Brain Research Bulletin, Vol. 24, pp. 41–48. © Pergamon Press plc, 1990. Printed in the U.S.A. 0361-9230/90 $3.00 + .00

Distribution of Parvalbumin Immunoreactivity in the Rat Septal Area

J. R. ALONSO, R. COVEÑAS, J. LARA AND J. AIJÓN[1]

Citología e Histología, Facultad de Biología, Universidad de Salamanca, 37008 Salamanca, Spain

Received 14 June 1989

ALONSO, J. R., R. COVEÑAS, J. LARA AND J. AIJÓN. *Distribution of parvalbumin immunoreactivity in the rat septal area.* BRAIN RES BULL **24**(1) 41–48, 1990.—The distribution of parvalbumin (PV)-containing neurons and processes in the septal area of the rat brain was studied using a monoclonal antibody and the avidin-biotin immunoperoxidase method. PV-immunoreactive neurons were mainly located in the medial septum/diagonal band complex and in the horizontal limb of the diagonal band of Broca, showing a high density of heavily immunostained neurons and fibers. Nonimmunoreactive cells surrounded by PV-positive cells and processes were observed in the same region, but no pericellular basket-like arrangements were found. On the contrary, the dorsal, intermediate, and ventral nuclei of the lateral septum were practically devoid of PV-positive neurons and processes. Thus, in these nuclei only a very low density of isolated neurons was labeled; these were specially scattered in the ventrolateral septal nucleus and in the dorsolateral septal nucleus just below the corpus callosum. Delicate PV-positive axonal plexuses were also observed in the dorsal and intermediate nuclei of the lateral septum. The immunopositive neurons displayed very different sizes and morphologies among the various septal nuclei and inside each of them, indicating that they do not belong to a single morphological class of neurons. Finally, the distribution of PV in the rat septal area is not directly related to cholinergic and GABAergic septal neurons.

Parvalbumin Calcium-binding protein Septal area Immunocytochemistry Rat

CALCIUM-binding proteins are a group of low molecular weight substances related to Ca^{2+}-mediated events and have a wide distribution and functionality. This group of substances includes, among others, calmodulin, present in all eukaryotic cells, troponin C, S-100 proteins, the human 28 KDa cerebellar calcium-binding protein, vitamin D- and vitamin K-dependent calcium-binding proteins, aequorin, calsequestrin, spasmin, and parvalbumin (12, 22, 34). Although these proteins are structurally related and show a common evolutionary origin (20), their distributions are quite different, suggesting different physiological roles (22).

Parvalbumin (PV), the first calcium-binding protein to be isolated and crystallized (19), has been detected in different concentrations in muscle and brain cells, and in few other locations (5). In the CNS, PV is scattered throughout the whole brain, but with a clear-cut localization in zones, layers, and neuronal types (6). Although there are several descriptions in the recent literature on the distribution of PV-positive neurons in the rat brain (11, 15, 28, 33), and a recent report (16) demonstrates that PV is present in some GABAergic septohippocampal neurons, a detailed study on the PV-immunoreactivity in the septal area is still lacking.

The septal area is an important relay station in the neuronal loop that interconnects the limbic telencephalon with the hypothalamus and the brain stem (13, 31, 37, 43). Thus, this region seems to influence various behavioral patterns and autonomic functions as well as motivational, emotional and associative processes (13). Immunocytochemical studies carried out in this region have demonstrated a high degree of heterogeneity in both the types and quantity of neuroactive substances (8, 17, 30, 31, 36, 39). This suggests complex interactions, including colocalization and excitatory and inhibitory connections, between the different immuno-identified systems.

The aim of the present work was to study the distribution of both PV-positive cell bodies and fibers in the different septal nuclei, comparing these observations with previous reports on the distribution of calcium-binding proteins and of other neuroactive substances, with special emphasis on the GABAergic system, since previous studies have emphasized the close relationship between PV and the inhibitory neurotransmitter gamma-aminobutyric acid (9, 11, 16, 24, 28, 33). We consider that the correlation of PV-distribution in certain neuronal types with the electrophysiological properties exhibited by such neurons, may be useful for interpreting the function of this protein in the CNS.

METHOD

Three adult male Sprague-Dawley rats with a body weight of 200–250 g and kept under standard laboratory conditions (dark/

[1]Requests for reprints should be addressed to Dr. J. Aijón, Citología e Histología, Facultad de Biología, Plaza de La Merced s/n, 37008 Salamanca, Spain.

