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Variations of nNOS and its mRNA in the neurons of the hypothalamic supraoptic nucleus in response to stress by immobilization

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SUMMARY

Using rats subjected to immobilization stress, the aim of the present study was to investigate the expression of neuronal nitric oxide synthase and of its mRNA in the magnocellular neurons of the supraoptic nucleus of the hypothalamus, which does not have parvocellular neurons or direct neurohaemal connections with the anterior lobe of the hypophysis.

nNOS expression was studied with immunohistochemistry using sheep anti-nNOS serum, and the intraneural detection of nNOS mRNA was accomplished using a non-isotopic *in situ* hybridization technique, employing a specific biotinylated probe. The acute stress elicited by restraint induced an increase in the overall size of the supraoptic nucleus, together with an increase in the number of magnocellular neurons expressing nNOS immunoreaction. The optical densitometry values of the nNOS immunoreaction, the nuclear areas of the immunoreactive neurons, and the density of neurons showing nNOS mRNA hybrids were higher in the supraoptic nucleus of the stress-treated animals than in the controls, suggesting that nitric oxide of nNOS origin plays an active role in the magnocellular neurons of the hypophyseal-hypothalamic system and the involvement of this system in the stress axis.

Key words: Nitric oxide – Stress – In situ hybridization – Immunohistochemistry – Optical densitometry – Morphometry

INTRODUCTION

In situations considered to be stressful, two neuroendocrine protection systems are activated: the sympatho-adrenergic system and the hypothalamic-pituitary-adrenal (HPA) axis. The first elicits a specific behavioural response that induces animals to fight or flee and, through the HPA axis, physiological changes and behavioural responses occur that prepare the animal to face the threat or stress successfully (Levine and Ursin, 1991; Rivier, 2002; Engelmann and Ludwig, 2004).

The magnocellular neurons of the paraventricular (nPV) and supraoptic (nSO) hypothalamic nuclei, integrated in the hypothalamic-neurohypophyseal system (HNS), synthesize and release the hormones vasopressin (AVP) and oxytocin (OT). Towards the middle of the last century, it was believed that the AVP released by these neurons was the main modulator of the HPA axis. Later,

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this function was attributed to the parvocellular neurons of the nPV that synthesise and release AVP and corticotropin releasing hormone (CRH), which- acting synergistically on the anterior lobe of the hypophysis- release ACTH (Guiles et al., 1982). In recent years, reappraisals have been made of the participation of the HNS in the stress axis (Engelmann and Ludwig, 2004; Orlando et al., 2008a) in view of the elevation of OT levels in blood after the application of different stressors and the fact that the magnocellular neurons of the HNS release AVP and OT into the extracellular space, independently of systemic release (Neumann et al., 1993).

Nitric oxide (NO) is a freely diffusible gaseous molecule and is considered to be a neurotransmitter/neuromodulator in the central nervous system (Zhang and Snyder, 1995); its synthesis requires nitric oxide synthase (NOS). The neuronal isoform of nitric oxide synthase (nNOS) is found at high concentrations in the magnocellular nuclei of the HNS (Luckmann et al., 1997), such that it was initially believed that NO would participate in the control of AVP and OT release into the blood stream (Summy-Long et al., 1993; Villar et al., 1994). Although some studies have implicated NO in stress (López-Figueroa et al., 1998; Massod et al., 2003; Engelmann et al., 2004; Orlando et al., 2007), its role in the HNS axis and stress-related behavioural changes has not been clearly defined. In order to study the involvement of NO during stress in the magnocellular neurons of the nSO, which does not possess either parvocellular neurons or direct haemoneural connections with the anterior lobe of the hypophysis, here we studied the changes in the immunohistochemical expression of nNOS and/or its mRNA by *in situ* hybridization after maintained restraint, which is considered to be a powerful physical and psychological stressor.

