Journal of Chemical Neuroanatomy, Vol. 6: 335–341 (1993)

Infrequent Cellular Coexistence of NADPH-Diaphorase and Calretinin in the Neurosecretory Nuclei and Adjacent Areas of the Rat Hypothalamus

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ABSTRACT

Colocalization of the calcium-binding protein calretinin and NADPH-diaphorase activity at the cellular level was studied in the magnocellular secretory nuclei of the rat hypothalamus using sequential immunocytochemical and histochemical staining of the same sections. A low degree of colocalization of these markers was observed in certain cellular subpopulations within all the areas considered (supraoptic, paraventricular, circular and both fornicals nuclei and in the hypothalamic area located between the supraoptic and paraventricular nuclei). However, since in the paraventricular nucleus both markers were expressed by different neuronal populations, the coexistence was almost non-existent in some subdivisions of this nucleus. This rare coexistence strongly suggests that NADPH-diaphorase and calretinin are related to different functions shared by restricted hypothalamic neuronal populations.

KEY WORDS: Calcium-binding protein Nitric oxide synthase Coexpression Magnocellular nuclei

INTRODUCTION

NADPH-diaphorase (ND) activity has been identified as a nitric oxide synthase (Hope *et al.*, 1991) and is a selective histochemical marker for specific neuronal populations in the central and peripheral nervous systems (Alonso *et al.*, 1992a,b, 1993; Arévalo *et al.*, 1992; Hedlich *et al.*, 1990; Mizukawa *et al.*, 1989; Roberts and Difiglia, 1988; Sandell *et al.*, 1986; Vaney and Young, 1988; Villalba *et al.*, 1988, 1989; Vincent, 1986; Vincent *et al.*, 1983a,b,c; Winsky and Jacobowitz, 1991, among others). ND is widely distributed within the magnocellular neurosecretory nuclei of the rat hypothalamus. It is especially abundant in the paraventricular (PVN), supraoptic (SON) nuclei, and in the area located between these two nuclei (area inter SON-PVN) (Arévalo *et al.*, 1992). The brain nitric oxide synthase has an absolute requirement for calcium. Nitric oxide release by ND-positive neurons is mediated by Ca^{2+}-sensitive N-methyl-D-aspartate gated channels (Bredt *et al.*, 1991; Garthwaite *et al.*, 1988; Kiedrowski *et al.*, 1992). The hypothalamus contains different populations of neurons expressing 'EF-hand' calcium-binding proteins, one of the most important systems that regulates intracellular Ca^{2+} concentrations in the brain. In a series of studies, we compared the distributions of ND and different neuropeptides and calcium-binding proteins within the hypothalamus (Alonso *et al.*, 1992a,b; Sánchez *et al.*, 1993). We have demonstrated a partial coexistence of ND and calbindin D-28k (CaBP) in the magnocellular neurosecretory neurons, with the exception of the area inter SON-PVN, where no neurons colocalized both markers (Alonso *et al.*, 1992a). However this coexistence is not general in the brain, since in the rat olfactory bulb, by contrast, ND and CaBP were expressed by different neuronal populations, even belonging to the same neuronal type (Alonso *et al.*, 1993).

Calretinin (CR), another calcium-binding protein, has a relatively similar molecular weight to CaBP (29–31 kDa for CR and 28 kDa for CaBP). Both proteins are highly homologous (with a 59% coincidence in the amino acid sequence between chicken CR and chicken CaBP), they are specific markers for particular groups of neurons (Celio, 1990; Pochet *et al.*, 1989; Résibois and Rogers, 1992; Rogers, 1991; Rogers and Résibois, 1992). Both are recognized simultaneously by several polyclonal antisera (Pochet *et al.*, 1989; Rogers, 1987) and are highly conserved during vertebrate evolution (Rogers, 1991). Recently prepared monoclonal antibodies have allowed the differentiation of the CaBP- and CR-immunopositive neuronal populations in the rat brain (Celio, 1990; Résibois and Rogers, 1992).

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0891–0618/93/060335–07 $08.50

Comparing the distributions of ND activity and CR in the rat hypothalamus, the presence of overlaps is evident. However, without a double-labelling study it is unclear whether ND and CR are colocalized in the same subset of neurons or, on the contrary, they label different cells with partially similar distributions.

