Computational study of the effect of forced exercise (swim stress) on the NADPH-diaphorase activity in the supraoptic nucleus

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SUMMARY

Nitric oxide synthesizing neurons of the hypothalamus show changes following a variety of experimental and pathological conditions, such as salt loading, hypotension, cholestasis, water deprivation, and several types of stress including immobilization, exposure to low temperature and changes of environment. Recently, our group has shown that forced exercise (swim stress) is an additional stressor for the nitrergic neurons located in the paraventricular nucleus of the hypothalamus. On the other hand it is currently well-known, by means of histochemical (NADPH-diaphorase), immunohistochemical and hybridization methods, that the supraoptic nucleus contains an important nitrergic population of neurons. In the present study, the effect of forced exercise on these neurons was investigated in a computational study of the NADPH-diaphorase positive population of the supraoptic nucleus. A significant increase in the number of positive neurons was observed following forced swimming, especially after 30 and 40 min. These data indicate: 1.– The influence of stress on the NADPH-diaphorase-activity in the supraoptic nucleus. 2.– The involvement of hypothalamic nitric oxide synthesizing-neurons in the response to different types of acute stressors. 3.– The excellence of a combination of morphological and computational tecniques for the detection of changes in plasticity of the hypothalamic neurons.

Key Words: Forced exercise – Computational techniques – Nitric oxide synthase – Hypothalamus

INTRODUCTION

The supraoptic (SON) and the paraventricular nuclei (PVN) contain a wide variety of neuropeptides, neurotransmitters and other bioactive molecules (Alonso et al., 1992 a,b; Arévalo et al., 1993; Torres et al., 1993; Vázquez et al., 1993; Siaud et al., 1994; Yamada et al., 1996; Crespo et al., 1998; Sánchez et al., 1994; 1998 a; 1999 a, b; 2000; Sánchez y Santos, 2000). Most of these neurones are capable of co-expressing several of these molecules simultaneously and change the chemical composition under different physiological conditions. An important population of these neurons express the neuronal isoform of nitric oxide synthase (NOS) (Alonso et al., 1992 a,b; Arévalo et al., 1993; Vázquez et al., 1993; Yamada et al., 1996; Crespo et al., 1998; Sánchez et al., 1994; 1996; 1998 a,b; 1999 a, b; 2000).

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It is now well known that a variety of physiological and pathological conditions produce changes in the expression of NOS in the nitrergic neuronal population of the PVN. Among others, salt loading, water deprivation and modification of food intake, hypothyroidism, ovariectomy and treatment with 17β-estradiol, adrenalectomy, hybernation, lactation, hypotension, cholestasis (see Yamada et al., 1996; Sanchez et al., 1998 a; 2000) promote plastic changes upon the NOS-neurons, especially of the PVN. However, little information is available about possible changes of the nitrergic population of the SON.

Our group has recently shown that forced exercise (swim stress) is an acute and potent stressor that activates neuronal NOS in the PVN (Sánchez et al., 1999 a) by means of the histochemical detection of the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (ND) histochemical method. However, no information is available on the possible effect of this type of acute stress, on the SON. Thus, the present study was undertaken to define whether or not forced swim stress promotes changes in the expression of the ND-activity in the SON.

Material and Methods

Animals and histology

Twenty adult male Wistar rats (275-325 g) divided into four groups were used for the present study. 1.– Normal animals (n=5), receiving no treatment. 2.– Normal animals (n=5), subjected to a forced swim in a 36ºC water in a 60 x 100 x 60 tank for 10 min. 3.– Normal animals (n=5), subjected to the same swim test for 30 min. 4.– Normal animals (n=5), subjected to the same swim test for 40 min. 60 min after the onset of swim stress animals were deeply 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 brains were removed and the hypothalamic regions were dissected out, postfixed 4 h at 4ºC in the same fixative solution and cryoprotected with 30% sucrose (v/v) in PB at 4ºC overnight. Thirty-micrometer coronal sections were cut using a cryostat and serially collected in cold (4ºC) 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; Crespo et al., 1998; Sánchez et al., 1994, 1998a, b, c; 1999 a b; 2000). Briefly, sections were incubated for 60-90 min at 37ºC in an incubation solution made up of 1 mM reduced ß-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.

