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Immunohistochemical demonstration of serotonin-containing nerve fibers in the cerebellum*

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Summary. The localization of serotonin (5-HT)-immunoreactive nerve fibers in the cerebellum of the rat and cat was investigated by means of the peroxidase-anti-peroxidase (PAP) method using highly specific antibodies to 5-HT.

Serotonin-containing nerve fibers were distributed throughout the entire cerebellum including the deep cerebellar nuclei, while 5-HT-positive neuronal somata were not detected in the cerebellum of either species. A different pattern of 5-HT innervation was found among the three layers of the cerebellar cortex. There were also interspecific differences in the pattern of distribution of 5-HT. In the rat, the pool of 5-HT nerve fibers mainly consisted of tangential elements, which were predominant in the molecular layer, while in the cat only a few 5-HT fibers were found in the molecular layer of the cerebellar cortex; dense networks of 5-HT nerve fibers were present in the granular layer. Some differences are evident in the pattern of distribution of 5-HT fibers in cerebellar regions classified on an anatomical and functional basis.

Key words: Serotonin-immunoreactive nerve fibers – Cerebellum – Cat – Rat – Immunohistochemistry

Current knowledge concerning terminal projections of serotonin (5-HT)-containing neurons has been obtained by use of several methods (Moore 1981): 1) fluorescence histochemical method introduced by Falck et al.; 2) biochemical determination of 5-HT content in small tissue samples; 3) autoradiographic tracing techniques; 4) autoradiography following superfusion or infusion of tritiated 5-HT; and 5) immunohistochemical methods.

However, little is known concerning 5-HT fibers in the cerebellum. Biochemical determinations revealed that both the cerebellar cortex and nuclei contain the least amount of 5-HT among various areas in the central nervous system (CNS) of the rat

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(Palkovits et al. 1974). Using the fluorescent histochemical method Hökfelt and Fuxe (1969) described for the first time fine varicose 5-HT fibers in the molecular layer of the anterior lobe of the rat cerebellum. However, the fluorescent histochemical techniques, both the original and the improved procedure, have a considerably lower sensitivity for 5-HT than for catecholamines.

Chan-Palay (1975) demonstrated 5-HT nerve fibers in the cerebellum of the rat and rhesus monkey using light- and electron-microscopic autoradiography. The terminals labeled by silver grains presumably belong to 5-HT neurons, however, absolute proof is lacking since terminals of non 5-HT neurons may also be labeled.

Immunohistochemistry using a specific antiserum against 5-HT has recently been introduced, and a detailed tracing of 5-HT-immunoreactive structures could be performed in the CNS of the rat by Steinbusch (1981). Despite this excellent technique, the cerebellar cortex hardly stained immunohistochemically for 5-HT, partially because of the low content of 5-HT in this area.

We presently report the immunohistochemical localization of 5-HT nerve fibers and terminals in the cerebellum of the rat and cat using a highly specific and sensitive PAP-technique.

Materials and methods

Antibodies were raised in male rabbits against an antigen prepared by coupling 5-HT to bovine thyroglobulin by means of a formaldehyde induced reaction (Takeuchi et al. 1982).

For immunohistochemical staining, 20 male Wistar rats weighing 200–250 g, and 8 male cats weighing 2.0–2.5 kg were used. The animals were anesthetized with Nembutal or ketamine chloride, and perfused transcardially with 4% paraformaldehyde, 0.4% glutaraldehyde (GA) and 0.2% picric acid in 0.1 M-phosphate buffer (PB). The brain was immersed in the fixative without GA for 48 h, cut on a cryostat (20 µm-thick frontal or sagittal sections), and then processed with a modified PAP-immunohistochemical method described by Kimura et al. (1981). The sections were incubated freely floating in the primary serum diluted with 0.3% Triton X-100 in PBS (phosphate buffered saline) at 1:32,000 for 24–48 h at 4° C. Then they were washed with 0.3% Triton X-100 in PBS, incubated in a goat-anti-rabbit IgG solution (1:200) for 3 h, rinsed, and incubated in rabbit peroxidase-anti-peroxidase (PAP) solution (1:200) for 90 min.

After the 3-3'-diaminobenzidine (DAB) coloration, the sections were treated with 0.1% osmium tetraoxide for 3 min to enhance the visualization of the reaction product. Following the immunohistochemical procedure, the specimens were counterstained with cresylviolet.

