Distribution of Serotonergic Neurons in the Central Nervous System:
A Peroxidase-Antiperoxidase Study with Anti-Serotonin Antibodies

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Distribution of serotonin (5-HT) neurons in the central nervous system (CNS) of various vertebrates was investigated with a highly sensitive immunohistochemical technique. Antibodies were raised in rabbits against an antigen prepared by coupling 5-HT to bovine thyroglobulin. 5-HT neurons were found to be distributed more widely and densely than has been heretofore described. Serotonergic neuronal somata are organized according to certain basic patterns, but there are interspecific differences with regard to the distribution of 5-HT fibers. The processes of 5-HT neurons form a dense plexus by ramification and anastomosis in almost all areas of the CNS, including the ventricular surfaces. In the light of our observations, Golgi's reticular theory may have to be revised.

KEY WORDS: Serotonin; Central nervous system; Immunohistochemistry.

Introduction
Current knowledge concerning the distribution of serotonergic neurons and fibers has been obtained by various approaches: a) formaldehyde-induced fluorescence histochemistry; b) biochemical determination of serotonin (5-HT); c) autoradiographic tracing methods; d) autoradiography following superfusion or infusion of tritiated 5-HT; e) immunohistochemistry using antibodies to enzymes, such as tryptophan hydroxylase or 3,4-dihydroxyphenylalanine (DOPA) decarboxylase; and f) immunohistochemical methods using antibodies raised against 5-HT itself. Among these methods, the last is considered to be most reliable, both in sensitivity and specificity.

The distribution of 5-HT in the central nervous system (CNS) of the rat was recently demonstrated by an immunofluorescence method using antibodies to 5-HT (5,7,11,12). We also introduced a highly specific as well as sensitive peroxidase-antiperoxidase (PAP) immunohistochemical technique for detection of 5-HT in the CNS, using antibodies to 5-HT (14), and a series of investigations on the distribution of 5-HT neurons and fibers in various vertebrates has been completed (6,10,13–16).

This article is a summary of a) the distribution of 5-HT neurons in several species; b) the distribution of 5-HT nerve fibers in areas such as the cerebellar cortex, where 5-HT fibers have not been previously demonstrated by immunohistochemistry; and c) the distribution of 5-HT fibers in the cerebral cortex. The concept of axonal ramification of 5-HT neurons and a revision of Golgi's reticular theory are proposed.

Materials and Methods
Preparation of the antibody. Serotonin-creatine sulfate was coupled to bovine thyroglobulin by a formaldehyde-induced reaction. After this procedure, dialysis and a centrifugation step were added. Antibodies were raised in male rabbits. Each was given subcutaneously a mixture prepared by emulsifying 0.5 ml of the antigen-containing solution (2 mg/ml) and complete Freund's adjuvant. Booster injections (0.5 ml of the antigen-containing solution and 0.5 ml incomplete Freund's adjuvant) were given every 10 days. The presence of antibodies in serum samples was evaluated mainly by immunohistochemistry.

Tissue preparation. The animals were anesthetized with Nembutal or ketamine chloride and perfused transectially with ice-cold phosphate-buffered saline (PBS; 0.9% NaCl in 0.1 M PB, pH 7.4) and subsequently with a fixative containing 4% paraformaldehyde.
Serotonin Neurons in Rat, Cat, and Monkey Brains

In the section titled "Distribution," the terms used in this report. "noreactivity" may be preferable, "5-HT neurons, cells, or fibers" are used.

Immunohistochemical staining. A modified PAP method was used. The sections were incubated while freely floating in the primary antisera diluted with PBS at 1:3,200 for 24-48 hr at 4°C. The sections were then stained with 0.3% Triton X-100 for at least 2 days before immunohistochemical staining.

Specificity of the immunohistochemical method used. The specificity of the method was documented as previously described (14). Briefly, the following five criteria were applied to test specificity, as proposed by Steinbusch (12): a) control experiments using preimmune sera; b) absorption with 5-HT; c) attempts to inhibit staining by this method was indeed highly specific for 5-HT.

Results and Discussion

Serotonin Neurons in Rat, Cat, and Monkey Brains

Distribution of the somata of 5-HT neurons was observed from the level of the caudal portion of the nucleus ruber to the level of the decussation of the pyramids in rat, cat, and monkey (Macaca fuscata). In agreement with previous data obtained with the formaldehyde-induced fluorescence method, in most of the 5-HT cells were found within the raphe nuclei, i.e., the nucleus linearis, nucleus raphe dorsalis, nucleus centralis superior, nucleus raphe pontis, nucleus raphe magnus, nucleus raphe obscurus, and nucleus raphe pallidus. However, many 5-HT cells were seen outside the raphe system throughout the lower brain stem. These areas are a) the periaqueductal gray lateral to the nucleus raphe dorsalis (Figure 2); b) the field of the lemniscus lateralis; c) the locus coeruleus complex, including the nucleus parabrachialis medialis and lateralis; d) the nucleus interpeduncularis; e) the pontine reticular formation medial to the nucleus motorius nervi trigemini; f) the pontine reticular formation dorsal to the nucleus olivaris superior; g) the dorsal pontine tegmentum around the genu nervi facialis; h) the area ventral to the nucleus nervi facialis; i) the nucleus interfascicularis hypoglossi; j) the nucleus reticularis lateralis; and k) the area ventral to the pyramid.