Computational study

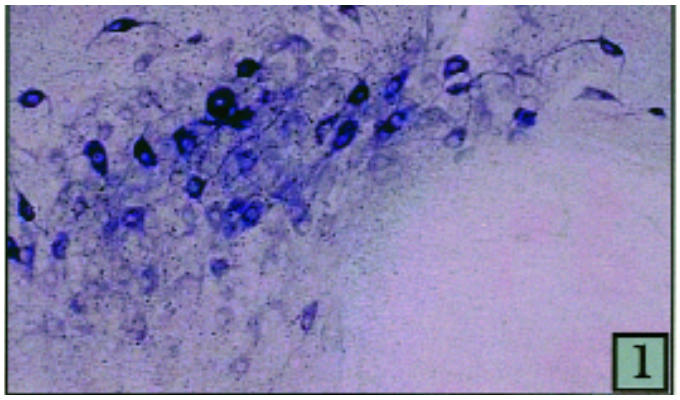
Calculation of the number of positive cells was carried out with an image analyzer system MIP-2. By means of a graphic tablet, we counted for all sections of each individual animal the number of the stained neurons. All the counts were always carried out by the same author. Only cells in which the nucleus 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). The mean and the standard error of the mean for each group were calculated by using the corrected average for each individual animal. The exact location of the positive neurons was determined by means of phase contrast microscopy (Sánchez et al., 1994; 2000).

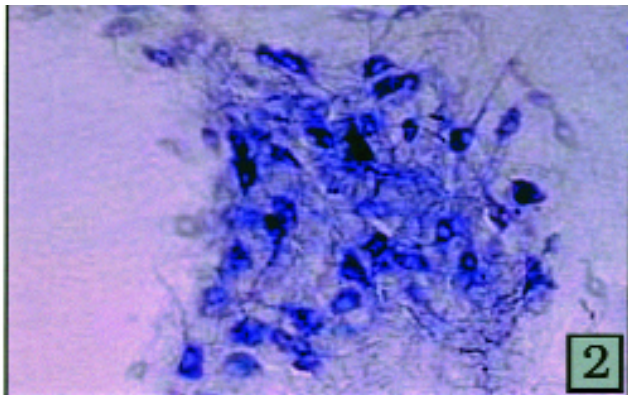
Statistical test

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

Neurons located both at the prechiasmatic and retrochiasmatic subdivisions displayed positive labelling. However, in the retrochiasmatic one, only a few cells (two or three) were stained. As a consequence, only the prechiasmatic (principal) subdivision was considered for the present study. In this subdivision, most of the stained neurons were located in the dorsal part, forming a dense cluster of ND-active neurons (Figs. 1 and 2). A few stained cells were present in the ventral part located close to the pial limit.





Figs. 1 and 2. *Normal rats.* Note the preferential location of ND-active neurons in the dorsal part of the prechiasmatic (principal) subdivision. Isolated positive neurons were situated in the ventral part. x 120.

Following swim stress for 10 min, a slight increase in the number of ND-neurons was observed (Fig. 3). The results obtained in the computational treatment in this group showed no significant statistical increase in the number of ND-neurons when compared to the normal group ($p > 0.01$) (Table 1).

