Eur J Anat, 12 (1): 73-80 (2008)

Variations of neuronal nitric oxide synthase after hypophysectomy suggest a neuroprotective effect of nitric oxide in response to damage of the neurons of the hypothalamic supraoptic nucleus in the rat

J.M. Riesco-López, J. Carretero, R. Vázquez, J.A. Juanes, E. Blanco, M. Rubio, M. Asensio and J.M. Riesco

Departamento de Anatomía e Histología Humanas, Facultad de Medicina, Universidad de Salamanca, 37007 Salamanca, Spain

SUMMARY

The hypothalamic-neurohypophyseal system offers an ideal model to study the processes of nerve degeneration/regeneration after lesions caused by cutting the hypophyseal stalk, or hypophysectomy. Additionally, the magnocellular neurons comprising it contain nitric oxide, and its enzyme, neural nitric oxide synthase, shows important variations in functional alterations of the hypothalamic-neurohypophyseal system. Currently, there is still some controversy as regards whether nitric oxide exerts neurodegenerative or neuroprotective effects in the nervous system. Here we performed a quantitative and qualitative study of the variations in the neuronal activity of nitric oxide synthase, using immunohistochemistry, and of its mRNA, by *in situ* hybridization, in the magnocellular neurons of the supraoptic nucleus of the hypothalamus of hypophysectomised rats with survival periods of 10-30 days. Our morphological and morphometric results and those pertaining to neuronal quantification and optical density point to a neuroprotective effect of nitric oxide on magnocellular neurons during the regenerative process of the supraoptic nucleus that occurs after hypophysectomy.

Key words: nNOS – Nitric oxide – Hypophysectomy – *in situ* hybridization – Immunohistochemistry – Optical densitometry – nNOS mRNA

INTRODUCTION

The recognition of nitric oxide (NO) as a neuronal messenger (Bredt and Snyder, 1992) led to investigations of the molecule and discovery of its mechanisms of synthesis, regulation and actions on certain organs, among them the central nervous system (CNS). The behaviour of NO in the CNS seems ambivalent, since it sometimes apparently functions as a neuroprotector, while at other times is

Submitted: December 21, 2007
Accepted: May 16, 2008

Correspondence to:
Dr. José M. Riesco. Dpto. de Anatomía e Histología Humanas, Facultad de Medicina, Universidad de Salamanca, Av. Alfonso X el Sabio s/n, 37007 Salamanca, Spain.
Phone: +34 923 29 45 46; Fax: +34 923 29 45 59. E-mail: jmrs@usal.es

appears to be a neurodegenerative substance (Lipton and Stamler 1994; Dawson and Dawson, 1995; Iadecola, 1997). These circumstances have led it to be referred to as "the two-headed Janus" or "Jekyll and Hyde" (Leong et al., 2002). However, its true mechanism of action remains to be elucidated.

Within the CNS, one of the structures in which NO is found is the hypothalamic-neurohypophyseal system (HNS). This system offers a paradigmatic model for the experimental study of the neuroendocrine and nervous systems owing to their accessibility, the size of their neurons, and the multiple functions in which they are involved (Hatton, 1997; Oliet et al., 2001; Gainer et al., 2002; Yuan et al., 2006). The HNS is essentially formed by two nuclei: the paraventricular nucleus (nPV) and the supraoptic nucleus (nSO). The nPV comprises mango- and parvocellular neurons and has been studied in some depth with respect to NO and its involvement in stress mechanisms. However, the nSO has received less attention in this field. It is formed exclusively by magnocellular neurons that project to the posterior lobe of the hypophysis, thus allowing study of a single cell type. In light of the above, since the nSO is an exclusively magnocellular nucleus, we were prompted to perform the present study in an attempt to investigate the behaviour of NO in it following hypophysectomy, a nerve lesion that cuts the fibres of the HNS and alters its function. To do so we analysed the interneuronal expression of the nNOS protein and of its mRNA.

Materials and Methods

Animals and treatments

For this study 16 adult (200-230 g) male Wistar rats from Charles River® were used. Twelve of them were hypophysectomised and allowed to survive for 10, 21 and 30 days. From reception to sacrifice the animals were stabled (4 animals per cage with no limits to their movements) under standard conditions (temperature ± 20ºC; RH 50 ± 5%; controlled photoperiod of 14/10 h light/darkness) and were fed with standard rat chow (Panlab®) and had drinking water *ad libitum*. At all times the animals were treated according to the EU directive on Animal Protection for Experimental and Scientific Purposes (86/609/C) and also according to current Spanish legislation in that 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 inmunohistochemical detection

To determine the nNOS positive supraoptic neurons, the biotinylated-streptavidin-peroxidase immunohistochemical method was used.