For further experiments both rats and cats were examined that had been pretreated with a monoamine oxidase inhibitor (MAOI, nialamide 300 mg/kg intraperitoneally). In addition, several rats were given 5,6-dihydroxytryptamine (5,6-DHT, 100–150 mg in 20 µl saline) into the lateral ventricle 5 days prior to sacrifice.

The specificity of the antiserum has been previously discussed (Takeuchi et al. 1982). Briefly, five criteria were used to test the specificity, as described by Steinbusch (1981): (1) control experiments using preimmune sera; (2) absorption test with 5-HT; (3) inhibition test with various substances that might possibly cross-react with the antibody; (4) pharmacological treatment with a 5-HT depletor, such as 5,6-DHT (Jonsson 1981); (5) comparison with data previously obtained by other techniques, such as the Falck-Hillarp fluorescence histochemical method.

From these tests, it was concluded that the antiserum employed in the present study is highly specific and cross-reacts only with 5-hydroxytryptophan at a level of less than 0.1%.

Results

Serotonin-immunoreactive (5-HT) nerve fibers are distributed throughout the entire cerebellum. Within the cerebellar cortex, a different pattern of 5-HT innervation is found among the three cortical layers. Species differences exist in the

5-HT innervation between the rat and cat. 5-HT-positive somata were not detected in the cerebellum of these two species.

Rat

In general, 5-HT-immunoreactive nerve fibers were found predominantly in the molecular layer, especially in frontal sections. In the single cortical layers the density of 5-HT fibers appeared relatively uniform irrespective of their phylogenetic history, i.e. neo-, paleo- or archicerebellar origin. In frontal sections of the vermis, 5-HT axons bifurcated in a T-shaped manner within the molecular layer and formed long tangential fibers parallel to the pial surface, mainly in the superficial layer (Fig. 1). Such tangential elements could be distinctly seen in frontal or horizontal sections of the vermis (Fig. 2), while few were found in sagittal sections. Occasionally, tangentially oriented 5-HT fibers running immediately beneath the surface appeared to extend out of the parenchyma to the pia. Vertical or oblique fibers were also observed in this layer, some reaching the leptomeninges (Fig. 1). In the sagittal plane, in contrast to the few tangential fibers, 5-HT-positive vertical fibers and dot-like profiles were readily observed.

In the molecular layer of the paraflocculus, flocculus and the lobulus semilunaris superior along the fissura posterior superior, relatively short vertical fibers were predominant; tangential fibers were rare in frontal sections. This difference among these areas did not seem to correlate to the anatomical subdivisions.

In the Purkinje cell layer, 5-HT fibers sometimes ran along the Purkinje perikarya during their course to the molecular layer. There was no evidence, at the light-microscopic level, that the 5-HT fibers terminate directly on the somata of Purkinje cells.

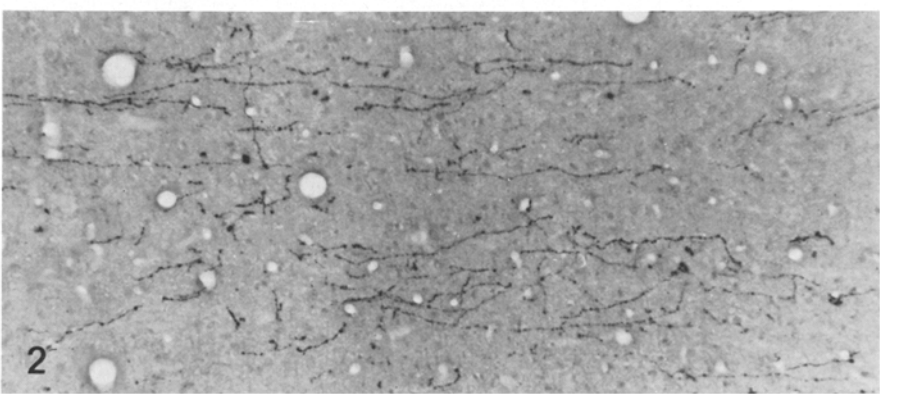
In the granular layer, the density of 5-HT fibers was less than that in the molecular layer; this was particularly evident when examined in frontal sections (Fig. 1). The 5-HT fibers, which were predominantly short and obliquely oriented, showed an irregular distribution. Occasionally, long tangential fibers with relatively large varicosities were seen adjacent to the Purkinje cell layer. The structure of the "mossy rosettes" described by Chan-Palay could not be detected in the neo-, paleo- and archicerebellum. The general appearance of the 5-HT fibers in this layer was similar in both frontal and sagittal sections, but the continuity of the fibers was usually more apparent in the sagittal plane.

