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CaBP D-28k and NADPH-diaphorase coexistence in the magnocellular neurosecretory nuclei

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Coexistence of the calcium binding protein calbindin D-28k and NADPH-diaphorase activity was studied in the magnocellular secretory nuclei of the rat hypothalamus using both immunocytochemical and histochemical techniques. Coexistence was found in all the nuclei considered (supraoptic, paraventricular, circularis and fornicals nuclei) with the exception of the hypothalamic area situated between the supraoptic and the paraventricular nuclei. Since both stainings are reliable markers, not based upon the physiological characteristics at a given moment, our study provides a further characterization of the neurons in the magnocellular neurosecretory nuclei.

Key words: Calbindin D-28k; NADPH-diaphorase; Coexistence; Magnocellular nuclei; Hypothalamus; Rat

Introduction

Calbindin D-28k (CaBP) is a highly specific intracellular calcium-binding protein located widely throughout the brain, where it is considered to function as a calcium buffer. It may also facilitate diffusion of Ca^{2+}, thus modulating the calcium gradients in neurons.[1] It is currently known that the central nervous system contains high activities of the enzyme nicotinamide adenine dinucleotide phosphate diaphorase, the presence of which can be detected histochemically. The neuronal NADPH-diaphorase (ND) is a nitric oxide synthase and so provides specific histochemical labelling for neurons producing nitric oxide throughout the brain.[2]

We have recently carried out studies on the distribution of CaBP[3] and ND[4] activity in the magnocellular neurosecretory nuclei of the rat hypothalamus. In general, we found that both the CaBP and ND stainings were present in the same nuclei. In view of the fact that both staining patterns were quite similar, in this study we have attempted to correlate both labellings and to determine the degree of coexistence of ND activity and CaBP immunostaining by successive incubations of the same sections.

Material and Methods

Seven adult female Wistar rats (230–280 g) were used. The animals were deeply anaesthetized using ketamine (Ketolar 50 mg kg^{-1}) and perfused through the ascending aorta with 100 ml Ringer solution followed by a fixative containing 4% paraformaldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.25 (PB).

After two hours, the brains were removed, the hypothalamic regions dissected out and postfixed at 4°C for a further two hours in the same fixative. Frontal sections 30 μm thick were cut on a cryostat and collected in cold (4°C) PB. Free-floating sections were processed for the demonstration of ND activity following the protocol described previously.[3,5] Briefly, the sections were incubated in a solution containing 0.08% Triton X-100, 1 mM reduced-β NADPH, 0.8 mM nitroblue tetrazolium in 0.1 M Tris buffer pH 8, at 37°C for 1–3 h. All reagents were obtained from Sigma. The course of the reaction was controlled under the microscope.

When the histochemical reaction was concluded, the sections were rinsed in PB and processed for immunocytochemistry as previously described.[4,6] The sections were incubated in primary antibody (McAB 300 anti-calbindin D-28k) diluted 1:2000 in PB containing 10% normal horse serum and 0.03% Triton X-100 for 48 h at 4°C. Thereafter, the sections were processed according to the avidin-biotin-immunoperoxidase method. Tissue-bound peroxidase was visualized by incubating the sections with 0.07% 3,3′ diaminobenzidine and hydrogen peroxide in 0.1 M Tris buffer (pH 7.6). After incubation, the sections were rinsed in PB, dehydrated, and mounted on gelatin-coated slides for examination by light microscopy.

Controls for the histochemical procedure included incubation without substrate (NADPH) or chromogen (nitroblue tetrazolium). In both cases, no residual reaction was observed. The use of primary antibody against calbindin D-28k has been fully characterized.[7] Controls of the specificity of the immunostaining procedure as described[6] were also carried out. No immunoreaction was observed.

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The number of reacting cells showing CaBP, ND and coexistence was calculated by analysing each nucleus (and the magnocellular subdivisions of the paraventricular nucleus) with the exception of the parvicellular subdivisions of the PV, given the scarcity of reacting neurons in that region (see Results). Calculation was carried out with an Apple digital planimeter connected to an RCA video system. Only cells in which the nucleus was present were considered.

Results

In the present study the nomenclature and nuclear boundaries proposed by other authors,[8,9,10] with small changes we have previously described,[3,4,11] have been used. Although the study is focused on the magnocellular neurons in the paraventricular nucleus, both well-known cellular types (magnocellular and parvicellular) were also considered.

Incubation with antiserum against CaBP and histochemistry for ND displayed four neuronal types: CaBP-positive-ND-negative, CaBP-negative-ND-positive, CaBP-positive-ND-positive and CaBP-negative-ND-negative. The latter was clearly present in the parvicellular part of the paraventricular nucleus, in which only a few neurons were positive.

