EXPERIMENTAL AND MORPHOLOGICAL STUDY OF THE INNERVATION OF CEREBRAL BLOOD VESSELS

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Fluorescence- and electron-microscopic study was performed on nerve terminals close to cerebral blood vessels. For work with the electron microscope, subdural perfusion fixation with potassium permanganate was carried out. Some aminergic terminal boutons containing large and small cored vesicles were observed contiguous to blood vessels in the cerebral cortex of the rat deprived of the bilateral superior cervical ganglion. Since these terminals are found in the rat after bilateral superior cervical ganglion excision, they probably originate from central catecholaminergic neurons in the brain stem. In addition to aminergic terminal boutons, non-aminergic nerve terminals containing non-cored vesicles also ended in the capillaries. These findings suggest that central aminergic and non-aminergic neurons might play some role in cerebral blood flow regulation. On the other hand, two types of axon terminal, aminergic and non-aminergic, were observed in the adventitia of the basilar artery. Observation of separated sections would seem to indicate that sometimes two types of nerve fibers make contact with each other. During observation of serial sections, however, fusions of synaptic vesicles in the two types of nerve terminals were observed at membrane apposing muscle cells respectively. These facts suggest that two different types of axon terminals independently release "transmitters" toward muscle cells.

The use of histofluorescence methods (4, 16, 19) has well demonstrated that sympathetic catecholamine(CA) nerves follow the pial arteries. Furthermore, several authors have found that central CA fibers from the CA neurons in the brain stem which have also been visualized by fluorescence histochemistry closely follow the path of small blood vessels in the brain. It has consequently been suggested that the central CA neurons play a significant role in the local microregulation of the cerebral blood flow (1, 2, 14). However, the studies lack direct proof of this.

On the other hand, the potassium permanganate fixation method developed by Richardson (22), Hökfelt (6) and Ochi et al. (17) has proved most suitable for the detection of granular vesicles in the sympathetic CA nerve terminals. Demonstration of CA nerve terminals in the central nervous system by potassium permanganate has been unsuccessful presumably because of their paucity of CA content. We modified this fixation method to suit the central nervous system and succeeded in identifying CA nerve terminals originating from the brain stem (15).

First of all, in order to determine whether central CA neurons really take part in the control of the cerebral blood flow, we attempted to obtain direct proof of central catecholaminergic innervation of cerebral blood vessels under electron microscopy by using the potassium permanganate method of perfusion fixation. We then examined the basilar arteries, which were richly innervated by peripheral CA neurons, under both electron and fluorescence microscopy. These results were compared with each other.

MATERIALS AND METHODS

Tissues from 80 adult albino rats weighing 200 g were observed by histofluorescence and electron microscopy. The forty of them in which the superior cervical ganglion(SCG) had been excised bilaterally 2 weeks previously were subjected for study of the central CA system. For the histofluorescence study, brains were analyzed with the glyoxylic acid(GA)-formaldehyde fluorescence method (11) as modified by Kimura and Tohyama (9). Animals were first perfused at constant flow (50–60 ml/min) for 20 sec with ice cold Krebs-Ringer bicarbonate buffer (with 1.8 g/l glucose added) which was saturated with a mixture of 95% O₂ and 5% CO₂. After perfusing the buffer solution we perfused the animals with an ice cold GA solution (pH 7.4) at constant flow (35 ml/min) for 7 min. This was prepared by dissolving 2% GA in a Krebs-Ringer bicarbonate buffer (initial pH 7.4) (with 1.8 g/l glucose added). The buffer was saturated with a mixture of 95% O₂ and 5% CO₂. The brains were rapidly taken out and immersed in 2% ice cold GA solution for 5 min. The specimens were then rinsed in Krebs-Ringer solution for 5 min, frozen in liquid propane, freeze dried for several days, and, finally, treated with paraformaldehyde for 1 hr at 80°C. For the histofluorescence study of the basilar artery, arteries were observed with the use of the GA immersion method (12).

For electron microscopic observation, subdural perfusion fixation with potassium permanganate was carried out as described by Maeda et al. (15). After perfusion of potassium permanganate solution, the tissue slices were dissected out and refixed in the same solution. The tissue slices were rinsed in Ringer solution, stained *en bloc* with 1% uranyl acetate and dehydrated and embedded in Epon 812. Ultrathin sections were made on a Porter-Blum ultratome and stained with lead citrate. The sections were examined under an HU-12 electron microscope. Observation with the electron microscope was for the most part carried out on serial sections.

