Dual Adrenergic and Cholinergic Innervation of the Cerebral Arteries of the Rat

AN ULTRASTRUCTURAL STUDY

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ABSTRACT

The innervation of the anterior cerebral artery of the rat was examined by electron microscopy and by the fluorescence method for localizing adrenergic nerves. Two groups of axon bundles were associated with the artery: one at the outer margin of the adventitia (periadventitial bundles) and the other within the adventitia or at the adventitia-media border (adventitial bundles). Periadventitial bundles consisted of nonmyelinated axons (0.1-2 μm diam), some of which contained synaptic vesicles; in some bundles, myelinated axons were seen. Adventitial axons often contained many synaptic vesicles and were free of Schwann cell sheath in areas apposed to smooth muscle cells. The closest observed approach of axon to muscle cell was 800 A. No nerve fibers penetrated the medial muscle. After fixation with glutaraldehyde plus osmium, large (1000 A) granular and small (500 A) agranular vesicles were seen within many axon profiles. Small granular vesicles were rare. After permanganate fixation, terminal axons contained (besides large granular vesicles) either predominantly small granular vesicles or exclusively small agranular vesicles. Two days after sympathetic denervation, no axons containing small granular vesicles and no fluorescent fibers were seen. Adrenergic fibers were readily identified after injection of rats with 6-hydroxydopamine; small vesicles of adrenergic axons contained highly opaque granular cores, even in osmium-fixed material. Axons containing small agranular vesicles after 6-hydroxydopamine were considered cholinergic. The density of granulation of the large vesicles of adrenergic, but not cholinergic, axons was considerably enhanced following 6-hydroxydopamine. Both adrenergic and cholinergic axons come into close relationship with smooth muscle cells.

ADDITIONAL KEY WORDS autonomic innervation of cerebral arteries axon bundle grouping osmium fixation sympathetic denervation

There has been considerable controversy concerning the motor innervation of cerebral arteries. Bayliss et al. (1) and Hill and MacLeod (2) reported no evidence of any vascular response suggesting the existence of vasomotor nerves supplying the vessels of the brain. Dunke and Schmidt (3) also found little effect of sympathetic stimulation on cerebral blood flow, as did Carlyle and Grayson (4), who concluded that non-nervous autoregulation is the most important factor in the control of cerebral blood flow. The view of these authors (1-4) and others that vasomotor nerves are of minor importance in the regulation of cerebral blood flow has been supported in recent reviews (5-7), but these conclusions have been recently challenged by James et al. (8), who implicated vasomotor nerves in the responses of cerebral vessels to changes in blood CO2 levels. Earlier than this, Hirthle (9) and Forbes and Cobb (10) had.
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observed clear responses of cerebral arteries to motor nerve stimulation. Forbes and Cobb observed a constriction of cerebral arteries in response to sympathetic stimulation and a dilatation, which was blocked by atropine, in response to parasympathetic stimulation. Meyer et al (11), using a preparation similar to that of Dumke and Schmidt (3), recently observed a 22 to 30% reduction in internal carotid blood flow when the cervical sympathetic nerve was stimulated.

By light microscopy, a dual sympathetic and parasympathetic innervation of cerebral arteries was indicated by Chorobski and Penfield (12), who traced nerve fibers from both the facial nerve and the superior cervical ganglion to these vessels. More recent studies have shown that noradrenaline is associated with nerve fibers in the adventitia of cerebral arteries (13, 14). These fibers degenerate after removal of the superior cervical ganglion (13, 14). However, a large number of fibers survive cervical sympathectomy (12); they are probably parasympathetic, cholinergic fibers arising from cranial nerves (10, 12) and some sensory fibers which accompany the cerebral arteries (12, 15). Acetylcholinesterase is associated with some of the nerve fibers of the cerebral arteries (16).

The electron microscope makes it possible to examine the relationship of motor nerves to their effectors. Pease and Molinari (17) studied the ultrastructure of the cerebral arteries in cat and monkey and concluded that the separation of the axons from the smooth muscle was too great for functional transmission to be likely. Sarnaastinghe (18) and Sato (19) both examined the innervation of the cerebral arteries in the rat, and although they reported close neuromuscular approaches, they did not distinguish sympathetic from parasympathetic fibers. Dahl and Nelson (20) identified adrenergic fibers in the adventitia of human cerebral arteries on the basis of the inclusion of granular vesicles. The source of these fibers was not examined.