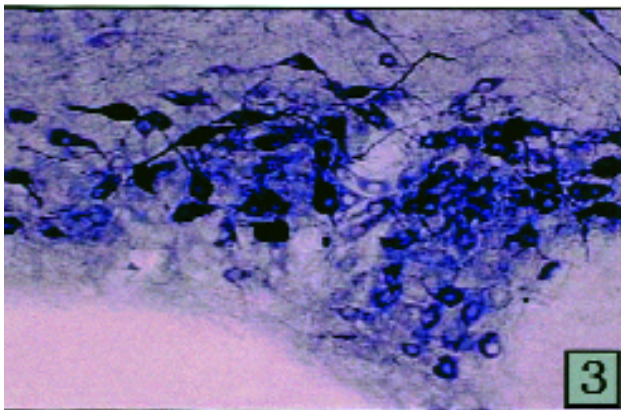


Fig. 3. *Forced exercise for 10 min.* Note the slight increase in the number and intensity of reaction of the ND-active neurons. x 120.

Table 1.- Mean ± standard error of ND-positive neurons obtained in the SON in the different groups of forced exercise (swim stress). * $p < 0.01$ when compared to the normal and the 10 min group.

Normal	10 min	30 min	40 min
478.4±15.3	488.4±15.7	565.4±16.3*	587.4±16.8*

Forced swimming for 30 min produced a marked increase produced a marked increase in the histochemical expression of ND-activity (Fig. 4). Data processing in this group revealed a statistically significant increase in the number of ND-neurons when compared to the normal and 10 min groups ($p < 0.01$) (Table 1).

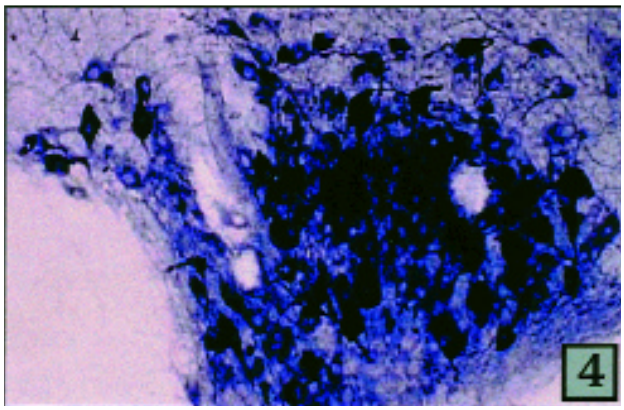
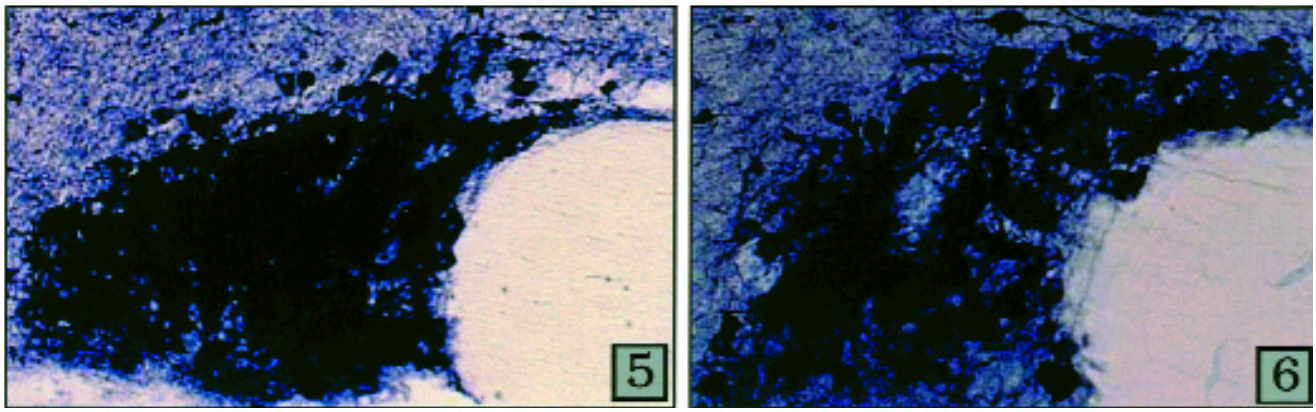


Fig. 4. *Forced exercise for 30 min.* Marked increase in the number and intensity of reaction of the ND-active neurons both in the dorsal and ventral parts of the principal subdivision. x 120.

