Calretinin immunoreactivity in the magnocellular neurosecretory nuclei of the rat hypothalamus

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Summary

The distribution of calretinin-immunoreactivity in the magnocellular neurosecretory nuclei of the rat hypothalamus was studied using a polyclonal antibody and the avidin-biotin immunoperoxidase technique. Calretinin-immunoreactive neurons were observed in the supraoptic and paraventricular nuclei. Additionally, we detected for the first time, immunoreactive neurons located in the circularis and fornicals nuclei, and isolated positive neurons situated in the hypothalamic area located between the supraoptic and paracentricular nuclei. When these results were compared to those obtained in previous studies for another two calcium-binding proteins: calbindin D-28k and parvalbumin, two major differences may be concluded: a) a different distribution of calretinin, especially in the paraventricular nucleus, and b) an expression of calretinin lower than calbindin D-28k and higher than parvalbumin in the magnocellular hypothalamic nuclei. Of special interest is the fact that calretinin is one of the few markers which demonstrates predominantly parvicellular neurons in the paraventricular nucleus. Although the exact biochemical function of these three calcium-binding proteins remains unknown, their uneven and characteristic distributions strongly suggest that specific neuronal populations in the hypothalamus may use alternatively different calcium-sequestering molecules.

Key words: calretinin – calbindin D-28k – parvalbumin – immunocytochemistry – Rat

Introduction

Calretinin (CR) is a 29–31 kDa calcium-binding protein which is mainly, if not exclusively, located in several types of neurons in the central and peripheral nervous systems (Rogers, 1987, 1991; Résubois and Rogers, 1992). It belongs to the “EF-hand” family, a group of proteins which bind calcium with dissociation constants in the micromolar range (Persechini et al., 1989).

The “EF-hand” family includes, among others, calbindin-D28k (CaBP) and parvalbu-
umin (PV), which are also selective markers for particular groups of neurons in the nervous system (Celio, 1990).

Calretinin has a primary aminoacidic sequence highly homologous to that of CaBP (59% between chicken CaBP and chicken CR; Rogers et al., 1990). Indeed, some polyclonal antisera against CaBP cross-react with CR in the mammalian and avian brains (Rogers, 1987; Pochet et al., 1989). However, both calcium-binding proteins have been separately conserved throughout tetrapod evolution as indicated by genetical, biochemical, and immunocytochemical evidences (Rogers, 1991).

In a previous work, we studied the distribution of PV and CaBP (using a monoclonal antibody which does not cross-react with CR) in the hypothalamic magnocellular neurosecretory nuclei of the rat (Sánchez et al., 1992). These distributions were very different since CaBP was expressed by a large number of neurons in most nuclei, whereas PV was only found in a restricted group of neurons in the circularis nucleus.

The information available on the anatomical distribution of CR in the neurosecretory hypothalamic nuclei is very limited since only a general study on the presence of this protein in the brain (Résoibois and Rogers, 1992; Rogers and Résoibois, 1992), and a radioimmunoassay study (Winsky and Jacobowitz, 1991) provide data on its presence in this zone. However, different areas such as several accessory magnocellular nuclei or the different subdivisions of the paraventricular nucleus have not even been considered.

Thus, the aim of this work is to analyze in detail the presence of CR in the magnocellular hypothalamic neurosecretory nuclei of the rat and to compare these results with previous data on the distribution of other calcium-binding proteins (CaBP and PV).

Material and Methods

Animals and tissue preparation. Six adult female Wistar rats (210–240 body weight) were used. After deep anaesthesia with ketamine (Ketolar 50 mg/kg body weight), the animals were perfused through the ascending aorta with 100 ml saline followed by 400 ml of a fixative mixture containing 4 % paraformaldehyde and 15 % saturated picric acid in 0.1 M phosphate buffer, pH 7.3 (PB).

After two hours, the brains were removed, a block containing the hypothalamic region was dissected out, and postfixed for two additional hours in the same fixative. These blocks were washed several times in PB and 30 µm thick sections were cut on a cryostat in a frontal plane. The sections were collected in cold (4°C) PB and then immersed in 30 % sucrose (v/v) for cryoprotection.

