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IMMUNOCYTOCHEMICAL STUDY OF PARVALBUMIN FIBERS AND CELL BODIES IN THE RAT HIPOTHALAMUS

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INTRODUCTION

In previous studies it has been suggested that calcium-binding proteins might play important roles in the CNS such as synaptic transmission and axoplasmic transport (7, 12). The first calcium-binding protein to be crystallized after its isolation from frog and carp muscle (10), parvalbumin (PA), has been localized not only in nervous tissue but also in non neuronal organs such as the spleen, kidney and ovary (2). In the mammalian CNS, PA has been detected by using biochemical and immunocytochemical techniques showing a broad distribution (4, 6, 8, 13). Thus, PA has been localized in the cerebellum, cerebral cortex, olfactory bulb, hippocampus, and thalamus (6, 8). Hitherto, scanty data are available on the distribution of PA in the hypothalamus (6). This last report describes clusters of PA immunoreactive neurons in the lateral preoptic area and in the premamillary nuclei (6). In addition, no data exist on the localization of PA fibers in this diencephalic region. This unique previous report on PA distribution in the rat hypothalamus was carried out by using a PA polyclonal antibody (6). The specificity of polyclonal antibodies has been a subject of controversy, due to the difficulties involved in purifying completely a neuroactive substance. Monoclonal antibody techniques provide, however, a means to obtain antibodies without a rigorously purified and characterized antigen, displaying a high specificity. In order to localize PA immunoreactive structures a monoclonal antibody against PA was used in the present study.

The aim of the present report was to re-examine the distribution of fibers and cell bodies containing PA in the rat hypothalamus by using a monoclonal antibody and to compare the findings with previous reports on the distribution of other calcium-binding proteins.

MATERIAL AND METHODS

Five adult rats were used in the present study. The animals were deeply anaesthetized with ketamine (50 mg/kg body weight) and perfused via the ascending aorta with 100 ml of 0.9% NaCl followed by a fixative containing 4% paraformaldehyde, 0.08% glutaraldehyde and 15% saturated picric acid in 0.1 M. phosphate buffer (pH 7.2). After removing the brain, the hypothalamus region was dissected out and stored for 2 h in glutaraldehyde-

free fixative. Then, 60 μm sections were cut with a vibratome and processed for immunostaining.

The sections were incubated with the monoclonal anti-PA antibody (Mc AB 235) and diluted 1:5000 in 0.1 M phosphate buffer for 48 h at 4 C. The free-floating slices were then washed in phosphate buffer and incubated with biotinylated antimouse immunogammaglobulin diluted 1:250 for 3 h at 20 C and Vectastain ABC reagent (1:250) for 2 h. Finally, the tissue-bound peroxidase was revealed using 3, 3' diaminobenzidine as the chromogen. The sections were dehydrate and mounted with Araldite between two plastic foils.

The monoclonal antibody used has been fully characterized (5). In addition, the results were controlled by omitting the PA antibody in the first incubation bath. In order to avoid a possible interference by endogenous peroxidases, these were ruled out by staining some sections beginning with the diaminobenzidine step. In both cases no reaction was visualized. Finally, the mapping was carried out according to the stereotaxic atlas of Albe-Fessard *et al.* (1).

Abbreviations

ahl : Area hypothalamica lateralis,
ahp : Area hypothalamica posterior,
ar : Nucleus arcuatus,
dm : Nucleus dorsalis hypothalami,
Fx : Fornix
mam L : Corpus mamillare lateralis,
mam M : Corpus mamillare medialis,
Ped : Pedunculus cerebri,
Pv : Nucleus paraventricularis hypothalami,
tmt : Tractus mamillo-thalamicus,
to : Tractus opticus,
vm : Nucleus ventromedialis hypothalami,
ZI : Zona incerta.

RESULTS

The distribution of both PA fibers and cell bodies in the rat hypothalamus is shown in Figure 1. We describe our observations following a caudo-rostral cartography of the hypothalamic region. In posterior regions, at A 4.6 (Fig. 1A), PA cell bodies were grouped in midline and lateral nuclei. In this respect, a large number of immunoreactive perikarya was found in the corpus mamillare medialis, whereas a scarce number was visualized in the corpus mamillare lateralis. Moreover, a high density of immunoreactive fibers were observed in the corpus mamillare lateralis, in the tractus mamillo-thalamicus and in a small region placed laterally to this tractus, whereas in the corpus mamillare medialis and above the corpus mamillare lateralis a middle density of PA fibers was found. In addition, a low number of PA processes was observed between the tractus mamillo-thalamicus and the pedunculus cerebri. Finally, no immunoreactivity was found in the area hypothalamica posterior.