Up to now, there is no study available on the presence of ND-activity in the CR-positive and negative neurons of a given region, although regions and nuclei with neurons expressing both markers are widely distributed throughout the brain. Therefore, the aim of this study is to carry out a sequential histochemical-immunocytochemical double-labelling of the same sections to determine whether ND and CR coexist within the neurons of the magnocellular neurosecretory nuclei of the rat.

MATERIALS AND METHODS

Five adult female Wistar rats (235–260 g body weight, supplied by Charles River, Spain) were used. The animals were perfused under deep anaesthesia (Ketolar, 50 mg/kg body weight) through the ascending aorta with 100 ml of a solution composed of 0.9% NaCl, 0.025% KCl and 0.05% $NaHCO_3$ in distilled water, followed by 400 ml of a fixative containing 4% paraformaldehyde, and 15% saturated picric acid in 0.1 M-phosphate buffer, pH 7.25 (PB).

After 2 h, the hypothalamic regions were removed, stored in cold fixative for 2–5 h and then placed in phosphate-buffered 30% sucrose (v/v) overnight at 4°C. Thirty-micrometer frontal sections were cut on a cryostat (−19°C) and serially collected in cold (4°C) PB. The sections were processed free-floating for the demonstration of ND activity as described elsewhere (Arévalo *et al.*, 1992). Briefly, sections were incubated for 60–90 min at 37°C in a solution containing 1 mM-β-NADPH (Sigma no. 1630), 0.8 mM-nitroblue tetrazolium (Sigma no. 6876), and 0.08% Triton X-100 in 0.1M-Tris–HCl buffer (pH 8.0). Endogenous ND reacts with nitroblue tetrazolium to produce a blue reaction product. The course of the reaction was monitored under the microscope, and was stopped by washing the sections three times in cold PB.

When the histochemical reaction was concluded, the sections were rinsed in PB and processed for immunocytochemistry as described previously (Alonso *et al.*, 1992a,b, 1993). Following a preincubation in 5% normal goat serum in PB for 24 h at 4°C, the sections were sequentially incubated in the following solutions: (1) a primary antiserum solution containing polyclonal rabbit anti-CR serum (diluted 1:2000 in PB; kindly donated by Dr J. H. Rogers, University of Cambridge, UK), 5% normal goat serum, and 0.5% Triton X-100, for 48 h at 4°C; (2) biotinylated goat anti-rabbit immunoglobulin G (1:200 in PB, Vector Labs, Burlingame, CA, USA) for 2 h at room temperature; and (3) avidin–peroxidase complex (1:225 in PB; Vector Labs) for 90 min at room temperature. Tissue-bound peroxidase was visualized by treating the sections with 0.07% 3,3′ diaminobenzidine (DAB) and hydrogen peroxide in 0.1 M-Tris buffer (pH 7.6), which results in a brown reaction product. The DAB reaction was terminated by rinsing the sections in PB. The sections were mounted on gelatin-coated slides, dehydrated in ethanol series, cleared with xylene, and coverslipped with Entellan.

Controls for the histochemical procedure included incubation without substrate (NADPH), incubation without the electron acceptor nitro blue tetrazolium in order to control for possible non-specific formation of colouration due to other reagents in the incubation solution, and heat denaturation of the enzyme by heating the tissue sections at 85°C for 5 min. No residual reaction was observed. The used primary antibody against CR has been fully characterized (Rogers, 1987) and previously used in different regions of the brain (Résibois and Rogers, 1992). In addition, the specificity of the immunostaining was controlled by omitting the antibody (first or second) in each incubation step in ND-stained sections. No residual immunoreactivity was observed.

In order to rule out possible variations in the CR-immunolabelling results by the previous ND histochemical procedure, the same study was carried out in another group of rats ($n = 4$) in which the ND reaction was developed after the CR-immunostaining. Similar results were obtained in this experimental group.