RESULTS

- I) Innervation of cerebral capillaries and arterioles
- a) Fluorescence microscopy

Two weeks after bilateral excision of the SCG, intensely fluorescent nerves in the adventitia of the pial arteries had disappeared and faint green fluorescent nerve terminals of varicose appearance were sometimes found contiguous to small blood vessels in the cerebral cortex. The fluorescent fibers approached the vessels, followed them for a small distance and then left them (Fig. 1) in the same manner

FIG. 1. Fluorescence micrograph of the cerebral cortex of the rat with excision of bilateral superior cervical ganglion. Faint green fluorescent varicose fibers are visible contiguous to small blood vessel (arrow). $\times 90$

as described by Edvinsson et al. (2). Therefore, the fluorescent nerve terminals close to the intracerebral small arteries, which were resistant to the bilateral excision of SCG, must be those originating from central CA neurons and having no relation to the sympathetic nervous system.

b) Electron microscopy

Varicose nerve terminals containing large and small cored vesicles were often observed in various areas of the cerebral cortex (somatomotor area) of 30 rats deprived of the bilateral SCG. Most of them showed no close relationship to the cerebral blood vessels. However, detailed observation under relatively high magnification revealed that there exist several aminergic terminal boutons contiguous to blood vessels in the brain tissue. The wall of these blood vessels usually had no muscle layers and therefore they were considered to be capillaries. It should be stressed that no aminergic terminal was found in the adventitia of arteries with muscle layer. These aminergic terminals, demonstrated with potassium permanganate, showed axonal bulging measured 500 to 800 nm in diameter (Fig. 2), large cored vesicles of 80–120 nm (arrow in Fig. 2–4) and small cored vesicles of 40–60 nm which contained central electron precipitate attributable to noradrenaline (NA) as suggested by Hökfelt and Jonsson (7). Serial sections showed that terminal boutons containing cored vesicles first appear in the surrounding perivascular end-

FIG. 2. Serial electron micrograph of the capillary in the cerebral cortex of the rat deprived of the bilateral superior cervical ganglion. Aminergic terminal bouton (*) containing large and small cored vesicles is seen contiguous to the capillary. In Figs. 2–1 to 2–7 an aminergic nerve terminal (*) gradually approaches the wall of the capillary and in Fig. 2–8 this terminal is finally located just contiguous to the basement membrane(b) of the capillary. Arrow in Fig. 2–4 indicates a large cored vesicle in the terminal. A missed section is presented by Fig. 2–3. A: astrocyte; E: endothelium; L: lumen of the capillary. $\times 21,000$

2-3

2-4

FIG. 3. Electron micrograph of the capillary in the cerebral cortex of the rat.
 3-a Varicosed nerve terminals (*) containing non-cored vesicles penetrate the end-feet of perivascular astrocyte (A) and approach the capillary. $\times 32,000$
 3-b Non-aminergic nerve terminal (*) shows contact with the basement membrane (B) of the capillary. E: endothelium; P: pericyte. $\times 41,000$

feet of the astrocyte as they approach the capillary wall, penetrating the cytoplasm of the astrocyte. Finally, these terminals were located contiguous to the basement membrane of the capillary (Fig. 2-8). Small cored vesicles found in the terminal sometimes aggregated close to the membrane apposite the basement membrane of the capillary (Fig. 2-8).

In addition to aminergic nerve terminals, terminals containing non-cored

FIG. 4. Fluorescence micrograph of the superior mesenteric artery (4-a) and the basilar artery (4-b) of the rat. The direction of noradrenaline fibers in the basilar artery (4-b) is mainly spiral, while those found along the mesenteric artery have a mesh-like appearance (4-a). $\times 60$

vesicles were also observed in the end-feet of the astrocyte which surrounded the capillary (Fig. 3-a). These non-aminergic terminals were also of a varicose appearance and penetrated the end-feet of the perivascular astrocyte, gradually approaching the basement membrane of the capillary (Fig. 3-a). Ultimately, the terminals touched the basement membrane of the capillary (Fig. 3-b).

II) Innervation of the basilar artery

a) Fluorescence microscopy

A very dense network of fluorescent nerves was found in the basilar artery (Fig. 4-b). The plexus was somewhat less dense in the basilar artery than in the

FIG. 5. Electron micrograph of the basilar artery. 5-a Two types of axon terminal, aminergic (a) containing cored vesicles and non-aminergic (c) containing non-cored vesicles, were observed in the adventitia, enclosed with the same Schwann's cell (s). $\times 34,000$ 5-b Axon terminals (a) containing cored vesicles were interconnected with thin fibers to form varicose terminal fibers (arrows), as observed under fluorescence microscopy (cf. Fig. 4-b). $\times 27,000$ 5-c Aminergic terminals (a) often show a close contact with non-aminergic axon terminals (c). However, fusion of vesicles cannot be found between contact membranes. m: muscle cell. $\times 27,000$

FIG. 6. Electron micrograph of the basilar artery. The fusion of the vesicles in the aminergic terminals (a) was observed at the membrane apposite the muscle cell (m) (thin arrows in Fig. 6-a and 6-b). In Fig. 6-b synaptic vesicles in non-aminergic terminal (c) also seem to aggregate toward muscle cells and some of them became attached to the contact membrane (thick arrows). $\times 34,000$

