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CALCIUM-BINDING PROTEINS CALBINDIN AND PARVALBUMIN IN THE SUPERFICIAL DORSAL HORN OF THE RAT SPINAL CORD

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Abstract—Neurons containing the calcium-binding proteins, calbindin or parvalbumin, were studied by immunohistochemistry in the superficial dorsal horn of the rat spinal cord. Calbindin-containing cells were found in laminae I, II and III, being more abundant in laminae I and II. Some of the neurons in lamina I containing calbindin projected to the supraspinal area. Parvalbumin-containing neurons were mainly distributed in laminae IIi and III. Calbindin and parvalbumin were not detected in the same cells. Some 75% of the neurotensin-like immunoreactive neurons contained calbindin, which corresponded to 13% of the calbindin-containing neurons. Calbindin was sometimes found in the same cells with substance P, enkephalin or somatostatin but less frequently (44–46% of the peptide-containing neurons). Parvalbumin was not found together with these peptides. Electron microscopy showed that the immunoreactive products of calbindin or paravalbumin were mostly in the dendrites or cell bodies. Immunoreactive axon terminals were relatively few. In rhizotomized animals, neurons containing one of these proteins in laminae II and III were found to receive direct inputs of primary afferent fibers.

These findings indicate that neurons containing these two proteins belong to different subpopulations of dorsal horn neurons. They may be important in primary afferent processing.

Two calcium-binding proteins, calbindin and parvalbumin, which are widely distributed in the CNS[1,6,20] and the peripheral nervous system,[4,8,9,20] are considered to play important roles in the storage and/or transport of intracellular Ca^{2+}.[1,6,13,15,20] The distribution and properties of these two proteins have been studied in the CNS, including the olfactory bulb, cerebral cortex, hippocampus and cerebellum[1,9,12,24,34] and the peripheral nervous system, including the dorsal root ganglia and enteric neurons.[4,5,29] These proteins have been found in the dorsal horn of the spinal cord in chickens[25] and rats,[39] and discussed in general distribution studies.[6,11,12] However, morphological details including their projections and fine structure of dorsal horn neurons containing calcium-binding proteins are still unclear.

The superficial dorsal horn, which contains many peptidergic neurons, is important in pain transmission systems. It is of particular interest to know the precise localization of the calcium-binding proteins calbindin and parvalbumin and their relationship with peptidergic neurons in this area. In the olfactory bulb, hippocampus and cerebellum, parvalbumin has been found in the same cells with GABA,[6,22,24,36] and Buchan[4] has reported that calbindin is present together with vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY) in enteric neurons. The coexistence of these proteins with neurotransmitters or neuromodulators might be a key to the understanding of the functional roles of these proteins. Here, we investigated the distribution, cytoarchitecture, projections and fine structures of neurons containing these calcium-binding proteins in the superficial dorsal horn of the rat spinal cord. We also examined whether these two proteins are to be found together with each other or with neuropeptides.

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Abbreviations: DAB, diaminobenzidine; ENK, [MET]-enkephalin Arg^6-Gly^7-Leu^8; FB, Fast Blue; FITC, fluorescein isothiocyanate; -LI, -like immunoreactive; NPY, neuropeptide Y; PAP, peroxidase–antiperoxidase; PB, phosphate buffer; PBS, phosphate-buffered saline; SP, substance P; VIP, vasoactive intestinal polypeptide.

EXPERIMENTAL PROCEDURES

Twenty male Wistar rats (from Kiwa Experimental Animals, Japan) (eight normal and 12 operated rats) weighing 60–100 g and aged 4–6 weeks were used.

Surgical procedures

All surgery was carried out with the rats under sodium pentobarbital anesthesia (40 mg/kg body weight, i.p.).

For the study of the coexistence of the two calcium-binding proteins with peptides, 10 μl of 1% colchicine was injected into the subarachnoid space of the lumbar spinal cord (L4) with a cannula connected to a Hamilton microsyringe. These four animals were killed 2 days later.

The second group of four animals was used for a study that combined the use of the retrograde tracer technique and immunohistochemistry. The first 0.5 μl of Fast Blue (FB, 2% solution in distilled water) was injected into the upper cervical spinal cord (C1) unilaterally with a glass micropipet

connected to a micromanipulator. These animals were killed 2 days later.

In the third group of four animals, dorsal roots were exposed at the level of L1 spine, and L2–L5 dorsal roots were cut with a pair of fine scissors. These animals were kept alive for 10 days for fluorescent immunohistochemistry or for 2 days for the electron microscopic study.

Tissue preparation

Both the normal and operated animals were deeply anesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.) and perfused transcardially with 60–80 ml of saline followed by 400 ml of Zamboni's fixative[40] for fluorescent immunohistochemistry. For the combination of FB tracing and immunohistochemistry, 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) was used. For the electron microscopic study, modified Samboni's fixative, consisting of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M PB (pH 7.4), was used.

Spinal cords were dissected out together with dorsal roots and ganglia, postfixed in the same fixative overnight at 4°C, and transferred to 30% sucrose in 0.1 M PB (pH 7.4). For the electron microscopic study, spinal cord segment L4 was dissected out and postfixed in the modified Zamboni's fixative without glutaraldehyde. Then the tissue was put into 15% sucrose in 0.1 M PB.