In the double-labelled sections, the blue-coloured reaction of ND histochemistry and the brown DAB reaction product of the immunoperoxidase were clearly distinguishable. The neurons that displayed both stainings showed cell bodies with both colours at the same focussing plane. The distribution of both labellings in the double-stained sections was similar in all parameters (including numbers of positive cells) to single-stained sections, with the only exception of a somewhat higher background staining, observed in brain tracts such as the fornix, corpus callosum, anterior commissure, or optic chiasma. Brightfield and darkfield analysis of these structures demonstrated that this staining is not associated with any particular cellular structure. The number of the reacting cells displaying ND, CR and coexistence was determined by analysing each nucleus (and all the magnocellular and parvicellular subdivisions of the PVN). Calculation of the number of reacting cells was carried out with an image analyser system MIP-2 (IMCO 10). By means of a graphic tablet, we counted for all sections of every individual animal the number of positive or immunoreactive neurons (by means of an optic pen connected to the graphic table and the image analyser we singled out the different types of cells). Calculation of the number of positive-reactive cells was always carried out by the same author (F.S.). Only cells in which the nucleus was present were

considered. The total number of stained neurons was corrected by a factor of 0.750 according to Abercrombie's formula (Abercrombie, 1946).

In the PVN we have considered all the positive or immunoreactive neurons located within the borders of every subdivision (magnocellular and parvicellular subdivisions), independently of their size. It is well known that in the PVN, although magnocellular and parvicellular subdivisions can be recognized, neurons with different sizes are detected in every particular subdivision (see Kiss *et al.*, 1991; Swanson and Kuypers, 1980, among others).

RESULTS

In the present study nomenclature and nuclear boundaries proposed by other authors (Peterson, 1966; Rhodes *et al.*, 1981) have been used with small changes (Alonso *et al.*, 1992a,b; Sánchez *et al.*, 1990, 1992). Although our studies focused on the magnocellular neurons, in the PVN both well-known types of subdivisions (magnocellular and parvicellular) were considered (Swanson and Kuypers, 1980). Additionally we considered the area inter SON-PVN which includes the neurons located between both nuclei, from the beginning of the prechiasmatic subdivision of the SON, up to the retrochiasmatic one excluding the cluster of neurons forming the CN.

The double-labelling procedure resulted in four staining neuronal-types: CR-positive ND-negative, CR-negative ND-positive, CR-positive ND-positive and CR-negative ND-negative. The latter group was clearly present in the dorsal and lateral parvicellular subdivisions of the PVN, in which only a few neurons were positive for either CR or ND.

Supraoptic nucleus

The four neuronal types, without any special distribution, were found in this nucleus. Thus, neurons expressing exclusively ND-activity, neurons expressing exclusively CR, cells colocalizing both markers, and CR-negative ND-negative neurons were detected (Fig. 1a and b). All four types were especially evident in the prechiasmatic subdivision and to a lesser extent in the retrochiasmatic one, due to the paucity of CR-stained cells in this latter subdivision, and the high predominance of ND-active neurons (Fig. 1b).

As in the rest of the nuclei the number of SON neurons demonstrating coexistence of both markers was low (7.5%) when compared to the numbers of neurons showing either ND- or CR-reactivity (Table 1).

Paraventricular nucleus

As in the SON, all four cellular types were present. CR-immunoreactivity was preferentially located at the level of the periventricular, anterior and medial parvicellular subdivisions (Fig. 1c–e). In contrast, ND-activity was especially located in the magnocellular subdivisions, and within these, in the posterior magnocellular subdivisions (Fig. 1c and d). Few ND-positive cells were observed in the parvicellular subdivisions, especially in the periventricular one, mainly in its anterior part and closely located to the wall of the third ventricle (Fig. 1e).

With the exception of the dorsal parvicellular subdivision, all the subdivisions of the PVN studied (magno- and parvicellular) showed very low levels (less than 1% of the stained neurons) of ND and CR colocalization (Table 1). PVN neurons showing coexistence of ND and CR were not found in all sections and in the rest, it was usual to find no-more than one or two neurons displaying both markers. The relatively highest levels of ND and CR colocalization were found in the commissural magnocellular and lateral parvicellular subdivisions (Table 1).

Magnocellular accessory nuclei

In the circular nucleus, although all staining types described were present (Fig. 1f), most neurons were exclusively ND-positive (Table 1). Additionally, this small nucleus showed the major degree of coexistence (12%) in comparison with the other hypothalamic neurosecretory nuclei. In the fornicals nuclei, the ND-positive population was also the type of stained neurons most frequently found (Fig. 1g, Table 1), and only a few cells showed coexistence of ND and CR (Table 1). The distribution patterns of stained neurons in both anterior and posterior fornicals nuclei were similar.

Numerous ND- and CR-stained neurons were present in the area inter SON-PVN, where 4.5% of these neurons showed both ND and CR reactivity (Fig. 1h). ND-positive neurons showed long stained processes, whereas the CR-immunopositive cells were smaller and, at most, only the proximal dendrites were labelled.