More rostrally, at A 4.8 (Fig. 1B), two well defined regions were observed showing PA immunoreactivity. The first was the nucleus arcuatus placed in the

midline, while the second PA-positive region extended ventro-laterally from the ventricle to the most ventral region of the pedunculus cerebri. In the nucleus arcuatus a high density of both PA fibers and cell bodies was observed, whereas in the region between the ventricle and the tractus mamillo-thalamicus a low number of PA fibers and perikarya was found. The tractus mamillo-thalamicus showed a high density of PA fibers. The region extending from the tractus mamillo-thalamicus to the ventral region of the pedunculus cerebri showed a middle density of PA fibers. In addition, a cluster of PA perikarya showing a high density was visualized below the tractus mamillo-thalamicus. This density was lower (middle density) in more ventro-lateral zones.

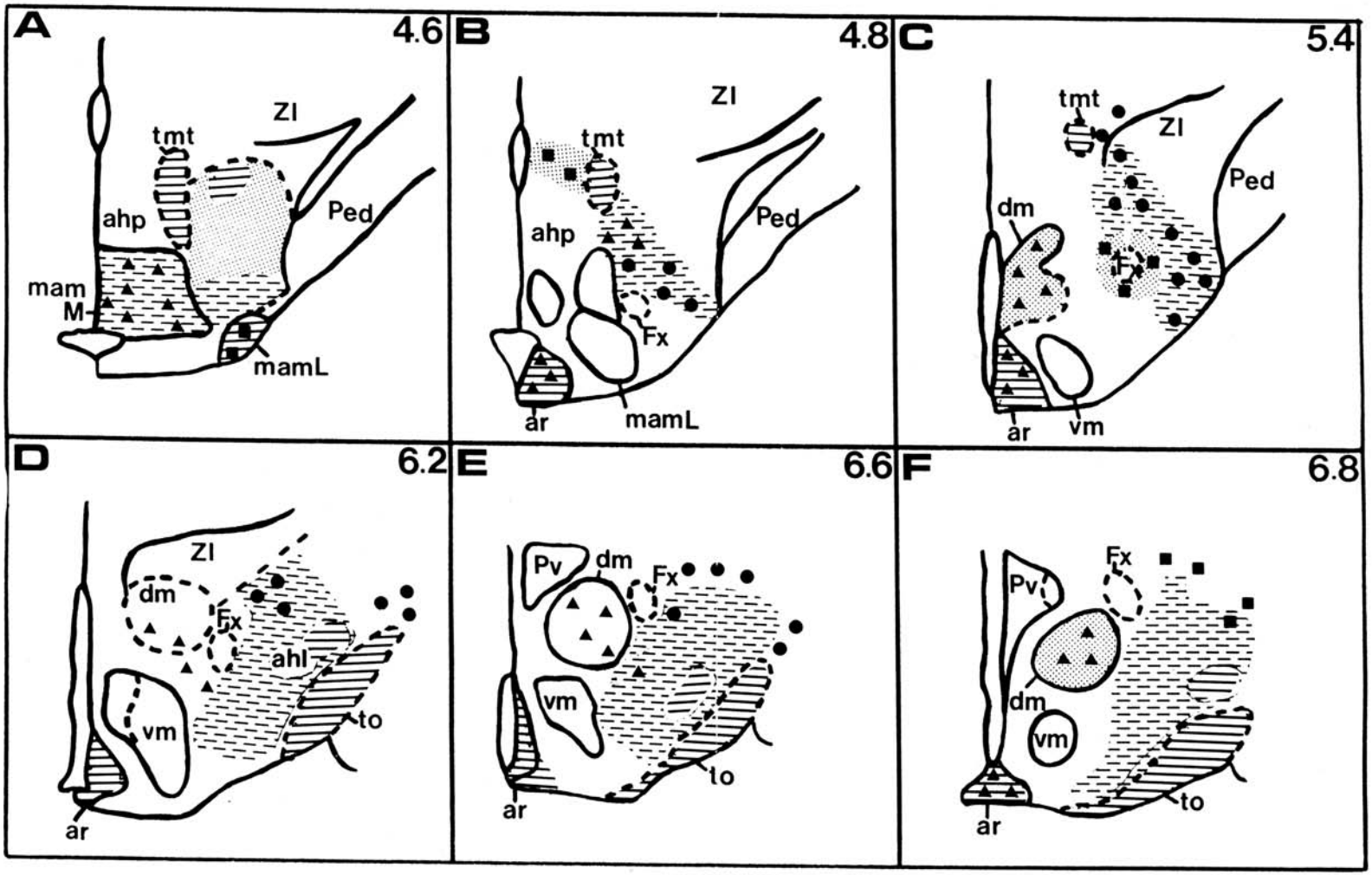
More rostrally, at A 5.4 (Fig. 1C), PA immunoreactive structures were observed in two nuclei placed in the midline region, the nucleus arcuatus and the nucleus dorsomedialis hypothalami. In both nuclei a large number of immunoreactive cell bodies was visualized. Fibers containing PA were scarce in the nucleus dorsomedialis hypothalami but quite numerous in the nucleus arcuatus. In comparison with the previous anterior level (A 4.8), the immunoreactivity found between the midline and the tractus mamillo-thalamicus disappeared at A 5.4. However, the immunoreactivity extending from the tractus mamillo-thalamicus to the ventral region of the pedunculus cerebri remained. In this region a middle density of PA fibers and cell bodies was observed, except around the fornix, where few cell bodies and fibers were visualized. Finally, a rich network of PA fibers was found in the tractus mamillo-thalamicus.