Immunohistochemistry

Analysis for distribution of calbindin and parvalbumin in the dorsal horn in relation to their projection. Frozen sections were cut frontally in a cryostat 20-μm-thick. They were incubated for 2 days at 4°C in a primary antiserum solution in 0.02 M phosphate-buffered saline (PBS) containing 0.3% Triton X-100 as follows: (1) anti-calbindin antiserum raised in rabbits and diluted in PBS (1:1000) or (2) anti-parvalbumin raised in sheep (diluted 1:500). These sections were washed in 0.02 M PBS and incubated overnight at 4°C in one of the following secondary antisera: (1) fluorescein isothiocyanate (FITC) anti-rabbit antiserum (Miles, diluted 1:1000) for the staining of calbindin and (2) FITC-conjugated anti-sheep antiserum (Cappel, diluted 1:500) for visualization of parvalbumin.

Morphological analysis of calbindin-like immunoreactive and parvalbumin-like neurons. To identify the morphological features of calbindin-like immunoreactive (-LI) and calbindin-LI neurons, frozen sagittal sections 70-μm-thick were cut from the L4 level of two rat spinal cords. The sections were processed to make parvalbumin and calbindin-like immunoreactivities visible with a peroxidase–antiperoxidase (PAP) technique. Anti-calbindin antibody raised in rabbits or anti-parvalbumin antibody raised in sheep was used for the primary antibody, anti-rabbit antibody raised in goats (Cappel, diluted 1:500) or anti-goat antibody raised in rabbit (Cappel, diluted 1:500) was used for the second antibody and PAP complex of rabbits (Cappel, diluted 1:500) or goats (Cappel, 1:500) was used for the tertiary antibody. These antibodies were diluted with 0.1 M PBS containing 0.3% Triton X-100 and 2% bovine serum albumin. Sections were incubated with a primary antibody for 48 h at 4°C, and with secondary and tertiary antibodies overnight at 4°C. After washing with PBS and 50 mM Tris–HCl buffer (pH 7.6), the antigens were visualized by 15 min incubation in 50 mM Tris–HCl buffer containing 0.03% diaminobenzidine (DAB) and 0.01% hydrogen peroxide at room temperature. The sections were then dehydrated and embedded in Epon. Some coronal sections 20-μm-thick were also processed with a PAP method.

Analysis of calbindin and parvalbumin in single dorsal horn neurons. Fluorescence double labeling was performed on sections from animals treated with colchicine. The sections were incubated in a mixture of two primary antisera raised in different species. To detect calbindin and parvalbumin in the same cells, a mixture of an anti-calbindin antiserum raised in rabbits (final dilution, 1:1000) and an anti-parvalbumin antiserum raised in sheep (diluted 1:500) was used for the primary antisera. The specificity of the antisera against calbindin and parvalbumin has been described previously.[19] The sections were incubated in a mixture of FITC-conjugated anti-goat antiserum raised in swine (TAGA; diluted 1:500) and biotinylated anti-rabbit IgG antiserum raised in donkeys (Amersham; diluted 1:500) overnight at 4°C. Then the sections were incubated with streptavidin conjugated with Texas Red (Amersham; diluted 1:500) overnight at 4°C.

Analysis of peptides and calbindin or parvalbumin in single dorsal horn neurons. To find if calcium-binding proteins were present in the same cell with certain peptides, either anti-calbindin or anti-parvalbumin antisera raised in sheep (final dilution, 1:500) was mixed with one of the following antisera: anti-neurotensin (produced by us, final dilution, 1:1000), [Met]enkephalin Arg^6-Gly^7-Leu^8 (ENK; provided by Dr Yanaihara, diluted 1:1000), substance P (SP; produced by us, diluted 1:1000) or somatostatin (produced by us, diluted 1:1000), all of which were raised in rabbits. The specificities of these antisera against neuropeptides have already been described elsewhere.[26] After the sections were washed in 0.02 M PBS, they were incubated in the same solution used in the fluorescent double-labeling studies. Cross-reactivity between the secondary antisera was checked by omission of one primary antiserum.

Electron microscopic study. Two normal and two dorsally rhizotomized animals were used in the electron microscopic study. Sections of the spinal cord (L4) were cut 40-μm-thick on an Oxford PL 1000 Vibratome, and immersed in 15% sucrose in 0.1 M PB for several hours. After being frozen in liquid nitrogen and thawed, the sections were processed by a PAP method as described above except that Triton X-100 was omitted from all solutions. These sections were then osmicated, dehydrated, and flat-embedded in Epon. Ultrathin sections were cut, placed on grids, and stained with lead citrate. Sections were observed under a JEM 100CX electron microscope.

RESULTS

Distribution of calbindin-like immunoreactive and parvalbumin-like immunoreactive neurons and fibers in the superficial dorsal horn (Figs 1 and 2)

Calbindin. Calbindin-LI neurons were found in all laminae of the lumbar dorsal horn (L4), and were most concentrated in laminae I and II. In lamina I, small- to medium-sized calbindin-LI neurons were observed. Medium-sized neurons were 17–21 μm in diameter, and some of them were similar in shape in Waldeyer cells, extending their processes mediolaterally. Small neurons were 10–14 μm in diameter. Calbindin-LI neurons in lamina II and those scattered in lamina III were all small (10–13 μm in diameter). Calbindin-LI neurons in other laminae were small to large (12–30 μm in diameter). In sagittal sections, dendritic arborizations of the calbindin-LI neurons in lamina IIo were mostly cylindrical and no dendrite processes were extended to lamina I. The dendrites of these cells extended from 60 to 100 μm in the rostrocaudal direction and 20–30 μm dorsoventrally. Dendritic arborizations of calbindin-LI neurons in lamina IIi were also cylindrical. The dendrites extended from 80 to 150 μm in the rostrocaudal direction, but only 10–20 μm dorsoventrally. Calbindin-LI neurons in lamina III had rostro-