In the present study, the electron microscope was used to examine the innervation of the anterior cerebral artery of rats. Various fixation techniques, combined with sympathetic denervation, were used to distinguish sympathetic and parasympathetic fibers. In addition, 6-hydroxydopamine (6-OHDA), which displaces noradrenaline from adrenergic axons and causes a specific, intense granulation of the vesicles in these axons (21-
A periadventitial nerve bundle consisting of nonmyelinated fibers of small diameter and a single myelinated axon (M). The bundle is surrounded by a perineural sheath (P), which also encloses some collagen (C). The axons are accompanied by Schwann cells (SC). Fixation in osmium; calibration, 1µ.

Materials and Methods

Rats were decapitated under ether anesthesia and the cranium was opened. The brain, together with the circle of Willis, was removed, and the anterior cerebral arteries were prepared for either fluorescent histochemistry or electron microscopy. The superior cervical ganglia were removed from 40 rats. The anterior cerebral arteries from these rats were examined 18, 24, 28, 30, 32, 36, 42 and 48 hours and 4 and 6 days after the operation. The effectiveness of the operation was always checked by fluorescent histochemical examination of arterial segments adjacent to those taken for electron microscopy.


Fluorescence Histochemistry

The vessels were examined both in section and in whole-mount stretch preparations. Tissue to be examined in sections was frozen in liquid propane and then placed in a freeze-drying unit for 2 days. The tissue blocks were treated for 1 hour in formaldehyde vapor (relative humidity 70%) at 80°C and then embedded in paraffin. Sections (10µ) were examined with a fluorescence microscope. Whole-mount preparations were stretched on glass slides and air-dried at room temperature before treatment with formaldehyde.

Electron Microscopy

Most of the material, from both control and operated animals, was fixed in 2% glutaraldehyde at 4°C in 0.1M cacodylate buffer at pH 7.3 for 2 hours. After washing for at least 4 hours in 0.1M...
An adventitial nerve bundle containing nonmyelinated axons. The bundle passes close to the outermost smooth muscle cells (SM) of the artery. Some axon profiles contain exclusively small (500 Å) agranular vesicles (N₁), others (N₂) contain both small, agranular vesicles and large (1000 Å) granular vesicles. There are areas free of intervening Schwann cell between some axon profiles and the adjacent muscle cells. Glutaraldehyde-osmium fixation; calibration, 0.5μ.

Vessels from rats that were injected with 250 mg/kg of 6-hydroxydopamine (6-OHDA) 1 hour before they were killed were examined with the electron microscope to differentiate sympathetic and parasympathetic fibers. These specimens were fixed in 1% OsO₄ for 2 hours or with glutaraldehyde followed by osmium (see above).

Thin sections were cut with a Huxley ultramicrotome, stained with uranyl acetate and lead hydroxide (26) and examined with an Hitachi 11 B electron microscope.

Results

FLUORESCENCE HISTOCHEMISTRY

After vessels were incubated in formaldehyde vapor at 80°C for 1 hour, green fluorescent fibers were seen on the cerebral arteries. The fibers were not seen in nonincubated specimens or after rehydration of the tissue. No fluorescent fibers were seen within the medial musculature of the arteries. Two groups of fluorescent nerves surrounded the arteries (Fig. 1A), one within the adventitia or at the adventitia-media border and the other at the outer limit of the adventitia. These are referred to below as the adventitial and periadventitial bundles, respectively. The periadventitial nerve bundles were larger and fewer in the two groups could also be seen in stretch.
Granulation of small vesicles. After glutaraldehyde and osmium fixation some axon profiles include small (500 Å) vesicles containing fine granules (arrows). These profiles contain predominantly small, agranular vesicles and some large, granular vesicles. The granules in the large vesicles vary in size and opacity. Calibration, 0.5 μ.

preparations by using the microscope focus to follow the nerves. Whole-mount preparations of the arteries showed that the fluorescent fibers formed a meshlike plexus over the entire length of the anterior cerebral artery (Fig. 1B).

After removal of both superior cervical ganglia, the fluorescent nerve fibers gradually disappeared from the cerebral arteries. No change in the adrenergic innervation was detected at 18 or 24 hours. The decrease in the fluorescent innervation was detectable after about 26 hours and all the fluorescent nerve fibers had disappeared by about 32 hours. Two days after the removal of both superior cervical ganglia, no fluorescent fibers were observed along the anterior cerebral arteries (Fig. 1C, D).

**ELECTRON MICROSCOPY**

**Osmium and Glutaraldehyde Fixation.**—The periadventitial bundles were enclosed in a perineural sheath (Fig. 2). Most of the axons in these bundles were small, 0.1 to 2 μ in diameter, and unmyelinated. However, a few myelinated fibers were also seen in some of the periadventitial bundles (Fig. 2). Both myelinated and nonmyelinated axons contained tubules, neurofilaments and mitochondria. Some axons of the periadventitial bundles contained vesicles. Many smaller bundles of nerve fibers branched from the periadventitial bundles and passed inward toward the medial muscle coat.

