Environmental temperature changes enhance the reduced nitotinamide adenine dinucleotide phosphate-diaphorase activity in the paraventricular nucleus of the rat hypothalamus

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Summary

The nitrergic neurons of the paraventricular nucleus of the hypothalamus show changes following a variety of experimental conditions. In the last year, it has been suggested that nitric oxide has a possible role in the regulation of hibernation in the toad. Based upon these studies we attempt to elucidate whether or not changes in the environmental temperature affect the histochemical expression of the nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity in the mammalian paraventricular nucleus. Cryostat sections were cut and processed for the hstochemical detection of the NADPH-diaphorase activity. Following exposure to low temperature (4°C) (12 and 24 hours), statistical analysis displayed a marked increase in the number of NADPH-diaphorase nurons, especially in the posterior magnocellular and the lateral parvicellular subdivisions. These data indicate that NADPH-diaphorase-activity in the paraventricular nucleus is influenced by the environmental temperature.

Key Words: Low temperature - Environment - Nitric oxide synthase - Hypothalamus - Magnocellular - Parvicellular.

Introduction

The mammalian hypothalamus plays an important role in the control of autonomic and endocrine processes. It is also implicated in diverse physiological and behavioral functions such as maternal behaviour, temperature regulation, energy balance and circadian rhythmicity (Armstrong, 1995). It is now well recognized that an important mechanism underlying the functional integrity of the hypothalamic surveillance system is neurochemical diversity (Armstrong, 1995; Sánchez et al., 1998a).

In this sense, the paraventricular nucleus (PVN), a complex cytoarchitectural and functional formation, contains a wide variety of neuropeptides, neurotransmitters and other bioactive molecules (Alonso et al., 1992 a,b; Arévalo et al., 1993; Torres et al., 1993; Sánchez et al., 1994; Siaud et al., 1994; Yamada et al., 1996; Crespo et al., 1998). The majority of these neurones, are capable of co-expressing several of these molecules simultaneously (see Sanchez et al., 1998a) and change the chemical composition under different physiological conditions.

An important population of PVN neurons express the neuronal isoform of nitric oxide synthase (NOS), as it has been demonstrated by means of immunohistochemistry (Yamada et al., 1996), in situ hybridization (Calzá et al., 1993; Ceccatelli and Eriksson, 1993; Ceccatelli et al., 1996) and the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase

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(ND) histochemical method (Alonso et al., 1992 a,b; Arévalo et al., 1993; Sánchez et al., 1994, 1996, 1998 a,b, 1999; Siaud et al., 1994; Crespo et al., 1998).

At present, it is well-known that NO plays a crucial role in the control of several functions exerted by the hypothalamic nuclei including a possible participation in the regulation of hibernation (González-Nicolini et al., 1998). In addition, in the toad it has been shown an increase in NOS activity following exposition to low temperature (González-Nicolini et al., 1998). However, no information is available about related environmental temperature changes of NOS in the mammalian hypothalamus.

Thus, the aim of the present study is to elucidate the possible effect of different periods of exposure to low temperature (3h, 12h, 24h) upon the ND-histochemical expression in the PVN.

Materials and Methods

Animals and histology

Twenty adult male Wistar rats (250-275 g) divided into four groups were used for the present study. 1.- Control animals (n=5), kept in a lab environment at a spring-summer temperature (23°C). 2.- Animals (n=5) exposed to low temperature (4°C) for 3 hours, in a refrigerated room. 3.- Animals (n=5) exposed to low temperature (4°C) for 12 hours, in the same experimental conditions. 4.- Animals (n=5) exposed to low temperature (4°C) for 24 hours, according to identical protocol. Animals were housed one per cage and were given free access to food and water. The light/dark schedule (12h/12h; lights on at 08.00h) was identical in all groups. Rats were left completely undisturbed during the experiments (controls animals at least 1 day prior to the time of sacrifice).

Following the experiments, animals were anaesthetized with ketamine (Ketolar, 50 mg/kg body weight, i.p.). After packing the heads with ice, the animals were perfused through the ascending aorta with 100 ml Ringer solution followed by 500 ml fixative solution containing 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.3 (PB). The hypothalamic regions were dissected out, postfixed 4 h at 4°C in the same fixative solution and cryoprotected with 30% sucrose (v/v) in PB at 4°C overnight. Thirty-micrometer coronal sections were cut using a cryostat and serially collected in cold (4°C) PB.