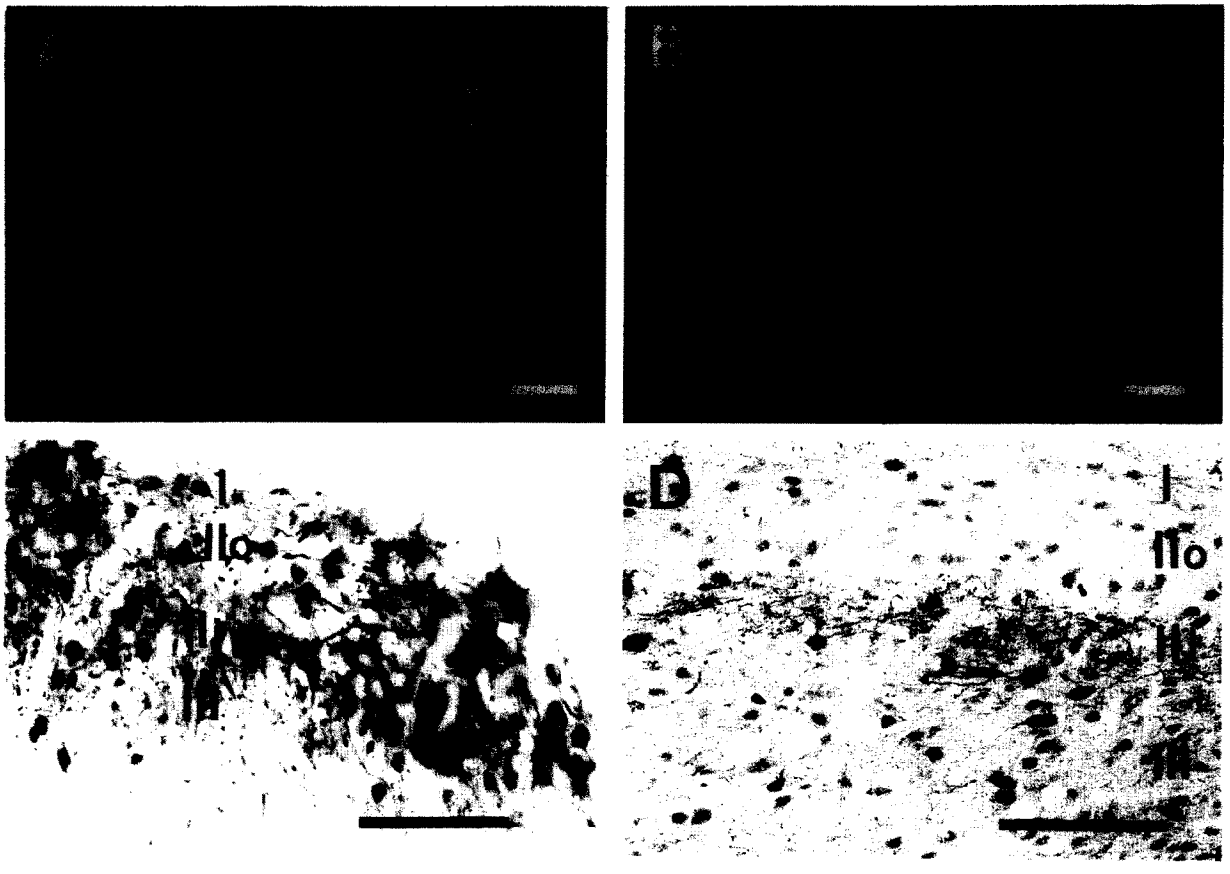

Fig. 1. Distribution of calbindin-LI (A and C) and parvalbumin-LI (B and D) neurons in the dorsal horn of the spinal cord. Roman numbers are used for the laminae of the spinal cord. Scale bars = 100 μm.

caudally oriented dendritic arbors which were confined to lamina III. A dense network of calbindin-LI fibers were found in layers I and II. Dorsal rhizotomy did not affect these fiber networks. These distribution patterns were similar at any spinal level.

Parvalbumin. Compared to calbindin, parvalbumin-LI neurons were few. Most of the parvalbumin-LI neurons were found in the inner part of lamina II (IIi), some were found in lamina III. They were all small (10–13 μm in diameter), and in sagittal sections, all of the parvalbumin-LI neurons in lamina IIo had cylinder-type dendritic arborizations; dendritic arbors of parvalbumin-LI neurons in lamina III extending rostrocaudally were confined in lamina III. Dense fiber-like staining was observed in lamina II, which was not affected by dorsal root rhizotomy. Processes of parvalbumin-LI neurons were not observed in the marginal layer.

Coexistence of calbindin and parvalbumin

The distribution of calbindin-LI and parvalbumin-LI cells overlapped in laminae IIi and III, so the coexistence of these two proteins was examined by a double-labeling method. However, no neurons containing both of these two calcium-binding proteins were found.

Coexistence of peptides with calbindin or parvalbumin (Fig. 3C–F)

The coexistence of these proteins with various peptides was investigated. The distribution of neurons immunoreactive to the four peptides (neurotensin, ENK, SP and somatostatin) examined in this study was identical to those reported in previous papers.[13,17,31,32] In brief, most neurotensin-LI neurons were found in laminae IIi and III, but some were found in lamina IIo. SP-LI neurons were mainly in lamina II, but a few were found in laminae I and III. The distribution of ENK-LI neurons was similar to that of SP-LI neurons, but there were more ENK-LI neurons. Somatostatin-LI neurons were mostly confined to lamina II.

