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DISTRIBUTION OF PARVALBUMIN-IMMUNOREACTIVITY IN THE RAT THALAMUS USING A MONOCLONAL ANTIBODY

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INTRODUCTION

In recent years calcium-binding proteins have received increased attention due to their involvement in the mediation of calcium-dependent events. In the CNS, calcium ions have important effects in synaptic transmission and axonal transport, both mechanism requiring the presence of specific calcium-binding proteins exerting regulatory roles.

In order to localize the distribution of calcium-binding proteins, several immunocytochemical and biochemical studies have been carried out in the mammal CNS (2, 3, 6, 11, 13, 18, 20, 25). Thus, a cartography of a protein, the vitamin D-dependent calcium-binding protein, has been performed in the rat CNS using immunohistochemical techniques (20). This calcium-binding protein has been detected in the rat brain using radioimmunoassay techniques (3). Moreover, calmodulin, another protein also belonging to the family of calcium-binding proteins, has been observed in the rat cerebellum, as well as in the basal ganglia of the mouse brain (26, 29).

Parvalbumin (PA) was the first calcium-binding protein to be crystallized after isolation from frog and carp muscle (21). It has been localized in nervous and non-nervous tissues. Thus, in the latter, PA structures were observed, e.g., in the spleen, kidney and ovary (4, 5). In the mammalian CNS, PA has been detected using biochemical and immunocytochemical techniques (6, 11, 13, 18, 25) showing a broad distribution. In addition, PA has been observed recently in the retina of rat, monkey and human (17). Thus, PA is a calcium-binding protein which is only found in a limited number of vertebrate tissues where it is restricted to a few distinct cell types, suggesting that PA may be involved in more specialized calcium-regulated functions in the CNS.

In the CNS of rodents, PA has been observed in the cerebellum, cerebral cortex, olfactory bulb, hippocampus and hypothalamus (11, 18). In addition, PA immunoreactive structures have been observed in the monkey thalamus (25), whereas in the human brain PA immunoreactivity was found in the hippocampus (6). However, hitherto, scarce data are available on the distribution of PA in the thalamus of the rat, since only the reticular nucleus has been reported to contain PA positive perikarya, whereas PA fibers were observed in the specific thalamic nuclei (11, 18). This latter study was performed using a PA polyclonal antibody.

The specificity of polyclonal antibodies used in immunocytochemical studies has frequently been a subject of controversy because of the difficulties involved in purifying completely a neuroactive substance. Monoclonal antibody techniques, however, provide an useful means to obtain antibodies without a rigorously purified and characterized antigen, displaying a high specificity. In order to exploit this advantage, the aim of the present report was to re-examine the localization of fibers and cell bodies containing PA in the thalamus of the rat using a monoclonal antibody.

MATERIAL AND METHODS

Seven adult male Sprague-Dawley rats (200-250 g body weight) kept under standard laboratory conditions were used in the present study. The animals were deeply anaesthetized with ketamine (50 mg/kg body weight) injected intraperitoneally and perfused intracardially 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 (28). Following this, the brains were removed, the thalamic region dissected out and stored in glutaraldehyde-free fixative for a further two hours. These blocks were washed in several changes of phosphate buffer and cut at 60 μm perpendicularly to the longitudinal axis of the brain using a vibratome (Campden Instruments). The sections were washed for 2 h in phosphate buffer.

Immunocytochemistry was performed using a monoclonal antibody against PA (10) and the avidin-biotin-peroxidase method (23). Free-floating slices were incubated with the anti-PA antibody diluted 1:5000 in 0.1 M phosphate buffer containing 1% normal horse serum for 48 h at 4° C. The sections were then carefully washed in phosphate buffer and incubated with biotinylated antimouse immunogamma globulin (Vectastain ABC Kit, Vector laboratories, Burlingame, U.S.A.) diluted 1:250 for 3 h at 20° C, and Vectastain ABC reagent (1.250) for another two hours. Tissue- bound peroxidase was visualized by incubating the sections with 3,3' diaminobenzidine (0.07%) and H_20_2 in Tris buffer 0.1 M (pH 7.6) for 5-15 min.