The adventitial nerve bundles were generally accompanied by Schwann cells, although axons devoid of a Schwann sheath, or only partly surrounded, were seen (Fig. 3). Some myelinated axons ran singly in the adventitia. Fewer myelinated axons were seen in the adventitia than in the periadventitial bundles. Those areas which were partly devoid of Schwann cell covering often contained many synaptic vesicles (Fig. 3). As they approached the muscle layer, a greater proportion of the axons had the appearance of terminal fibers, i.e., axons devoid of Schwann sheath and containing synaptic vesicles and mitochondria. The closest approach of nerve fibers to muscle cells observed was 800 Å (Fig. 8). There was always a basement membrane intervening between the axon and muscle cell membranes. The muscle cell surface at the closest approach of the nerve fibers showed no synaptic specialization. These axons were particularly crowded with synaptic vesicles and mitochondria. Small axons containing neurotubules but no synaptic vesicles were also seen close to the muscle within adventitial nerve bundles. These probably represent sections through intervaricose regions of the terminal fibers (15).

With osmium or glutaraldehyde fixation, two types of axon terminal could be distinguished according to the nature of the vesicle population. Terminals of the first type con-
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A small adventitial nerve from the anterior cerebral artery of a rat killed 18 hours after bilateral removal of the superior cervical ganglia. One axon (N1) is at an early stage of degeneration. The mitochondrion (M) and the vesicles are still recognizable. The cytoplasm has darkened considerably. A second fiber (N2) contains some small granulated vesicles but does not yet show definite sign of degeneration. The intact fiber (N3) contains only small agranular vesicles. Calibration, 0.5μ.

Intact nerve profiles containing small agranular vesicles and sometimes also large granular vesicles were often enclosed in the same Schwann cell sheath as the osmiophilic remains of degenerated sympathetic fibers. There were a few unusual axons in the adventitia, with many small mitochondria and electron-opaque bodies in their cytoplasm (Fig. 6). The electron-dense bodies in these axons were about 1000 to 5000 A in diameter. They often contained membranous structures and were membrane bound. Although the axons were observed as close to the smooth muscle cells as 1μ, they seldom contained synaptic vesicles. They were generally accompanied by Schwann cells, but were sometimes

FIGURE 5
A small adventitial nerve from the anterior cerebral artery of a rat killed 18 hours after bilateral removal of the superior cervical ganglia. One axon (N1) is at an early stage of degeneration. The mitochondrion (M) and the vesicles are still recognizable. The cytoplasm has darkened considerably. A second fiber (N2) contains some small granulated vesicles but does not yet show definite sign of degeneration. The intact fiber (N3) contains only small agranular vesicles. Calibration, 0.5μ.
An unusual axon in the adventitia contains an accumulation of mitochondria and large osmiophilic bodies. Few vesicles can be observed. This axon is close to a muscle cell (M) and is not accompanied by a Schwann cell. Calibration, 0.5 μm.

These axons were still seen after sympathetic denervation.

Permanganate Fixation.—Fixation with potassium permanganate clearly distinguished two populations of small vesicles (Fig. 7): those with an electron-lucent interior and those with a distinct electron-dense core. On the basis of these differences, the terminal axons were easily divided into two classes. In about 50% of the profiles containing small synaptic vesicles, the vesicles were exclusively agranular and in others they were predominantly granular. There were large granular vesicles in both types of axon. Frequently, the same Schwann cell sheath contained axons of both types (Fig. 7). Axon profiles containing small granular vesicles were also in the
Vesicle types after potassium permanganate fixation. Two axon profiles are ensheathed in the same Schwann cell (SC). Nearly all of the small vesicles in one axon profile (N1) contain granular cores, while the small vesicles in the other axon profile (N2) are exclusively agranular. Both axons are devoid of Schwann cell where they oppose the smooth muscle cell (SM). Calibration, 0.5μm.

Appearance after 6-Hydroxydopamine.—In tissue from rats injected with 6-OHDA 1 hour before sacrifice, almost all the small vesicles of about 50% of the terminal axon profiles had dense cores (Fig. 9). Adjacent axons often contained a large number of small vesicles, all of which were agranular. No other class of nerve ending was seen. The granulated vesicles were readily identified after fixation with osmium or glutaraldehyde plus osmium from animals treated with 6-OHDA. Dense-cored vesicles were observed in nerves of the periadventitial bundles and in those close to smooth muscle cells. In axons containing small granular vesicles after 6-OHDA treatment, the granulation of the large vesicles was more prominent. There was no apparent change in the large granular vesicles in fibers whose small vesicles remained agranular after treatment with 6-OHDA.