Some of the calbindin-LI neurons had immunoreactivity for neurotensin, SP, ENK or somatostatin. The percentages of cells in which there was coexistence is shown in Table 1. About 75% of neurotensin-LI neurons were also immunoreactive to calbindin; this was the highest percentage. All neurons with both calbindin and one of these four peptides were small (10–14 μm in diameter), except for medium-sized SP-LI neurons in lamina I (17–21 μm in diameter) also stained for calbindin. This pattern of coexistence was the same at any spinal level.

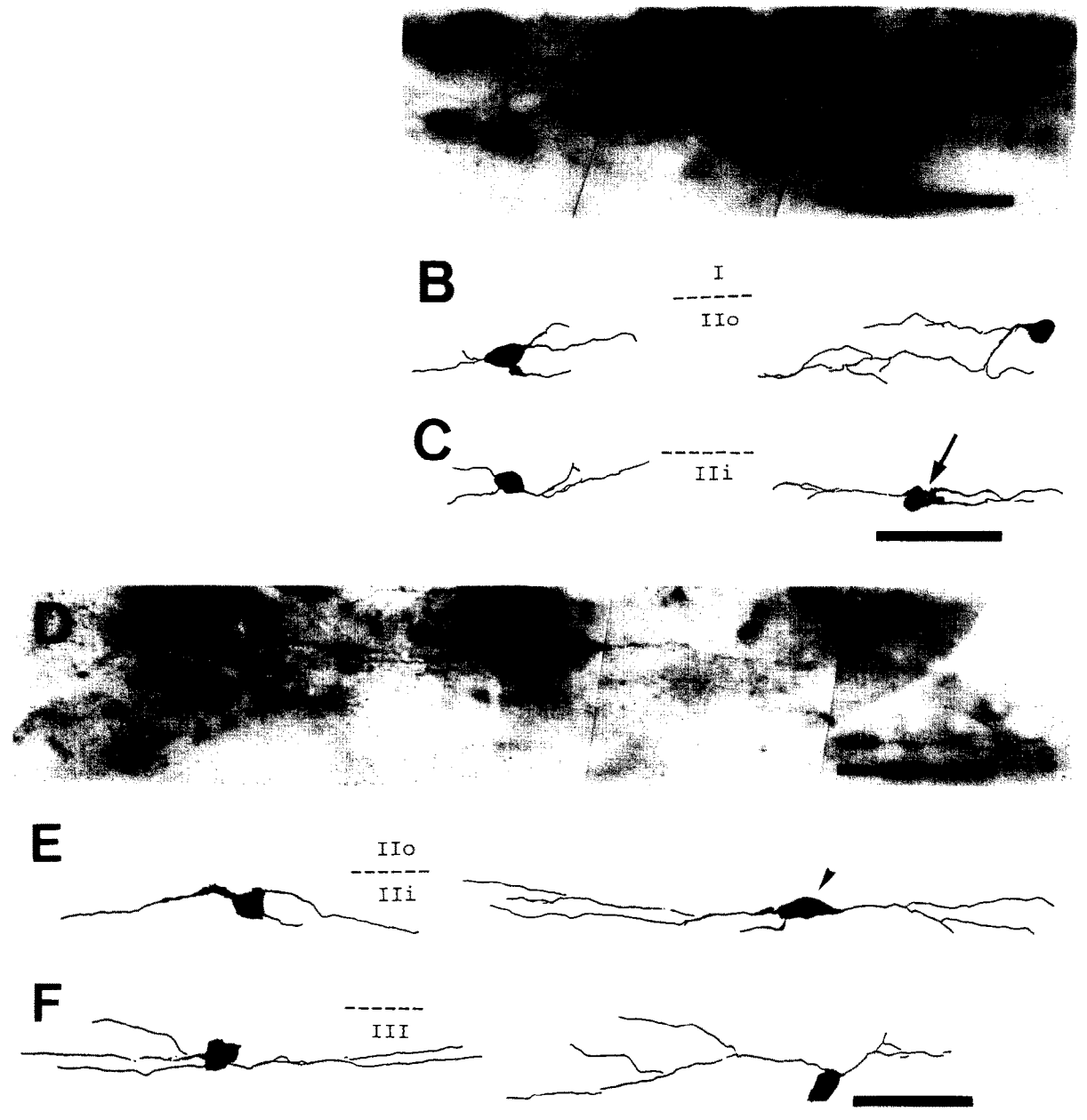

Fig. 2. Microphotographs and camera lucida tracing of calbindin-LI (A, B and C) and parvalbumin-LI (D, E and F) neurons in the sagittal sections. (A), (C) Calbindin-LI neurons in lamina IIi. Arrows indicate the same calbindin-LI neuron. (B) Calbindin-LI neurons in lamina IIo. (D), (E) Parvalbumin-LI neurons in lamina IIi. Arrowheads indicate the same parvalbumin-LI neuron. (F) Parvalbumin-LI neurons in lamina III. Scale bars = 50 μm.

No parvalbumin-LI neurons were immunoreactive to any of these peptides.

Immunohistochemistry and the retrograde tracer method (Fig. 3A, B)

FB-labeled cells were found in all of the laminae except for laminae II and III of the dorsal horn. In lamina I, the FB-labeled cells were 10–22 μm in diameter and elongated mediolaterally. FB-labeled cells in other laminae were 15–30 μm in diameter and most of them were round except for larger (30 μm) spindle-shaped cells located in lamina IV. About 32% (73/192) of the calbindin-LI neurons identified in lamina I were labeled with this dye. Although such neurons included both large Waldeyer-type cells and small cells, more of the larger calbindin-LI neurons including lamina III were labeled with FB than small ones. Calbindin-LI neurons in other laminae were not labeled with FB. No parvalbumin-LI neurons in superficial dorsal horn were labeled with FB.