The most rostral levels (A 6.2-6.8) (Fig. 1D-F) showed a similar distribution of PA immunoreactivity. In the midline region the only nucleus labeled was the nucleus arcuatus. In the caudal zone of this segment (A 6.2-6.6) (Fig. 1D-E) a high density of PA fibers was found, whereas more rostrally, at A 6.8 (Fig. 1F), a high number of both perikarya and fibers containing PA was localized. Laterally, the region placed between the tractus opticus, the fornix and the nucleus dorsomedialis hypothalami showed a middle density of PA fibers. In addition, islets located near the tractus opticus with a high number of PA processes were also observed (Fig. 1D-F). Clusters of immunoreactive perikarya from middle (A 6.2-6.6) (Fig. 1D-E) to low (A 6.8) (Fig. 1F) densities were observed dorsolaterally to the fornix or dorsally to the tractus opticus. In the nucleus dorsomedialis hypothalami a high number of PA cell bodies was observed ventrally (A 6.2) (Fig. 1D) or throughout the nucleus (A 6.6-6.8) (Fig. 1E-F). At A 6.2 and A 6.6 (Fig. 1D-E) the cluster of PA perikarya extended ventrally. In addition, scarce PA fibers were found in the nucleus dorsomedialis hypothalami at rostral level (A 6.8) (Fig. 1F). Moreover the whole tractus opticus showed a high PA immunoreactivity (Fig. 1D-F).

More rostrally, (from A 7.0 to A 7.9) (not shown) a moderate cluster of PA immunoreactive cell bodies was found in the area preoptica lateralis. Finally, no PA immunoreactivity was visualized in the area preoptica medialis, area hypothalamica anterior, area hypothalamica posterior and nuclei supraopticus, suprachiasmaticus and paraventricularis hypothalami.

Fig. 1. – *Summary of the distribution and relative density of PA fibers and cell bodies in the rat thalamus.*