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FIG. 7. Schematic illustration of central and peripheral dual innervation of cerebral blood vessels. A: astrocyte; B: basement membrane; E: endothelium; F: fibrocyte; LC: locus coeruleus; M: muscle cell; SCG: superior cervical ganglion.

superior mesenteric artery (Fig. 4-a). Furthermore, there is a marked difference in the direction of their nerve fibers. The direction of adrenergic fibers in the basilar artery is mainly spiral, while that in the mesenteric artery is mesh-like.

b) Electron microscopy

Two types of axon terminal were observed in the adventitia of the basilar artery. The first contained large and small cored vesicles, while the second contained non-cored vesicles (Fig. 5-a). These nerve terminals, both aminergic and non-aminergic, were visible in outer layer of adventitia, enclosed by a Schwann's cell, or in inner layer close to muscle cell without a Schwann's cell. The terminal boutons containing small and large cored vesicles were interconnected with thin fibers to form varicose terminal fibers which run spirally (Fig. 5-b). The bulge containing the cored vesicles sometimes showed a close contact with axon terminals in which non-cored spherical vesicles were present (Fig. 5-c). The fusion of the vesicles in the NA terminals was observed at the membrane apposite the muscle cell and it appeared that their content was released to the muscle cell (Fig. 6).

However, it should be noted that there was no cored vesicle that touched the membrane apposite the non-aminergic terminals. In non-aminergic terminals, synaptic vesicles seem to aggregate toward smooth muscle cells. It is likely that both nerve terminals independently release "transmitters" to smooth muscle cells of the basilar artery.

DISCUSSION

Numerous attempts have been made to study the autonomic nerves accompanying the cerebral vessels (4, 5, 20). Since Willis first found nerve fibers on the anterior and posterior cerebral arteries, many authors have demonstrated that two types of axon terminals end in the adventitia of the cerebral arteries (10, 15, 18). One of them, containing cored vesicles, is thought to be adrenergic and to originate from SCG. The second, containing non-cored vesicles, is probably a cholinergic terminal of unknown origin. The role of these nerve fibers accompanying cerebral blood vessels is controversial. Recent pharmacological, physiological and anatomical data suggest the possibility of axo-axonic interaction such as presynaptic inhibition between aminergic and cholinergic nerve fibers (3, 24). However, with the use of electron microscopy we observed that cored vesicles in the NA terminals abutting on the membrane apposite the muscle cell of the basilar artery, and synaptic vesicles in the non-aminergic (probably cholinergic) terminals aggregated toward muscle cell. These findings suggest that "transmitters" in both type of terminals are independently released to muscle cells.

Although several authors have discussed the central CA neuron system (1, 2, 14), insufficient attention has been paid to this area. With the use of electron microscopy Rennels and Nelson demonstrated axon terminals close to capillary pericytes in the feline brain (21). However, even in the investigations which suggested that the central CA neuron system could possess important cerebro-vascular innervation, direct proof was lacking (1, 2, 14). With the use of electron microscopy we succeeded in demonstrating aminergic nerve terminals which abut on the basement membrane of the cerebral blood capillaries. Since they are found in the rat subjected to bilateral SCG excision these terminals probably originate from central CA neurons. The terminals demonstrable in the brain are considered to be mostly noradrenergic. NA fibers to the cerebral cortex in the rats are exclusively supplied by the locus coeruleus (LC) (13, 18, 23, 25). Therefore, the NA nerve terminals must originate from LC. The cerebral blood capillaries in other regions of the brain might be supplied by other neurons which innervate such regions.

Although the physiological role of NA cannot be surmised from morphology, the present study suggests that the central NA neurons might play some role in the local microregulation of the cerebral blood flow. The sympathetic CA fibers following the cerebral arteriole are much more frequently observed in lower vertebrates, whose aminergic neuron system remains immature in the brain (26, 27, 28), than in the case of mammals. Therefore, it is likely that the sympathetic system might play a more important role in the regulation of blood flow in the lower vertebrate brain while during the evolutionary process towards mammals the local cerebral blood flow regulation may be influenced by the central CA

neurons together with the sympathetic nerves.

Non-aminergic nerve terminals with empty vesicles also made contact with cerebral blood capillaries. These terminals come from brain tissue, penetrate the astrocyte and touch the basement membrane of the capillaries. They are probably cholinergic nerve terminals originating from unknown cholinergic sites in the central nervous system.

Finally, it is very interesting that cerebral blood vessels have both central and peripheral dual innervation of aminergic and cholinergic fibers. The innervation of the cerebral blood vessels is schematically drawn in Fig. 7.

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HISTOCHEMICAL STUDY OF THE CENTRAL MONOAMINE NEURON SYSTEMS IN THE TORTICOLLIS OF CAT

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Spasmodic torticollis is a tonic and phasic involuntary contraction of neck muscles, the etiology of which is unknown. Foltz et al (3) and Mizawa (5) re-

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