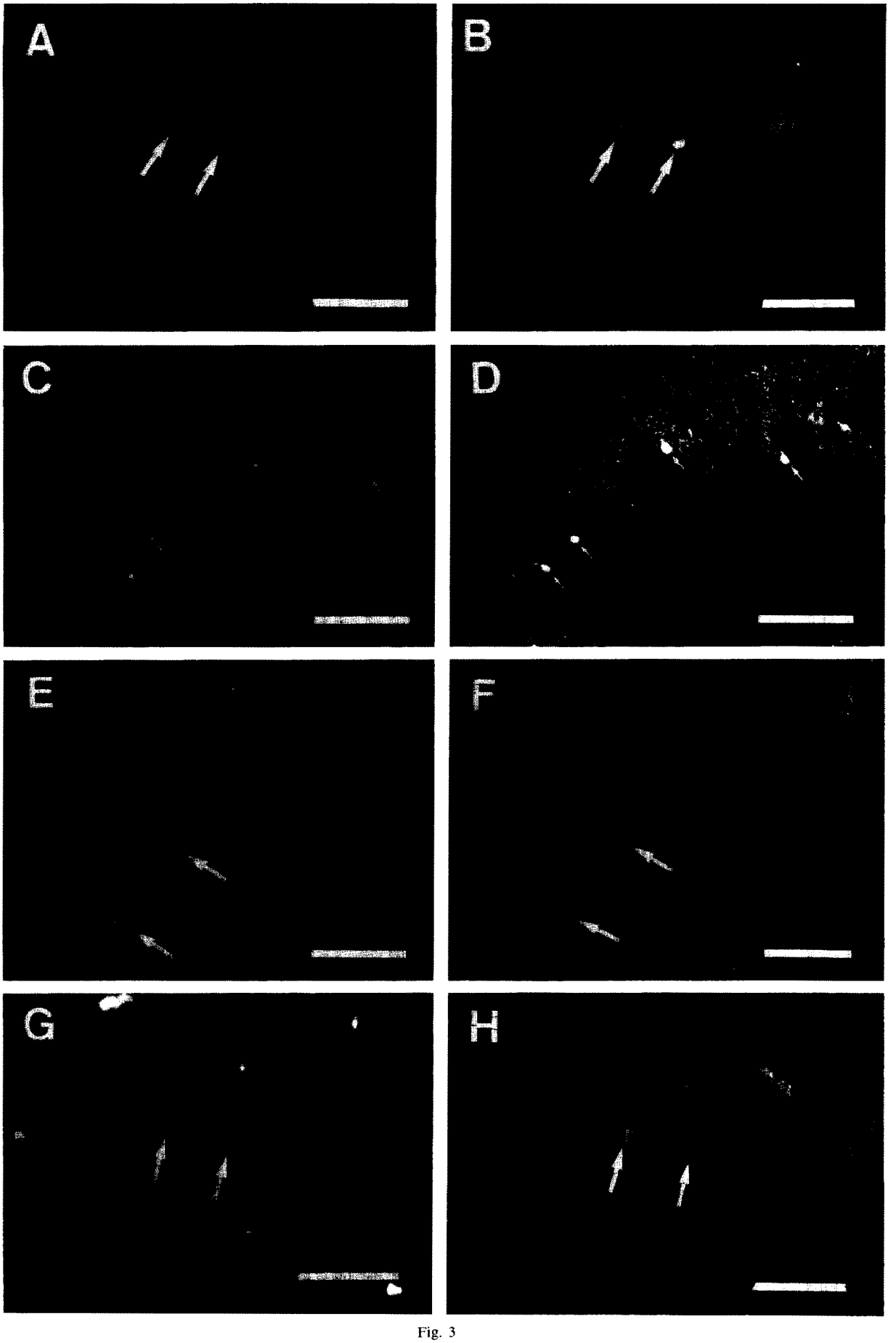

Fig. 3

Table 1. The number of neurons showing coexistence of calbindin with peptides in the superficial dorsal horn

(a)	Name of peptides	Neurotensin	ENK	SP	Somatostatin
(b)	Calbindin-LI neurons counted	911	629	897	611
(c)	Peptidergic neurons counted in the same area	154	203	203	140
(d)	Calbindin-LI neurons containing peptides	115	86	93	65
(e)	% of (d)/(b)	12.6	13.7	10.3	10.6
(f)	% of (d)/(c)	74.6	45.8	44.4	46.4

Electron microscopic study

Calbindin (Fig. 4). Numerous peroxidase DAB reaction products were observed in laminae I and II. Some were also found in lamina III. Most of the calbindin-LI structures were in cell bodies, dendrites and the nuclear matrix; immunoreactive axons and axon terminals were relatively few. The reaction was stronger in the dendrites than in the perikaryal cytoplasm (Fig. 4A). In dendrites, immunoreactive products were associated with microtubules. In lamina I, medium-sized (17–21 μm in diameter) neurons elongated mediolaterally were observed. In contrast, calbindin-LI neurons in laminae II and III were all small, and their nuclei occupied a large portion of their cell bodies. There was no difference in size and shape between neurons in lamina II and those in lamina III. In laminae I, II and III, both large (2.3–5.5 μm; Fig. 4C) and small (0.6–1.3 μm; Fig. 4D) immunoreactive dendrites were postsynaptic to unlabeled terminals, which were 0.4–1.4 μm in diameter and contained either round or pleomorphic vesicles. Most of these synapses were asymmetric with prominent postsynaptic densities (Fig. 4C–E), and some symmetric synapses were also observed. Some of the immunoreactive dendrites contained synaptic vesicles (Fig. 4E). Some calbindin-LI perikarya were postsynaptic to unlabeled terminals. Most of the calbindin-LI axon terminals were filled with small clear vesicles, but on some occasions large dense-cored vesicles were also observed. Theses calbindin-LI axon terminals made synaptic contacts with immunonegative small dendrites (Fig. 4F,G) in lamina II.