Materials and Methods

Animals and treatments

Eight adult male Wistar rats (200-230 g) from Charles-River®, stabled (4 animals per cage, with no limits to their movements) under standard conditions (temperature 22 ± 2ºC; RH 50 ± 5%; controlled photoperiod of 14/10: light/dark) and fed with standard rat chow (Panlab®) and water *ad libitum* were used. Four of the animals were subjected to immobilization stress for 4 hours, after which they were killed immediately. The animals, which had been taken to the experimentation room 2 hours previously, were placed face-down on an immobilization plate, restraining their four limbs at moderate tension on the plate with adhesive tape. The other four animals were not subjected to immobilization and formed the control group. In all cases the animals were treated according to the EU directive on Animal Protection for Experimental and Scientific Purposes (86/609/C) and also according to Spanish legislation in this regard.

The animals were sacrificed by decapitation under ether anaesthesia and their brains were carefully removed, fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4), and embedded in paraffin. Coronal serial sections (7 μm) were obtained and processed for study.

nNOS immunohistochemical detection and in situ hybridization procedure

The immunohistochemical detection techniques for nNOS and the *in situ* hybridization procedure have been described in detail in a previous work (Riesco-López et al., 2008). Briefly, to determine nNOS in the magnocellular neurons of the nSO the biotinylated-streptavidin-peroxidase immunohistochemical method was used. The sections were incubated with sheep polyclonal anti n-NOS serum (K205; Herbison et al., 1996), diluted 1:100.000. Then, biotinylated rabbit anti-sheep IgG (Caltag®, 1:150) and streptavidin-biotinylated horseradish peroxidase complex (Caltag®, 1:150) were successively applied and the reactions were developed in freshly prepared solutions of 3-3' DAB.

Controls included the substitution of the primary antibody by normal rabbit serum or TBS, as well as omission of the secondary antibody; after both tests, no immunoreactivity was detected.

The expression of mRNA for nNOS was demonstrated by non-isotopic *in situ* hybridization. To accomplish this, a highly specific biotinylated probe was developed by mixing the two probes reported by Lau et al. (2003) and Engelmann et al. (2004), corresponding to the respective 797-841 and 349-393 sequences of rat nNOS mRNA (Bredt et al., 1991): B-ggc ctt ggg cat gct gag ggc cat tac cca gac ctg tga ctc tgt and B-tac att ggg ttg gat ctg ctg aac ccc aaa cgt gtt ctc ttc cat.

(Access numbers: NM 052799, XM 346438, XM 346438; the sequence was derived from U67309.1 and replaced gi:34872537). According to the database consulted, specificity was for an error < $3x10^{17}$ for rat nNOS mRNA.

Sections were acetylated and then dried at 55ºC in an incubator. Prehybridization of the tissue sections was carried out by immersion in 200 µl of omnibuffer. Then, 20 µl of probe diluted in omnibuffer (0.02 ng/µl) was added; following this, the sections were covered with hybridization chambers (CoverWell, Sigma ®) and hybridization was carried out in a Hybaid OmnoSlide ® thermocycler.

After hybridization, the sections were rinsed in 1x saline-sodium citrate (SSC) and hybrids were identified by the immunocytochemical detection of biotin. The immunocytochemical reaction was developed by successive incubation in biotinylated goat anti-mouse IgG (1:200 in TBS, Caltag®) and streptoavidin-peroxidase complex (1:250 in TBS, Caltag®). Visualization of the reaction was accomplished using freshly elaborated 3-3' DAB. To enhance the reaction, in some sections visualization was amplified using the tyramide amplification-kit (Dako®).

Controls: RNase pre-treatment, substitution of antisense probe by omnibuffer and hybridization with the sense probe, sequence: -cag gaa ccc gta cga ctc ccg gta atg ggt ctg gac act gag aca, were carried as reaction controls; the results were negative in all cases.