In the medullary white matter, 5-HT fibers were rare in frontal sections, but some 5-HT fibers were seen in the medulla of the paleo- and archicerebellum. In sagittal sections, long 5-HT fibers with a few varicosities ran parallel to the medullary bundle and some even entered the granular layer. This was most evident in the archicerebellum.

5-HT fibers were also present in the cerebellar nuclei and formed fine but dense networks in the neuropil of the entire area of each nucleus. These networks were denser in the lateral (nucleus dentatus) and medial nuclei (nucleus fastigii) than in the interpositus nucleus (Fig. 3). In addition, in an area medial to the lateral nucleus, dense 5-HT fibers were seen in frontal sections.

Almost all 5-HT fibers in the cerebellum possessed varicosities (0.5–2.0 μm in diameter) at several μm intervals and were similar to those seen in other areas in the CNS. Few smooth and relatively straight 5-HT fibers were found in the cerebellum.


W
G
P
M
1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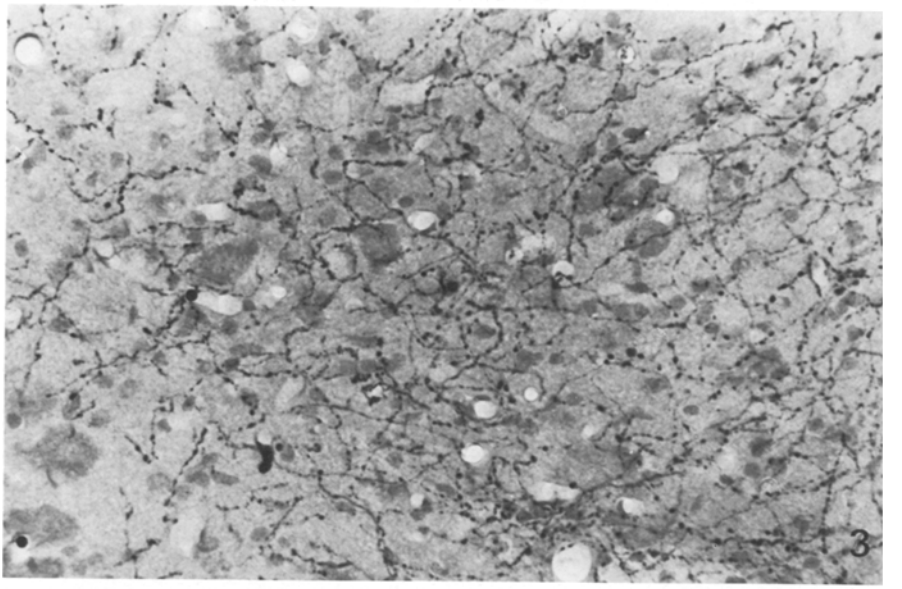