Synaptic inputs to calbindin-LI dendrites from degenerated axons were found in laminae II and III (Fig. 4E). Some of these degenerated axons were glomerular type terminals.

Parvalbumin (Fig. 5). In laminae II and III, the peroxidase DAB reaction products were scattered throughout the cytoplasm of the somata, dendrites and nuclear matrix, but they were very few in the axon terminals. Dendrites tended to be stained more intensely than perikaryal cytoplasm. Parvalbumin-LI neurons were, like other neurons in laminae IIi and III, small and most of their somata were occupied by nuclei. Parvalbumin-LI dendrites were postsynaptic to unlabeled terminals, which were 0.4–1.0 μm in diameter and had round or pleomorphic vesicles, making exclusively asymmetric synapses. At times, parvalbumin-LI dendrites containing vesicles were presynaptic to unlabeled dendrites and at the same time made synaptic contacts to an unlabeled dendrite (Fig. 5D). In laminae II and III, degenerated terminals with diameters of 0.4–0.8 μm were observed to make synaptic contacts on parvalbumin-LI dendrites.

DISCUSSION

General considerations

The distribution of calbindin-LI and parvalbumin-LI in the dorsal horn of the rat spinal cord reported here was in good agreement with studies by Celio and Heizmann,[6] Garcia-Segura *et al.*[12] and Yamamoto *et al.*[39] Compared to avians, calbindin-LI neurons in the dorsal horn of the spinal cord of the rat were more numerous, presumably a species difference. The present study further demonstrated characteristic features of calbindin-LI and parvalbumin-LI neuronal elements at both light and electron microscopic levels.

Coexistence of calbindin and paravalbumin

The coexistence of calbindin with parvalbumin was not found in the dorsal horn of the rat spinal cord. In previous reports, calbindin and parvalbumin had similar distributions in the CNS.[1,3,10,12,30,34] Few reports have been made of the coexistence of these two proteins,[21] but it is reasonable to assume that most Purkinje cells contain both of these proteins because all Purkinje cells contain parvalbumin[9] and most of them are calbindin-positive.[1,12] Recently, it has been demonstrated that most of the parvalbumin-LI dorsal root ganglion cells contain calbindin-LI.[5] However, in the dorsal horn of the rat spinal cord, neurons containing calbindin and those containing parvalbumin belong to different subpopulations.

Projection of calbindin-like immunoreactive neurons in lamina I

In lamina I, some of the neurons containing calbindin projected to the upper CNS. These may be

Fig. 3. Calbindin-LI (A) and FB-labeled (B) neurons in lamina I. Some 32% of the calbindin-LI neurons in lamina I were labeled with FB dye. Calbindin (C and E) and neurotensin (D and F) were found in the same neurons, and coexistence of calbindin (G) and ENK (H) was found in the single neurons. Arrows indicate neurons containing both substances. Scale bars: 100 μm (A–D); 50 μm (E–H).