Image analysis: morphometric and densitometric analyses

The cellular and nuclear areas of nNOS-positive neurons were calculated using the ImagenJ program (NIH, USA) from 250 cells per animal, as was the total area occupied by the nSO in 5 frontal sections, separated by 100 µm from each other (Bregma: -0.9 to -1.3 of the stereotaxic atlas of Paxinos and Watson, 1998). Using the same program, densitomet-

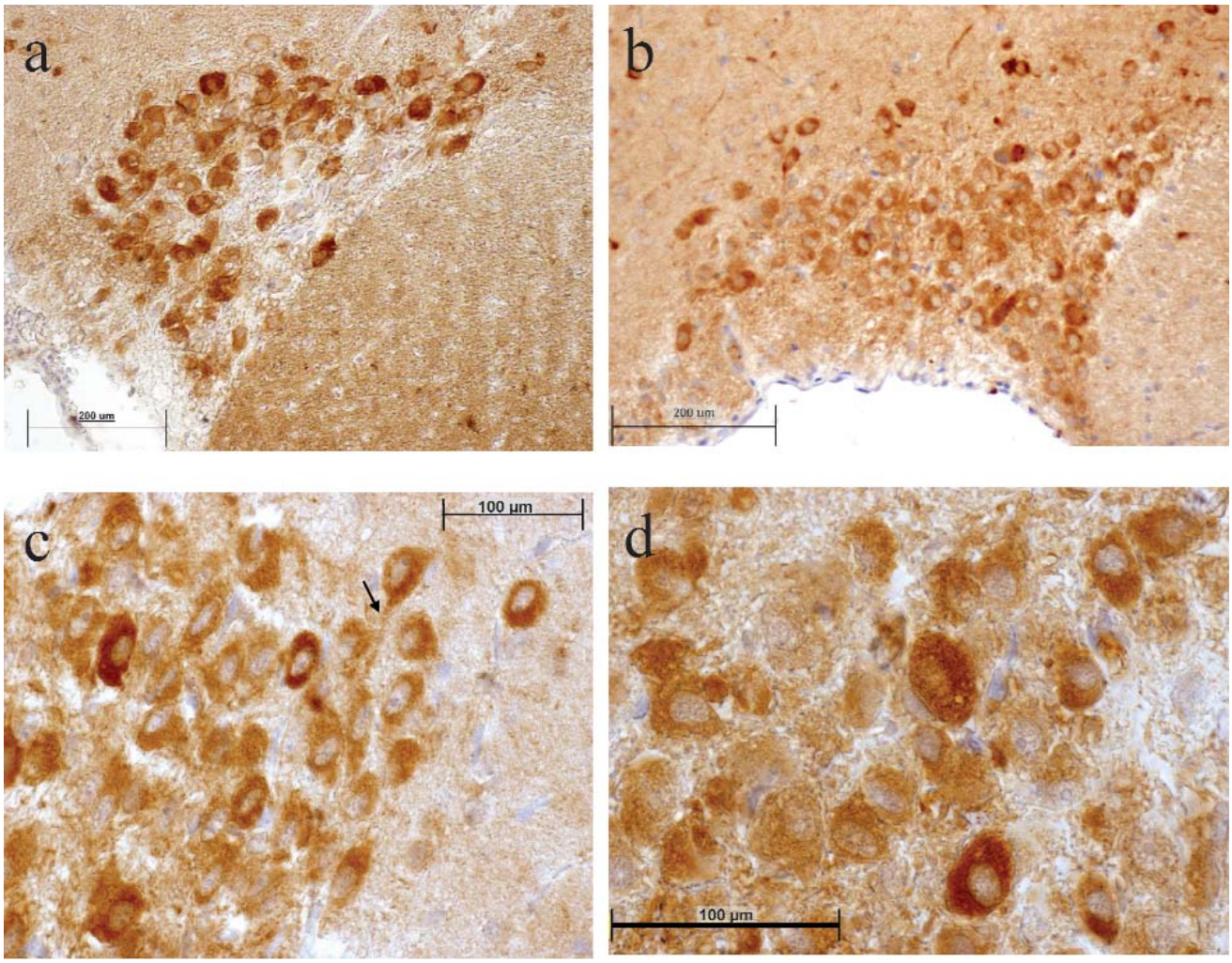

Fig. 1. Micrographs of coronal sections of supraoptic nuclei showing the morphology and immunoreaction of the neurons to nNOS in control animals (**a**) and rats subjected to restraint-induced stress over 4 hours (**b-d**), all with a high and qualitatively similar reaction intensity. Note the increased number of neurons extending across the surface of the nSO in the restrained animals (**b**), distribution of the immunoreaction through processes (**c**, arrow) and the eccentricity of the nuclei in neurons with a strong immunoreactive product at the opposite pole (**d**).

