Serotonin-containing nerve fibers in the rat spinal cord: electron microscopic immunohistochemistry.

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Abstract

The ultrastructure of the serotonin (5HT) system in the spinal cord of rats was studied by an immunohistochemical peroxidase-antiperoxidase (PAP) method. Under the light microscope, 5HT immunoreactive staining was observed as brown-colored dots in the anterior horn, lateral horn, posterior horn and pericentral canal region. These positively staining dots were probably indicative of 5HT immunoreactive varicosities and nerve terminals. At the ultrastructural level, 5HT immunoreactive nerve fibers appeared as darkly stained varicosities with PAP positive large electron dense vesicles (80-100 nm), as well as small clear vesicles (30-40 nm) finely coated with PAP immunoreactive products. In the anterior horn, some of the 5HT immunoreactive structures were clearly nerve terminals forming asymmetric synaptic contact with soma or dendrites of the anterior horn cells. In the lateral horn, posterior horn and pericentral canal region, however, only 5HT positive varicosities were detected.

KEYWORDS: spinal cord, serotonin, immunohistochemistry, ultrastructure.

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Since the introduction of the histofluorescence method by Falck *et al.*(1), many investigations have been carried out on the distribution of monoaminergic nerve fibers in the mammalian spinal cord (2-5). However, it is very difficult to detect the serotonin (5HT) containing nerve fibers in the spinal cord by this fluorescent method. Furthermore, the method is not useful for ultrastructural investigations. Ultrastructural investigations on 5HT containing nerve fibers have also been undertaken with various methods involving potassium permanganate fixation (6), autoradiography with ^{3}H-5HT (7-11) and uptake of false transmitter, 6-hydroxytryptamine (12). An excellent immunohistochemical method using antibodies against 5HT itself was introduced recently by Steinbush (13) and Takeuchi *et al.*(14). They used the peroxidase-antiperoxidase (PAP) immunohistochemical procedure with highly specific antibodies against 5HT in order to investigate the 5HT system in the central nervous system. Using this PAP method, Kojima *et al.*(15) studied the detailed distribution of 5HT nerve fibers in the dog spinal cord at the light microscopic level. Here we used the PAP method with antibodies against 5HT and elucidated the detailed ultrastructure of 5HT containing nerve fibers in the rat spinal cord.

Materials and Methods

Ten rats of both sexes were used in this experiment. After anesthesia with Nembutal, exper-

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imental animals were perfused via the heart with 0.1 M phosphate buffered saline (PBS)(pH7.4), followed by a fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.4% glutaraldehyde in PBS at 4°C. After perfusion, the thoracic segment of the spinal cord was promptly removed and sliced into blocks which were kept in a postfixative containing 4% paraformaldehyde and 0.2% picric acid in PBS for 2 days at 4°C. Transversely and longitudinally cut sections of 80-100 μm thickness were prepared with a Vibratome (Oxford) and stored in PBS. For visualization of the 5HT immunoreactivity, the following modification of Takeuchi's procedure (14) was used. The sections were preincubated in 3% normal goat serum in PBS for 2 h, and then rinsed with PBS twice. The preincubated sections were incubated in primary antiserum against 5HT (1 : 15,000 dilution) for 48 h at 4°C, and then rinsed with PBS twice. These sections were incubated in goat antirabbit IgG (1 : 100 dilution) for 2 h at room temperature, and then rinsed with PBS twice. The final incubation was carried out with rabbit peroxidase-antiperoxidase complex (Cappel, 1 : 100 dilution) for 2 h at room temperature, followed by two rinses with PBS. The sections were rinsed with Tris buffer (pH 7.4), and then treated with 3,3' diaminobenzidine (DAB) containing H_2O_2 (0.003%) in Tris buffer solution for 10-15 min at room temperature. After this immunohistochemical procedure, the sections were postfixed for one hour in 1% osmium tetroxide, dehydrated in a graded series of acetone solutions and horizontally embedded in EPON 812 resin on a plastic slide. By this method, immunoreactive nerve fibers were clearly visible through the embedding media under the light microscope. Ultrathin sections were cut on an ultramicrotome (MT5000, Sorvall) and examined without any additional staining under an electron microscope (JEOL 100CX). The preparation of the 5HT antiserum and the specificity of the immunohistochemical method have previously been documented (14).