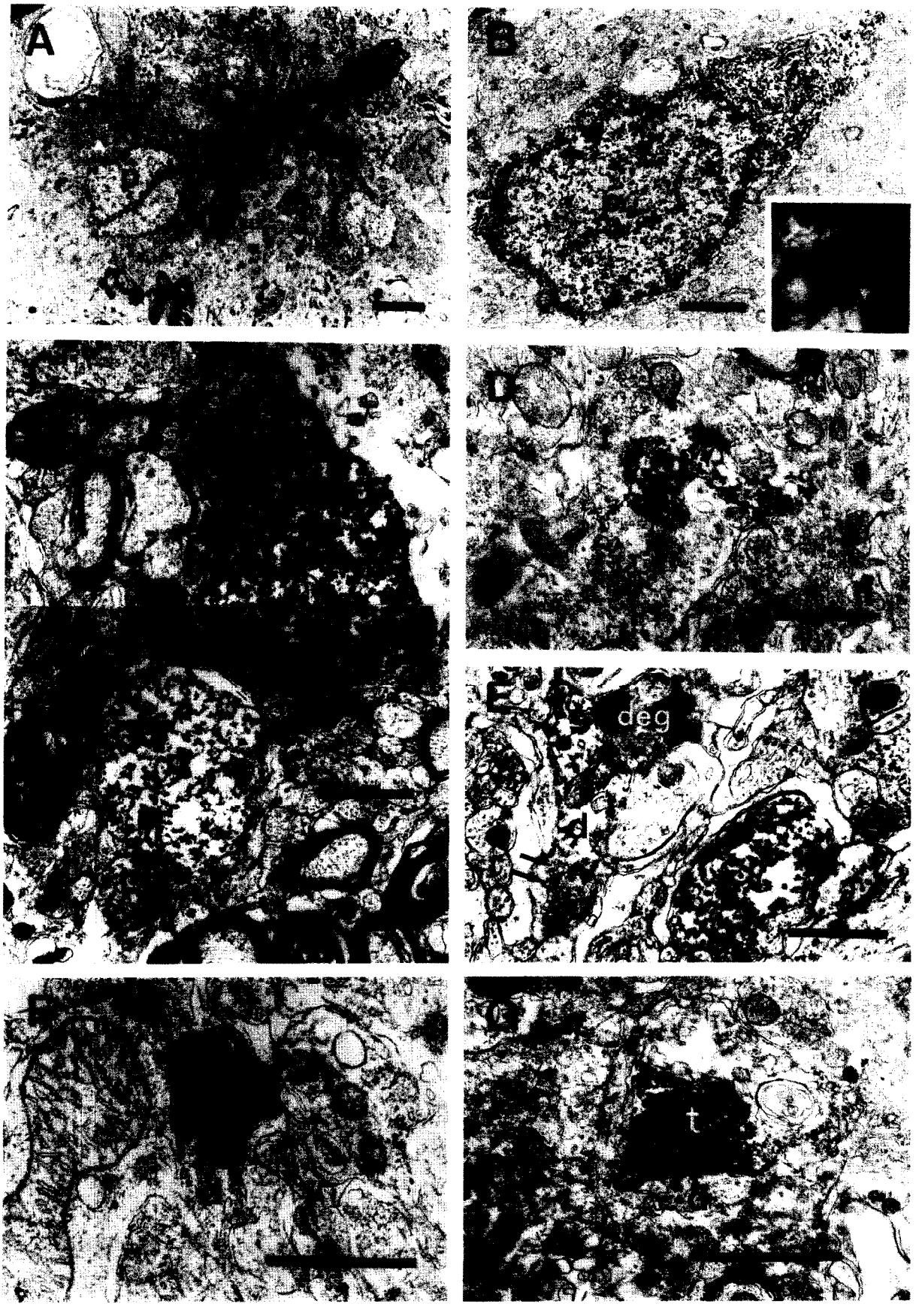

Fig. 4. Electron microscopic observation of calbindin-LI structures. (A) Calbindin-LI neuron in lamina I (p). (B) Calbindin-LI neuron in lamina II (p). (C), (D) Calbindin-LI dendrites (d) making synapses (arrowheads) to unlabeled terminals (t) in lamina II. (F). Calbindin-LI dendrite (d) making a synapse (arrowheads) to a degenerating terminal (deg) after rhizotomy in lamina II. This dendrite has vesicles (arrows). (F), (G) Calbindin-LI axon terminals (t) to unlabeled dendrites (d). Scale bars: 10 μm (A); 2 μm (B); 1 μm (C–G).

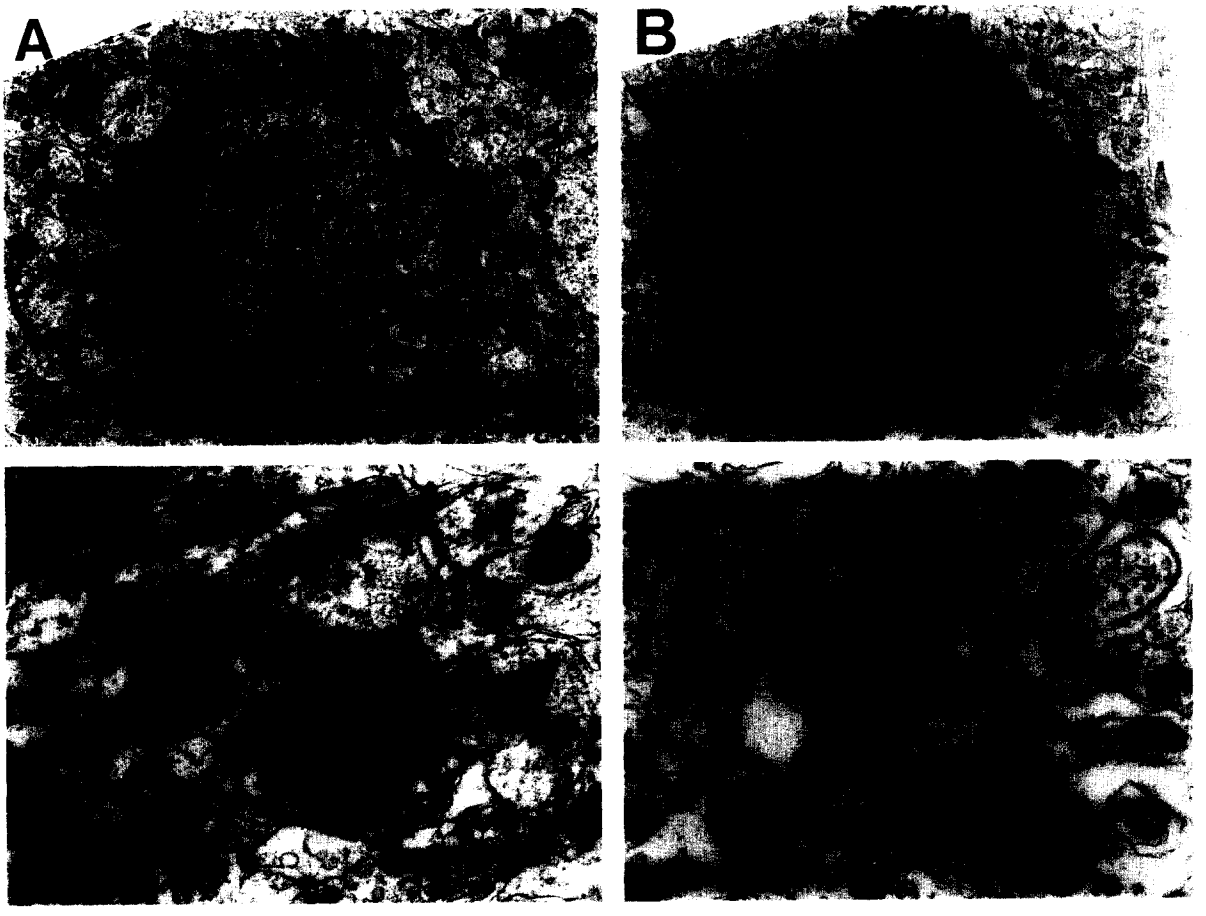

Fig. 5. Electron microscopic observations of parvalbumin-LI structures. (A), (B) parvalbumin-LI neuron (p) in lamina II (A) and lamina III (B). (C) Parvalbumin-LI dendrite (d) making a synapse (arrowheads) to unlabeled terminals (t) in lamina II. (D) Parvalbumin-LI dendrite (d) making a synapse (arrowheads) to a degenerating terminal (deg) after rhizotomy in lamina II. This dendrite has synaptic vesicles (arrows) and makes a synaptic contact to an unlabeled dendrite (u). Scale bars: 2 μm (A, B); 1 μm (C, D).