The monoclonal antibody 235 against PA has been fully characterized in a recent paper (10), demonstrating its use in the qualitative detection of PA by immunohistochemistry, in the quantitation of PA by radioimmunoassay and in the detection of PA on immunoblots (We used the same antibody kindly provided by Prof. Marco R. Celio). In addition, the specificity of the immunostaining was controlled by omitting the PA antibody in the first incubation bath. In this case, no residual immunoreactivity was found. Moreover, possible interference by endogenous peroxidases was ruled out by staining some sections beginning with the diaminobenzidine step. No reaction was visualized. Finally, vibratome sections of the olfactory bulb, hypothalamic and hippocampal regions of the same rats were cut and processed for immunostaining together with the thalamic sections since the former three regions contain a characteristic pattern of PA immunoreactivity (11 13, 18), and were used as additional controls of the immunostaining procedure. Mapping was carried out according to the stereotaxic atlas of Albe-Fessard *et al.* (1). The same atlas was used for the terminology of the thalamic nuclei.

Abbreviations used in Figure 1.

AD , nucleus anterior dorsalis;
AM , nucleus anterior medialis;

AV , nucleus anterior ventralis;
CL , nucleus centralis lateralis;
CM , nucleus centrum medianum;
GLD , nucleus geniculatum laterale dorsalis;
GLv , nucleus geniculatum laterale ventralis;
Hbl , nucleus habenularis lateralis;
L , nucleus lateralis;
LA , nucleus lateralis anterior;
lem , lemniscus medialis;
LP , nucleus lateralis posterior;
MD , nucleus medialis dorsalis;
MV , nucleus medialis ventralis;
NCM , nucleus centralis medialis;
Pt , nucleus parataenialis;
PV , nucleus paraventricularis;
Re , nucleus reuniens;
Ret , nucleus reticularis;
Rh , nucleus rhomboidens;
Sm , stria medullaris;
Thp , tractus habenulo-interpenduncularis;
tmT , tractus mamillo-thalamicus;
VA , nucleus ventralis anterior;
VL , nucleus ventralis lateralis;
VM , nucleus ventralis medialis;
VP , nucleus ventralis posterior;
ZI , zona incerta.

RESULTS

Fibers and cell bodies containing PA are widely distributed throughout the thalamus of the rat (Fig. 1). Immunoreactivity was found at almost all thalamic levels, among others, including the following nuclei: habenularis lateralis, geniculatum laterale dorsalis, centrum medianum, geniculatum laterale ventralis, centralis medialis, ventralis posterior, ventralis lateralis, reticularis, anterior ventralis, anterior dorsalis and lateralis posterior (Fig. 1A-D).

PA-immunoreactive fibers were observed in several thalamic nuclei such as the centrum medianum, ventralis medialis, anterior ventralis, ventralis medialis, anterior medialis, rhomboidens, medialis ventralis, anterior dorsalis, lateralis anterior, parataenialis and medialis dorsalis (Fig. 1A-D).

PA-immunoreactive cell bodies were mainly found in nuclei localized laterally in the thalamus such as reticularis, geniculatum laterale ventralis, ventralis posterior, ventralis anterior and ventralis lateralis (Fig. 1A-D), although in more medial nuclei, e.g. habenularis lateralis and centralis lateralis, fewer immonoreactive cell bodies were also found (Fig. 1, A,B).