Results

A large number of 5HT immunoreactive brown-colored dots were observed in the rat thoracic cord under the light microscope.

Fig. 1 Light micrograph observed by transillumination of the surface of an EPON embedded transversely sectioned block of the anterior horn. Many 5HT immunoreactive nerve fibers are observed as brown-colored dots and varicosities around the anterior horn cells. * : anterior horn cells. Bar = 100 μm.

Fig. 2 Light micrograph observed by transillumination of the surface of an EPON embedded transversely sectioned block of the lateral horn. The most dense distribution of 5HT immunoreactive nerve fibers is observed around the intermediolateral nuclear cells. * : intermediolateral nuclear cells. LF : lateral funiculus. Bar = 100 μm.

Fig. 3 Light micrograph observed by transillumination of the surface of an EPON embedded longitudinally sectioned block of the pericentral canal region. A dense distribution of the 5HT immunoreactive nerve fibers is observed in the subependymal region (arrows). The right side of this picture is of a part more posterior than that of the left side. In the more posterior part, there are crossing 5HT immunoreactive varicosities (arrowheads). * : central canal. Bar = 100 μm.

These positively staining dots were probably indicative of 5HT immunoreactive varicosities and nerve terminals. These 5HT immunoreactive nerve fibers were distributed in networks around the anterior horn cells (Fig. 1). In the lateral horn, the most dense aggregation of 5HT immunoreactive nerve fibers was observed around the nucleus intermediolateralis (Fig. 2). In the pericentral canal region, a dense distribution of 5HT immunoreactive nerve fibers was observed lining the central canal, especially in the posterior gray commissure (Fig. 3). In the posterior horn, the fine 5HT immunoreactive nerve fibers were distributed in the substantia gelatinosa.

Under the electron microscope, 5HT immunoreactive large electron dense cored vesicles (80-100 nm in diameter) as well as PAP reactive products lightly coating small clear vesicles (30-40 nm in diameter) were observed. If the immunoreaction was strong, the product was also seen on the axoplasmic membrane and outer mitochondrial mem-