These immunohistochemical investigations were carried out on several pharmacologically untreated species (13,14). Therefore, we need not discuss the effects of drugs such as monoamine oxidase inhibitors, which are often used for histochemical detection of 5-HT neuron systems. 5-HT cells had been noted previously in areas other than those mentioned above. For instance, Chan-Palay (3), as well as Beaudet and Descarries (1), used autoradiography and found evidence of 5-HT cells within the diencephalon. Steinbusch performed immunofluorescence histochemical studies and reported a number of 5-HT cells in the area postrema of rats pretreated with colchicine (12). Since the specificity of identification of 5-HT by the autoradiographic method is sometimes questionable (12), the distribution of 5-HT cells revealed in our studies may be a better reflection of their true distribution in the CNS.

Although the population of 5-HT cells in a corresponding area differed among the three species tested, the 5-HT neuron system in the CNS is organized according to rather basic pattern, with some minor differences. In addition to the anatomical stability in the organization of 5-HT cells, the morphological characteristics of 5-HT cells are similar in corresponding areas of each species. For example, prominent bilateral clusters of 5-HT cells were found laterally to the nucleus raphe dorsalis and had large and intensely stained somata with distinct processes in all three species used in our studies. 5-HT neurons are located not only within the specific nuclear structures or the raphe system, but are extensively distributed in further lateral areas. The wide as well as the scattered distribution of 5-HT neurons in different species warrants a reconsideration of the terminology of 5-HT neuron groups (B groups in the rat) introduced by Dahlström and Fuxe (4).

Serotonin Nerve Fibers in the Cerebellum

Little is known of the presence of 5-HT fibers in the cerebellum. Biochemical studies revealed that the cerebellar cortex, as well as nuclei, contains the least amount of 5-HT among
Figures 1–5
The typical pattern of 5-HT distribution mentioned above, which was observed in most portions of the cerebellar cortex, varied somewhat within some lobules, particularly in the molecular layer, despite the uniform structure of the cerebellar cortex. For instance, in the parafocculus and flocculus, tangential fibers were seldom found, whereas short oblique and straight fibers were characteristically observed in these areas. In the vermis, 5-HT axons made up tangential fibers parallel to the pia mater. These may be the same as the “parallel fiber-like 5-HT axons” described by Chan-Palay (2). In the granular layer, the density was less than that observed in the molecular layer, and short oblique fibers were seen. However, varicosities of 5-HT fibers were not always located in the cerebellar islands, revealed as eosin bodies by counterstaining. In the medullary white matter, 5-HT fibers were rare in frontal sections, but a few 5-HT fibers were seen in the medulla of the paleo- and archicerebellum.

The highest density of 5-HT fibers was observed within the granular layer. In the plexiform layer, the density of 5-HT fibers was high, and tangentially oriented fibers were predominantly observed in the molecular layer, and the density of 5-HT fibers in each cortical layer appeared relatively uniform across all phylogenetic subdivisions (neo-, paleo-, and archicerebellum). In frontal sections of the vermis, 5-HT axons made up tangential fibers parallel to the pia mater. These may be the same as the “parallel fiber-like 5-HT axons” described by Chan-Palay (2). In the granular layer, the density was less than that observed in the molecular layer, and short oblique fibers were seen. However, varicosities of 5-HT fibers were not always located in the cerebellar islands, revealed as eosin bodies by counterstaining. In the medullary white matter, 5-HT fibers were rare in frontal sections, but a few 5-HT fibers were seen in the medulla of the paleo- and archicerebellum.

The typical pattern of 5-HT distribution mentioned above, which was observed in most portions of the cerebellar cortex, varied somewhat within some lobules, particularly in the molecular layer, despite the uniform structure of the cerebellar cortex. For instance, in the parafocculus and flocculus, tangential fibers were seldom found, whereas short oblique and vertical fibers were prominent. In the cat, the distribution pattern was quite different. In the molecular layer of the cat cerebellum, only a few 5-HT fibers were found, but dense networks of 5-HT fibers were present in the granular layer (Figure 3). In the cat, 5-HT fibers possessed varicosities 0.5-2.0 µm in diameter, and some were observed to make direct contact with the dendrites of the Purkinje cells. In the monkey, 5-HT fibers displayed a pattern similar to the distribution seen in the cat.

In the cerebellar nuclei, 5-HT fibers were densely distributed, and interspecies differences were not distinct.