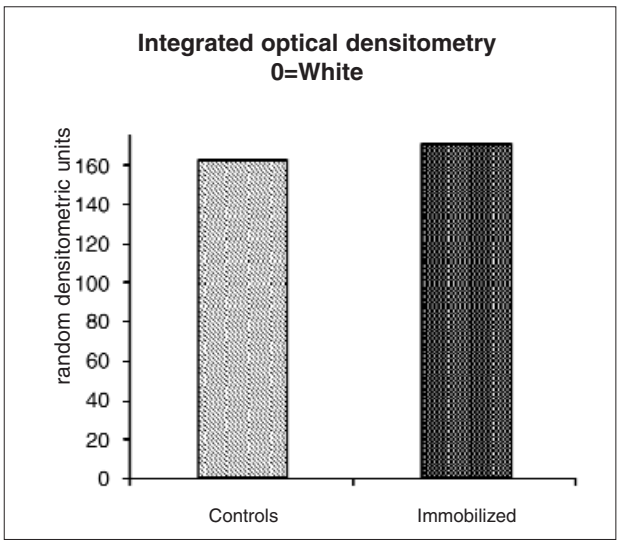

Fig. 2. Values of integrated optical densitometry, expressed as arbitrary units, of the immunohistochemical reaction to nNOS. Note the similar values for the control and stressed animals.

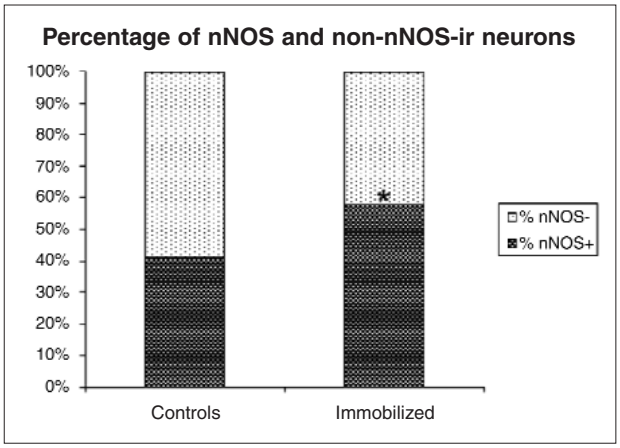

Fig. 3. Percentage of nNOS and non-nNOS-ir neurons in control and stressed animals. * $p<0.05$ *vs.* the percentage of nNOS-ir neurons in the controls.

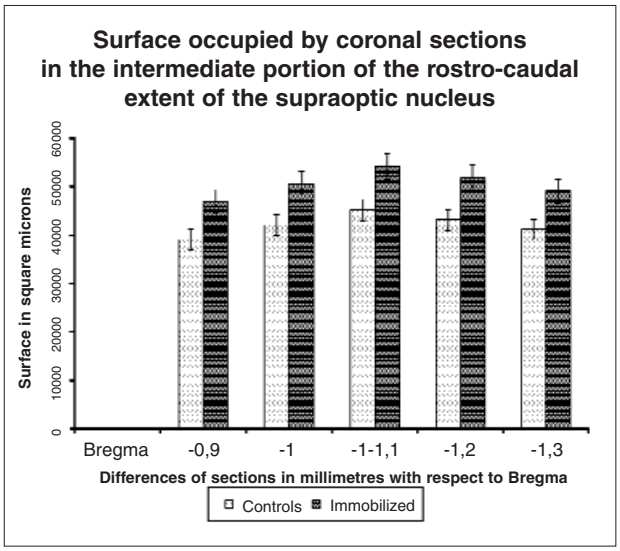

Fig. 4. Means ± SEM of the areas occupied by nSO in frontal sections taken at distances of -0.9 to -1.3 with respect to Bregma.

ric mean grey values were calculated from the cytoplasm of 100 nNOS-positive cells per animal and densitometric profiles were obtained. Morphometric and densitometric analyses were carried out from digital microphotographs obtained under homogeneous conditions of colour temperature.