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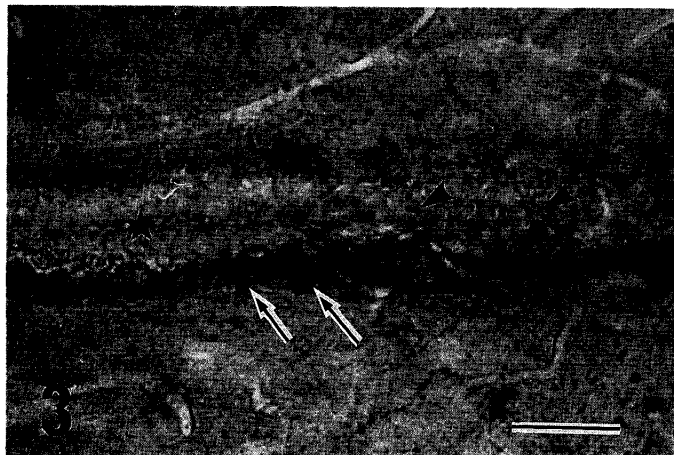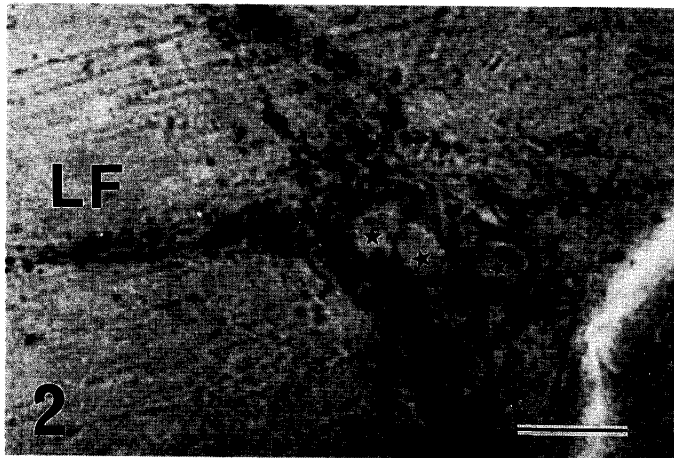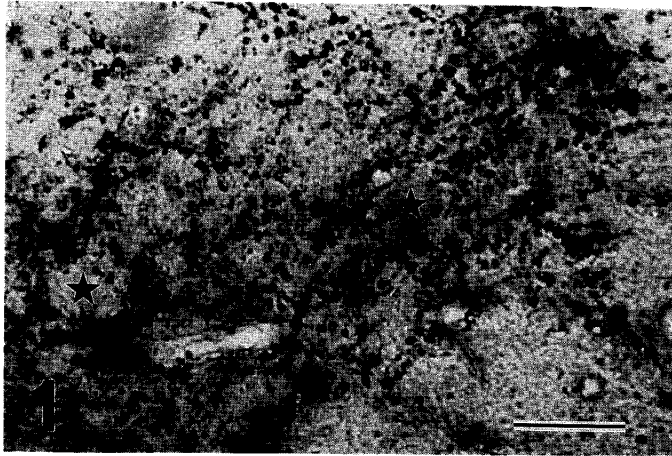
branes of the 5HT immunoreactive nerve fibers.

In the anterior horn, 5HT immunoreactive nerve fibers were observed in the vicinity of anterior horn cells which sometimes made synaptic contact with their somata (Fig. 4) and dendrites (Fig. 5a, 5b). A large number of these synapses were of the asymmetric type. The 5HT immunoreactive axodendritic synaptic terminals were often observed beside other non-5HT immunoreactive presynaptic terminals (Fig. 5a, 5b).

In the posterior horn, a large number of small 5HT immunoreactive nerve fibers were observed, but it was difficult to observe a typical synaptic contact which was positive for 5HT immunoreactivity.

In the lateral horn, a large number of 5HT immunoreactive nerve terminals and fibers were detected close to the intermediolateral nuclear cells (Fig. 6a). In the longitudinal sections, 5HT immunoreactive nerve terminals contained many large electron dense vesicles as well as small clear vesicles which were coated with fine PAP products (Fig. 6a, 6b, 6c). These terminals seemed to characteristically encapsulate the neighboring dendrites (Fig. 6c).

In the pericentral canal region, a large number of 5HT immunoreactive nerve fibers were present in the vicinity of the subependymal cell layer (Fig. 7). In this region, we observed no typical synaptic specialization of the 5HT immuno-positive varicosities (Fig. 8).