Supraoptic nucleus (SON): Three neuronal types, without any particular distribution, were clearly present in this nucleus: (1) neurons expressing exclusively ND activity (Figs. 1a and b). (2) neurons expressing exclusively CaBP-immunoreactivity (Figs. 1a and b) and (3) neurons in which it was possible to observe colocalization of both ND and CaBP (Figs. 1a and b). These three neuronal types were especially clear in the prechiasmatic subdivision (as shown in Figs. 1a and b). In the retrochiasmatic subdivision these neuronal types were also present, but due to the paucity of neurons only a few stained cells were detected.

Paraventricular nucleus (PVN): The four aforementioned neuronal populations were present in this nucleus. Taking into account the different subdivisions of this nucleus, in the commissural magnocellular subdivision most of the neurons were ND positive and only some of them presented CaBP reaction or colocalization (Fig. 1c). In the posterior magnocellular subdivision, the results obtained were comparable to those observed in the commissural subdivision (Fig. 1d). With regard to the parvicellular subdivisions, the stained neurons were predominantly found at the periventricular subdivision level. In this zone, the majority of neurons were CaBP-reactive, and a few of them displayed ND activity or colocalization (Fig. 1e). In the remaining parvicellular subdivisions only a few stained cells appeared, the main neuronal type being CaBP-negative-ND-negative.

Table 1. Number (mean ± s.e.m.) and percentages of NADPH-diaphorase active (ND), calbindin D-28k immunostained (CaBP) and double-labelled (Coexistence) neurons in the hypothalamic magnocellular neurosecretory nuclei

Nucleus	Labelling		
	ND	CaBP	Coexistence
Supraoptic	412.12±28.72 29.73%	238.66±9.90 17.22%	735.32±31.31 53.05%
Paraventricular (Commissural)	38.04±2.08 60.32%	11.67±0.88 18.51%	13.35±2.17 21.17%
Paraventricular (Posterior)	539.31±38.19 56.16%	254.43±18.19 26.49%	166.56±21.86 17.35%
Circularis	31.56±2.02 66.69%	6.33±0.82 13.38%	9.43±0.76 19.93%
Fornical anterior	13.40±0.91 41.01%	8.65±0.82 26.52%	10.60±0.29 32.47%
Fornical posterior	15.29±0.78 40.94%	9.35±1.45 25.03%	12.71±1.20 34.03%
Area inter SON-PVN	228.11±14.84 74.76%	77.02±10.97 25.24%	0 0%

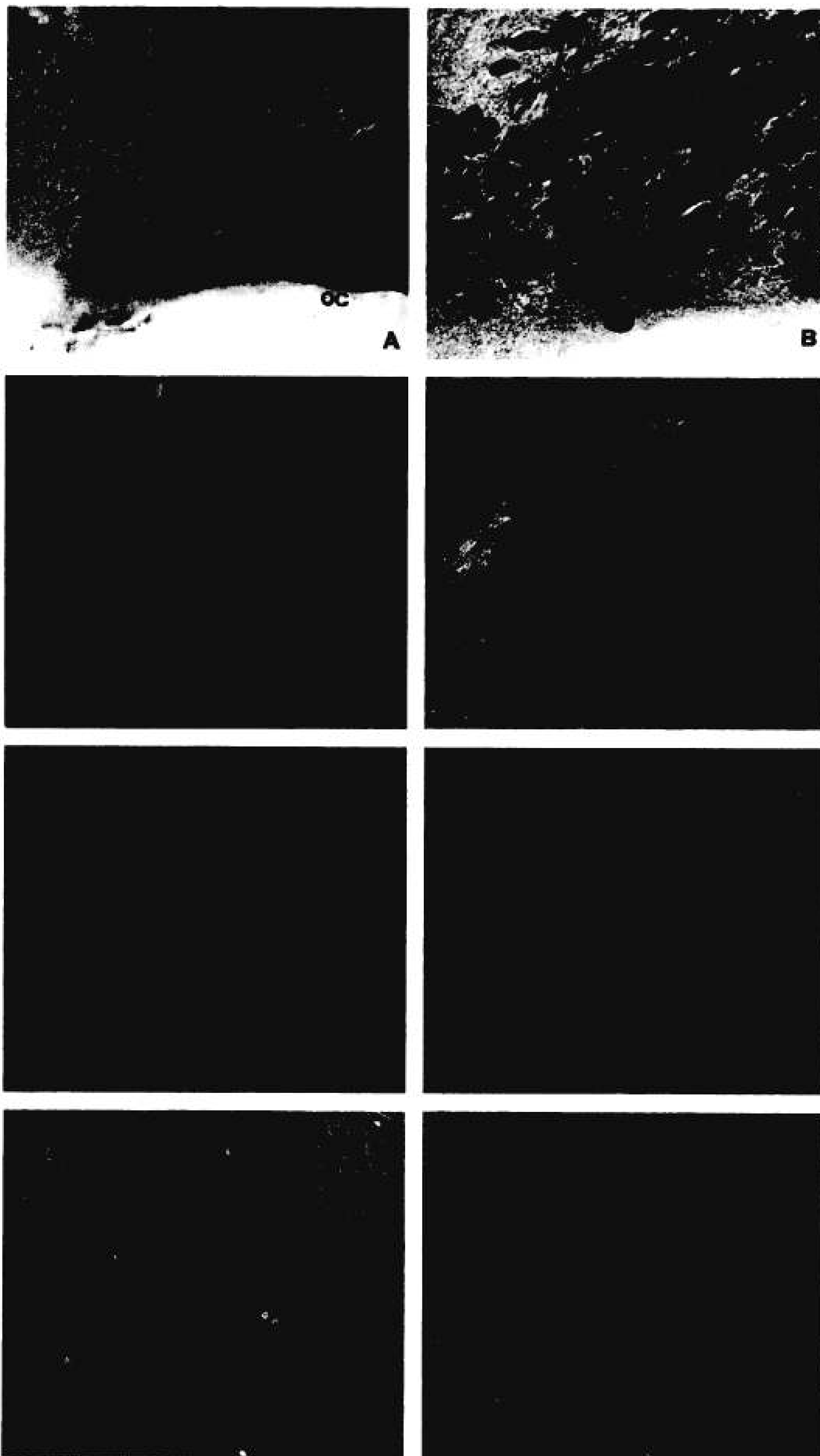
Magnocellular accessory nuclei: In the circularis nucleus the three stained neuronal types were present, with a strong predominance of the neuronal type expressing exclusively ND activity (Fig. 1f). In both fornicals nuclei (anterior and posterior) ND-active, CaBP-stained and double-labelled neurons were found (Fig. 1g). The distribution patterns of stained neurons in both nuclei were very similar.