PA cell bodies are indicated by closed triangles, circles and squares, their shape being related to the density of cell bodies (▲ high density: > 10 cell bodies; ● middle density: 5-10 cell bodies; ■ low density: 1-5 cell bodies). PA fibers are represented as follows: ≡ high density; ≡ middle density; ⁝⁝ low density. The frontal (anterior) level of each section is indicated in the upper right corner.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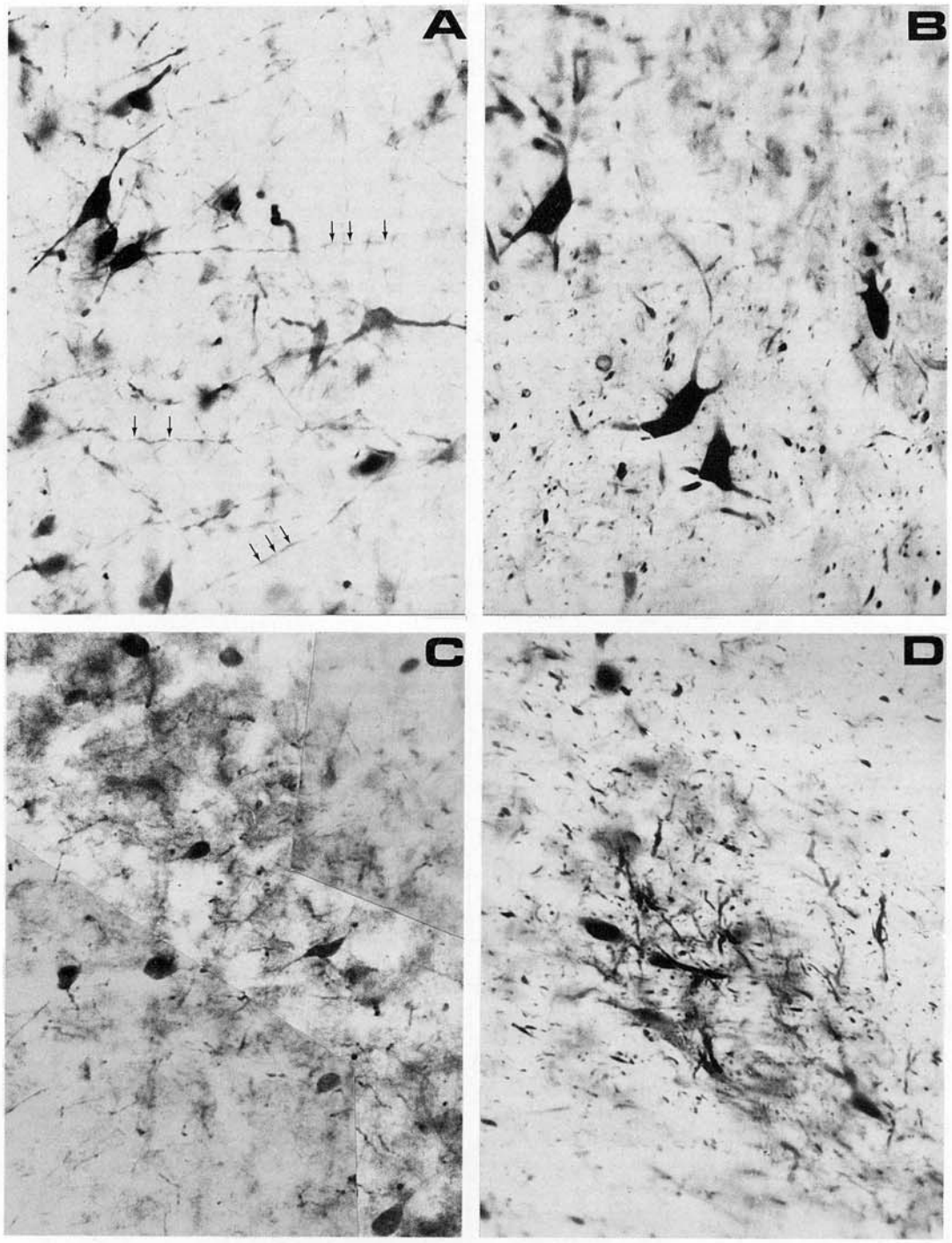

Fig. 2. – *PA immunoreactive cell bodies and fibers in the hypothalamus of the rat.*

A. Cluster of immunoreactive neurons in the nucleus dorsomedialis hypothalami. Many long PA processes are also observed (arrows) (×200).
B. Immunoreactive cell bodies and fibers placed dorsally to the fornix (×200).
C. Group of immunoreactive perikarya in the nucleus arcuatus (×200).
D. PA cell bodies and fibers placed dorsally to the tractus opticus (×200).

Clusters of PA immunoreactive cell bodies and fibers in different hypothalamic areas are shown in Fig. 2.