Fig. 4   Electron micrograph of a 5HT immunoreactive nerve terminal in the anterior horn (unstained). This terminal, which contained PAP positive large electron dense vesicles, forms an axo-somatic synaptic synapses with the anterior motor neuron. SOM : soma of the anterior motor cell. Bar = 0.5 μm.
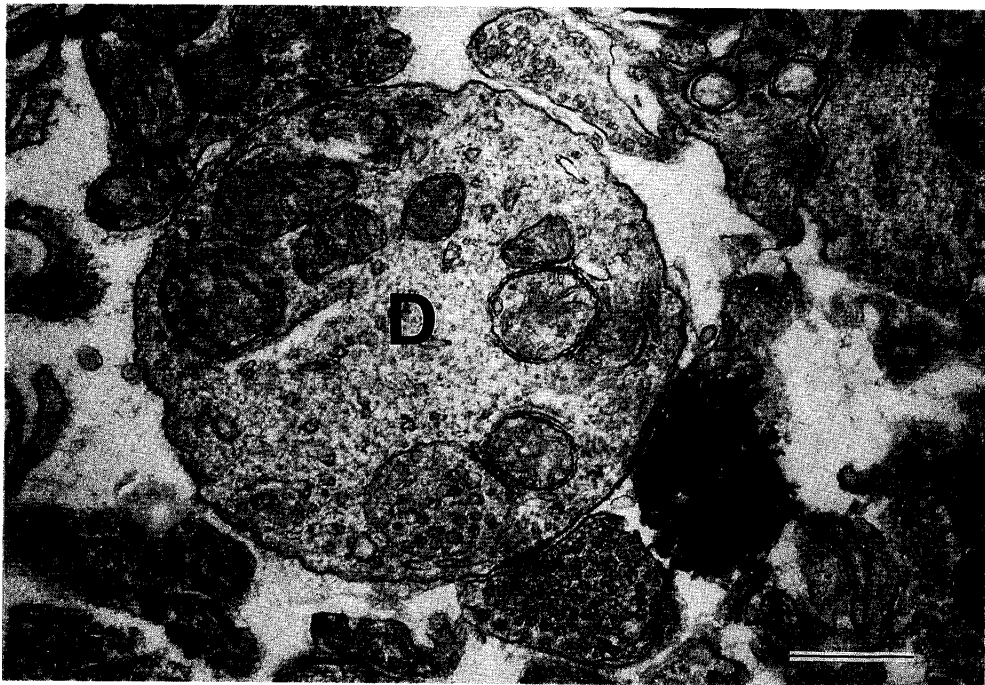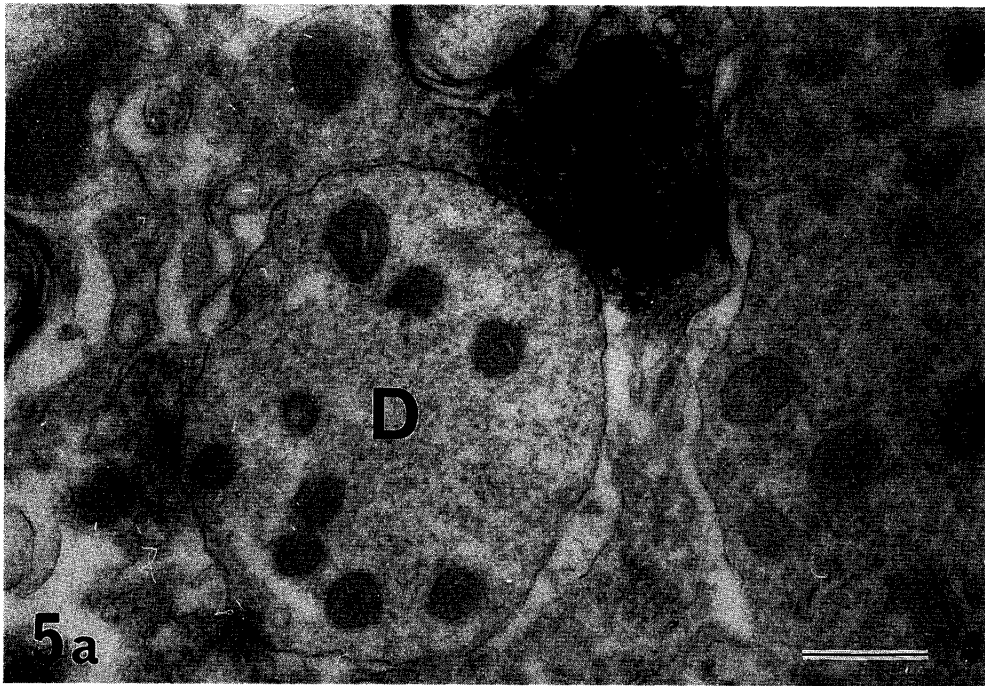