In the thalamic nuclei in which PA-immunoreactive cell bodies were observed, at the same rostro-caudal level, perikarya were observed both grouped in some particular regions of the nuclei or extending throughout the whole nuclei. Thus,

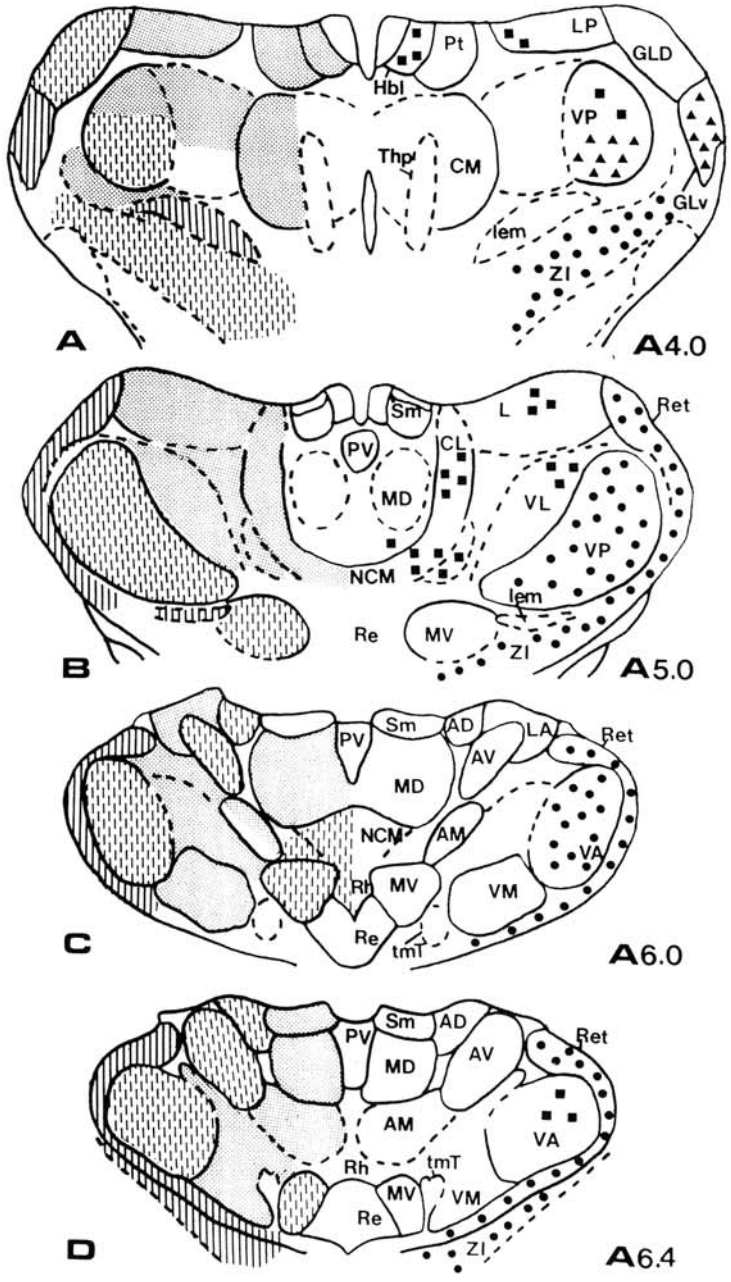


Fig. 1 – *Distribution of PA fibers and cell bodies in the rat thalamus taking into account their relative densities.*