Immunohistochemistry. Calretinin immunoreactivity was detected in the free-floating sections with a rabbit antiserum against chick CR and the avidin-biotin-immunoperoxidase method. The procedure was as follows: 1) Preincubation with 5 % normal goat serum in PB for 1 h at 4°C; 2) Anti-CR serum (1:2000 in PB with 0.5 % Triton X-100 and 5 % normal goat serum) for 48 hours at 4°C; 3) Biotinylated goat anti-rabbit immunoglobulin G (Vector Labs., 1:200 in PB) for 2 h at room temperature; and 4) Avidin-peroxidase complex (Vector Labs, 1:225 in PB) for 90 min at room temperature. Tissue-bound peroxidase was revealed with 0.07 % 3,3' diaminobenzidine and 0.003 % H2O2 in 0.1 M Tris-HCl buffer (pH 7.6) under visual control.

The primary antibody used has been fully characterized (Rogers, 1987, 1989) and previously used in different regions of the brain (Résoibois and Rogers, 1992). In addition, the specificity of immunostaining was controlled by omitting the antibody (first or second) in each incubation step. No residual immunoreactivity was observed. The exact location of the immunostained neurons was determined by staining one out of four sections with cresyl violet and by phase contrast microscopy.

Results

For the present study, the nomenclature and nuclear boundaries of the magnocellular neurosecretory nuclei proposed by Peterson (1966) and Rhodes et al. (1981) have been followed. Additionally, for the paraventricular nucleus, the subdivision described by Swanson
and Kuypers (1980) has been used. However, according to Peterson (1966) and Rhodes et al. (1981), as in previous papers (Sánchez et al., 1992, 1993) the anterior and medial magnocellular subdivisions have been considered as a whole called commissural. Since this nucleus is composed of not only magnocellular neurons but also of parvicellular ones, both well-known cellular types were considered. Thus, the following nuclei have been considered in our present study: Supraoptic, paraventricular, circularis and fornicals (anterior and posterior). Additionally, the hypothalamic area situated between the paraventricular and supraoptic nuclei has also been considered.

Incubation with antiserum against CR showed positive neurons in all the nuclei (and all subdivisions of the paraventricular nucleus) considered, including the hypothalamic area situated between the supraoptic and paraventricular nuclei.

Neuronal Typology: In all aforementioned nuclei, the distribution of the immunoreactive neurons was homogeneous; however, neurons with differences in their staining and morphology were detected. CR-immunoreactivity was always prominent in the cell bodies, and in some neurons in the dendritic and axonal processes.

Neurons were mostly round or oval shaped; however, especially at the level of the parvicellular periventricular subdivision of the Paraventricular nucleus, pyramidal shaped neurons with two long well-stained prolongations were detected. In general, they were highly variable in size, but the majority were small and their processes (if visible) could be followed for very short distances.

Location: Supraoptic nucleus: Most CR-positive neurons were located at the level of the prechiasmatic subdivision (Fig. 1a), whereas in the retrochiasmatic one, only few isolated stained cells were observed (Fig. 1b).

In the prechiasmatic subdivision, the neurons were located occupying all the rostrocaudal extension. They were predominantly situated in the dorsal part, forming dense clusters of strongly-stained neurons (Fig. 1a). By contrast, in the ventral part, only a few weakly-reactive neurons were seen (Fig. 1a). Most cells located in the dorsal part were pyramidal shaped with clear prolongations normally oriented perpendicularly to the chiasma. The presence of stained fibres coursing parallelly to the chiasma was also noted (Fig. 1a).

In the retrochiasmatic subdivision, only isolated piriform neurons with one weakly-stained dendrite were observed (Fig. 1b).

Paraventricular nucleus: Both types of neurons in this nucleus (magnocellular and parvicellular) displayed CR-immunoreactivity. Most positive neurons were located in the parvicellular subdivisions, especially in the periventricular, anterior and medial parvicellular subdivisions (Fig. 1c−e). In the dorsal and lateral parvicellular subdivisions, only some isolated CR-positive neurons were found. In all parvicellular subdivisions, the neurons had round somata with small CR-immunostained prolongations (Fig. 1d), with the exception of those cells located in the periventricular subdivision where it was possible to observe weakly-stained processes running parallelly to the third ventricle wall (Fig. 1e).