Figs. 5a, 5b Electron micrographs of 5HT immunoreactive terminals in the anterior horn (unstained). These terminals, which contained PAP positive large electron dense vesicles and many fine PAP products, show axo-dendritic asymmetrical synapses. D : dendrite of the motor neuron. Bars = 0.5 μm.
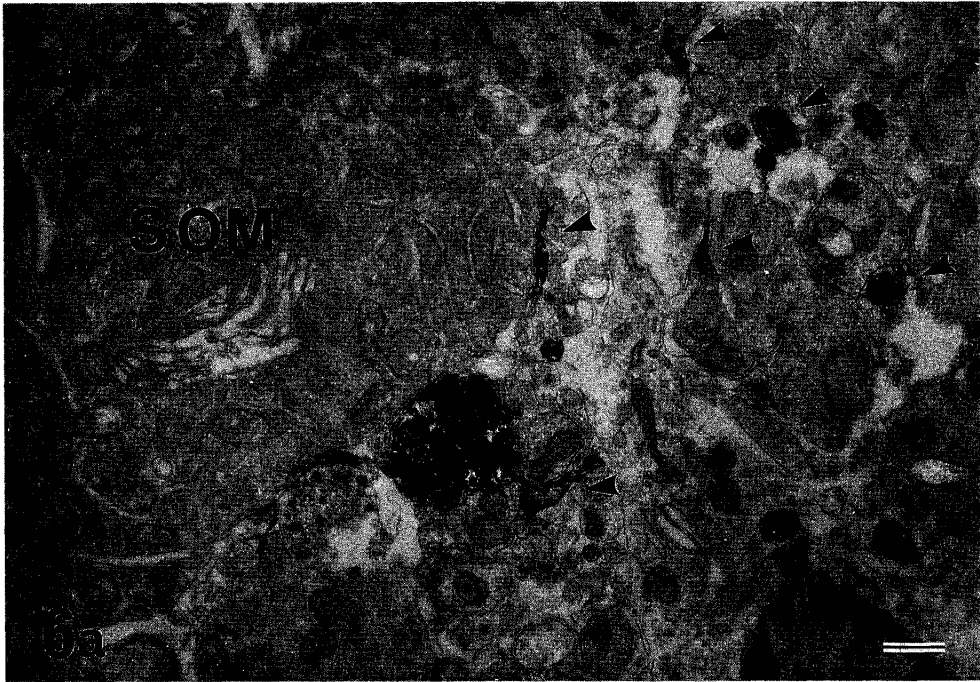
Figs. 6a, 6b and 6c Electron micrograph of 5HT immunoreactive nerve fibers and terminals in the lateral horn (unstained). Bars = 0.5 μm.