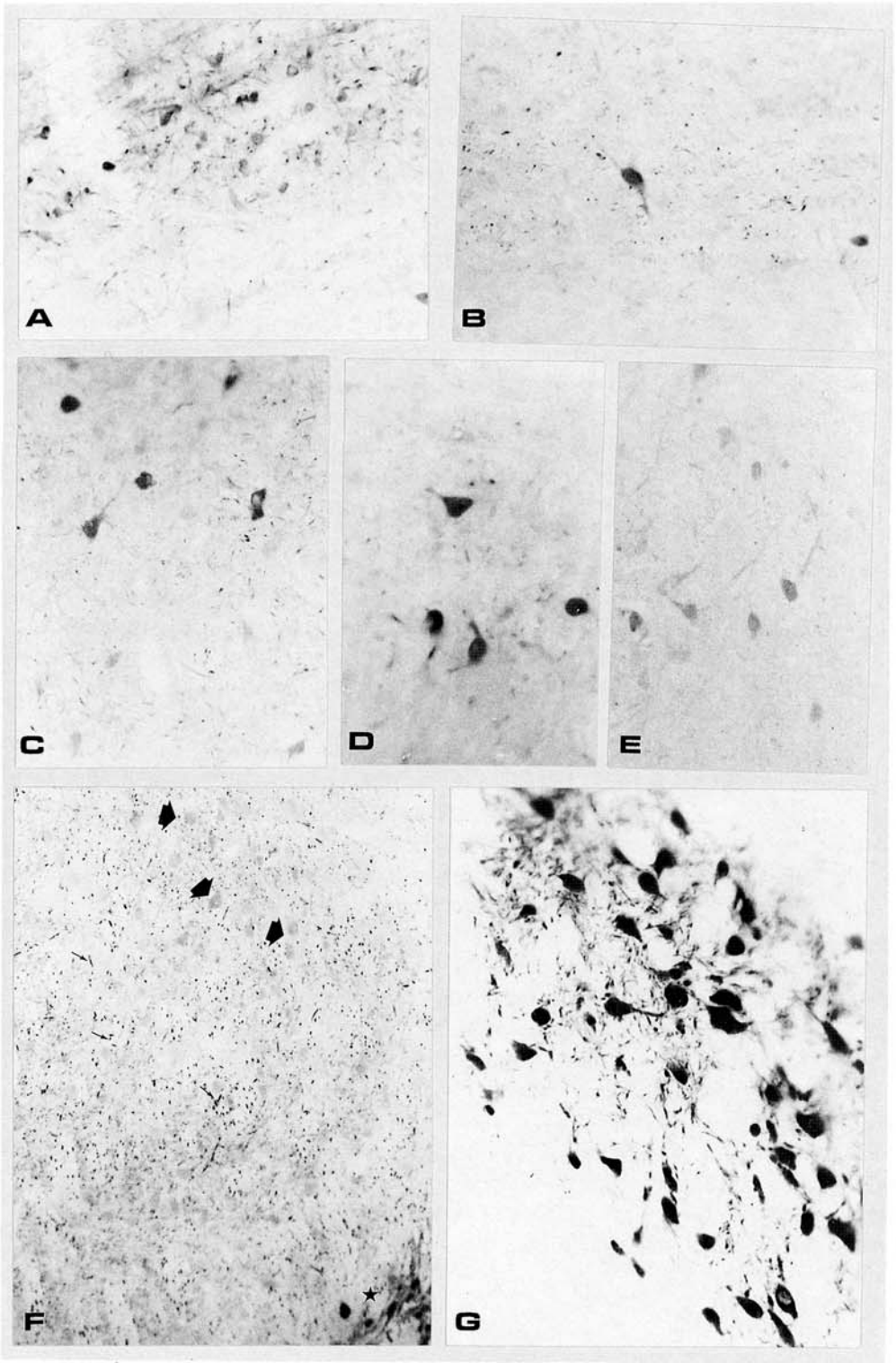
PA cell bodies are indicated by closed circles, triangles and squares (● high density: > 30 cell bodies; ▲ middle density: 10-30 cell bodies; ■ lower density; < 10 cell bodies). PA fibers are represented as follows: ||||| high density; ⁞⁞⁞ middle density; ⋮⋮⋮ low density). The frontal level of each section is indicated in the lower right corner.