Similar results were obtained in the group of animals exposed to forced exercise for 40 min (Figs. 5 and 6). In some slices, the increase in the intensity of ND-activity did not allow observation of the cellular limits among the different cells located in the nucleus. the computational study showed a significant increase in the number of ND neurons when compared to the normal and 10 min groups ($p < 0.01$) (Table 1).

DISCUSSION

NO plays an important role in the maintenance of autonomic homeostasis of the body. NO-producing neurons are activated in the brain during exposure to different levels of environmentally



Figs. 5 and 6. *Forced exercise for 40 min.*Marked increase in the number and intensity of reaction of the ND-active neurons. The chromogen deposit does not allow observation of the cellular limits among the different positive cells. x 120.

produced stimulations. A primary component of the mammalian response to stress is known to involve activation of the hypothalamic-pituitary-adrenocortical axis (Campeau et al., 1994; Cullinan et al., 1995; 1996). Stress related inputs converge upon the neurons of the PVN and, to a lesser extent of the SON. These neurons synthesize and release several secretagogues (corticotropin releasing factor and vasopressin) serving as the origin of a cascade of events resulting in the release of glucocorticoids from the adrenals. These neurons also coexpress the neuronal isoform of NOS (Torres et al., 1993; Vázquez et al., 1993; 1996; Yamada et al., 1996; Sánchez et al., 1998 a).

Our results show that exposure of animals to forced exercise (an acute stressor) leads to the activation of significant proportions of ND-neurons of the SON. Different experimental conditions including, hypotension (Krukoff 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), lactation (Ceccatelli and Erickson, 1996), hypothyroidism (Ueta et al., 1995), ovariectomy and chronic treatment with estradiol (Ceccatelli et al., 1996; Sánchez et al., 1998 b), adrenalectomy (Sánchez et al., 1996), exposure to low temperature (Sánchez et al., 1999 b), darkness and change of environment (Sánchez et al, 2000), are also currently known to produce changes in the expression of ND/NOS in neurons located in the hypothalamus. In a previous study, Sanchez et al. (1999 a) showed that forced exercise (swim stress) produces an increase in the ND-activity of the magno- and parvicellular neurons located in the PVN.

It is currently known that some acute stressor increases fos expression in the SON (Cullinan et al., 1995; 1996). These findings indicate that different classes of stressors access the SON and modify the neuronal content of several molecules, in good agreement with our findings. Stress is a mechanism of activation of NOS-hypothalamic neurons, activating a cascade of events resulting in the adrenocorticotropin mediated release of glucocorticoids, which have profound effects on the HPA axis. Moreover, there is a relatively restricted set of forebrain afferent sources in position to provide inputs to the hypothalamus in response to different acute stressors that may affect the NOS neurons situated not only in the PVN but also in the SON.

It would be of potential interest to characterize the ND-neurons responding to stress by performing double-staining experiments for the different neuropeptides present in the SON and closely related to stress. However, it is currently known that in the SON the degree of coexistence among ND/NOS and vasopressin is very low (Sánchez et al., 1994, 1998 a; Yamada et al., 1996). In addition, experimental conditions affecting the hypothalamic-pituitary-adrenal axis have shown that in the PVN, the vasopressinergic population of neurons involved in response to specific changes of the axis, such us adrenalectomy, is different to the ND-population (Sánchez et al., 1996; 1998 a).

For the first time our data demonstrate, by means of a combined computational/histochemical study that forced exercise enhances the expression of ND activity in the SON, adding a new dimension to our knowledge of the regulation of the expression of this enzymatic activity in the hypothalamus. In addition, the possibility that ND-activity in the SON may be used as a marker of neuronal activity in response to stress should be considered.

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