6a. Electron micrograph of a transverse section. A 5HT immunoreactive nerve terminal characteristically containing PAP positive large electron dense vesicles is in contact with the soma of a neuron of the intermediolateral nucleus. Many small nerve fibers and terminals are also observed (arrowheads). SOM : soma of the intermediolateral nuclear cell.

6b. Electron micrograph of a longitudinal section. 5HT immunoreactive nerve terminals contain many PAP positive large electron dense vesicles as well as many small clear vesicles which are coated with fine PAP products.

6c. Electron micrograph of a longitudinal section. 5HT immunoreactive varicosities are observed around dendrites. These terminals also contain many large electron dense vesicles filled with PAP reactive products. D : dendrite.
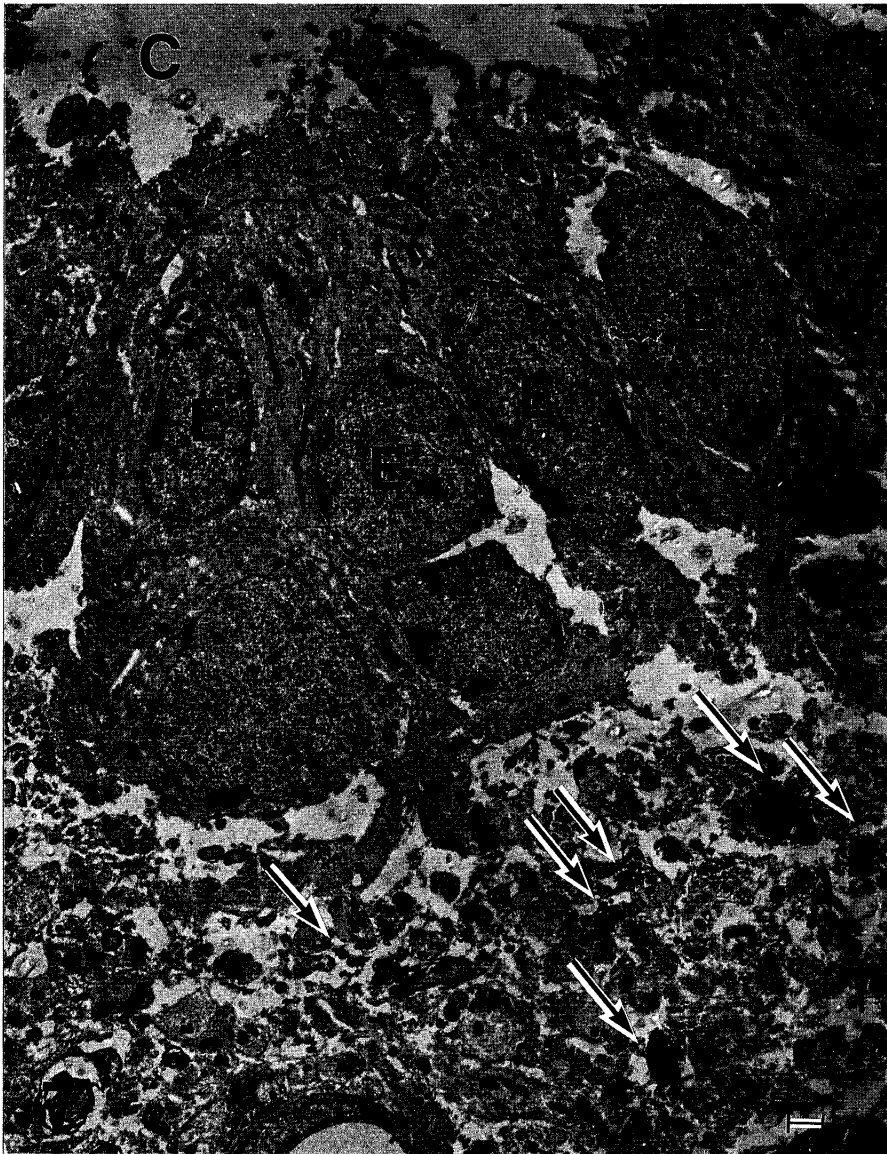

Fig. 7 Electron micrograph of a transverse section of the pericentral region (unstained). In the subependymal region, many 5HT immunoreactive nerve terminals are observed (arrows) (unstained). C : central canal. E : ependymal cell. Bar = 1 μm.
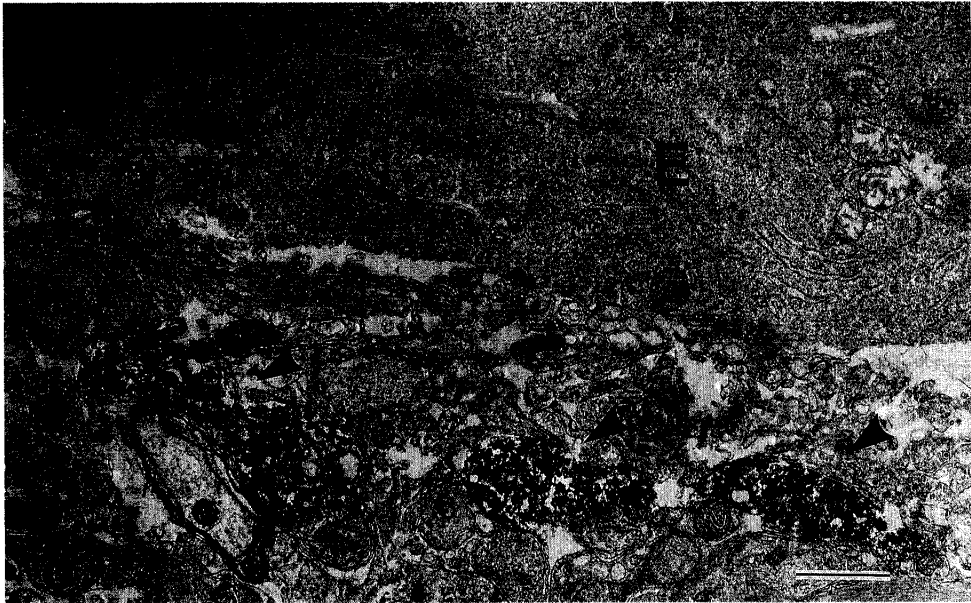
Fig. 8   Electron micrograph of a longitudinal section of the pericentral region (unstained). 5HT immunoreactive nerve varicosities containing numerous large electron dense vesicles filled with PAP products and many clear vesicles coated by fine PAP products are observed (arrowheads). There are no synaptic specialization of these varicosities. E : ependymal cell. Bar = 1 μm.