Histochemical procedure

Sections were washed in 0.1 M Tris-HCl buffer pH 8.0, and processed free-floating for ND histochemistry as previously described (Alonso et al., 1992 a,b; Arévalo et al., 1993; Sánchez et al., 1994, 1998b; 1999; Crespo et al., 1998). Briefly, sections were incubated for 60-90 min at 37°C in an incubation solution made up of 1 mM reduced β-NADPH (Sigma #N1030), 0.3 mM nitro blue tetrazolium (Sigma #N0870) and 0.08% Triton X-100 in 0.1 M Tris-HCl buffer, pH 8.0. The course of the reaction was controlled under the microscope. When the histochemical reaction was concluded, sections were washed in 0.1 M Tris-HCl buffer, pH 8.0, dehydrated in ethanol series and mounted with coverslips using Entellan. Controls for the histochemical procedure included a) omission of the substrate β-NADPH in the incubation media; b) omission of the chromogen nitro blue tetrazolium; c) denaturation of the enzyme by heating the tissue at 84°C for 5 min; and d) substitution of β-NADPH by NADP (Sigma #N0505). For all controls no residual activity was observed.

Numerical data

Calculation of the number of positive cells was carried out with an image analyzer system MIP-2. By means of a graphic tablet, we counted for all sections of each individual animal the number of the stained neurons. All the counts were always carried out by the same author (FS). Only cells in which the nucleus was present were considered. Total numbers of stained neurons were corrected according to the Abercrombie's formula by a factor of 0.750 (Sánchez et al., 1994; 1998b; 1999; Crespo et al., 1998). The mean and the standard error of the mean for each group were calculated by using the corrected average for each individual animal. In the different subdivisions of the PVN all positive neurons showing the nucleus have been considered as "magnocellular" or parvicellular" independently of their size, depending exclusively on which subdivision (magno- or parvicellular) they were located in. The exact location of the positive neurons was determined by means of phase contrast microscopy (Sánchez et al., 1994; 1998b; 1999; Crespo et al., 1998).