DISCUSSION

The distribution pattern of ND observed in this study coincides with the distribution of this enzymatic activity in the hypothalamic magnocellular neurosecretory nuclei described previously (Alonso *et al.*, 1992a,b; Arévalo *et al.*, 1992; Pow, 1992; Sagar and Ferriero, 1987; Sánchez *et al.*, 1993; Vincent, 1986). Previous studies from our group that have considered the number of ND-positive neurons in the hypothalamic neurosecretory nuclei (Alonso *et al.*, 1992a,b; Sánchez *et al.*, 1993) are in close agreement with the present observations. However, those studies were preferentially focused on the magnocellular nuclei and magnocellular subdivisions of the PVN (Alonso *et al.*, 1992a; Sánchez

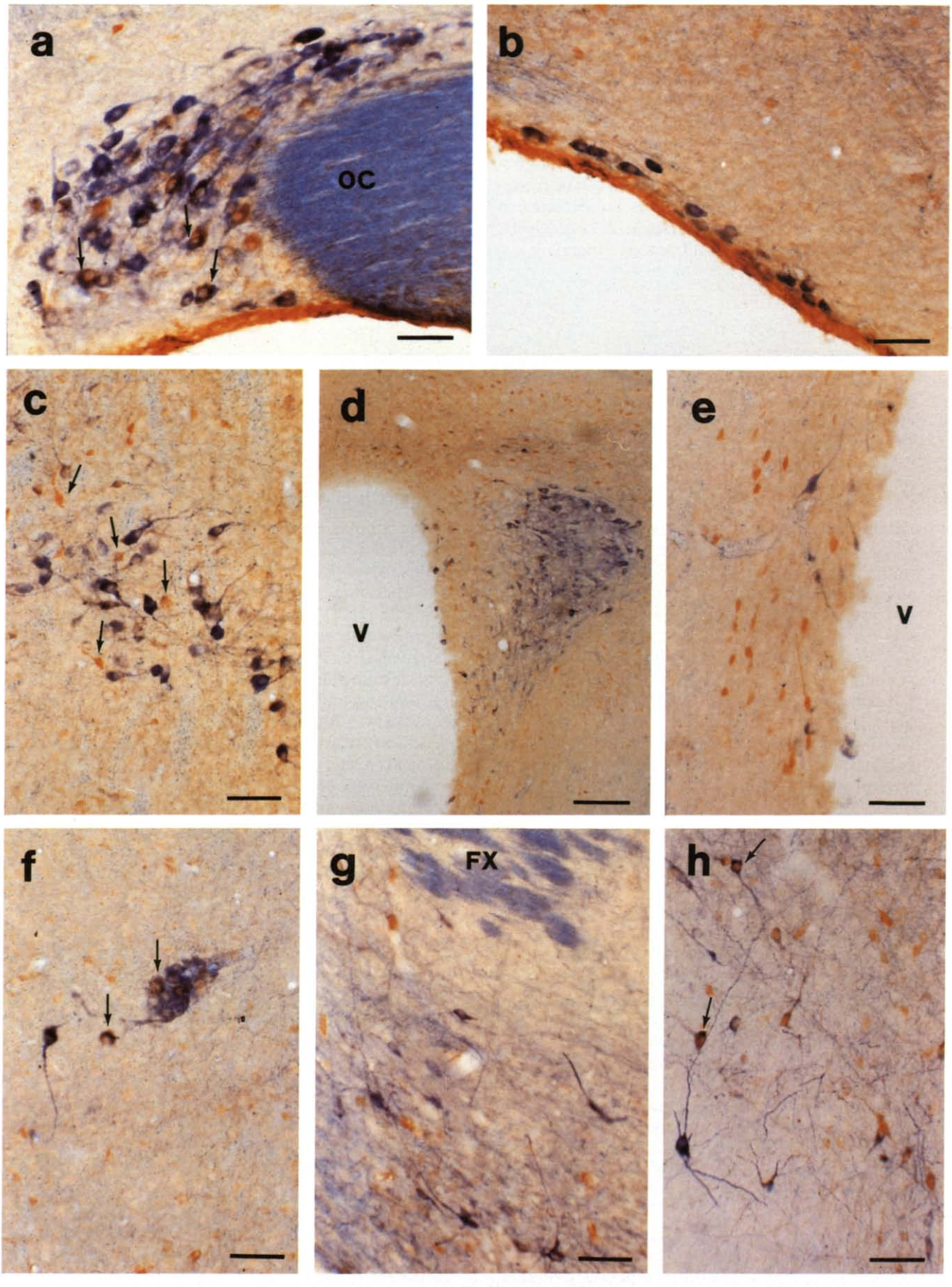
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Table 1. Number (mean ± standard deviation) and percentages of NADPH-diaphorase active (ND), calretinin-immunostained (CR) and double-labelled (coexistence) neurons in the hypothalamic neurosecretory nuclei

