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Article in Biomedical Research · October 1996
DOI: 10.2220/biomedres.17.359

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NADPH-DIAPHORASE AND GnRH: ANATOMICAL RELATIONSHIP IN THE RAT HYPOTHALAMUS

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ABSTRACT

In order to check possible neuroendocrine functions of nitric oxide, coexistence of gonadotropin-releasing hormone (GnRH) immunoreactivity and NADPH-diaphorase (ND) activity was studied in the rat hypothalamus using combined immunocytochemical and histochemical techniques. No coexistence was found in any hypothalamic nuclei. However, in specific areas such as the organum vasculosum of the lamina terminalis, the medial preoptic nucleus, and the periventricular area of the third ventricle GnRH-immunoreactive and ND-active elements were observed in close anatomical relationship. On the basis of the topographical distribution and morphological characteristics of labelled elements, it is possible to conclude that in normal male animals both markers are expressed in different cells and fibres, but, based in its close proximity, the released nitric oxide may be effective in influencing GnRH elements.

Nitric oxide (NO) is a neuronal messenger involved in diverse and important functions in the nervous system (31). At present the distribution of the brain nitric oxide synthase (NOS) is well-known in most areas of the brain as assessed by biochemical, histochemical, immunohistochemical and molecular biological techniques. In aldehyde-fixed brain sections, NADPH-diaphorase (ND) has been identified as an accurate marker for NOS-containing neurons (17). ND-positive neurons are located throughout the hypothalamus, including the areas where the gonadotropin-releasing hormone (GnRH)-immunoreactive neurons are present (5, 9).

Several studies have suggested a role for NO in the control of hypothalamic luteinizing hormone secretion through the regulation of the GnRH neurons activity (12, 23, 24, 26, 27, 32). Despite this functional link, very little is known about the anatomical relationship between NOS- and GnRH-systems in the intact hypothalamus. Moreover, some controversy exists both in untreated and animals under several experimental conditions. In normal animals very low partial coexistence (1 colocalizing neuron out of 370 neurons) (15), or absence of colocalization was found (16). In steroid-treated ovariectomized immature rat (9) and ovariectomized rats (16) no coexistence has been found. However, under certain conditions GnRH neurons may express NOS since GT1 cells (transformed immortalized neurons) have been reported to possess NOS mRNA (22, 24), suggesting therefore the presence of posttranscriptional regulation. Additionally, with the exception of the paper of Grossman et al. (15) in which both sexes were considered, the rest of the studies have been focused only in female rats.

The aim of the present study is to determine whether GnRH-neurons are able to express ND activity in the hypothalamus of intact male animals, and the degree of anatomical relationship between GnRH- and ND-positive elements, by means of a histochemical ND and immunocytochemical GnRH double labelling staining of the same sections.

MATERIAL AND METHODS

Four adult male Wistar rats, weighing between 210 and 270 g were used in the present study. After...
ND-positive (blue) and GnRH-immunoreactive (brown) labelled neurons and fibres in the rat hypothalamus. Scale bar: 1, 3, 4 and 5, 10 μm; 2, 5 μm

Fig. 1 Panoramic view of the OVLT in which a clear predominance of GnRH-fibres can be observed. ×100

Fig. 2 Higher magnification of the OVLT. Note the deposits of GnRH-immunoreaction. ×200

Fig. 3 ND positive neurons and GnRH-immunoreactive fibres in the periventricular area. ×100

Fig. 4 GnRH-immunoreactive fibre running dorsally to the supraoptic nucleus. ×100

Fig. 5 ND-positive neurons in the supraoptic nucleus. ×100
deep anaesthesia with ketamine (Ketolar, 50 mg/kg body weight), the animals were perfused through the ascending aorta with 100 ml Ringer solution followed by a fixative mixture of 4% paraformaldehyde and 2% picric acid in 0.1 M phosphate buffer, pH 7.3 (PB). After perfusion, the brains were dissected out, trimmed, and postfixed for two additional hours in the same fixative. The blocks containing the hypothalamic region were cryoprotected with sucrose and cut on a cryostat along the frontal plane at 30 μm thickness.

The sections were collected in cold (4°C) PB and processed free-floating for the demonstration of ND activity as described elsewhere (1, 2). The incubation medium was made up of 0.08% Triton X-100, 1 mM reduced β-NADPH and 0.4 mM nitroblue tetrazolium in 0.1 M Tris-HCl buffer (pH 8). All reagents were from Sigma.

After the histochemical reaction was finished, the sections were processed for immunocytochemistry as previously described (3, 4, 30). The sections were first incubated with an antibody to GnRH (1:5,000; clone BKL2) (28) for 48 h at 4°C. After thorough washing, sections were incubated with biotinylated antimouse immunoglobulin (1:250; 2 h at room temperature) followed by avidin-peroxidase complex (1:225; 90 min at room temperature), both from Vector Labs (Burlingame, CA, U.S.A.). Sections were then developed in 0.05% 3,3′-diaminobenzidine in 0.1 M Tris-ClH buffer (pH 7.6) plus H2O2. Sections were briefly washed in PB, mounted on gelatin-coated slides, dried overnight at room temperature, dehydrated through graded alcohols and xylene, and coverslipped with entellan.

The used primary antibody against GnRH has been characterized and it detects GnRH in quantities as small as 4 pg/tube on RIA analysis (28). In rat hypothalamic extracts this antibody binds a single peak eluting at a position corresponding to synthetic GnRH on size exclusion chromatography, and the major peak of immunoreactivity corresponded to synthetic GnRH on reverse phase HPLC (28). Control sections in which primary antibody was substituted with normal horse serum (20%) or with PB were negative.