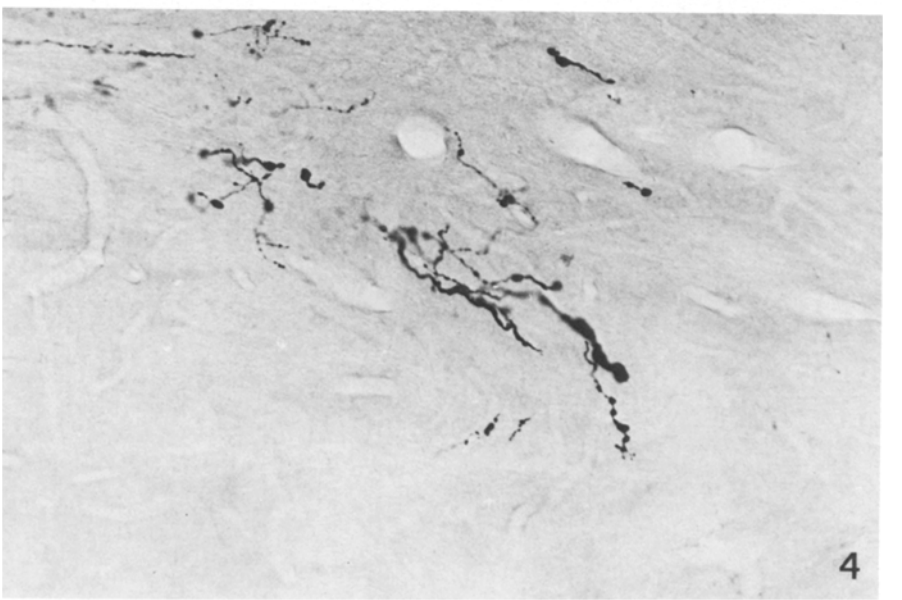
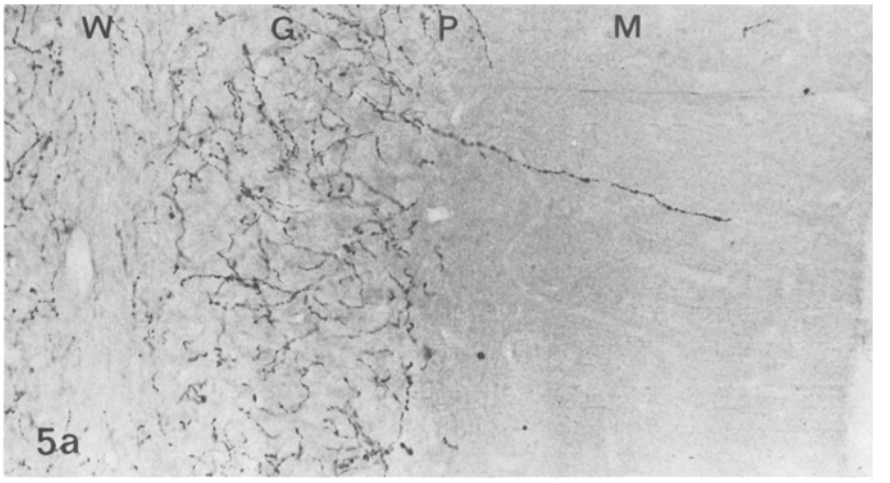


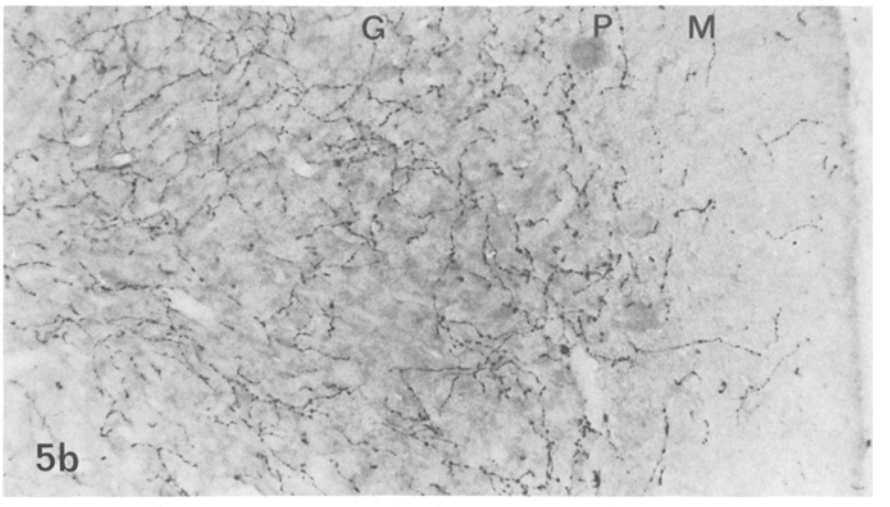
Fig. 1. Vermal zone of the rat cerebellum, frontal section. 5-HT fibers are predominant in the molecular layer but rare in the granular layer. There are no mossy rosettes in the granular layer. *M* molecular layer, *P* Purkinje cell layer, *G* granular layer, *W* white matter. ×260

Fig. 2. Molecular layer of the rat cerebellum, vermal zone, horizontal section. Tangential 5-HT-fibers are numerous ×130

Fig. 3. Numerous fine 5-HT nerve fibers within the nucleus dentatus of the rat, frontal section. ×130

Fig. 4. White matter of rats treated with 5,6-DHT intraventricularly, frontal section. Swollen and degenerated 5-HT nerve fibers in the proximal portion. ×210