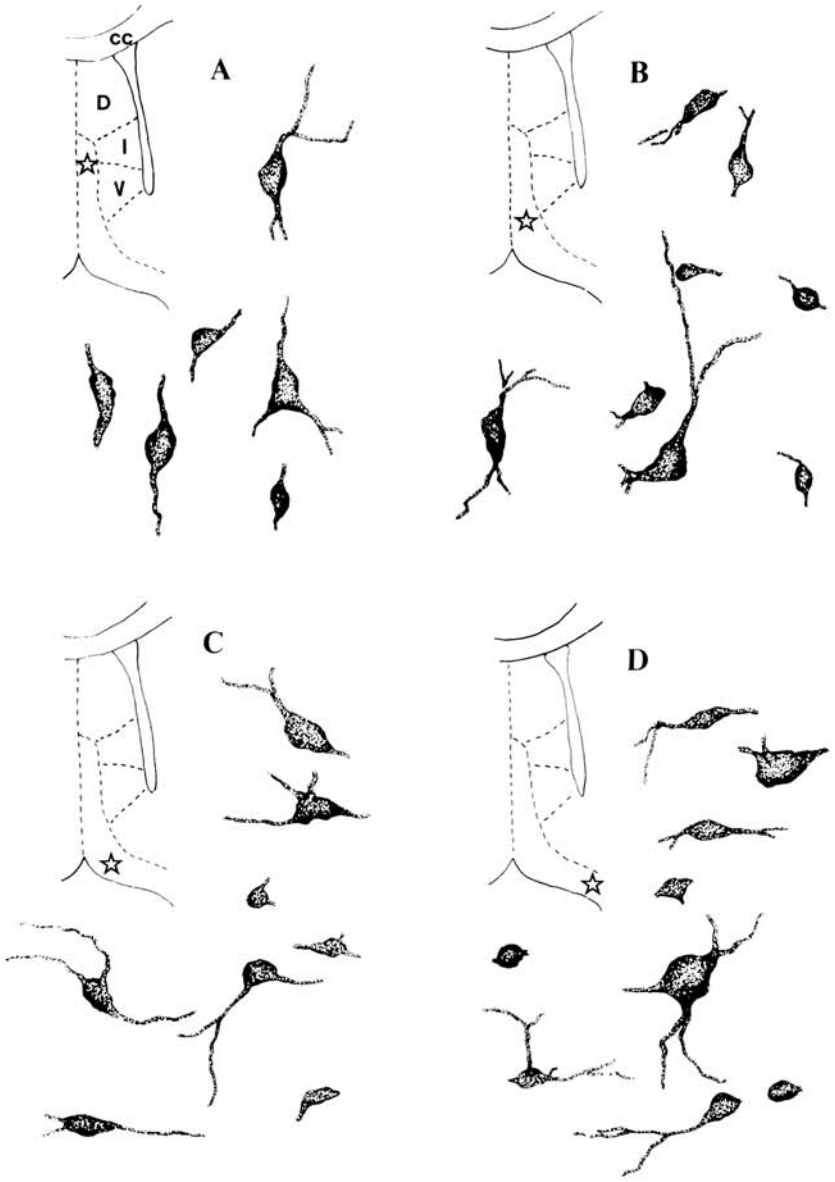

FIG. 1. Representative PV-positive cell bodies from the four locations marked on the insets (stars). (A) Dorsal region of the MSDB. (B) Ventral region of the MSDB. (C) Horizontal limb, close to the midline. (D) Horizontal limb, distant from the midline. Note the high variability of both sizes and shapes, suggesting a high degree of heterogeneity in the PV-positive neuronal population. (cc: corpus callosum; D: dorsal nucleus of the lateral septum; I: intermediate nucleus of the lateral septum; V: ventral nucleus of the lateral septum.)

light cycle set at 12/12 hours, 20°C, and rat chow and tap water ad lib) were used for the present study. The animals were deeply anaesthetized with intraperitoneal injections of ketamine (50 mg/kg body weight) and perfused intracardially via the ascending aorta with 40 ml of Ringer solution followed by the fixative for immunocytochemistry described by Somogyi and Takagi (41) (4% paraformaldehyde, 0.08% glutaraldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.2). After perfusion, the brains were removed, the septal area dissected out and stored in glutaraldehyde-free fixative for two additional hours. These blocks were washed in several changes of phosphate buffer and cut at 40 μm perpendicularly to the longitudinal axis of the brain using a vibratome (Campden Instruments). The sections were throughly washed in phosphate buffer, and stored overnight at 4°C.