RESULTS
Since the localization of ND and GnRH in the hypothalamus of adult rats have been previously described in detail (5, 7, 8, 19) and the observed distribution of both markers is coincident with the previous papers, the description is limited only to what is essential for comparison of both systems.

GnRH-immunoreactive material was identified in the tissue sections as deposits of brown granular product. The most abundant GnRH-immunoreactive elements in the rat hypothalamus were varicose fibres (strings of varicosities with occasional branches) (Figs. 1–4). GnRH-immunoreactive fibres were observed in the preoptic anterior hypothalamic area and in the periventricular stratum of the third ventricle. A few GnRH-immunopositive neurons were observed located at the level of the medial preoptic area.

In the anterior preoptic hypothalamic area, GnRH-immunoreactive fibres were observed densely packed in the organum vasculosum of the lamina terminalis (OVLT) (Figs. 1 and 2). Tracts of GnRH-fibres were found to terminate in the OVLT and in the median eminence. In the periventricular stratum of the third ventricle, the fibres were located in close contact with the ependymal lining of the third ventricle. These fibres course both longitudinally along the third ventricular wall (Fig. 3) as well as transversely through the ependyma onto the ventricular surface (Fig. 3). GnRH-immunoreactive fibres were also observed along the upper surface of the optic chiasma (Fig. 4), and finally some fibres were concentrated in the median eminence.

Histochemical ND staining was observed mainly in neurons and to a lesser extent in fibres. ND-positive cells occupied most of the hypothalamic nuclei (Fig. 1), being especially abundant in the magnocellular neurosecretory nuclei (Fig. 5). ND-stained cell bodies were frequently surrounded by GnRH-fibres (Fig. 3), however, no clear evidence of contacts was seen.

Coexistence of GnRH and ND was not observed in any of the studied hypothalamic structures. Both markers were therefore thought to be expressed in two different morphological systems. On analyzing all hypothalamic areas, GnRH-positive fibres seem to be thicker than those observed after ND staining (Figs. 3 and 5).

On considering the comparative topographic distribution of both systems, it was evident that the GnRH-immunoreactive system was widely extended in the preoptic region while the ND-positive system was mainly located more caudally, especially at the level of the magnocellular neurosecretory nuclei. However, in specific parts of the hypothalamus both systems overlap, showing an intermingling in the distribution of both populations of
labeled elements. This was especially evident in the area occupied by the OVLT (Fig. 1), in the medial preoptic nuclei, and in the periventricular area (Fig. 3). Within these areas the most striking relationship was found at the level of the OVLT that ND cells were completely surrounded by GnRH-immunoreactive fibres (Fig. 1). This anatomical relationship becomes less prominent more caudally, especially at the level of the posterior part of the periventricular zone where the GnRH system was less represented.

DISCUSSION

In the course of an on-going research, we and other groups have tried to identify the chemical nature and neurotransmitter content of ND-containing neurons in the rat hypothalamus. Previous studies included preferentially different neuropeptides (4, 10, 11, 33, 34, among others), and calcium-binding proteins (3, 6). The degrees of colocalization varied for the different substances without existence of a general coexistence.

In the present study, we have observed that GnRH and ND do not coexist in the rat hypothalamus. However cells expressing ND-activity and thus, capable of releasing NO are located in close relationship to GnRH elements (both cell bodies and fibres). Since NO diffuses freely across membranes, it is plausible that the control of NO upon GnRH release is done transneuronally and not by cosynthesis of both elements in the same neurons. These data are generally consistent with studies performed by Bhat et al. (9) and Herbison et al. (16) who found the absence of coexpression of both markers. By contrast, Grossman et al. (15) found some elements coexpressing both substances and under pathological circumstances GnRH-neurons may express NOS (22, 24).

The secretion of GnRH is modulated by a complex system, which includes the feedback actions of gonadal steroids and inputs from a wide variety of neurotransmitters and neuromodulators (see 13). In this sense, as indicated in the introductory remarks, there is sufficient evidence demonstrating that NO is an important regulator of GnRH release (12, 23, 24, 26, 27, 32). According to Herbison et al. (16) it seems that NO acts not only at the level of GnRH-terminals but also in the cell bodies to regulate the LH secretion. Although no coexistence has been found in this study, a close anatomical relationship between ND-active and GnRH-immunoreactive elements is evident. This fact is especially important since NO may be effective in influencing neural elements as far as away 200 µm from its point of origin (37).

Another important problem when evaluating GnRH-expression and, in consequence, its possible coexistence with other neuronal markers, is that the ability to detect GnRH-immunoreactive perikarya and fibres varies greatly depending on the composition and mode of fixation, immunocytochemical technique and antibody, sex of the animal, and experimental conditions (8, 14, 20, 21). It has been clearly established that experimental treatments such as manipulations of the gonadal environment, and colchicine injections, may give rise substantial changes in the visualization of GnRH-immunoreactive elements in the rat hypothalamus (19). Then, it cannot be excluded that after axonal transport blocking or other experimental conditions, some ND-positive cells would demonstrate GnRH-immunostaining. In addition, ND-staining has been described as activity-dependent in several experimental situations involving the hypothalamus (11, 18, 25, 29, 35, 36), and many treatments including chemical and mechanical injury elicit the production of inducible NOS in both neurons and glial cells (38). Thus, further experiments involving experimental manipulations should be performed to elucidate the relationship between both markers.

The authors express their gratitude to Dr. P. Herion for kindly providing the GnRH antibody and to Miss E. L. Shorten for kindly revising the language style. This work was supported by the Junta de Castilla y Leon (SA40-95) and DGICYT (PB94-1388).

Received 2 July 1996; and accepted 29 July 1996

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