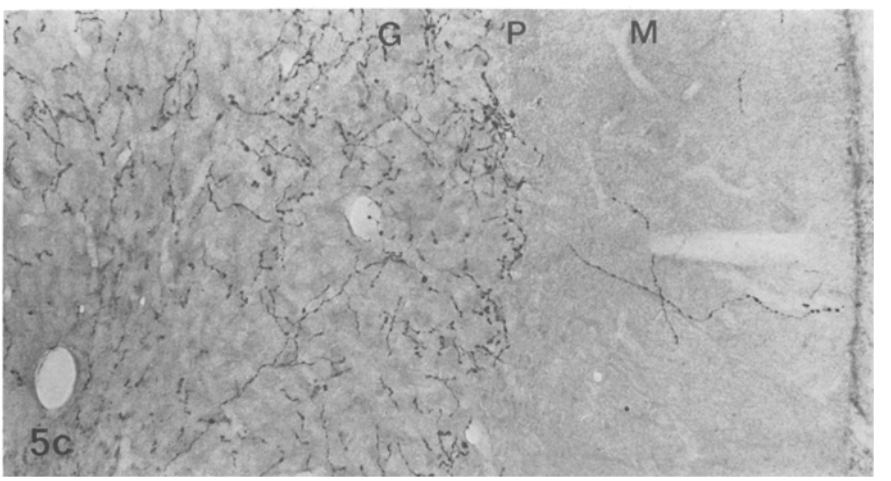


Fig. 5a–c. Cerebellum of the cat, frontal section. Tangential 5-HT fibers are rare in the molecular layer, but in the granular layer a dense reticulum of 5-HT nerve fibers is present. **a**: ×130. In the molecular layer of the flocculus, relatively short tangential, vertical and oblique fibers are seen. **b**: ×130. Some 5-HT nerve fibers reach the leptomeninges. **c**: ×130