Free-floating sections were incubated with a monoclonal antibody (McAB 235) against parvalbumin (10). The anti-PV antibody diluted at 1:5000 in 0.1 M phosphate buffer containing 1% normal horse serum and 0.1% NaN_3, for 48 hr at 4°C. Thereafter, the sections were washed in phosphate buffer and processed according to the avidin-biotin immunoperoxidase (ABC) method as described by Hsu *et al.* (25). Briefly, the sections were incubated with biotinylated anti-mouse immuno-gammaglobulin

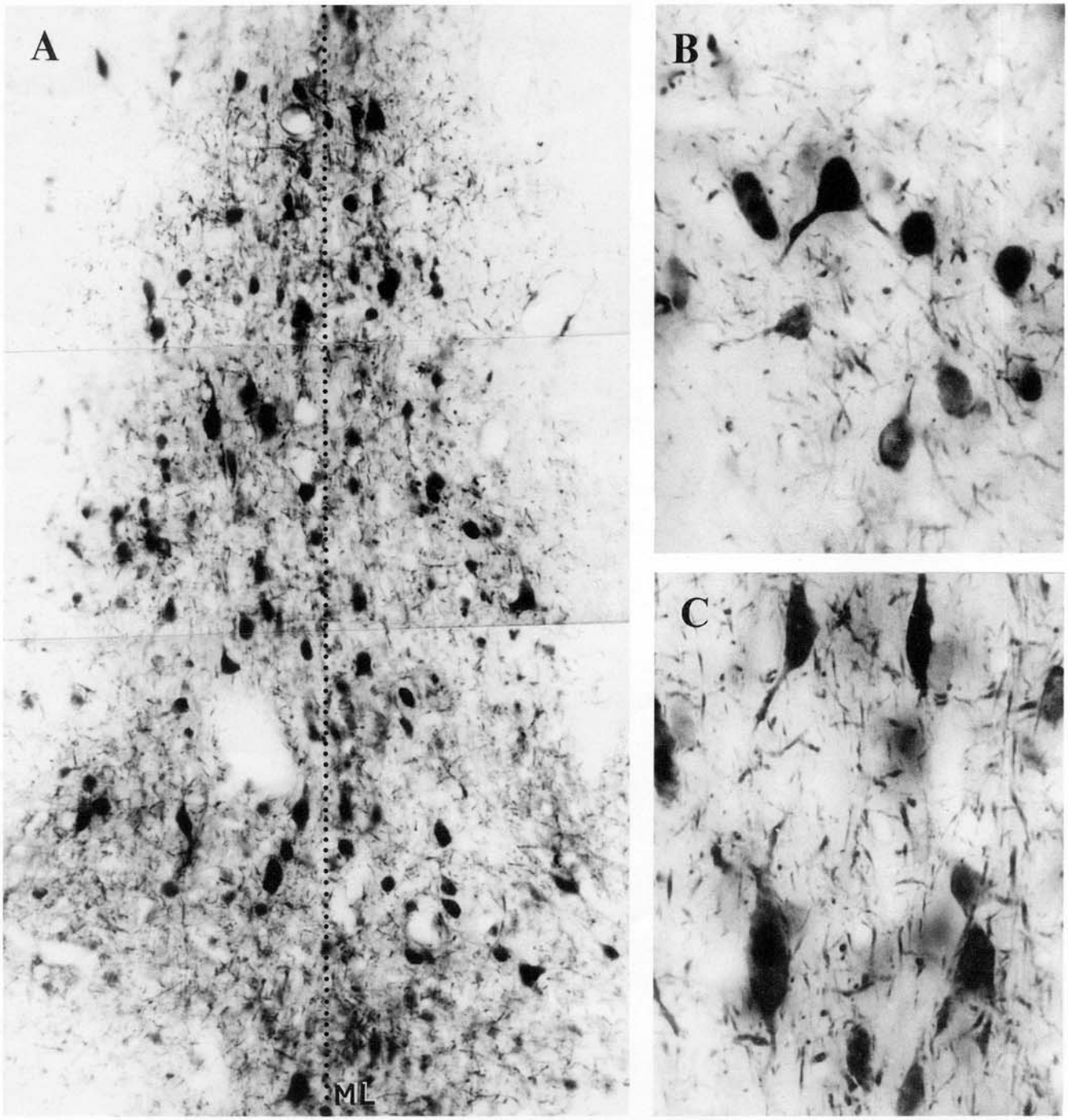

FIG. 2. (A) Composite photograph of a transverse Vibratome section of the rat septum. Numerous PV-positive neurons and fibers were seen in the dorsal and ventral regions of the medial septal nucleus/diagonal band complex. (ML: midline) (×194). (B) PV-immunoreactive neurons in the dorsal region of the MSDB. Note their small, round or oval-shaped somata. (×437). (C) PV-immunopositive neurons in the horizontal limb of the diagonal band. Note that most of the neurons show fusiform, medium-sized cell bodies. Somata and processes are oriented along the medio-lateral axis. (×437).

(Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) diluted 1:250 for 3 hr at 20°C, and Vectastain ABC reagent (1:250) for two additional hours. Tissue-bound peroxidase was visualized by incubating the sections with 3,3′ diaminobenzidine (0.07%) and H_2Q_2 in Tris buffer 0.1 M (pH 7.6) for 5–10 min. Finally, the sections were dehydrated in increasing ethanol series and flat-mounted with Araldite between two plastic foils. This procedure will allow us a future reexamination of the same neurons and processes using electron microscopy.

McAB 235 against PV has been fully characterized in a recent

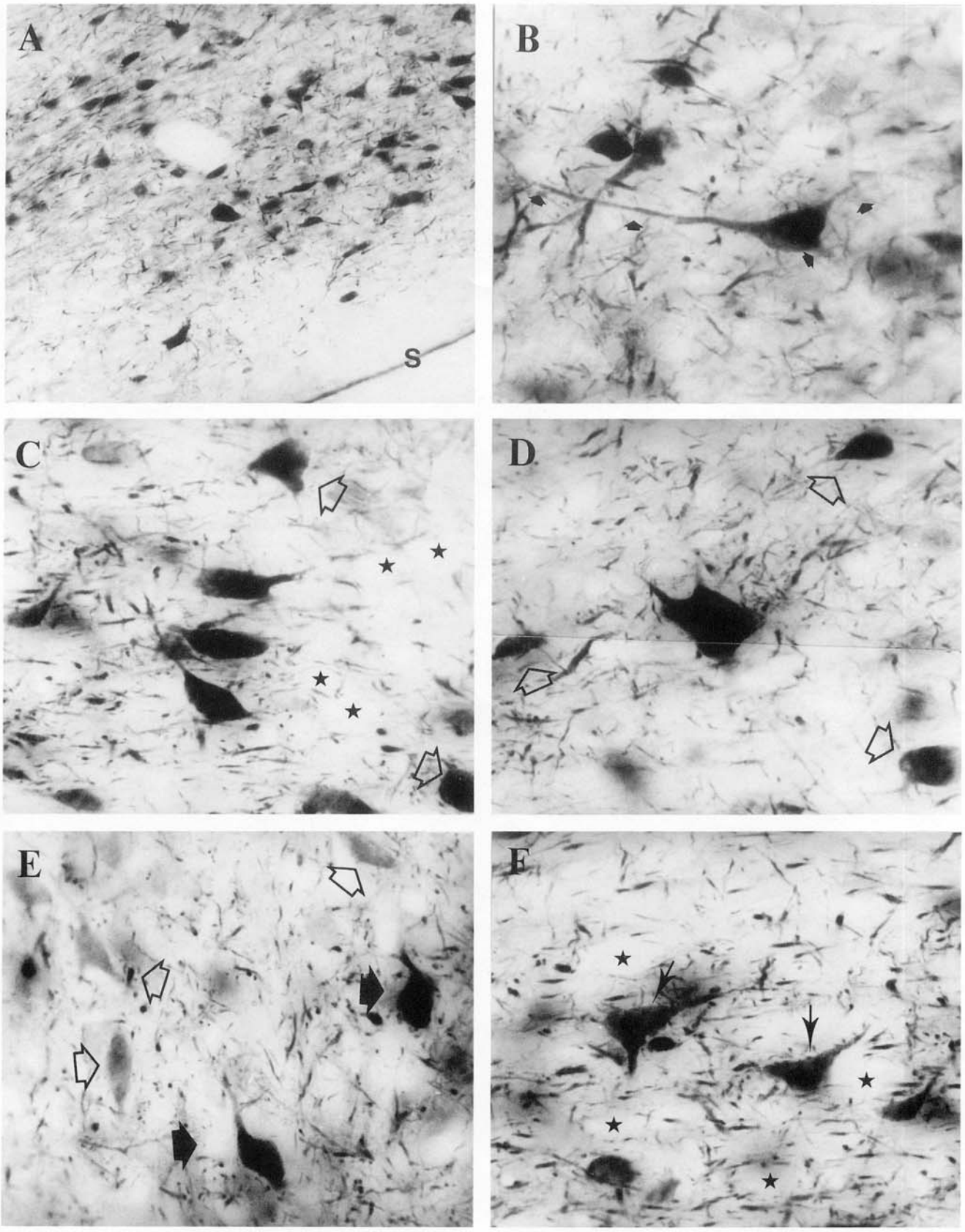
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FIG. 3. Parvalbumin immunoreactive neurons in the horizontal limb of the diagonal band. (a: ×160; b–f: ×437). (A) General view showing the distribution of PV-immunoreactivity. Numerous immunoreactive neurons are observed. Note that the zone near the ventral surface (s) of the septum is practically devoid of PV-positive cell bodies and processes. (B) Middle-sized neuron whose dendrites can be followed for some distance (arrows). (C) Immunoreactive fusiform neurons with horizontally oriented cell bodies and dendrites. Other PV-positive neurons with different morphologies (open arrows) and nonimmunoreactive cell bodies (stars) can also be observed in the same region. (D) Large, heavily immunostained multipolar neuron and smaller PV-positive neurons (open arrows). (E) Strongly (arrows) and slightly (open arrows) immunoreactive neurons in the horizontal limb of the diagonal band, close to the midline. (F) Group of strongly immunoreactive cell bodies with nearby scattered nonimmunoreactive neurons (stars). Some of the labeled neurons show puncta-like structures (arrows).