No small granular vesicles or large vesicles whose cores showed increased opacity were seen in the vascular nerves of sympathecto-
Close approach of a single axon to the smooth muscle. An axon containing only small vesicles of the agranular type is seen close to a smooth muscle cell (SM) of the media of the artery. Basement membrane intervenes between axon and muscle cell membrane. SN = Schwann cell nucleus. Potassium permanganate fixation; calibration: 0.5 μm.

Discussion

This work has clearly shown a dual adrenergic and nonadrenergic innervation of the anterior cerebral arteries of the rat. Two types of nerve fiber can be distinguished by their vesicle inclusions in tissue fixed in permanganate or, after treatment with 6-OHDA, in osmium or glutaraldehyde. The first type contained many small granular vesicles and degenerated after cervical sympathectomy. Fluorescent, noradrenaline-containing fibers were detected around the cerebral arteries; after sympathectomy, these fibers also degenerated. This suggests that the axons containing small granular vesicles are adrenergic. Previous studies on organs innervated by adrenergic nerves have also indicated that the small granular vesicles are sites of storage of noradrenaline (27–30). The second type of fiber contained small vesicles that were exclusively agranular and a few large granular vesicles. These fibers were not affected by sympathetic denervation. In this situation, intact axons, which were observed after the degeneration of adrenergic nerves and contained exclusively small, agranular vesicles, can be regarded as cholinergic (31, 32). These probably enter the cranial principally with the facial nerve, as described by Chorobski and Penfield (12). Both adrenergic and cholinergic axons were observed within 1000 Å of the outer muscle cells of the media of the anterior cerebral artery in this study. The closest neuromuscular approaches observed in other blood vessels are between 600 and 4000 Å (31, 33, 34), so terminal axons at comparable distances can presumably be regarded as involved in neuromuscular transmission. A structural basis for functional transmission to the cerebral arteries from both adrenergic (sympathetic) and cholinergic (parasympathetic) nerves is thus suggested by our results.

Hagen and Wittkowski (15) identified adrenergic fibers associated with the cerebral vessels on the basis of the inclusion of large (700 to 1100 Å) granular vesicles. However, axons containing large granular vesicles persist after adrenergic denervation, and it must be concluded that both adrenergic and cholinergic fibers contain a small proportion of large granular vesicles. The properties of these vesicles probably differ, since treatment of the animal with 6-OHDA increased the opacity of the granules of the large vesicles seen in adrenergic fibers but did not affect those of the cholinergic axons (22, 23).

It is interesting to note that cholinergic and adrenergic fibers were observed within the same process of a Schwann sheath. Tranzer and Thoenen (35) saw this type of relationship in the innervation of the cat iris, and...
Burn (36) suggested that cholinergic fibers in this situation may be of sympathetic origin. He suggested that sympathetic stimulation might release acetylcholine from cholinergic sympathetic fibers, which would then act on the accompanying adrenergic fibers to release norepinephrine to act on the effector tissue. However, in the present study, Schwann cells containing degenerating adrenergic and intact cholinergic fibers were seen after bilateral removal of the superior cervical ganglia. Thus, if the cholinergic fibers are of sympathetic origin, there must be an intervening ganglion between the superior cervical ganglion and the cerebral arteries.

The physiological significance of the two groups of nerve bundles (periadventitial and adventitial) associated with the cerebral arteries is puzzling. From their close relationship with the muscle of the blood vessel, it seems likely that the nerves of the adventitial plexus innervate the smooth muscle cells of the artery. The axon profiles of the periadventitial bundles may represent preterminal fibers passing to more peripheral effector sites. However, the presence of some vesicle-containing profiles in the periadventitial bundles and particularly their bright fluorescent appearance (37) are not typical of preterminal fibers. A possible role for the periadventitial nerves could be the uptake of catecholamines released from fibers closer to the arterial smooth muscle to prevent overflow into the tissue of the brain.
The unusual axons containing large electron-dense bodies and accumulations of mitochondria are possibly sensory. Hagen and Wittkowski (15) showed that, after losing their sheath, myelinated axons in the cerebral arteries have a similar appearance. In the present experiments these axons were observed in the adventitia and sometimes close to the arterial muscle cells. Myelinated axons were most often seen in the periadventitial bundles, and only few single myelinated axons were seen in the adventitia. It would be necessary to examine the tissue in serial section to determine the nature of the unusual axons.

References
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