the other hand, hyperthyroidism more than doubled the prevalence of NOS transcripts in the PVN after a similar time. These data point to an important role of NO in the control of the hypothalamo-pituitary thyroid axis.

Finally, the coexistence of ND and somatostatin (Alonso et al., 1992 a; Yamada et al., 1996) suggests a possible participation of NO in the hypothalamic control of growth hormone secretion. In addition, an inhibitory effect of NO upon the release of somatostatin has been reported (Aguila, 1994).

In conclusion, the wide presence of ND/NOS activity in the PVN, the different coexistences with bioactive molecules, the comparisons with other neuroactive substances present in the nucleus (without coexpression) and the regulation of the expression of the enzyme by different hormones clearly indicate that NO-producing neurons of the PVN are a key system in the functioning of this nucleus, although the exact physiological significance of this gas in the PVN remains unknown.

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