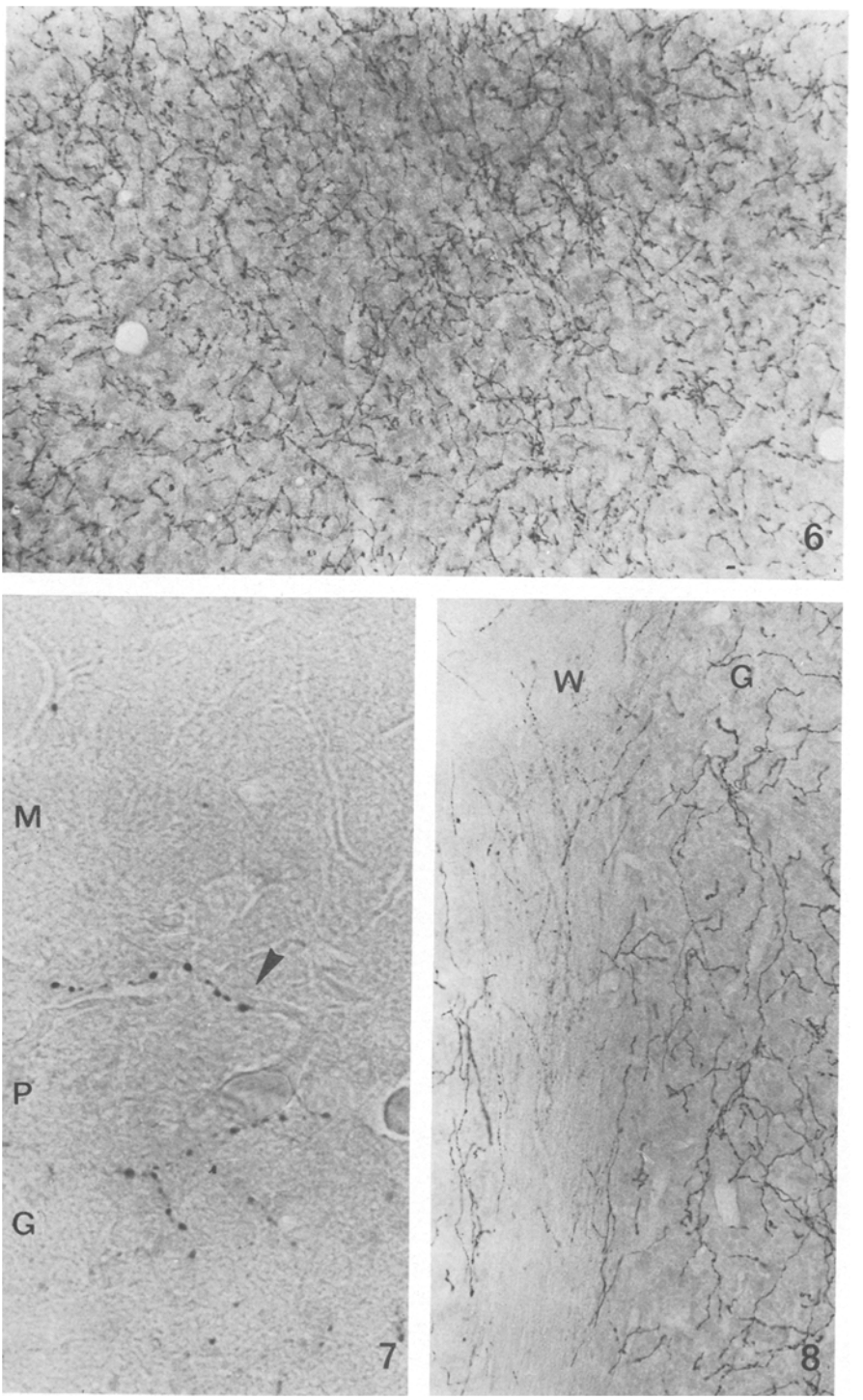


Fig. 6. Granular layer of the cat, horizontal section. Numerous 5-HT nerve fibers form a dense reticulum. ×130

Fig. 7. Cerebellum of the cat, frontal section. Some 5-HT varicosities appear to establish contacts with dendrites of Purkinje cells (*arrow*). ×520 (For abbreviations see Fig. 1)

Fig. 8. Cerebellum of the cat, sagittal section. Long 5-HT nerve fibers in the white matter, dense fiber reticulum in the granular layer. ×130

Cat

In the molecular layer of the cat the density of 5-HT fibers was generally less than in the rat brain (Fig. 5a). In the neocerebellum, the tangential fibers were much fewer than in the rat. These particular fibers sometimes almost reached the surface of the pia mater (Fig. 5c). As seen in the rat, 5-HT fibers possessed varicosities 0.5–2.0 μm in diameter, and some were seen to make direct contact with the dendrites of the Purkinje cell, thereby suggesting the existence of axo-dendritic synapses (Fig. 7).

In contrast to the molecular layer, numerous 5-HT fibers were observed in the granular layer (Fig. 5a–c). These fibers were irregularly distributed within the layer, and the majority ran in an oblique fashion forming a dense reticulum (Fig. 6).

In the medullary white matter, a few 5-HT fibers were seen to extend parallel to the medullary bundle, some branching at right angles to the granular layer (Fig. 8).

In the paraflocculus and flocculus, 5-HT fibers in the molecular layer showed a similar density to that seen in the rat. Relatively short tangential, vertical and oblique fibers were apparent (Fig. 5b). The distribution pattern of 5-HT fibers of the granular layer was essentially identical to that in the vermis.

In all cerebellar nuclei, the 5-HT fibers were distributed in a similar manner to that in the rat.

Pharmacological experiments

An additional immunohistochemical study was carried out in animals pretreated with nialamide, a monoamine-oxidase inhibitor (MAOI). The number of 5-HT fibers slightly increased in all layers, however, the patterns of distribution were fundamentally the same as in the untreated animals.

On the other hand, numerous 5-HT immunoreactive fibers disappeared in animals pretreated with 5,6-dihydroxytryptamine (5,6-DHT). The most extensive change was seen in the superficial cortices; swellings 4–8 μm in diameter suggested the terminal degeneration of 5-HT axons, as observed mainly in the medullary white matter (Fig. 4). These pharmacohistochemical studies confirmed the specificity of the immunohistochemical technique used in the present study.

Discussion

In the present study, details of the distribution of 5-HT-immunoreactive nerve fibers in the cerebellum of the rat and cat were demonstrated without any pharmacological pretreatment of the animals. To visualize 5-HT nerve fibers in areas poor in 5-HT, a specific and sensitive histochemical technique is required. The histofluorescence method that has contributed remarkably to the observation of catecholamine neurons has essentially two disadvantages when used for demonstration of 5-HT neurons: 1) The rapid photodecomposition of β-carboline, the product of 5-HT fluorophore, under the fluorescence microscope; 2) the fact that the method requires pretreatment with MAOI in combination with or without 5-hydroxytryptophan, a precursor of 5-HT, in order to increase 5-HT fluorescence.