Nucleus	ND	CR	Coexistence
SON	470.2 ± 19.8	228.4 ± 14.1	56.6 ± 4.9
	62.3%	30.2%	7.5%
PVN (commissural)	65.4 ± 4.8	18.2 ± 2.6	0.6 ± 0.8
	77.7%	21.6%	0.7%
PVN (posterior)	667.4 ± 51.7	27.2 ± 5.1	0.4 ± 0.5
	96.0%	3.9%	0.1%
PVN (periventricular)	41.4 ± 7.98	112.6 ± 10.9	0.8 ± 0.8
	26.7%	72.8%	0.5%
PVN (anterior)	12.0 ± 3.1	105.2 ± 14.1	0.8 ± 0.4
	10.2%	89.2%	0.6%
PVN (medial)	24.8 ± 8.92	129.1 ± 9.7	0.4 ± 0.54
	16.1%	83.7%	0.2%
PVN (dorsal)	7.1 ± 3.1	6.2 ± 2.4	0
	53.4%	46.6%	0%
PVN (lateral)	17.2 ± 5.9	13.8 ± 3.0	0.2 ± 0.45
	55.1%	44.2%	0.7%
CN	28.8 ± 2.8	15.4 ± 2.0	6.0 ± 1.3
	57.4%	30.6%	12.0%
AFN	23.0 ± 2.8	11.4 ± 1.5	1.6 ± 0.7
	63.9%	31.7%	4.4%
PFN	19.4 ± 2.1	9.6 ± 2.2	2.1 ± 1.2
	62.4%	30.9%	6.7%
Area inter SON-PVN	271.6 ± 10.6	84.2 ± 7.1	16.6 ± 3.5
	72.9%	22.6%	4.5%

AFN, Anterior fornical nucleus; CN, circular nucleus; PFN, posterior fornical nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus.

et al., 1993) or in the periventricular parvicellular subdivision of the PVN (Alonso *et al.*, 1992b). As CR is mostly expressed in the parvicellular subdivisions, the present study adds a quantitative study of all parvicellular subdivisions of the PVN. By considering together all data about the presence and distribution of ND-activity in the hypothalamic neurosecretory nuclei, it is clear that in the PVN, the majority of ND-positive neurons were located at the level of the magnocellular subdivisions, whereas CR-positive cells were preferentially located in the parvicellular ones (especially in the anterior, medial and periventricular subdivisions). In addition, the present data show that even in the nuclei and subdivisions where the distributions of both markers overlap, the degree of ND and CR colocalization at the single cell level was low.

Although ND is widely used as a reliable marker for neuronal nitric oxide synthase, recent evidence indicates that the ND staining pattern can be modified in response to different types of stimuli. Solodkin *et al.* (1992) found a bilateral increase in ND-staining in the lumbar spinal cord after unilateral hindpaw inflammation. Pyramidal neurons of the rat neocortex which normally do not express ND-activity, demonstrated strong ND-reactivity after stab lesions (Kitchener *et al.*, 1993). In the posterior pituitary (Sagar and Ferriero, 1987) and SON (Pow, 1992), the distribution pattern of ND-activity also showed activity-related changes. These changes may be mediated by reciprocal interactions between nitric oxide and intermediate-early genes. In this sense, it has been observed that both nitric oxide synthase-immunoreactive neuronal processes and ND-positive neuronal processes appose Fos-positive neurons in the dorsal horn of the spinal cord (Lee *et al.*, 1993). In addition, N^{ω}-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, produces a dose-related suppression of Fos expression induced by mechanical noxious stimulation (Lee *et al.*, 1992). Therefore, variations in the expression of ND and therefore in the degree of coexistence of ND and CR in the different