paper (10), showing its use in the quantitation of PV by radioimmunoassay, in the detection of PV on immunoblots, and in the qualitative detection of PV by immunohistochemistry. In addition, the specificity of the immunostaining was controlled by omitting the PV 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 septal sections, since the three former regions contain a characteristic pattern of PV immunoreactivity (11, 15, 28, 33), and was therefore used as an additional control of the immunostaining procedure.

The size of neurons was measured with a Zeiss ocular micrometer.

RESULTS

The septal area may be subdivided into different nuclei, which differ in both their cytoarchitecture and connections. However, the boundaries between these nuclei are not very sharp and different terminologies have been used. In this study, we follow the parcellation of this region as proposed by Swanson and Cowan (43) and Köhler and Chan-Palay (30), referring to both the medial septal nucleus and the vertical limb of nucleus of the diagonal band as the medial septal nucleus/diagonal band complex (MSDB) as suggested by Bialowas and Frotscher (7) (Fig. 1A).

Light microscopic examination of sections treated with McAB 235 against PV showed a strong and highly specific immunolabeling of perikarya, proximal dendrites, and fibers in the rat septal area. This immunostaining was predominantly confined to neurons located in the MSDB and in the horizontal limb of the diagonal band (Figs. 2A, 3A). Other regions, such as the dorsal, intermediate, and ventral nuclei of the lateral septum, showed very scarce PV-positive cell bodies (Fig. 4A–C). In some cases, the PV-immunoreaction product filled the dendrites over long distances (Fig. 3B), although not providing a complete Golgi-like appearance as was observed with the same antiserum in sections of the olfactory bulb or hippocampus, or in the striatum or cortical regions in the same septal sections.

Medial Septum-Diagonal Band Complex (MSDB)

This region showed numerous perikarya densely filled with DAB reaction product (Figs. 1A–B, 2A–B). These cells were located around the midline area, showing a very high density in all regions of the MSDB. However, there were differences in their size and immunostaining. Thus, in the dorsal part of the MSDB medium-sized neurons showing strong immunoreactivity, and small cells displaying a lighter degree of immunolabeling were found (Figs. 1A, 2B). In addition, numerous thick fibers orientated along the dorsoventral axis were seen in this region. The largest cells were normally fusiform with a maximum diameter of 24–28 μm, whereas the smaller ones were round or oval-shaped with a maximum diameter of 16–21 μm (Fig. 1A). In the ventral region of the MSDB, the cells were slightly larger and their dendrites did not show a predominant orientation, as observed in the dorsal region (Fig. 1B). In addition, negative neurons were observed scattered between the PV-positive neurons. These unlabelled cells were completely surrounded by PV-positive processes. However, no basket-like arrangements were observed. In some cases, PV-positive and negative neurons were surrounded by PV-immunopositive puncta (Fig. 4D), although it was not possible to elucidate whether these were terminals or transversally cut PV-processes.

Different morphologies of PV-immunostained processes could be identified. Thus, there were abundant thick processes coursing vertically in the proximity of the midline and very fine axonal plexuses with more irregular and ramified trajectories which could not be followed far from the MSDB. The degree of staining of the thicker processes was considerably stronger and on some occasions, they could be presumably identified as dendrites (Fig. 2A). Finally, in some occasions the presence of varicosities in a PV-labeled process, which are characteristic of some neurons in the MSDB, could be seen.