the following nuclei displayed PA immunolabeled neurons confined to a restricted area of the nucleus: in the dorsal-most part of the nucleus ventralis lateralis, in the dorso-lateral region of the nucleus ventralis anterior (at A 6.4), in the region nearer to the midline of the nucleus lateralis posterior as well as in the middle region of the nucleus centralis lateralis PA perikarya were observed, whereas cell bodies showing PA-positive immunostaining were localized throughout the whole of the following nuclei: the habenularis lateralis, geniculatum laterale ventralis, ventralis posterior, reticularis, ventralis anterior (at A. 6.0) and in the zona incerta (Fig. 1A-D). In all the thalamic nuclei in which PA perikarya were found, these were small, showing in general a rounded appearance, although fusiform immunoreactive cell bodies were also visualized. PA immunoreactivity was not observed on glial cells. The labeling of the positive neurons in the thalamic region was practically confined to the perikarya and proximal processes, and no Golgi-like immunolabelings were seen as in the case of the hippocampal formation and hypothalamic region of the same sections and in sections of the olfactory bulb.

Finally, in the thalamic nuclei habenularis medialis, paraventricularis, reuniens (Fig. 1A-D) and geniculatum mediale (not shown) no PA-immunoreactive structures were observed.

In posterior thalamic regions (A 4.0) (Fig. 1A), PA cell bodies were grouped mainly in nuclei placed laterally such as geniculatum laterale ventralis (Fig. 2A) and ventralis posterior. In the latter, scarce immunoreactive perikarya were observed on its dorsal region. On the contrary, in the ventral region of the nucleus ventralis posterior and in the nucleus geniculatum laterale ventralis a middle density of PA perikarya was visualized. In addition, a low number of neurons was observed in the nucleus habenularis lateralis and in the medial-most part of the nucleus lateralis posterior (Fig. 2B). Finally, a rich cluster of PA cell bodies was found in the zona incerta. At this level (A 4.0) PA-immunoreactive fibers were widely distributed throughout the thalamus, except in nuclei close to the midline. Thus, the nucleus geniculatum laterale ventralis showed the densest network of PA fibers, whereas the nucleus geniculatum laterale dorsalis and the ventral region of the nucleus ventralis posterior had an intermediate density. The remaining nuclei habenularis lateralis, parataenialis, lateralis posterior, centrum medianum and the dorsal region of the nucleus ventralis posterior showed the lowest density of PA fibers. On the other hand, strongly immunoreactive fibers were observed in the lemniscus medialis, whereas in the zona incerta a middle density was found. Finally, a low number of PA processes was observed between the nuclei centrum medianum and ventralis posterior.

More rostrally, at A 5.0 (Fig. 1B) the pattern of immunoreactive structures is quite similar to that referred to the previous anteriority. Thus, immunoreactive perikarya were observed in lateral nuclei such as the reticularis, ventralis posterior and ventralis lateralis, as well as in the zona incerta. In the nucleus ventralis lateralis (Fig. 2C), scarce PA cell bodies were found. However, in the other three nuclei a high number of immunorective neurons was observed. In addition, a low number of cell bodies was visualized in the nuclei centralis lateralis (Fig. 2D)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and lateralis (Fig. 2E). On the other hand, a rich network of immunoreactive fibers was observed in the nucleus reticularis and lemniscus medialis, whereas the nuclei lateralis, centralis lateralis, centralis medialis and ventralis lateralis had a low density. In other thalamic nuclei such as the medialis ventralis and ventralis posterior the density of PA fibers was intermediate.

At A 6.0 (Fig. 1C), except the nuclei paraventricularis and reuniens, the remaining thalamic nuclei showed PA immunoreactive structures. PA perikarya were only observed laterally in the nuclei reticularis and ventralis anterior (Fig. 2F), whereas a large number of immunoreactive cell bodies was seen in both nuclei. Fibers containing PA were found in the following nuclei: the reticularis (high density); ventralis anterior, medialis ventralis, centralis medialis, anterior ventralis, rhomboidens and anterior dorsalis (middle density); the anterior medialis, ventralis medialis, lateralis anterior and medialis dorsalis showed a low density.