The autoradiographic method has also yielded extensive data on both pathways of projections and location of 5-HT neurons (Moore 1981); however, the light-

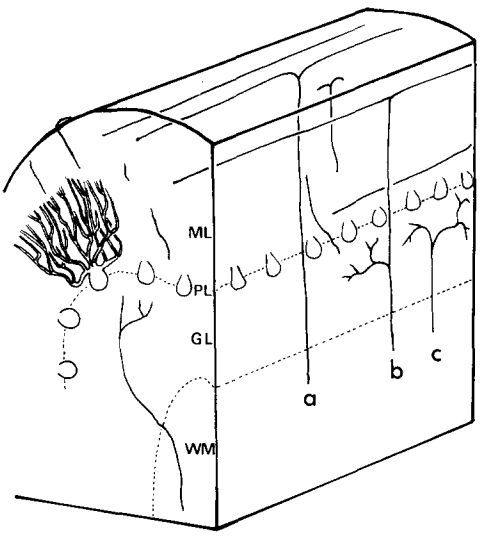


Fig. 9. Schematic representation of typical 5-HT afferents in the rat cerebellum. Tangential "parallel fiber-like 5-HT axons" are not identical with classical "parallel fibers". In the granular layer, "mossy rosettes" are missing. Apparently three types of 5-HT afferents exist (*a, b, c*). *ML* molecular layer, *GL* granular layer, *PL* Purkinje cell layer, *WM* white matter

micrographs leave several questions open to discussion. Moreover, the specificity for identifying 5-HT neurons by this method is sometimes questionable. Tritiated 5-HT can be taken up by nerve cells other than 5-HT neurons, especially when a high concentration is used for intraventricular infusions (Steinbusch 1981). For example, 5-HT-containing neuronal somata in the rat brain as demonstrated by autoradiography (Chan-Palay 1977) differ significantly in their distribution from the pattern obtained by immunohistochemistry (Steinbusch 1981; Takeuchi et al. 1982). In addition, the autoradiographic method is suitable only for study of the periventricular region due to the limitations of penetration and diffusion (Chan-Palay 1975).

Recent technical modifications in the fixation and immunohistochemical staining procedure introduced by Kimura et al. (1981), as well as a high titer of our specific antiserum to 5-HT, enable demonstration of 5-HT neurons in the CNS with a high degree of specificity and sensitivity.

The pattern of the 5-HT innervation of the cerebellum as revealed in this study differs among the cerebellar layers and the animal species. The distribution of 5-HT afferents in the cerebellar cortex of the rat is summarized in Fig. 9. In the molecular layer, the tangential fibers are predominant and are particularly abundant in the superficial layer. A few vertical and oblique fibers are also present. Some of the 5-HT fibers apparently reach the leptomeningial surfaces, as has been observed in other areas of the CNS (unpublished data). The tangential fibers demonstrated in frontal and horizontal sections may be compared to the "parallel fiber-like 5-HT axons" described by Chan-Palay (1975). In the granular layer of the rat cerebellum, relatively short, oblique fibers were seen, but no 5-HT-immunoreactive mossy fiber

rosettes were found in the granular layer of any cerebellar lobules. In addition, varicosities of 5-HT fibers in the granular layer are not always located in the cerebellar islands, as demonstrated by intensely staining structures (eosin body) when using eosin as counterstain. Thus, rosettes in the granular layer may not be present in the cerebellum of untreated animals. The silver grains in the mossy fiber rosettes become visible only after the infusion of tritiated serotonin.

As was also reported by Voogd (1967), 5-HT afferent axons in the cerebellum do not join the classical mossy or climbing fibers. From these data, three possibilities can be deduced regarding the types of 5-HT afferents in the rat cerebellum: 1) 5-HT fibers terminating in the molecular layer without branching *en route* into the granular layer (Fig. 9a); 2) 5-HT fibers dispersed in both the molecular and granular layers (Fig. 9b); 3) 5-HT fibers terminating only in the granuiar layer (Fig. 9c).