The antigenic capacity of the tissue was reactivated by means of immersion of the sections in a thermostatted bath in citrate buffer (10 mM, pH 6.0) for 15 min at 96ºC, and endogenous peroxide was inactivated with H_2O_2 in methanol and non-specific reactions of the secondary antibody by incubation in normal pig serum (Dako®, diluted 1:30). Following this, sections were incubated overnight at 4ºC with sheep anti nNOS antibody (K205; Herbison et al., 1996), diluted 1:100.000, tested with several methods and previously assayed for immunocytochemistry. (Weruaga et al., 2000; Carretero et al., 2003). Biotinylated rabbit anti-Ig G from sheep (Caltag®, 1:150) and streptavidin-biotinylated horseradish peroxidase complex (Caltag®, 1:150) were successively applied at room temperature for 40 and 30 min respectively. Reactions were developed in freshly prepared solutions of 3-3'DAB (0.025% in TRIS buffer, 0.05 mol/l, pH 7.4 containing 0.003% of H_2O_2) for 5 min. Finally, the tissue sections were contrasted with Mayer acid haematoxylin and mounted with Aquatex (Merk®).

Controls included 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.

In situ hybridization procedure

mRNA expression for nNOS was demonstrated by non-isotopic *in situ* hybridization, modifying previous protocols from our laboratory (Herraez-Baranda et al., 2005; Rodríguez et al., 2005).

For this purpose, a highly specific biotinylated probe was developed by mixing the two probes reported by Lau et al. (2003) and Engelmann et al. (2004), corresponding respectively to the 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, the sequence was derived from U67309.1 and replaced gi:34872537). According to the data base, the specificity was for an error < $3x10^{-17}$ for rat nNOS mRNA.

Sections were acetylated to avoid the appearance of false positives (0.25% acetic anhydride and 0.1M triethanolamine in 0.9% saline; pH 8.0) and then dried at 55ºC in an incubator for 2 hours. Prehybridization of tissue sections was carried out by immersion for 1 hour in 200 µl of omnibuffer (Omnibuffer, Wak-Chemie Medical GMBH, Steinbach, Germany) at room temperature in a humidity chamber. Twenty µl of probe diluted in omnibuffer (0.02 ng/µl) were added; then the sections were covered with hybridization chambers (CoverWell, Sigma ®) and hybridization was carried out overnight at 37ºC in a thermocycler (Hybaid OmnoSlide ®).

After hybridization, the chambers were taken out and the sections were rinsed twice in 1x saline-sodium citrate (SSC) for 30 minutes at 55ºC, and two washes of 30 minutes in 1x SSC at room temperature.

Hybrids were detected by immunocytochemical detection of biotin. The sections were incubated overnight in mouse monoclonal antibody against biotin (1:250 in 0.05M TBS plus 0.8% of NaCl, pH 7.4) in humidity chambers at 4ºC. After 2 washes in TBS (0.05M, pH 7.4), the immunocytochemical reaction was developed successively at room temperature by incubation over 45 minutes in biotinylated goat anti-mouse IgG (1:200 in TBS, Caltag®) and streptavidin-peroxidase complex (1:250 in TBS, Caltag®) for 45 minutes. Visualization of the reaction was accomplished using freshly elaborated 3-3' DAB (Sigma®, 0.025% in TRIS buffer 0.05 mol/l, pH 7.4, containing 0.003% of H_2O_2) for 5 min. To enhance the reaction, in some sections visualization was amplified using the tyramide amplification-kit (Dako®). Slides were counterstained with Mayer's haematoxylin and mounted with Aquatex (Merck®).

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 implemented as a reaction control. 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 ImageJ program (NIH, USA) from 250 cells per animal (1000 cells per treatment) and the total area occupied by nSO was determined in five frontal sections, separated from each other by 100 µm (Bregma: -0.9 to -1.3 of the stereotactic atlas of Paxinos and Watson, 1998). Using the same program, mean grey densitometric 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 and following stabilization of the input grey levels from 37 to 223 using the Adobe Photoshop 7.0® program.