Finally, at rostral level (A 6.4) (Fig. 1D) a low number of PA neurons was localized in the nucleus ventralis anterior, whereas a large number was found in the nucleus reticularis (Fig. 2G) and zona incerta. In the nucleus ventralis anterior, PA immunoreactive processes can be observed on PA cell bodies. Moreover, the densest network of immunoreactive fibers was observed in the nucleus reticularis and zona incerta, whereas in the nuclei anterior dorsalis, anterior ventralis, ventralis anterior and medialis ventralis a moderate density was observed. In the nucleus anterior dorsalis, PA immunoreactive processes were observed surrounding cell bodies not containing PA. The nucleus anterior medialis, nucleus medialis dorsalis, stria medullaris and the region between the nuclei ventralis anterior and medialis ventralis showed the lowest density of PA immunoreactive fibers.

DISCUSSION

In the present study, using a monoclonal antibody, cell bodies and fibers positive for PA have been localized for the first time in some nuclei of the rat thalamus. In this sense, we observed immunoreactive perikarya in nuclei such as the ventralis anterior, habenularis lateralis, lateralis posterior, geniculatum laterale ventralis, ventralis posterior, lateralis, centralis lateralis and ventralis lateralis, hitherto unreported to contain such PA-positive neurons (11, 18). In addition, PA fibers were observed in nuclei such as the anterior dorsalis, centralis medialis, anterior

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

A. Group of immunoreactive perikarya in the nucleus geniculatum laterale ventralis (100x).
B. Two immunoreactive neurons in the nucleus lateralis posterior (200x).
C. Immunoreactive cell bodies in the nucleus ventralis lateralis (200x).
D. Cluster of immunoreactive neurons in the nucleus centralis ventralis (200x).
E. PA cell bodies showing a weak immunoreactivity in the nucleus lateralis (200x).
F. PA immunoreactive cell bodies in the nuclei ventralis anterior and reticularis (star). Note that in the first nucleus the neurons show a weak immunoreactivity (arrow head). Many PA processes are also observed (arrows) (100x).
G. Cluster of immunoreactive cell bodies in the nucleus reticularis (200x).

medialis, lateralis posterior, anterior ventralis, habenularis lateralis, rhomboidens, centrum medianum, centralis lateralis and parataenialis, in which this immunoreactivity has not been previously observed (11, 18). We found a large number of PA immunoreactive cell bodies in the nucleus reticularis, as well as PA fibers in the lemniscus medialis and in the specific thalamic nuclei. These observations are similar to those found in previous works on the distribution of PA in the rat CNS (11, 18). In sum, it seems that the distribution of PA fibers and cell bodies is broader in the thalamus of the rat in comparison with previous reported results (11, 18). Technical considerations such as the fixative used and the embedding procedure could be the origin of this difference, since in both works (11, 18) the rat brains were perfusion-fixed with Bouin fluid and embedded in paraffin.

In comparison with a previous study on the distribution of PA immunoreactive structures in the monkey thalamus (25), it seems that in general the distribution of PA fibers in the monkey thalamus is quite similar to that found in the rat. Thus, for example in both rat and monkey PA fibers have been observed in the nuclei habenularis lateralis, lateralis posterior, anterior medialis, geniculatum laterale dorsalis, centrum medianum, reticularis, anterior ventralis, anterior dorsalis, ventralis medialis and zona incerta. In addition no PA immunoreactive fiber was found in both animals in the nuclei lateralis dorsalis and paraventricularis. However, some differences can be observed: thus, in the nuclei centralis medialis and rhomboidens, we have observed PA immunoreactive fibers in the rat, but in both nuclei no immunoreactive fiber has been found in the monkey. By contrast, in the monkey PA fibers have been observed in nuclei such as parafascicularis, geniculatum mediale, habenularis medialis, as well as in the tractus opticus, in which we have not visualized immunoreactive fibers in the cat. In addition, Jones and Hendry (25) have described the localization of PA immunoreactive perikarya in the thalamus of the monkey. In comparison with our findings in the rat it seems that in the monkey PA immunoreactive cell bodies are more widespread distributed. Thus, in both rat and monkey PA immunoreactive cell bodies have been observed in the nuclei habenularis lateralis, lateralis posterior, zona incerta, reticularis, centralis lateralis, and ventralis anterior, whereas in the monkey PA immunoreactive perikarya were additionally observed in the following nuclei: geniculatum laterale dorsalis, centrum medianum, anterior ventralis, anterior dorsalis, medialis dorsalis, parafascicularis, geniculatum mediale and habenularis medialis but not in the rat. Finally, neither in the monkey nor the rat PA immunoreactive perikarya were observed in the nuclei centralis medialis, rhomboidens, anterior medialis, ventralis medialis, lateralis dorsalis and paraventricularis.