Statistical analyses

The results obtained were processed statistically using GraphPad Prism 4. The results are expressed as arithmetic means ± standard errors of the mean. The differences observed were compared using analysis of variance, accepting $p<0.05$ as significant for the Bonferroni test.

RESULTS

Morphology and immunohistochemistry

In the control rats, the nNOS-immunoreactive (nNOS-ir) magnocellular neurons were oval and were distributed in parallel lines throughout the dorsal and central regions of the nSO, showing an intense immunostaining that was homogenously distributed throughout the perikaryon (Fig. 1a). The nNOS-ir neurons of the restrained animals showed polymorphic characteristics and a tendency for their nuclei to be eccentric. They were found densely packed throughout the nSO, with the exception of a narrow ventral band close to the pial basement membrane (Fig. 1b). The immunostaining encompassed not only the perikaryon but also the neuronal processes (Fig. 1c). In the nNOS-ir neurons with eccentric nuclei, strongly reactive zones were observed at the pole opposite the perikaryon (Fig. 1d).

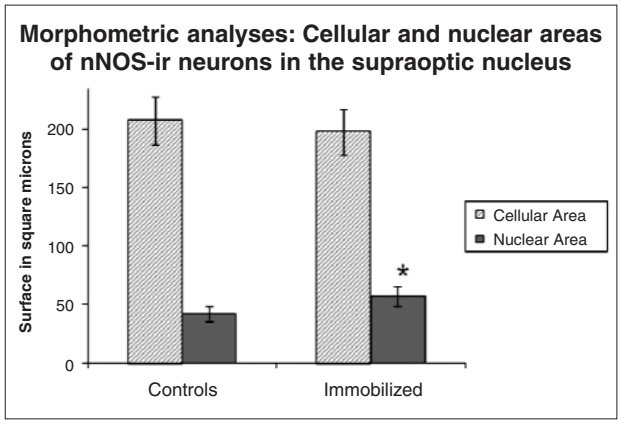

Fig. 5. Graphic representation of the values corresponding to the cell and nuclear areas (means ± SEM) of the nNOS-ir neurons in the nSO of control and stressed rats. *$p<0.05$ *vs.* control values.