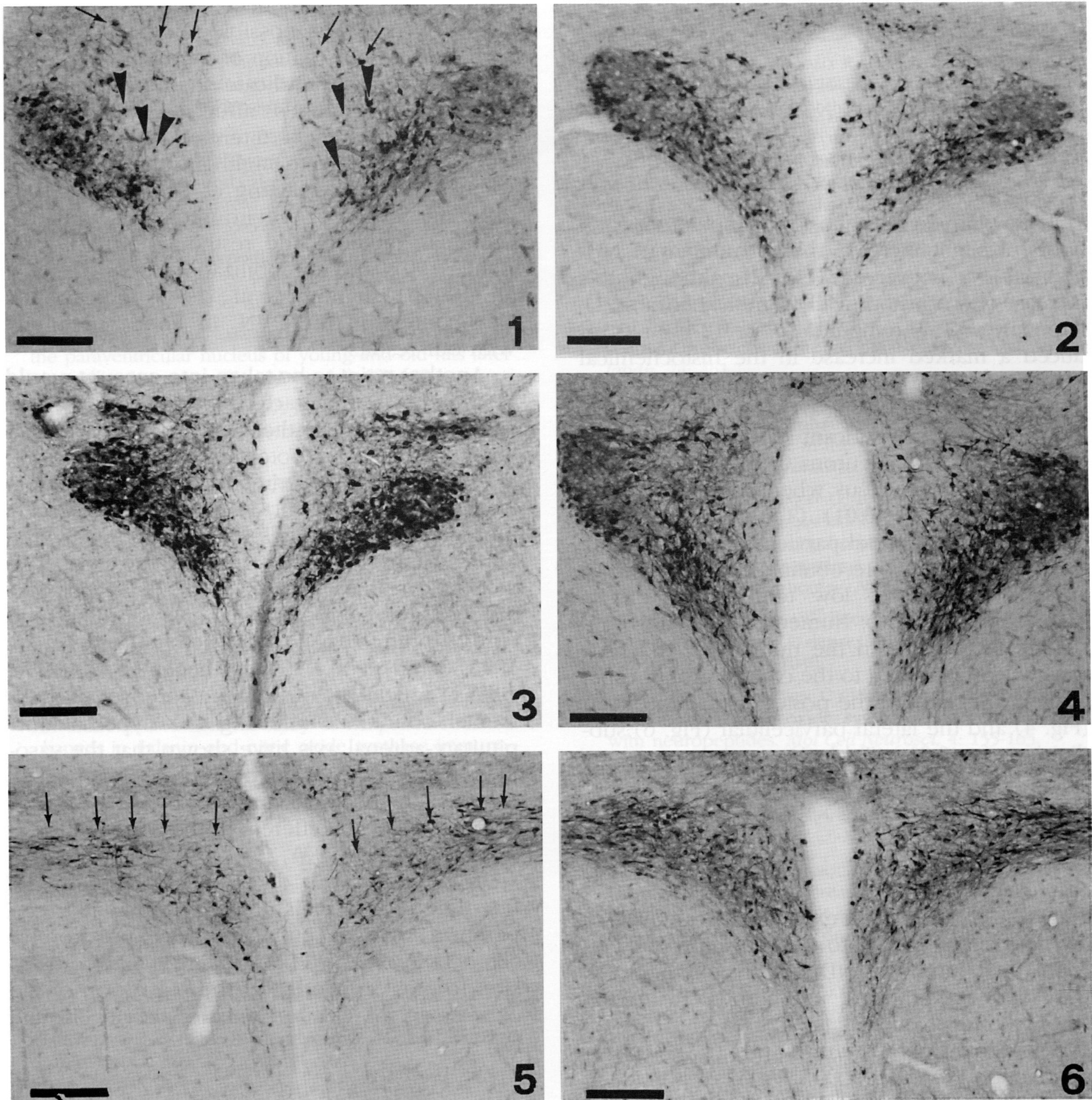
Statistical test

The values of the parameters obtained were compared statistically using the ANOVA test. Values of $p<0.01$ for the Fisher PLSD and Scheffe test were considered significant. In both the text and the tables, data represent the mean and standard error of the mean.

Results

For the present analysis the nomenclature and nuclear boundaries proposed in previous papers from our group have been considered (Alonso et al., 1992 a,b; Arévalo et al., 1993; Sánchez et al., 1994, 1998b; 1999; Crespo et al., 1998).

All subdivisions and neuronal types of the nucleus (magno- and parvicellular) displayed positive labelling. In the magnocellular subdivisions, most of the stained neurons were located in the posterior one, forming a dense cluster of ND-active neurons (Fig. 1). A few stained cells were present in the commissural subdivision. In the parvicellular subdivisions ND-positive neurons were also observed. ND-stained neurons were located close to the wall of the third ventricle in the parvicellular periventricular subdivisions (Fig. 1). In the anterior and medial parvicellular subdivisions a few ND-stained neurons were seen (Fig. 1 and 5). In the dorsal parvicellular subdivision a group of neurons formed a small cluster located close to the dorsal part of the third ventricle (Fig. 1). In the lateral one, ND-stained neurons were also present (Fig. 5).



Figs. 1 to 6.– NADPH (ND)-active neurons in the PVN in the different experimental groups (x 50). Scale Bar: 250 μm.
Fig. 1.– Control rat. Note the preferential location of neurons in the magnocellular posterior subdivision. A group of neurons is situated at the level of the dorsal parvicellular subdivision (arrows). In the medial parvicellular subdivision a few scattered cells are also present (arrowheads).
Fig. 2.– Exposure to low temperature 3 h. Note the slight increase in the number and intensity of staining of the ND-positive neurons.
Fig. 3.– Exposure to low temperature 12 h. Marked increase of ND-staining when compared to figure 1.
Fig. 4.– Exposure to low temperature 24 h. Similar characteristics to those described in image 3.
Fig. 5.– Control rat. Lateral (arrows) and periventricular subdivision.
Fig. 6.– Exposure to low temperature 24 h. Marked increase of ND-staining, at the level of the lateral parvicellular subdivision, when compared to figure 5.