In comparison with a previous study on the distribution of another calcium-binding protein in the rat CNS (20), the vitamin D-dependent calcium-binding protein (ViDCaBP), it should be noted that fibers containing PA have been found in nuclei in which ViDCaBP has been localized, e.g., habenularis lateralis, anterior ventralis, anterior dorsalis, lateralis and reticularis. However, in other nuclei such as geniculatum mediale, habenularis medialis, paraventricularis, parafascicularis and reuniens, ViDCaBP fibers were observed (20), although no PA-immunoreactive

fibers was found. On the contrary, in the present study we observed PA fibers in the following nuclei: centralis medialis, anterior medialis, ventralis anterior, rhomboidens, lateralis posterior, centrum medianum and centralis lateralis, in which no ViDCaBP processes were observed (20). It seems that the distribution of ViDCaBP cell bodies is wider in comparison with the PA perikarya localization in the thalamus of the rat. In this sense, in many thalamic nuclei (habenularis medialis, anterior ventralis, anterior dorsalis, parataenialis, parafascicularis, paraventricularis, reuniens, geniculatum mediale) ViDCaBP cell bodies were observed. In these nuclei, no visualized perikarya containing PA were seen. However, we observed numerous immunoreactive PA neurons in the nuclei geniculatum laterale ventralis and reticularis, in which no cell body containing ViDCaBP was visualized. In conclusion, it seems that the immunohistochemical localization of PA in the rat thalamus described here differs from that of ViDCaBP (20). Until now, data are lacking about the functional significance of this different distribution of both calcium-binding proteins.

Similarities between the distribution of PA positive neurons and those containing the inhibitory neurotransmitter GABA in the mammalian CNS have been reported (9, 19). Perikarya containing both PA and GABA have been described in the cerebral cortex, pars reticulata of the substantia nigra, basket cells of the hippocampus, Purkinje, basket and stellate cells of the cerebellum and periglomerular cells in the olfactory bulb (9, 11). On the contrary, this co-localization was not observed in certain regions of the CNS. Endo *et al.* (18) have shown in the rat basal ganglia that PA exists in a specific population of neurons that differ from those containing GABA. In the rat thalamus PA and GABA are known to co-exist in neurons of the reticular nucleus (see 11). The possible co-localization of GABA and PA in other thalamic nuclei in which we have described PA perikarya is a question to be elucidated.

Studies on the distribution of PA and cytochrome oxidase activity should also be carried out in the rat thalamus in order to know their possible correlation, since Braun *et al.* (7, 8) have demonstrated in the brain of the zebra finch this correlation of areas containing PA and high cytochrome oxidase activity, suggesting a high oxidative metabolism among such PA-controlled calcium mechanisms. The distribution of fibers and cell bodies containing methionine-enkephalin (12, 15) and the localization of angiotensin II perikarya (14) has been recently described in the cat and rat thalamus. However, a clear relationship between cell bodies containing methionine-enkephalin or angiotensin II and PA structures cannot be suggested. In addition, processes containing methionine-enkephalin are localized in nuclei along the midline, in which scarce or no PA immunoreactivity was observed, whereas PA structures are abundant in the lateral thalamic nuclei, in which methionine-enkephalin fibers are absent.