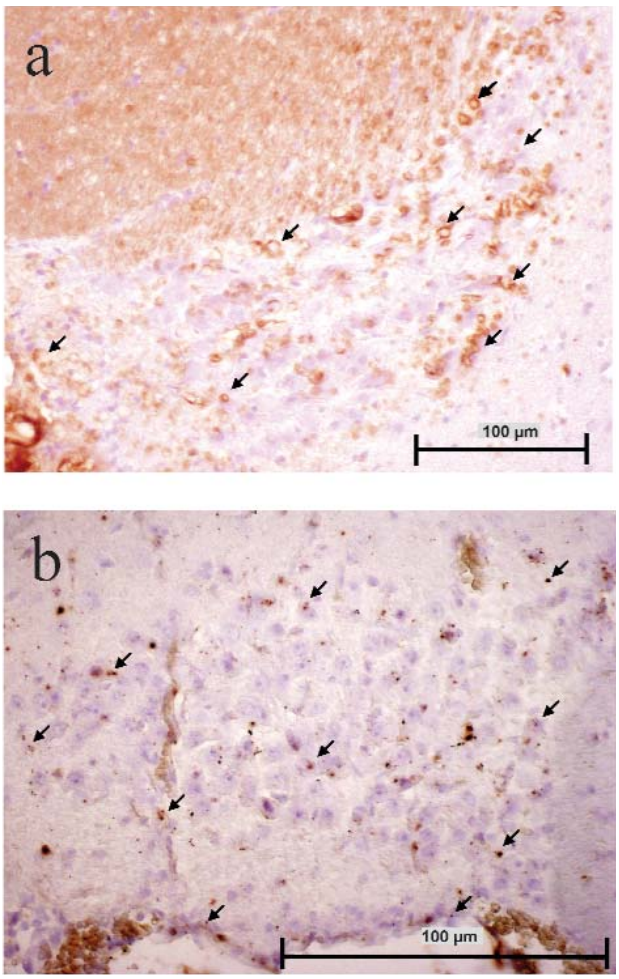

Fig. 6. Photomicrographs showing signs of hybrids for nNOS mRNA in the perikaryon of nSO neurons in a control animal (**a**) and a restrained rat (**b**). Note that the *in situ* hybridization signal for nNOS mRNA is amplified in (a) because tyramide was used as a signal enhancer. In the animals subjected to stress, an increase is seen in the number of neurons with signals of hybrids, extending across the surface of the nSO, including the pial basement membrane.

Densitometry, neuronal quantification and morphometry

The randomized densitometry values of the immunoreaction to nNOS were high in all the animals (Fig. 2), and even higher in the restrained animals (mean value: 171 random units; white = 0) *vs.* the values obtained in the control animals (163 random units; white = 0).

The percentage of nNOS-ir neurons with respect to the total number of magnocellular neurons present in the nSO was 56% in the restrained animals as compared with 38% in the control rats (Fig. 3), the difference being highly statistically significant ($p<0.001$).

The surface of the coronal sections of the nSO in the intermediate zone of its rostro-caudal distribution had a mean value of 50564 ± 8752 µm^2 in the restrained animals *vs.* 41950 ± 5298 µm^2 in the control animals with significant p values of < 0.05 (Fig. 4).

The morphometric values (Fig. 5) corresponding to the cell areas of the nNOS-ir neurons were very similar in both the restrained animals (198 ± 39 µm^2) and in the control rats (208 ± 29 µm^2); this was not the case of the nuclear areas, which had significantly higher values in the restrained animals (57 ± 9.5 µm^2) with respect to the controls (42 ± 9.6 µm^2).

In situ hybridization

The detection of nNOS mRNA by immunocytochemical detection with biotin revealed the presence of hybrids in the form of drops or spots in the perikaryon of magnocellular nSO neurons distributed throughout the nucleus in both the restrained and control animals (Figs. 6a, 6b). The coronal sections of the nSO of the restrained animals showed a greater density of neurons with hybrids than the controls. The distribution of the neurons with hybrids was homogeneous in the restrained animals, while in the control rats the neurons expressing nNOS mRNA were concentrated in the dorsal and ventral regions of the nSO (Fig. 6). In all the animals studied, a striking observation was the presence of clusters of neurons with hybrids in the perichiasmatic region of the nSO and in cells located next to the pial basement membrane.

Discussion

For several decades it has been known that exposure to different stressor agents elicits the activation of the sympathetic-adrenergic system and of the hypothalamic-pituitary-hypophyseal axis, in whose regulation the parvocellular neurons of the nPV participate, affording in an increase in plasma levels of adrenergic substances, prolactin, opioids, and corticoids. In recent years, interest in the involvement of the HNS in stress has increased owing to the observation of the presence of OT in blood, released from the magnocellular neurons of the HNS when the animals are challenged by different stressors (Neumann, 2002), and especially in view of the finding that the release of OT and AVP during stress mainly occurs in the neuropil of the nSO from the somata and dendrites of the magnocellular neurons (Wotjak et al., 1998; Engelmann et al., 1999; Engelmann and Ludwig 2004; Orlando et al., 2008b).

Upon studying responses to stress it is important to take into account the type of stress applied, the most commonly used being physical-emotional stressors (forced swimming, restraint, exposure to ether vapours or shaking-floor conditions) and the quality of the stressors; i.e., whether they are applied in acute and moderate situations (only once and for short times), or in chronic, severe stressful conditions (repeated and long-lasting exposures).

It has been reported that both the mode and the quality of the stress elicit a hormonal response and the secretion of neurotransmitters and modulators in the neurohypophyseal magnocellular system (Neumann, 2002; Engelmann and Ludwig 2004; Engelmann et al., 2004). Additionally it has been described that acute stress hardly affects the parvocellular neurons in the regulation of ACTH (Ma and Lightman, 1998; Aguilera and Rabadán-Diehl, 2000).