5-HT afferent axons in the cerebellum apparently do not join the classical mossy or climbing fibers. Among the three systems—mossy fibers, parallel fiber-like system, and a diffuse system—proposed by Chan-Palay (2), as regards the distribution of 5-HT afferent axons within the cerebellar cortex, according to our observations, only the parallel fiber-like axons appear to be serotonergic.

Serotonin Nerve Fibers in the Cerebral Cortex

In the cerebral cortex of the rat, 5-HT fibers spread throughout all layers, but the manner of distribution of these fibers differs among the neocortex, mesocortex, archicortex, and paleocortex, essentially supporting the findings reported by Liddon et al. (7).

Neocortex. In all layers, 5-HT fibers formed a dense arborizing plexus (Figure 4). Layer I displayed the highest density of 5-HT fibers, most of which were tangentially oriented. From layers II to V, tortuous 5-HT fibers formed a dense plexus and showed almost the same pattern of distribution, both in their high density and variable orientation. In these layers, thick and straight fibers were relatively rare. In layer VI, the majority of 5-HT fibers ran parallel to the boundary of the white matter.

In the rat, the fundamental manner of 5-HT innervation was fairly stable throughout the neocortex, but in the monkey, certain areas of the neocortex (for example, the primary visual cortex) displayed a notable laminar pattern.

Mesocortex (cingulate cortex). In layer I, the density of 5-HT fibers was similar to that of the neocortex, and most ran in a rostrocaudal direction. From layers II to V, 5-HT fibers were relatively sparse and ran almost vertically. Some thick and straight fibers were characteristically observed in these layers of the cingulate cortex. In layer VI, the density of 5-HT fibers was high, and tangentially oriented fibers were predominant.

Archicortex (hippocampus). In the hippocampus, there was a relatively restricted laminar pattern of 5-HT innervation. In the alveus, there were few 5-HT fibers, whereas in the stratum oriens and radiatum, many 5-HT fibers spread out in varied orientations. Between these two layers, the stratum pyramidale, which is composed of densely packed cells, had only a few fibers running vertically or radially among the pyramidal cells. The stratum lacunosum and molecular revealed a very high density of 5-HT fibers. Particularly in the latter, many tangentially oriented fibers and dots, representing frontally sectioned 5-HT fibers, were predominant. Interspecific differences in the distribution pattern of 5-HT fibers in the hippocampus were not evident.

Paleocortex (olfactory bulb). In the rat, 5-HT fibers were present within all layers in the olfactory bulb, except for the olfactory nerve layer, and the restricted laminar patterns of 5-HT innervation were in marked contrast to the uniform pattern of a dense arborizing plexus in the neocortex of the rat. The highest density of 5-HT fibers was observed within the glomeruli. In the plexiform layer, the density of 5-HT fibers was moderately low and the orientation variable. There was no specific relation of the 5-HT fibers to the mitral cell layer. In the granular layer, some 5-HT fibers ran parallel to the laminae of granular cells, whereas others penetrated each lamina to the outer portion, with ramifications.

In the olfactory bulb, there were also some species-related differences in the pattern of 5-HT distribution within the respective layers. The most notable difference appeared in the glomerular layer. In the rat and cat, a very dense, diffuse plexus of 5-HT fibers was found in the glomerulus, whereas in the monkey the distribution of 5-HT fibers was sparse and uneven.
Axonal Ramifications of 5-HT Neuron

We wish to propose a new concept concerning the morphology of 5-HT neurons, a conclusion we reached following immunohistochemical investigations on 5-HT neuron systems in the CNS of various vertebrates (9).

5-HT fibers repeatedly ramify and anastomose, to form a dense and extensive reticulum that is distributed throughout the CNS. This reticulum is apparently different from the so-called "telodendron." 5-HT fibers are varicose, and the varicosities are usually 0.5–4.0 µm in diameter. By light microscopic examination, these varicose fibers anastomose to form ring structures of various shapes and sizes. (Electron microscopy reveals no 5-HT-positive structures within the intercellular spaces.) The ring-like structures formed by 5-HT fibers constitute an axonal reticulum that is present in almost all areas of the CNS. In the area of inferior colliculus, a ring of 5-HT fibers is no more than 8 µm in diameter (Figure 5). In the supraependymal area, there is a plexus of 5-HT fibers (Figure 1). The 5-HT fibers penetrate the ependymal layer and form a supraependymal 5-HT plexus on all surfaces of the ventricles. However, density of the 5-HT fibers differs to some extent among various areas. In some areas where the supraependymal 5-HT plexus is dense, a terminal bar-like ring of 5-HT fibers is observed. In such cases, the diameter of a ring is about 15–18 µm, corresponding to the width of the ependymal cells. Our immunohistochemical technique was also applied to the serotonergic raphe nucleus of the newborn rat and mouse in vitro. Cultures of tissue from both species demonstrate a dense 5-HT immunoreactive plexus, indicating the existence of a syncytial network of 5-HT neurons (17). Judging from the morphological features of 5-HT neurons demonstrated by our immunohistochemical technique, Golgi's reticular theory may have to be revised.

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Literature Cited