spinothalamic or spinomesencephalic cells,[38] which are important in the perception and transmission of nociceptive information.[18] Although we cannot exclude the possibility that some of the calbindin-LI neurons in laminae II and III were projecting neurons in this experiment, most of calbindin-LI neurons in laminae II and III may be propriospinal neurons. The findings indicated that calbindin was involved in both projecting and propriospinal neurons in the dorsal horn of the spinal cord.

Morphological analysis

In laminae II and III, both calbindin-LI and parvalbumin-LI cells were observed. In lamina II, at least two kinds of neurons have been identified: stalked cells and islet cells.[7,14,37] Stalked cells are either nociceptive or wide dynamic range neurons, and islet cells are nociceptive if in lamina IIo and low-threshold mechanoreceptive if in laminae IIi and III.[2,14,37] In spite of the differences in the patterns of their dendritic arborization, calbindin-LI cells in laminae II and III were similar to islet cells[2] because calbindin-LI neurons in these laminae lack spines and stalk-like dendrites travelling into lamina I. In addition, dendrites of the calbindin cells here contain synaptic vesicles and this is one of the characteristics of islet cells.[2,14] Here, we further showed that some of the calbindin-LI islet cells contained neurotensin.

At least some of the calbindin-LI cells in laminae II and III had synaptic vesicles in their dendrites. Moreover, their dendritic processes were confined to lamina IIi. These findings suggested that these parvalbumin-LI cells are islet cells, which seem to be inhibitory neurons.[23] It is interesting that parvalbumin-containing neurons in hippocampus and some other regions contain GABA, an inhibitory neurotransmitter. It is likely that parvalbumin is a marker of inhibitory neurons in some regions of the CNS.

Electron microscopic studies

Calbindin-LI and parvalbumin-LI fibers were not apparently affected by dorsal rhizotomy. This is understandable, because most of them were found to be dendritic processes in the following electron microscopic study. Our observations that immunostaining was more intense in dendrites than in cell bodies, and

that it was weak in the axon terminals in both calbindin-LI and parvalbumin-LI neurons may mean that these proteins play a role mainly in postsynaptic fields rather than in presynaptic sites, as intracellular second messengers or cell protectors by sequestering free intracellular calcium. In addition, in calbindin-LI dendrites receiving synaptic inputs, the postsynaptic thickness was very dense and prominent. This may be partly due to the deposition of immunoreactive materials. Sloviter suggested that these proteins protect cells in the hippocampal formation.[34] The intense staining of the nuclear matrix observed here was also described by Heizmann and Celio,[16] Stichel *et al.*[36] and DiFiglia *et al.*,[8] and DiFiglia *et al.*, who used the postembedding method, suggested that calbindin may be found in the nuclear fraction and may bind with Ca^{2+}.

Coexistence of calbindin with peptides

We found that some neurons containing calbindin are also immunoreactive to the neuropeptides neurotensin, SP, ENK or somatostatin, all of which have important roles in sensory transmission in the superficial dorsal horn. These findings indicate that calbindin-LI cells consisted of various subpopulations in terms of peptide expression. However, neurons containing one peptide (e.g. SP) are not uniform in terms of concomitant calcium-binding proteins. Peptidergic neurons that lack calbindin-LI may have other unknown calcium-binding proteins.

Among these peptidergic neurons, neurotensin-LI neurons were most closely related to calbindin-LI neurons; 75% of neurotensin-LI cells were calbindin-LI. Cross-reaction of the antibodies we used was ruled out in control experiments and by the finding that in periaqueductal gray matter in the midbrain, where both calbindin and neurotensin are abundant, the coexistence of these two substrates was not found by the same double-staining procedure (unpublished observation). Thus, a close relationship between calbindin and neurotensin is not a ubiquitous phenomenon in the CNS, but a feature characteristic of the superficial dorsal horn of the spinal cord.

Neurotensin is highly concentrated in the neurons of superficial dorsal horn and may be involved in the modulation of sensory information from the periphery.[27,33,35] Calbindin in these neurotensin-LI neurons might also play a role in the modulation of sensation by influencing the neuronal activity. In addition to the association between calbindin and neurotensin found in this study, GABAergic neurons are parvalbumin-LI in some brain regions such as the hippocampus and lateral geniculate body.[6,22,24,36] That immunoreactivity of parvalbumin in cultured cells decreased after high K^+ evoked transmitter release[28] strongly suggests that calcium-binding proteins are involved in the neural transmission mechanism. Buchan[4] showed that calbindin is to be found with VIP and NPY in the enteric neurons, and colocalization of calbindin and VIP in the hippocampus was suggested by Sloviter.[34] These findings taken together suggest that there are transmitter-specific Ca^{2+}-dependent releasing systems to which both calbindin and parvalbumin are closely related, although Braun *et al.*[3] concluded that both proteins are not transmitter-specific from their observations in the visual nuclei.

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