Statistical analysis

The results obtained were processed statistically using GraphPad Prism 4. The results are expressed as arithmetic means ± error standard 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 untreated animals, the nSO displayed immunoreactive nNOS-ir neurons distributed throughout the rostro-caudal part of the nucleus and arranged in parallel rows located in its dorsal and central regions (Fig. 1a). The neurons not reactive to nNOS showed characteristics typical of magnocellular neurons and were mainly located in the ventral zone of the nucleus, close to the limiting pial membrane. After hypophysectomy, this arrangement was lost and the nNOS-ir neurons became disordered and were accompanied by abundant spongiosis and vascular dilation (Fig. 1 b-c). The non-nNOS-ir neurons usually showed alteration in the perikaryon and, in some cases, piknotic nuclei (Fig. 1c). All these morphological characteristics were more evident at 10 days after hypophysectomy.

The nNOS-ir neurons of the untreated animals had a large perikaryon with large centred nuclei. Ten days after hypophysectomy, the nNOS-ir neurons exhibited an exiguous perikaryon with an indented border that contained a large centred nucleus and a conspicuous nucleolus (Fig. 1 b-c), while at 30 days post-hypophysectomy the perikaryon was voluminous, with a homogeneous border and an

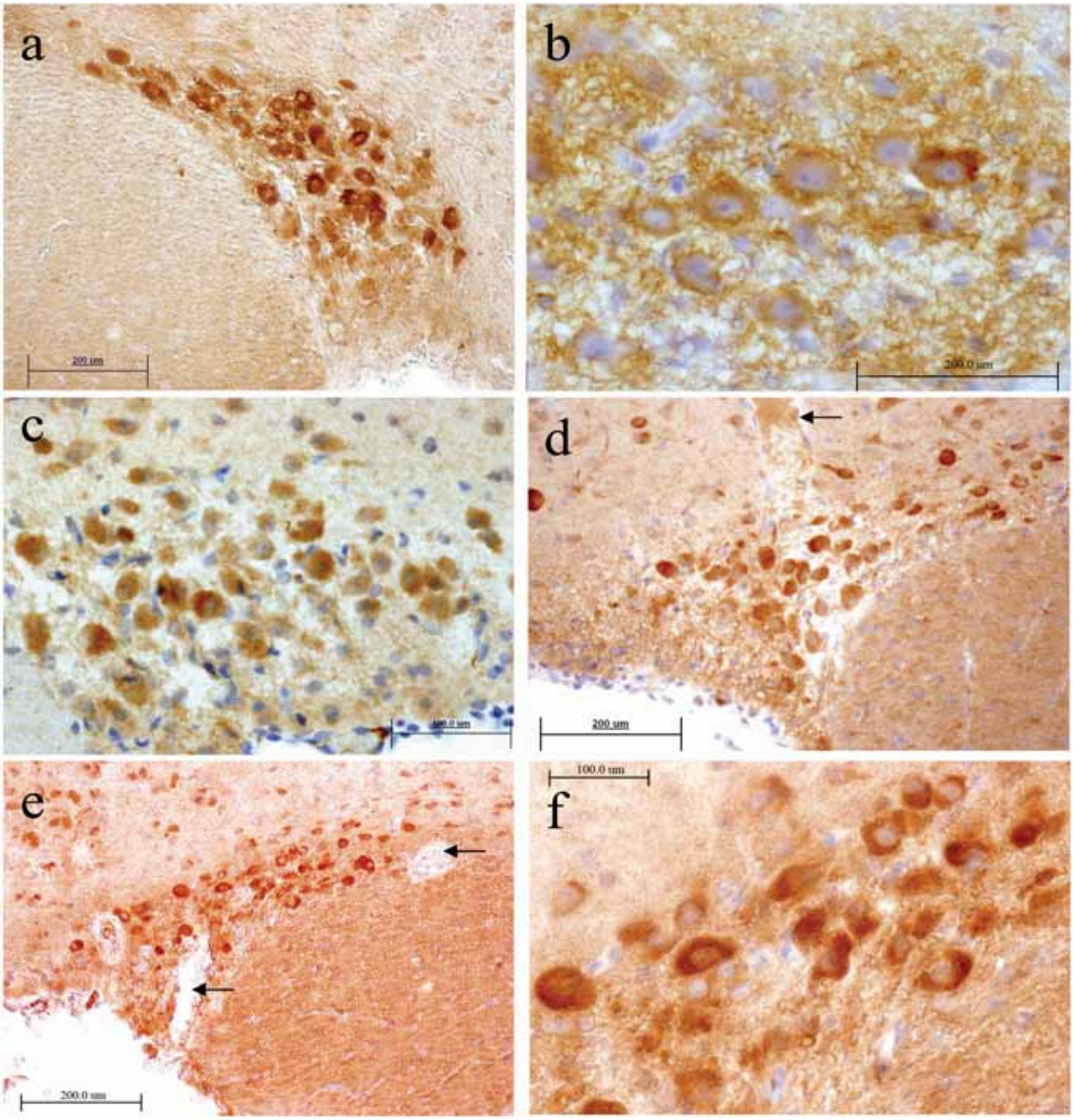


Fig. 1. Micrographs of coronal sections of supraoptic nuclei showing the morphology and immunoreactivity of nNOS neurons in untreated (a) and hypophysectomised (b) animals sacrificed at 10 (b-c), 21 (d) and 30 (e-f) days. Neuronal destruction and disorganisation are evident at 10 days and are accompanied by spongiosis and a glial reaction in the neuropil. Images of vascular dilation (arrow) were very frequent at 21 and 30 days. The intensity of the nNOS reaction was less prominent in the hypophysectomised than in the untreated animals. The disorganisation and irregularity of the perikaryon seen in the neurons of the nSO at the start of hypophysectomy disappeared as survival time progressed.

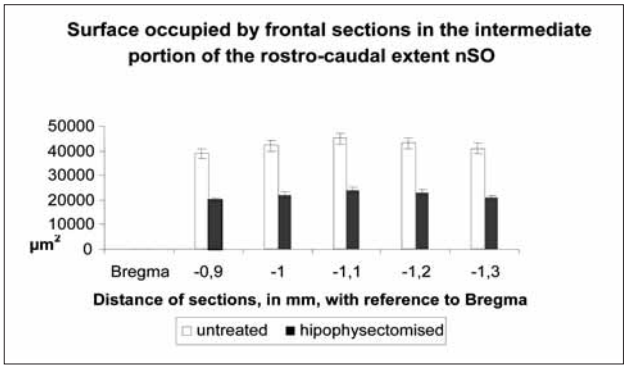


Fig. 4. Marked decrease (47.6%) in the areas of the intermediate frontal sections of nSO of hypophysectomised with respect to untreated animals.

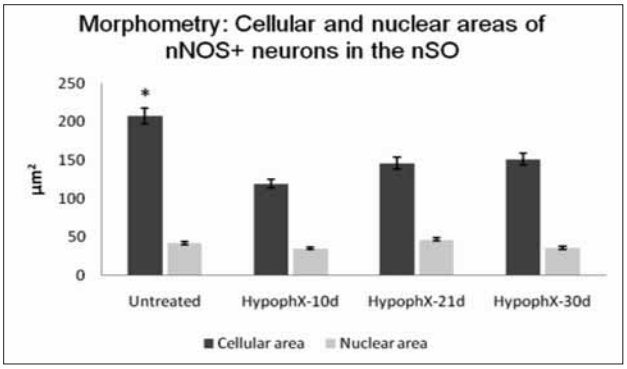
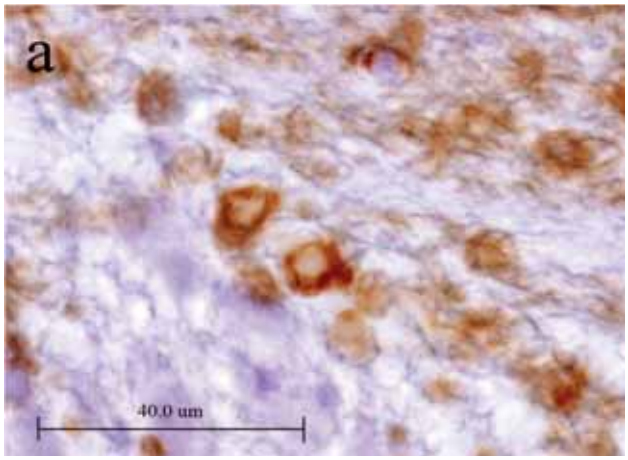
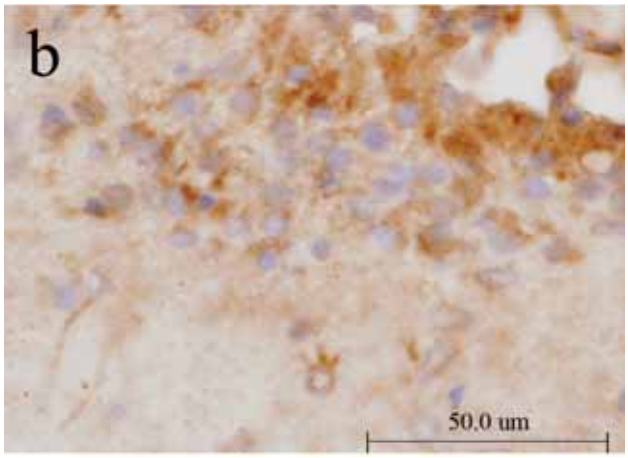