Horizontal Limb of the Diagonal Band

As reported for the MSDB, numerous strong-immunolabeled cells were found in the horizontal limb (Figs. 1C–D, 2C, 3). The largest PV-positive cells of the septal area were observed in this nucleus (35–42 μm of maximum diameter). These cells, located at a distance from the midline, were polygonal neurons, with three to five thick dendritic trunks (Fig. 3D). Surrounding these neurons, in the most lateral part of the horizontal limb of the diagonal band, medium size (27–33 μm maximum diameter), fusiform neurons with their longitudinal axis parallel to the ventral surface of the septum were also observed (Figs. 2C, 3C). These cells were the most frequent neurons in this zone. Finally, a third group of small neurons (17–22 μm maximum diameter) was also found showing round, ovoid or fusiform perikarya (Fig. 1C); most of these neurons displayed the lowest immunostaining intensity. Differences in the immunostaining even in neurons belonging to the same morphological type were observed (Fig. 3E).

PV-negative neurons were also found scattered between the labeled neurons of the horizontal limb of the diagonal band (Fig. 3F). Due to the high density of PV cells and processes, they could sometimes be identified as intermediate-size fusiform neurons and as small round neurons.

Lateral Septum

Three nuclei may be differentiated in the lateral septum: dorsal, intermediate and ventral (1). The dorsal nucleus of the lateral septum showed a small number of PV-positive cells located in its dorsal region, just below the corpus callosum (Fig. 4A). Some