Discussion

The distribution of 5HT containing nerve fibers in the spinal cord of various species has been extensively investigated by form-aldehyde-induced fluorescence histochemistry at the light microscopic level (2, 3). Nu-merous methods have also been used to inves-tigate the ultrastructure of these fibers. The potassium permangate fixation (6) does not preserve the ultrastructure of the axonal terminals well. Uptake of false neurotrans-mitters, such as 5-hydroxydopamine or 6-hydroxytryptamine (12), is not specific enough for indolamines. The autoradiographic meth-od using ^{3}H-5HT was thought to be the best method to analyse specifically labeled struc-tures prior to the introduction of the immuno-histochemical method (11). By using the PAP immunohistochemical method, as dem-onstrated in the present experiment, we can easily and exactly correlate the findings at the light microscopic level with those at the electron microscopic level. The present ex-periment clearly shows that the most char-acteristic feature of 5HT-containing nerve fibers is the presence of PAP immunoreac-tive large electron dense cored vesicles (80–100 nm in diameter) and small clear vesicles (30–40 nm in diameter) which are coated with fine electron dense PAP products. Within the strongly reacting 5HT nerve fibers, addi-tional electron dense PAP reaction products are associated with the mitochondrial mem-branes and plasma membrane. It should be noted that no detergent was used in the pres-ent immunohistochemical procedure since detergent is known to destroy the ultrastruc-tural morphology (16, 17).

In the anterior horn, 5HT immunoreactive nerve terminals sometimes showed asymmet-ric synaptic specialization. In the other re-gions, we seldom found such typical synaptic contacts. This result is consistent with the

data of monoaminergic terminals labeled by false neurotransmitter (18). Descarries *et al.* (9, 10), using an autoradiographic method, reported very few synaptic specializations involving 5HT-labeled terminals in both the cerebral cortex and hypothalamus of the rat. Maley and Elde (19), using immunohistochemistry, also reported a lack of genuine synaptic contacts involving 5HT in the nucleus of the solitary tract of the cat. On the other hand, Ruda *et al.* (20), using autoradiography in the dorsal horn of the medulla and immunohistochemistry (21) in the dorsal horn of the cat, reported a large percentage of 5HT profiles exhibiting synaptic contacts in both cases, which is disagreement with our present data. Our data are consistent with those of Maxwell *et al.* (22), who reported that terminals stained immunohistochemically by 5HT seldom formed conventional synaptic junctions in the rat dorsal horn. This inconsistency may be depend on differences in species.

Considering the functions of 5HT in the central nervous system, it is interesting that the degree of synaptic specialization is different from region to region. It is a possible view that 5HT released from the definite synapses acts mainly as a neurotransmitter in the anterior horn and in other regions, while it may be released from the non-synaptic varicosities and/or nerve fibers to act as a neuromodulator affecting the general activity of neighboring neurons.

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