Fig. 6. Morphometric values in untreated and hypophysectomised animals. Asterisk: $p<0.05$ untreated *vs.* hypophysectomised animals.





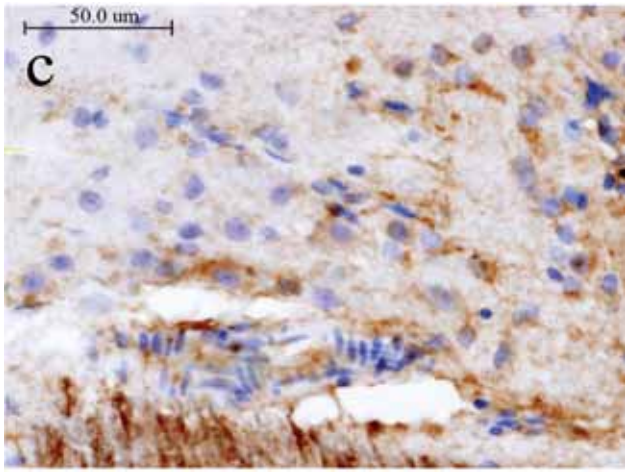


Fig. 2. In situ nNOS mRNA hybridization in the perikaryon of neurons corresponding to the supraoptic nuclei of an untreated animal (a), a hypophysecto-mised animal with a survival of 21 days (b) and of 30 days (c). Note the lower expression of hybrids in the hypophysectomised animals.

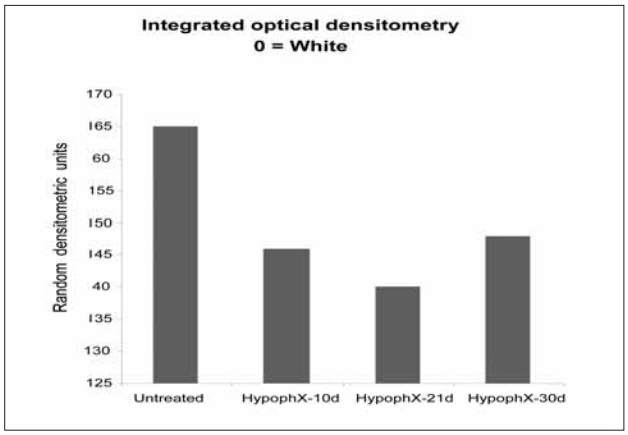
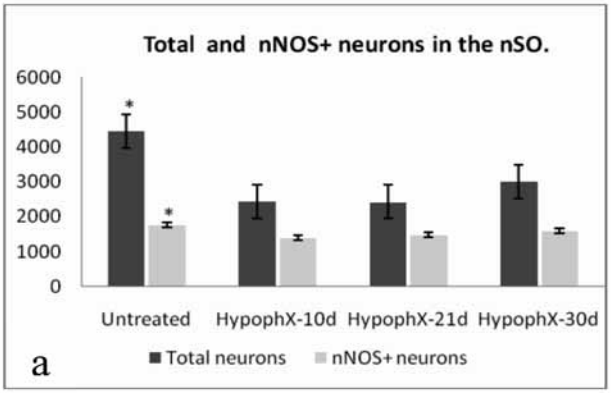


Fig. 3. Random values corresponding to optical density.