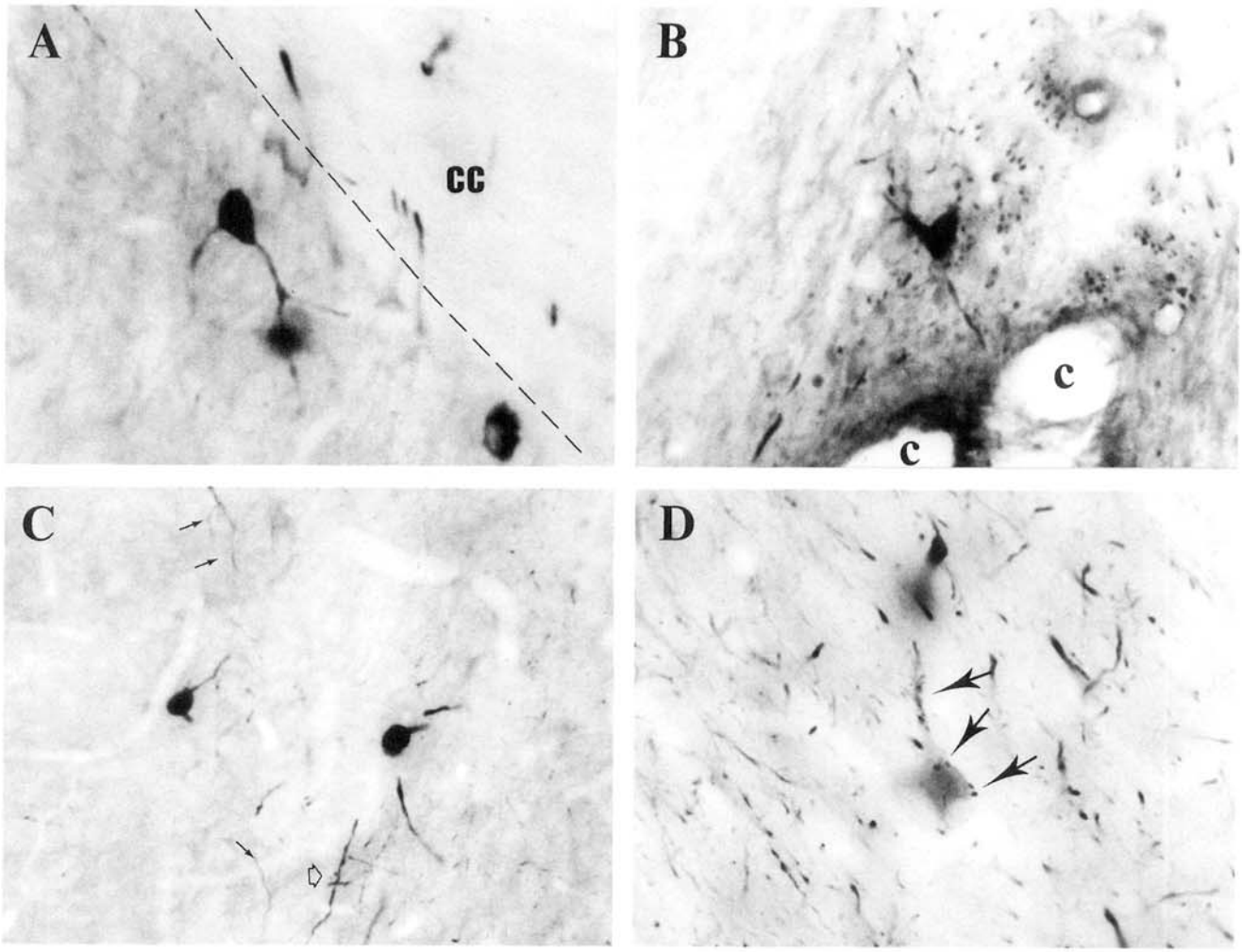

FIG. 4. (A) Small PV-positive neurons in the dorsal nucleus of the lateral septum, just below the corpus callosum (cc) (×466). (B) Isolated PV-immunoreactive neuron in the midline area of the dorsal nucleus of the lateral septum (c: cavities of the cavum septi pellucidi) (×374). (C) Two small PV-positive neurons in the ventral nucleus of the lateral septum. Scarce ramified PV-labeled processes can be seen (arrows). A thicker varicose process is also observed (open arrow) (×252). (D) Slightly immunoreactive neuron with puncta-like structures impinging on its cell body and apical dendrite (arrows). Horizontal limb of the Diagonal Band (×509).

PV-positive processes were observed in this region but the fibers of the corpus callosum were not labeled. The PV-immunostained cells were small and had strongly labeled, round or ovoid somata showing three to five dendrites coursing ventrally into the middle of the nucleus. The proximal segments of the dendrites did not bear spines (Fig. 4A, B). In addition to the thick PV-processes which in some cases could be identified as dendrites arising from these dorsally situated cell bodies, fine varicose axonal plexuses were also observed in the dorsolateral septal nucleus showing a slight degree of immunoreactivity, although it was possible to follow their courses for long distances in this region.

The rest of the lateral septum was also practically devoid of PV-positive elements. In the intermediate nucleus of the lateral septum, we only observed the presence of very scarce and isolated neurons and some fibers with no predominant orientation. Both perikarya and processes were mainly located near the lateral ventricles. In the third lateroseptal nucleus, the ventral nucleus, small neurons with a round, oval or polygonal perikarya and one to four thin dendrites arising from it, were observed (Fig. 4C). They were specially located in the ventral zone of the nucleus and did not form clusters. Their dendrites and axonal processes could be followed for long distances, but did not extend out from the nucleus.

Finally, other closely related brain regions proximal to the septal area, such as the striatum and the anterior hippocampal rudiment, also showed a characteristic pattern of PV-immunostaining, with PV-positive perikarya, fibers and puncta, and other PV-negative elements.

DISCUSSION

The present report describes the location and characteristics of PV-containing neurons and fibers in the rat septal area. Data on the presence of other calcium-binding proteins in the rat septal area are scarce. In this sense, PV and S-100 proteins antisera clearly marked different cellular population in the CNS (15). Additionally, vitamin D-dependent calcium-binding protein or calbindin (CaBPD-28K) has been detected in different brain regions using

both radioimmunoassay and immunocytochemical techniques (2, 3, 16, 18). Using the former, it has been shown that the septum contains an intermediate-low concentration of CaBPD-28K in comparison with other regions of the rat CNS (3). Using immunocytochemistry, CaBPD-28K has been observed in both neuronal perikarya and fibers of all the septal nuclei, including both lateral and medial septum (18). According to the afferent and efferent septal pathways, this protein was also found in the fimbria and in the rostral segment of the fornix, but the medial forebrain bundle was negative (18). Since CaBPD-28K (18) and PV are both present in the MSDB, and because no cross-reactivity between these calcium-binding proteins has been observed (Celio, Heizmann, and Roth, unpublished observations), a close interaction of both proteins may be suggested. However, preliminary observations on serial sections stained for PV and for CaBPD-28K suggested disimilar distributions.

Our own and previous observations (9, 11, 22) indicate a clear delimitation of PV-positive neuronal populations even in discrete brain areas, suggesting a specific function for PV in the CNS. In this sense, the results of the present report demonstrate that PV immunoreactivity is localized in the somata, dendrites and axonal processes of specific cell types in particular nuclei of the septal area. Thus, it seems to be an excellent neuronal marker in this region, since it was present in specific neurons but absent from other elements such as astro- and oligodendroglial cells.