As we have visualized in the rat thalamus, most of the PA perikarya observed in the CNS (11, 18) are small or medium size. Berchtold *et al.* (6) have speculated that these neurons are interneurons, although Endo *et al.* (18) have described large PA neurons in the superior vestibular nucleus and in the medial superior

olivary nucleus. Additionally, PA has been observed in Purkinje cells (11, 18). Hitherto, we have no data suggesting that PA neurons in the thalamus of the rat are local and/or projecting neurons. However, PA cell bodies localized in the nucleus reticularis and in the zona incerta could be interneurons, since in such regions both PA fibers and perikarya are dense. Alternatively, these regions may send distant PA projections and receive PA afferents. Additional experiments should be carried out to elucidate this question. Finally, in some nuclei such as anterior dorsalis and ventralis anterior we have observed PA processes on cell bodies containig PA. This observation is in agreement with Celio and Heizmann (11), since these authors have also observed immunoreactive processes impinging on the motoneurons in the ventral horn.

Like other calcium-binding proteins, PA may play a role in many calcium-dependent phenomena in nervous tissue such as synaptic transmission or axoplasmic transport (16, 24, 27), but at present it is not possible to correlate the presence of PA in neuronal cells with a definitive function, due to its broad but clear-cut distribution. However, it has been pointed out that PA in skeletal muscle play the role of a relaxing factor capable of removing calcium ions from calcium-binding proteins (22). It has also been suggested that PA could play a similar role in specific subpopulations of neurons as it does in skeletal muscle. On the other hand, the selective staining of some thalamic neurons by PA antisera indicates that this protein is a marker of a distinct neuronal population, suggesting that apart from the general functions indicated, PA must be involved in more specific functions. Accordingly, the widespread distribution of PA in the rat thalamus suggests that it might be involved in several physiological functions, e.g., the presence of PA immunoreactive structures in the stria medularis, nucleus geniculatum laterale and nucleus ventralis posterior could indicate a possible role of the PA in the limbic, visual and somatosensory systems. One possible approach to elucidate the role of PA in neurons is a systematic mapping of PA positive neurons in the CNS and the search for a correlation with known functional mechanisms. The observation that in some neuronal populations, such as the periglomerular cells of the rat olfactory bulb, only one group of neurons shows a high degree of immunostaining, whereas the rest was practically unlabeled, suggests that the elucidation of PA physiology is not an easy question and could be related with the high immunocytochemical heterogenity observed in this neuronal population.

SUMMARY

1. The distribution of parvalbumin cell bodies and fibers in the thalamus 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 ventralis posterior, reticularis, ventralis anterior and zona incerta, whereas the nuclei habenularis lateralis, lateralis posterior, lateralis, centralis lateralis and ventralis

lateralis had the lowest density. In the nucleus geniculatum laterale ventralis, the density of parvalbumin cell bodies was intermediate. In all these thalamic nuclei, small, round or fusiform immunoreactive cells with short immunolabeled dendritic processes were observed.

2. The densest network of immunoreactive fibers was observed in the nuclei geniculatum laterale ventralis, reticularis and zona incerta. The nuclei geniculatum laterale dorsalis, ventralis posterior, medialis ventralis, ventralis anterior, anterior ventralis, anterior dorsalis and rhomboidens contained a moderate number of parvalbumin fibers, whereas the nuclei lateralis posterior, habenularis lateralis, parataenialis, centrum medianum, lateralis, centralis lateralis, ventralis lateralis, medialis dorsalis, anterior medialis, ventralis medialis and lateralis anterior had the lowest density of immunoreactive fibers. In addition, a large number of immunoreactive fibers was found in the lemniscus medialis and a scarce number in the stria medullaris.

3. No immunoreactive structure was observed in the nuclei habenularis medialis, paraventricularis, reuniens and geniculatum mediale.

4. Thus, perikarya and fibers containing parvalbumin are widely distributed throughout the thalamus of the rat, suggesting that parvalbumin might play a role, directly or indirectly, in limbic, visual and somatosensory mechanisms.

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