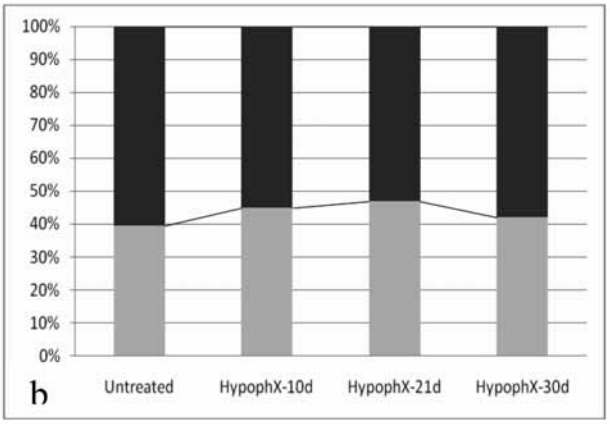


Fig. 5. a) Quantitative analysis of the absolute number of total neurons and of nNOS-expressing neurons. Asterisk: $p<0.01$ hypophysectomised *vs.* untreated animals; b) Relative distribution of nNOS-positive neurons with respect to the percentage of total neurons in each group, with a relative % increase of nNOS-positive neurons in the hypophysectomised animals.

eccentric nucleus (Fig. 1 e-f). At 21 days of survival, the nNOS-ir neurons displayed intermediate morphological characteristics between both groups (Fig. 1 d).

In situ hybridization

With *in situ* hybridization, the nNOS mRNA expression appeared uniformly distributed throughout the rostro-caudal sections of the nSO in the treated animals (Fig. 2a), in comparison with the reduced presence of hybrids in the neurons of the nSO at the different times of survival. However, at 30 days, the expression of RNA hybrids became more prominent (Fig. 2b) than at 10 and 21 days (Fig. 2c).

Densitometry

In the nNOS-ir neurons of the untreated animals, the reaction was intensely and homogeneously distributed throughout the perikaryon, while in the hypophysectomised animals, the intensity of the nNOS reaction was more reduced and showed a tendency towards polarisation as the time post-hypophysectomy pro-

gressed and the nuclei became more eccentric. No significant changes were observed in the densitometric values of nNOS between the different times of survival after hypophysectomy (Fig. 3).

Morphometry

The surfaces of frontal sections of the nSO, measured in the intermediate portion of its rostro-caudal arrangement, decreased after hypophysectomy by up to 47.6% of the values corresponding to the untreated animals (Fig. 4).

The global quantification of the neurons present in the nSO and that corresponding nNOS-ir neurons of all the animals studied is reflected in terms of absolute cellular density (Fig. 5a), and, proportionally, in percentages (Fig. 5b). The total number of neurons in the nSO decreased from 4466 ± 376 in the untreated animals to 2483 ± 270 in the hypophysectomised rats, with a mean neuron loss of 44.4% ($p<0.001$). In percentage terms, the neurons of the nSO underwent a dramatic reduction after hypophysectomy (Fig. 5a). However, in comparison with the total number of surviving neurons the percentage of nNOS-ir neurons was significantly higher than that of the untreated animals (Fig. 5b; $p<0.05$), although there were no significant differences in the number of nNOS-ir neurons with respect to the post-hypophysectomy survival time.

The values of the cellular and nuclear areas of the nNOS-ir neurons in the nSO were modified after hypophysectomy (Fig. 6). The cellular areas were significantly lower after hypophysectomy ($p<0.001$), especially at 10 days of survival, remaining very similar at 21 and 30 days of evolution. The nuclear areas were not significantly different between the untreated animals and the hypophysectomised rats, regardless of the time of evolution after hypophysectomy (Fig. 6).

Discussion

The aim of the present report was to study the effect of hypophysectomy on the neurons of the nSO and the possible neuroprotective role of NO. Thus, one striking morphological observation derived from hypophysectomy, regardless of the final survival time, was the loss of neurons in the nSO, eliciting a reduction in the central size of the nucleus -close to 48%- with respect to the untreated animals. This was also observed by Yulis and Rodríguez (1982). The main cause of the reduction in nuclear size was the massive destruction of magnocellular neurons as a result of a retrograde degeneration, which was seen at 10 days of survival and which persisted with no significant changes up to 30 days post-hypophysectomy. The percentage of neuronal loss detected here -about 40%- is similar to that reported in other publications in adult hypophysectomised rats (Moll and De Wied, 1962; Kawashima et al., 1966; Yulis and Rodríguez, 1982; Kawamoto and Kawashima, 1985, 1987). Other phenomena accompanying nerve lesions, such as spongiosis and a glial reaction in the nSO have been reported by Rasmussen (1940) and Vázquez et al. (1993).