Restraint is probably the most severe stressor of among those that have a predominantly emotional component (Armario et al., 2004). In the present study we applied an acute long-lasting stressor, with a restraint time longer than those used by other authors (Krisch, 1978; Iványi et al., 1991; De Oliveira et al., 2000; Masood et al., 2003). However, according to the findings reported in the literature, there are no significant differences in nNOS mRNA expression dependent upon the time of application of the stress; not even between the application of acute and chronic restraint (De Oliveira et al., 2000).

Our results highlight the increase not only in the number of nNOS-ir neurons but also in the intensity of immunostaining with respect to the control animals. This up-regulation of the nNOS protein was accompanied by a widespread diffusion of nNOS mRNA in the magnocellular neurons throughout the rostro-caudal axis of the nSO, although it was also expressed in the neighbourhood of the pial basement membrane in structures belonging to glial elements.

The pronounced activation of nNOS-ir neurons during restraint-induced stress was also patent in the increase detected in their cellular and above all nuclear, areas, with a nucleus/cytoplasm ratio indicative of intense cellular activity. These findings, together with the high number of neurons expressing a high intensity of immunoreactivity to nNOS suggest an increase in NO synthesis.

As early as 1970, Vázquez and Palomero reported morphological signs of strong activity in the nSO neurons of guinea-pigs subjected to stress and hunger. Later, it was observed that restraint increased the levels of nNOS mRNA in the parvocellular neurons of the nPV (Kishimoto et al., 1996; Krukoff y Khalili, 1997; De Oliveira et al., 2000), and at our laboratory we have observed an increase in NADPH-diaphorase levels in the nPV (Sánchez et al., 1999a, b). The increase in nNOS immunoreactivity in the nPV has been reported after the application of different types of stress, such as restraint (Cullinan et al., 1995; Krukoff and Khalili, 1997), exposure to ether vapours, and Pavlovian fear conditioning (Campeau et al., 1994) and forced swimming (Engelmann et al., 2002, 2004; Orlando et al., 2007). However, there is little information available concerning the behaviour of NO in the nSO, although the results of the few works that have been published are in agreement with our own. In this sense, since Krukoff and Khalili (1997) reported an increase in nNOS mRNA and Engelmann et al. (2004), working with adult rats subjected to forced swimming stress, described an increase in nNOS expression in the nSO.

The role of NO in hormone release in the magnocellular neurons of the nSO during stress is a debatable and somewhat conflictive issue (Engelmann et al., 2004; Nomura et al., 2005; Orlando et al., 2007). For Stern and Ludwig (2001), Engelmann et al. (2002) and Orlando et al. (2008b) NO would inhibit the excitability of AVP- and OT-producing neurons, in part due to an enhancement of GABA-ergic afferences.

In mice knocked-out for nNOS subjected to forced swimming stress, the hybridization signal for AVP in the nSO, but not that of OT, was greater in the mice not manipulated genetically, which suggests that NO would inhibit transcription of the AVP gene and would help to maintain constant the plasma levels of AVP and OT during exposure to acute stress (Orlando et al., 2007, 2008b). The NO could also cause an inhibition or a delayed release of OT into the bloodstream. This assumption is in agreement with the results obtained under pharmacological manipulations (Orlando et al., 2008b), pointing to a selective facilitative effect of nNOS inhibitors on OT release *versus* AVP release in dehydrated animals (Summy-Long et al., 1993) and in response to salt-loading (Ventura et al., 2005).

Despite the recently published information concerning nNOS and magnocellular neurons in the stress axis, further studies would be needed to elucidate the local action of NO with respect to magnocellular neuronal modulation and the cellular and molecular mechanisms responsible for the dual action of NO in models of magnocellular activation in situations of physical stress.

In the present study, acute and prolonged restraint caused an increase in the parameters studied in all the experimental animals: the overall size of the nSO; the number of neurons expressing nNOS protein and its mRNA; an elevation in the values of optical densitometry of the nNOS immunoreaction and of the nuclear areas of the reactive neurons. All the foregoing evidence points to an active role for NO in the magnocellular neurons of the HNS and to the participation of this system in the stress axis.

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