Fig. 1. NADPH-diaphorase- (blue) and calretinin-immunolabelled (brown) neurons in the hypothalamic magnocellular nuclei. FX, Fornix; OC, optic chiasma; V, third ventricle (scale bar: a,b,c,e,f,g: 100 μm; d: 250 μm). (a) Prechiasmatic subdivision of the SON, showing the predominance of ND-active neurons mainly located in the dorsal part. Note the presence of several neurons showing coexistence (arrows), and the absence of active-immunoreactive neurons in the ventral part of the subdivision. 100X. (b) Retrochiasmatic subdivision of the SON, showing a predominance of ND-active neurons. 100X (c) In the commissural subdivision of the PVN, most neurons were ND-active. Only a few CR-stained neurons (arrows) were detected. 100X. (d) Panoramic view of the posterior magnocellular and medial and periventricular parvicellular subdivision of the PVN. In the magnocellular subdivisions most neurons were ND-active, whereas in the medial parvicellular subdivision CR-immunoreactive cells predominated. 40X. (e) Periventricular parvicellular subdivision of the PVN. Most stained neurons were CR-immunoreactive. 100X. (f) In the CN, several neurons displayed ND-CR coexistence (arrows). 100X. (g) Predominance of ND-active cells in the anterior fornicals nucleus. 100X. (h) In the hypothalamic area between the SON and the PVN, scattered labelled neurons, some of them showing ND-CR coexistence (arrows) were detected. 100X.

hypothalamic nuclei may be expected after exposing the animals to adequate stimuli.

The distribution of CR-immunoreactive neurons in the hypothalamic magnocellular neurosecretory nuclei has been described previously using immunohistochemistry (Jacobowitz and Winsky, 1991; Résibois and Rogers, 1992; Rogers and Résibois, 1992) or radioimmunoassay (Winsky and Jacobowitz, 1991) techniques. Our data describing the distribution of CR cells in the PVN and SON are in agreement with these studies. No information is available about the distribution of CR in the accessory magnocellular nuclei.

In the magnocellular neurosecretory nuclei, ND has been found to colocalize with CaBP (Alonso *et al.*, 1992a), somatostatin (Alonso *et al.*, 1992b), vasopressin and oxytocin (Sánchez *et al.*, 1993). In other brain areas, the location of ND has been compared with classical neurotransmitters such as acetylcholine and γ-aminobutyric acid (Hedlich *et al.*, 1990; Roberts and Difiglia, 1988; Schober *et al.*, 1989; Vaney and Young, 1988; Vincent, 1986; Vincent *et al.*, 1983b,c) and with several neuropeptides (Roberts and Difiglia, 1988; Sandell *et al.*, 1986; Scott *et al.*, 1987; Sharp *et al.*, 1987; Villalba *et al.*, 1988, 1989; Vincent, 1986; Vincent *et al.*, 1983a). Despite the coexistences described, only partial coexistence have been found, with the exceptions of nitric oxide synthase and citrulline, a side product in nitric oxide synthesis, indicating that they are independent systems.

By comparing the degree of colocalization of ND and CR with that of ND and CaBP (Alonso *et al.*, 1992a), although both colocations occur in all the magnocellular neurosecretory nuclei, the degree of coexistence for ND-CR is clearly lower than that for ND-CaBP. It is coincident with the regional distribution of these markers, since CR is preferentially expressed in the parvicellular subdivisions, whereas CaBP is more abundant in the magnocellular subdivisions, where most ND-active neurons are observed (Alonso *et al.*, 1992a,b; Arévalo *et al.*, 1992; Sanchez *et al.*, 1992, 1993).

The selective topographical distribution and the low degree of coexistence between ND and CR found in the present study suggest that both chemical markers identify independent systems. The existence of different populations of neurons in some hypothalamic nuclei and divisions expressing either CR or ND, but not both, provides a useful tool for studying the variations in the distribution pattern of ND-activity after different experimental conditions.

ACKNOWLEDGEMENTS

This work has been supported by a grant from the University of Salamanca to J. R. A. by the Junta de Castilla y León (SA-55/09/92), 'Acciones Concertadas' (HA-135) and by the PB86/0213 and PB91/0424 DGICYT research projects. The authors want to express their gratitude to Miss E. L. Shorten and Mr G. H. Jenkins for revising the manuscript.

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Accepted 28 July 1993