The pattern of 5-HT innervation, especially in the molecular layer of the cerebellar cortex of the rat, differs somewhat within the different lobules, despite a widely uniform structure of the cortex. These differences, however apparently do not correlate to the anatomical and phylogenetic subdivisions of the cerebellum. The cerebellar cortex is also classified into several rostro-caudally longitudinal zones according to physiological data (Goodman and Simpson 1961; Voogd 1967). We, therefore, analyzed immunohistochemical data on the basis of physiological classifications. The morphological and functional subdivisions of the rat cerebellum are shown in Fig. 10a, and schematic representations of 5-HT-immunoreactive fibers demonstrated in frontal sections are presented in Fig. 10b, c. The typical pattern of innervation shown in Fig. 9 s observed in the vermal zone of the archicerebellum (Fig. 10A), in the vermal zone of the paleocerebellum (B, D), and in the vermal zone of the neocerebellum (C), all lobules of the paravermal zone (F) and the neocerebellum of the lateral zone, except for areas along the fissura posterior superior (I). On the other hand, in the archicerebellar paravermal zone (the flocculus, G), in the area along the fissura posterior superior (H) and in the paleocerebellar lateral zone (the paraflocculus, J) the pattern of distribution differed greatly from that in the above-mentioned areas. In the latter areas, tangential fibers were seldom found in the molecular layer, while short oblique and vertical fibers were distinct. In addition, long 5-HT fibers were often present, particularly in the medullary white matter of the flocculus and paraflocculus. However, prominent differences were not evident in the sagittal plane among various lobules of the cerebellum.

The diagram proposed by Chan-Palay (1975) to summarize the distribution of 5-HT afferents within the cerebellar cortex includes three systems: (i) mossy fibers, (ii) parallel fiber-like elements, and (iii) a diffuse fiber system. This concept is attractive, however, we failed to find any evidence to support the above-mentioned diagram, except that the parallel fiber-like axons were shown to be serotonergic.

No morphological evidence is available supporting the concept that serotonergic neurons of the brainstem directly terminate on the somata of Purkinje cells. Light microscopically, the varicosities of 5-HT fibers in the cat cerebellum appeared to contact the dendrites of Purkinje cells. Whether or not these varicosities establish synaptic contacts with the dendrites, remains to be determined in further immuno-electron-microscopic investigations.

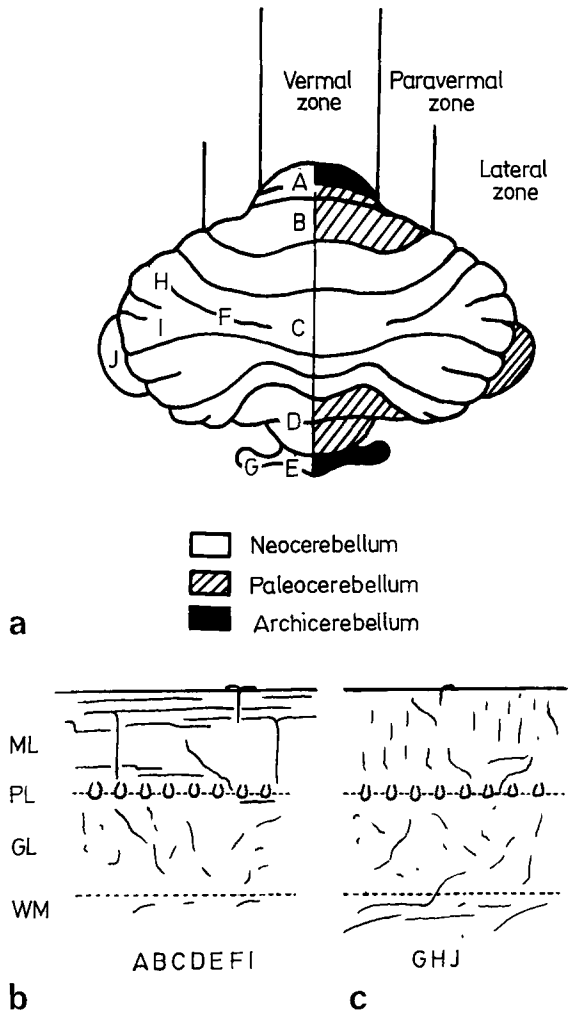


Fig. 10. **a** Schematic representation of morphological and functional subdivisions of rat cerebellum (modified after Goodman and Simpson 1961). **b, c** Schematic representation of 5-HT afferents in the rat cerebellum. A typical distribution pattern is observed in areas A–F and I (see Fig. 10b). In the flocculus (*G*), lobulus semilunaris superior of the lateral zone (particularly along the fissura posterior superior) (*H*) and paraflocculus (*J*), the distribution pattern of 5-HT fibers differs strikingly in the molecular layer (c)

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