In both magnocellular subdivisions, only some scattered immunoreactive neurons were found.

Circularis nucleus: A few stained neurons were detected (Fig. 1g). As in the supraoptic nuclei, neurons with different staining intensities were seen. However, no specific distribution of these neurons within the nucleus was observed. The cells showed morphological characteristics of magnocellular neurons: oval or pyramidal shapes and one or two stained processes.

Fornicals nuclei: In both anterior and posterior fornicals nuclei CR-immunoreactive neurons were seen. They surrounded the fornix, especially in its ventral part (Fig. 1f).

Area between supraoptic and paraventricular nuclei: Apart from the nuclei considered, it was possible to observe numerous CR-immunoreactive neurons located in the hypothalamic
Fig. 1. Calretinin immunoreactive neurons in the magnocellular neurosecretory nuclei. (Fx Fornix, OC Optic chiasma, V 3rd ventricle)

a) Prechiasmatic subdivision of the SON. Note the presence in the dorsal part of a group of strongly-stained neurons (arrows), whereas in the ventral part only slightly stained neurons can be detected (arrowheads). x100.

b) Retrochiasmatic subdivision of the SON. Only isolated immunoreactive neurons (arrow) located close to the pial limit are observed. x100.

c–e) Paraventricular nucleus.

c: Panoramic view showing the preferential location of the immunoreactive neurons at the level of the periventricular parvicellular subdivision. x40.

d: In the anterior parvicellular subdivision, a large number of well-stained round neurons is detected. x100.

e: Close to the 3rd ventricle, in the periventricular parvicellular subdivision, several neurons showing well-stained processes are observed. x200.

f) Anterior fornical nuclei. Several well-stained neurons especially located in the ventral part of the fornix are observed. x100.

g) Small cluster of immunoreactive neurons in the circularis nucleus. x100.

h) In the hypothalamic area located between the supraoptic and paraventricular nuclei numerous CR-immunoreactive neurons with different morphologies and immunoreaction intensities are present. x100.
area between the supraoptic and the paraventricular nuclei. They formed a scattered population of neurons with different morphologies (round, oval, and piriform) and reaction intensities, with or without stained processes (Fig. 1h).
Discussion

The main aim of this study has been to identify the types of neurons that express CR immunocytochemically in the magnocellular neurosecretory nuclei of the rat hypothalamus and to compare this pattern of distribution with those of other calcium-binding proteins especially CaBP and PV, whose distribution has been studied in detail previously (Sánchez et al., 1992).

Several major differences can be observed: Firstly, it can be noted that the pattern of distribution of the neurons expressing immunocytochemically CR in the hypothalamus was rather different to those of the two other calcium-binding proteins mentioned (Sánchez et al., 1992). Of special interest is the observed distribution in the paraventricular nucleus, where CR-immunoreactive neurons were preferentially located in the parvicellular subdivisions, whereas CaBP-immunoreactive cells were mainly located at the level of the magnocellular subdivisions. In this nucleus, no PV-immunoreactive neurons have been detected. In the prechiasmatic subdivision of the supraoptic nucleus, the distribution of CR-immunoreactive neurons was more restricted (mainly in the dorsal part) than the wider distribution found for CaBP (all parts). As in the paraventricular nucleus, no PV-reacting neurons were detected in this nucleus.

Secondly, although all nuclei contain CR-immunoreactive neurons, the number of CR-stained cells seems to be clearly lower than the neurons expressing CaBP. However, when compared with PV, the number of cells expressing CR is clearly higher due to the low expression of PV by magnocellular hypothalamic neurons (only detected in the circularis nucleus; Sánchez et al., 1992).