Table 1.– Number (mean±S.E.M) of NADPH-diaphorase (ND)-cells in the different subdivisions of the PVN.

Subdivision	Control	3h	12h	24h
Magnocellular				
Commissural	90±4	98±4	109±5*	115±6*
Posterior	793±31	803±30	907±29*	943±31*
Parvicellular				
Anterior	19±3	22±3	29±2*	32±3*
Medial	29±5	32±4	40±3*	45±3*
Periventricular	38±5	40±5	51±3*	54±4*
Dorsal	17±2	17±2	20±3	21±3
Lateral	34±3	39±3	59±4*	68±4*

3h, 12 h and 24: animals exposed to low temperature for 3, 12 and 24 h, respectively. *$p<0.01$ when compared to control animals.

Following exposure to low temperature for 3 hours, a slight increase in the number of ND-neurons was observed (Fig. 2), although this increase was not statistically significant (Table 1). Exposure to low temperature for 12 hours produced a marked increase in the histochemical expression of ND-activity in the different subdivisions of the PVN (Fig. 3). Cell counts in this group showed a significant statistical increase in the number of ND-neurons in the different subdivisions of the nucleus when compared to the control group ($p<0.01$) (Table 1), with the exception of the dorsal parvicellular subdivision.

Similar results were obtained in the group of animals exposed to low temperature for 24 hours (Figs. 4 and 6). Numerical data showed a significant increase in the number of ND neurons when compared to the control group, especially at the level of the posterior magnocellular (Fig. 4) and the lateral parvicellular (Fig. 6) subdivisions ($p<0.01$.) (Table 1).

Discussion

Our results show that exposure of animals to low temperature leads to the activation of significant proportions of ND-neurons of the PVN. Different experimental conditions including, among others, hypotension (Krukoff et al., 1997), cholestasis (Swain et al., 1997) water deprivation (O'Shea et al., 1996), salt loading (Villar et al., 1994), modification of food intake (O'Shea et al., 1996), immobilization stress (Calzá et al., 1993; Kishimoto et al., 1996), swim stress (Sánchez et al., 1999a), lactation (Ceccatelli and Erickson, 1996), hypothyroidism (Ueta et al., 1995), ovariectomy and chronic treatment with estradiol (Ceccatelli et al., 1996; Sánchez et al., 1998b) and in lesser degree adrenalectomy (Sánchez et al., 1996) are also currently known to produce changes in the expression of ND/NOS in the neurons located in the PVN.

With the exception of the toad, in other species no information is available about a possible effect of exposure to low temperature upon the ND-neurons located in the PVN (González-Nicolini et al., 1998). Our results agree with the observed increase in NOS immunoreactivity found in the magnocellular hypothalamic region of the toad when compared to animals kept in a natural environment at a spring-summer temperature (González-Nicolini et al., 1998). These authors also indicate a possible role of NOS in the regulation of hibernation of these animals.

These findings indicate that environmental temperature changes affects the PVN and modify the neuronal content of several molecules. It is important to highlight that other environmental changes acting as stressors such as differing levels of environmental stimulation (minimal, medium and restraint) (Krukoff and Kahili, 1997) and immobilization stress (Calzá et al., 1993; Kishimoto et al., 1996) increase the NOS-content in the PVN, in good agreement with our results.

Another point to be taken into account would be the chemical characterization of the ND-neurons responding to these environmental changes. It would be of potential interest to perform double-staining experiments for the different neuropeptides present in the PVN especially those related with stress (i.e.: vasopressin and corticotropin releasing factor). However, it is well-known that in the PVN the degree of coexistence among ND/NOS and vasopressin and corticotropin releasing factor is low (Torres et al., 1993; Siaud et al., 1994; Sánchez et al., 1994, 1996; Yamada et al., 1996). Moreover, experimental conditions affecting the hypothalamic-pituitary-adrenal axis have shown that the vasopressinergic population of neurons involved in response to specific changes of the axis, such as adrenalectomy, is different to the ND-population (Sánchez et al., 1996).

In summary, our data demonstrate, for the first time in mammals, that environmental temperature changes enhance the expression of ND activity in the PVN, adding a new dimension to our knowledge of the regulation of the expression of this enzymatic activity in the PVN.

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