DISCUSSION

In comparison with previous studies on the localization of PA in the mammalian CNS (4, 6, 8), it seems that the distribution of this protein is wider in our study; in the previous work, in fact, clusters of PA perikarya were reported only in the lateral preoptic area and the premamillary nuclei (6). These observations are partially in agreement with our findings since we found PA cell bodies only in the former region. In the present report, in which a PA monoclonal antiserum was used, both cell bodies and fibers containing PA were observed for the first time in the following nuclei of the rat hypothalamus: arcuatus, dorsomedialis hypothalami and area hypothalamica lateralis. In addition, immunoreactive PA fibers were observed in nuclei such as the corpus mamillare medialis and corpus mamillare lateralis as well as the tractus mamillo-thalamicus and tractus opticus, in which such immunoreactivity was never reported previously. This discrepancy with previous findings could be due to the specificity of the antisera, since Celio and Heizmann (6) used polyclonal antisera. Alternatively, it could be attributed to differences in the fixative used or in the embedding procedure, since in the latter study (6) the rat brains were fixed with Bouin fluid and embedded in paraffin.

The immunohistochemical localization of PA in the rat hypothalamus described in the present study differs from that of another calcium-binding protein in the rat CNS, the vitamin D-dependent calcium-binding protein (ViDCaBP) (9), since no PA immunoreactivity was observed in nuclei in which ViDCaBP has been localized, e.g., area hypothalamica anterior, suprachiasmaticus, supraopticus, paraventricularis, area preoptica medialis and area hypothalamica posterior. On the contrary, we found PA immunoreactive structures in the nucleus dorsomedialis hypothalami and the area hypothalamica lateralis, in which no ViDCaBP immunoreactivity was visualized (9). Finally, ViDCaBP was observed in the tractus mamillo-thalamicus and the area preoptica lateralis (9), in which PA immunoreactivity was also observed.

We don't have sufficient information as to whether PA-positive perikarya in the hypothalamus of the rat belong to local or projecting neurons. Most of the PA cell bodies observed in the CNS (6, 8) are small- or medium-sized and Berchtold *et al.* (3) have speculated that these hypothalamic neurons may be identified as interneurons. In the rat hypothalamus, the PA cell bodies found in the nucleus arcuatus could be attributed to interneurons, since in this region both PA fibers and cell bodies are dense. Alternatively, this region may send efferent PA projections and receive PA afferents. However, it can be hypothesized that PA perikarya in the nucleus dorsomedialis hypothalami contribute to long efferent projections, since this nucleus contains numerous PA perikarya but shows eventually a relatively low density of PA fibers. Moreover, the size of the neurons in both nuclei suggests that PA cell bodies could play a role as interneurons or projecting neurons, in

agreement with the suggestion of Berchtold *et al.* (4), since PA cell bodies in the nucleus arcuatus are quite small, whereas those found in the nucleus dorsomedialis hypothalami are larger in size. This latter finding is in agreement with the results by Endo *et al.* (8), who also described such large PA neurons in the superior vestibular nucleus and the medial superior olive.

The selective staining of some hypothalamic neurons by PA antisera indicates that this protein represents a marker of a distinct neuronal population, suggesting that in addition to the general functions of the calcium-binding proteins in synaptic transmission and axoplasmic transport (7, 11, 12), PA might be involved in more specific functions. Thus, the presence of PA in the hypothalamic nuclei arcuatus, dorsomedialis hypothalami, mamillary, area hypothalamica lateralis as well as in the tractus opticus and tractus mamillo-thalamicus suggests that PA could be directly or indirectly involved in neuroendocrine, limbic and visual functions. Although it is not possible, so far, to correlate the presence of PA immunoreactive neurons with a definitive function, a systematic mapping of PA positive neurons in the CNS is necessary to search for a correlation with known functional mechanisms.

SUMMARY

The distribution of parvalbumin (PA) cell bodies and fibers in the hypothalamus of the rat was studied using a monoclonal antibody and the avidin-biotin-peroxidase method. The densest clusters of immunoreactive perikarya were observed in the nuclei mamillare medialis, arcuatus and dorsomedialis hypothalami, whereas the corpus mamillare lateralis had the lowest density. The densest network of immunoreactive fibers was observed in the corpus mamillare lateralis and nucleus arcuatus. The corpus mamillare medialis contained a moderate number of PA fibers, whereas the nucleus dorsomedialis hypothalami had the lowest density of immunoreactive fibers. In addition, a large number of immunoreactive fibers was found in the tractus opticus and the tractus mamillo-thalamicus. Essentially, the distribution of PA in the rat hypothalamus after using a monoclonal antibody seems to be broader in comparison with previous studies carried out in the same diencephalic region of the rat.

The presence of PA in several nuclei of the rat hypothalamus suggests that this protein could be directly or indirectly involved in neuroendocrine, limbic and visual functions.

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