Finally, numerous stained neurons were located in the hypothalamic area between the SON and the PVN. An important finding was the absence of colocalization in this area. Thus, only ND- or CaBP-stained neurons were present (Fig. 1h), with a clear predominance of ND-active cells. The number and percentages of single- and double-labelled cells are shown in Table 1. In summary, the major degree of coexistence was found to occur in the SON while the circularis and the posterior magnocellular subdivision of the PVN showed a lower number of double-stained neurons.

Discussion

Our results show that the combined use of both labels allows a fairly good delimitation of hypothalamic neuronal populations, providing a more precise definition of them. Both stainings (CaBP and ND) share two characteristics: they are widely expressed by specific neuronal populations throughout the brain and they provide a Golgi-like filling of these neurons, showing not only the neuronal bodies and proximal dendrites, but frequently the complete dendritic arbor-

FIG. 1. **A, B: Supraoptic nucleus.** Note that the three neuronal types found in this study are present in the prechiasmatic subdivision. CaBP-positive neurons, ND-positive neurons, and CaBP-ND-positive neurons. A: × 107 B: × 214. **C to E: Paraventricular nucleus.** In the commissural (C) and posterior (D) magnocellular subdivisions predominate the ND-positive neurons. In the periventricular parvicellular subdivision (E) neurons showing coexistence were present (arrowheads). C–E: × 214. **F to H: Accessory nuclei.** In the circularis (F) and the anterior fornical nuclei (G) the three neuronal types were present. In the hypothalamic area between the paraventricular and the supraoptic nuclei (H) no coexistence was found (small arrows: CaBP-positive; large arrows: ND-positive) F: × 107 G–H: × 214.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A
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ization and long portions of the axon. Thus, they are excellent neuroanatomical tools since they stain neurons in a non-aleatory way, providing, at the same time, some insights into their biochemical nature.

It is well known that CaBP-containing neurons colocalize with VIP and neuropeptide Y in the enteric nervous system[12] or with somatostatin in the dorsal horn of the spinal cord.[13] ND has recently been shown to be a nitric oxide synthase[2] and which is present in neurons that also contain enkephalins, somatostatin, avian pancreatic polypeptide and GABA.[14] However, the functional significance of these colocalizations remains unclear. With respect to ND and CaBP we have demonstrated that, although both labels are present in the same zones, their coexistence is only partial.

After the combined use of both labels we have demonstrated that it is possible to differentiate between four chemically-identified subpopulations in zones whose neuronal populations were previously considered more homogeneous (although different projections and immunoreactivities with specific parcellations have been described). This is important since both markers have been studied in certain pathologies: ND is considered as a specific marker for neurons resistant to ischemia[15] or excitotoxins,[16] and these neurons are selectively spared in certain pathologies such as Huntington's disease[17] and aluminum-induced neurofibrillary degeneration;[18] CaBP is present in significantly lower concentrations in Alzheimer brains.[19] However, CaBP is located in multiple neuronal subpopulations that exhibit a differential vulnerability in Alzheimer's disease.[20] Therefore, the combination of CaBP immunocytochemistry and ND histochemistry provides a further parcellation of these chemically-identified populations throughout the brain.

Conclusion

The selective pattern of neuroanatomical distribution found in our study with CaBP and ND suggest that they are not involved in a general regulatory mechanism, but rather in specific physiological functions shared by restricted neuronal cell populations.

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