Acetylcholine and GABA are the most important neurotransmitters present in the neurons of the MSDB and the horizontal limb of the diagonal band (7, 8, 32). Choline acetyltransferase immunocytochemistry provides a staining of fusiform cholinergic perikarya in the MSDB and horizontal limb similar, at light microscopic level, to the PV-labeling reported in our study. GAD and GABA immunocytochemistry also demonstrates abundant neurons in the MSDB (8, 30, 44). These cells are dispersed between the cholinergic neurons and their sizes and morphologies also resemble the characteristics of the PV-positive neurons (32). However, in no case does the distribution of PV in the rat septal area mimic the distribution of either GABA nor acetylcholine. Thus, no cholinergic perikaryon may be observed in the lateral septum, where some PV-neurons were found. In addition, Freund (16) has observed that PV is absent from the non-GABAergic, presumably cholinergic, septohippocampal afferents, which arise from neurons located in the MSDB. On the other hand, although it has been demonstrated (16) that some GABAergic septohippocampal neurons located in the MSDB are PV-positive, GAD immunocytochemistry also demonstrates numerous GABAergic neurons in the dorsal, intermediate and ventral nuclei of the lateral septum which were not immunolabeled for PV in the present study. Thus, it is possible to conclude that there is a population of GAD-positive and PV-positive septal neurons, as well as another population of GAD-positive but PV-negative cells.

In a similar way, neither does the distribution of PV immunoreactivity in the septal area correlate with the distribution in the same region reported for methionine- and leucine-enkephalin, substance P, corticotropin releasing factor, tyrosine hydroxylase, 5-hydroxytryptamine, or dopamine (4, 17, 35, 39).

The functions of PV remains unclear. Thus, although it has been demonstrated that PV is restricted to distinct neuronal populations, it is hitherto unknown which functional similarities are present between these widely distributed neurons. The broad distribution of PV inside the immunolabeled cells agrees well with biochemical data indicating that PV is a water-soluble protein (22) possibly implicated in a widespread intracellular control of calcium (45). In this sense, it has been suggested (3, 42, 45) that the action of calcium-binding proteins may be an intracellular buffering of calcium, contributing to the maintenance of an intracellular ionic calcium concentration level. Another hypothesis is that the action of PV might be to remove Ca^{2+} ions from other calcium-binding proteins (15). Thus, it has been proposed (23) that PV acts in skeletal muscle as a relaxing factor capable of removing Ca^{2+} ions from troponin C, then passing these ions to the sarcoplasmic reticulum. It has also been suggested, and proved for some PV-positive neurons such as the Purkinje cells, that PV might be a marker for neurons showing calcium action potentials (22). However, other Ca^{2+} spike producing cells such as the small neurons in the spinal ganglia, are not labeled using the same antisera (11). Finally, another possibility is that the presence of a calcium-binding protein may privilege a given neuron for certain Ca^{2+}-dependent processes, such as synaptic transmission, axoplasmic transport, calcium-dependent mechanisms of functional plasticity, and long-term neuronal viability (14, 22, 26, 29). However, these possibilities do not clearly explain why PV is only present in some types of neurons or why, even more interesting, they appear in a partial population of a neuronal type.

The close relationship between PV and GABA has prompted workers to study the peculiar electrophysiological characteristics of GABAergic cells (9). However, a direct connection is not evident. In one sense, a cross-reactivity of the PV antiserum with GABA-synthesizing enzymes may be excluded in view of the different molecular weights, high specificity and presence of neurons staining for PV and not for GABA and viceversa (11). In this sense, the distribution of PV is different from that of known GABA-receptor subtypes (9). Other data such as the characteristic high firing rates of the GABA-positive neurons (40) have indicated that PV may enable the neuron to fire in rapid repetition and to recover more quickly from an extensive excitatory input as has been observed in most GABAergic neurons (38). Thus, typical characteristics of PV-positive neurons such as fast firing rates, narrow action potentials, small amplitude fast afterhyperpolarisation and lack of accommodation has been observed in slices of the guinea pig MSDB (21), where we have described an extent and varied population of PV-positive neurons. Further studies on the immunocytochemistry and electrophysiology of the PV-positive neurons located in the three different nuclei of the lateral septum would provide important additional information. Finally, the relation between PV and the fast firing rate and quick recovering after a massive excitatory input is supported by the presence of GABA-positive but PV-negative neurons, such as the striatonigral projection neurons and the cerebellar Golgi cells, which are metabolically less active, firing at low rates (9,27).

It has been suggested that one approach to elucidate the physiological roles of PV in neurons may be a systematic mapping of the hitherto incompletely known distribution of PV-positive neurons in the CNS, searching for a correlation with known functional mechanisms (45). Therefore, the results described in this report may be useful in clarifying the role of PV in the CNS, broadening its known distribution to a new region with clear-cut and disimilar patterns of GABA and PV immunoreactivities.

ACKNOWLEDGEMENTS

We are greatly indebted to Professor M. R. Celio (University of Kiel) for kindly providing the monoclonal antibody against parvalbumin, and Professor M. Frotscher (University of Frankfurt) for his continuous support. This work was partially supported by the Junta de Castilla y León, DAAD, Ministerio de Educación y Ciencia (PM88-0154) and a Twinning Grant to J. R. Alonso from the European Science Foundation.

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