In absolute terms, the number of nNOS-ir neurons was lower in all groups of hypophysectomised animals. However, the percentage of immunoreactive neurons with respect to the total number of surviving neurons was greater than that observed in the untreated animals. Previous work carried out on hypophysectomised animals reported a biphasic nitrergic response, with an increase in nNOS-ir neurons and in reaction intensity with respect to the values obtained in untreated animals (Wu and Li, 1993; Yu 1997; Lukacova et al., 2006). This began on the first day post-hypophysectomy and persisted for 7 days, thereafter followed by a period of gradual remission, with values very similar to or even below those of the untreated animals as from 14 days (Yuan et al., 2006). Something similar occurs in spinal cord lesions caused by avulsion of the nerve roots (Wu and Li, 1993; Yu, 1997; Lukacova et al., 2006). This nitrergic increase observed during the first days post-lesion could be related to the toxic effects of NO, produced in large amounts, which that would interfere with cell proteins or the formation of reactive oxygen species (Dawson and Dawson, 1996; Lukacova et al., 2006). Wu et al. (1994) observed a coincidence between the high induction of NO and the death of spinal motor neurons in spinal cord lesions due to root avulsion. The excess NO would originate not only from the up-regulation of nNOS but also from the formation of inducible NO in neutrophils and in the glia after cerebral ischaemia (Iadecola et al., 1995) or in other circumstances (Garcion et al., 1998; Weldon et al., 1998; Takeuchi et al., 1998). The high proportion of glia in the central nervous systems and its active proliferation could generate large amounts of NO (Leong et al., 2002). In our study, the spongiosis and glial reaction were very pronounced at 10 days post-hypophysectomy, such that the glia could be an important source of NO in the fist few days after lesion. Nevertheless, further studies meas-

uring the true amount of NO released in neurons and in the glia in the nSO following hypophysectomy would be required to elucidate all these aspects. Such studies would have been unthinkable a few years ago but are now possible thanks to new technological advances that allow NO to be detected at nanomolar level (Leong et al., 2002). Regarding the down-regulation of nNOS in the nSO seen 10 days after hypophysectomy, the factors responsible for this decrease are unknown, but target reinnervation by regenerated axons or the release of neurotrophic factors derived from the glia have been suggested (Hammarberg et al., 1996).

The relative increase in the number of nNOS-ir neurons and above all the fact that at 10 days post-hypophysectomy the nNOS-positive neurons did not show phenomena of involution, which the nNOS-positive neurons did, suggest a neuroprotective role for NO. In recent years, many authors have explored the anatomical and biochemical aspects of the cytotoxic and cytoprotective effects of NO (Wink et al., 1996; Chiueh, 1999; Andoh et al., 2000; Melino et al., 2000; Lukacova et al., 2006; Marsala et al., 2007). Most have concluded that NO acts as a cell protector not only in the brain but also in other organs such as kidney and liver (Marsden and Ballermann, 1990; Billiar et al., 1990), probably by acting as a hydroxyl radical scavenger that protects against oxidative injury (Mohanakumar et al., 1998; Chiueh, 1999).

The morphometric results –the marked decrease in cellular and nuclear areas in nNOS-positive neurons at 10 days post-hypophysectomy– suggest an exiguous functional activity; the opposite of that observed at 21 days, when the large nuclear size accompanied by cellular areas smaller than those observed in untreated animals would indicate a strong synthesis of neurosecretory material but with an important outflow towards the axons and the recently formed pseudolobe (Raisman, 1973; Kawamoto, 1985; Scott et al., 1995). In turn, the morphometric data observed at 30 days post-hypophysectomy suggest a stabilisation in the process of synthesis and a release of neurosecretory material in the nSO of hypophysectomised animals. These data are consistent with the ultrastructural observations and findings concerning the localisation of chemical messengers obtained in hypothalamic magnocellular neurons during different developmental stages after hypophysectomy (Raisman, 1973; Meister et al., 1990).

Study of the use of *in situ* hybridisation reveals that the nNOS mRNA followed a course parallel to that of the protein detected by immunofluorescence. Hypophysectomy, with an evolution of 10-30 days, down-regulated the expression of the nNOS gene in the nSO of the rats, suggesting that the regulation of nNOS in the nSO after hypophysectomy would take place at transcriptional level.

The morphological and morphometric data analysing the importance of the nitrergic neurons and the mRNA hybrids corresponding to nNOS in the untreated and hypophysectomised animals suggest a neuroprotective effect of NO on the neurons of the nSO, a morphofunctional adaptation occurring at around 30 days after hypophysectomy.

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