Thirdly, the morphological characteristics of CR-neurons are quite different from the CaBP-immunoreactive ones. As we pointed out in the results, with the exception of the parvicellular periventricular subdivision of the paraventricular nucleus, most CR-positive neurons were round or oval shaped, and they did not show stained prolongations, whereas CaBP-immunoreactive neurons, by contrast, normally had pyramidal or polygonal shapes and long well-stained prolongations (Sánchez et al., 1992).

Finally, with regard to the size of the CR-immunoreactive neurons the observed variability of these neurons agreed with previous data (Rézibois and Rogers, 1992).

Our results agree with previous reports indicating that CR and CaBP are, in general, expressed by different neuronal populations in the avian (Rogers, 1987) and mammalian (Pochet et al., 1989; Rézibois et al., 1990) brains. In agreement with the above mentioned differences, the distributions of CR and CaBP showed in general topographical terms, a low degree of overlapping indicating a possible co-expression of both proteins in a low number of hypothalamic neurosecretory neurons. Different authors (Pochet et al., 1989; Rogers et al., 1990; Rogers, 1991) have described the hypothalamic nuclei as one of the scarce brain regions showing CR-CaBP double-stained cells; however, the double-labelled cells in the magnocellular neurosecretory nuclei were mainly restricted to the supraoptic and paraventricular nuclei, and they were less frequent than the single-stained cells (Rogers and Rézibois, 1992). Given the restricted distribution of PV in the hypothalamus (Sánchez et al., 1992), the only zone where a coexistence between PV and CR could be expected is the circularis nucleus.

With regard to the nuclear distribution of CR-positive neurons, previous studies have described the presence of this type of neurons in the magnocellular neurosecretory nuclei. In fact, Rézibois and Rogers (1992) in a general study of the distribution of CR in the rat brain have described positive neurons in the supraoptic and periventricular nuclei. These findings are in agreement with our results since the periventricular nucleus, according to Swanson and Kuypers (1980) has been included as a part of the paraventricular nucleus (parvicellular periventricular subdivision). By contrast, we have found several CR-positive neurons located in the rest of the paraventricular nucleus subdivisions, not described by these authors, even though they described immunoreactive fibres in the same subdivisions.
In agreement with our immunocytochemical results is the paper by Winsky and Jacobowitz (1991) in which the presence of CR in the supraoptic and periventricular nuclei was biochemically detected by radioimmunoassay. However, this technique does not allow the characterization of the positive elements nor it is useful in the small nuclei (i.e. circularis and fornicals) included in our work.

Additionally, we have found CR-immunoreactive neurons in several accessory nuclei such as circularis and fornicals where they have not been previously described.

It still remains unknown why the distribution pattern of calcium-binding proteins is so complex (a typical mammal would contain, only from the “EF-hand” family, at least eleven subfamilies and multiple isotypes from within each subfamily (Kretsinger et al., 1991)); and a single neuron can colocalize at least three of these “EF-hand” calcium-binding proteins, e.g. calmodulin, CaBP and CR). On the other hand, the search for a common functional denominator for all these neurons expressing one of these neuron-type-specific calcium-binding proteins has been ineffective: the positive cells include a wide range of electrophysiological (Rogers, 1991) and neurochemical (Alonso et al., 1992) types.

Although such general function for CR in all neurons where it is expressed remains unclear, in our study the possibility of synaptic interactions between CR and CaBP-immunoreactive neurons is evident. In addition, there is a segregated distribution with a wide distribution of CR in the parvicellular subdivisions of the paraventricular nucleus (anterior, medial, and periventricular), preferentially involved in the control of the adenohypophysis; whereas CaBP-positive neurons are especially located at the level of the magnocellular subdivisions (Sánchez et al., 1992) whose neurons are mainly implicated in the control of the posterior lobe.

Due to the well-known refractoriety of the parvicellular system to be immunocytochemically shown with several antibodies (i.e. vasopressin) without experimental manipulations such as previous administration of colchicine, or adrenalectomy, the staining with CR of the aforementioned parvicellular subdivisions of the paraventricular nucleus is interesting. Thus, the use of CR antibody may be a useful tool to visualize without experimental pretreatment an abundant population of parvicellular neurons, especially at the level of the periventricular, anterior, and medial subdivisions.

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