The identification of adrenergic and cholinergic nerve endings in the trabecular meshwork

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There are nerves and nerve endings in the trabecular meshwork, but their structure does not reveal whether they are adrenergic or cholinergic in function. Tranzer and Thoenen7 have shown that the administration of 5-hydroxydopamine, a false sympathetic transmitter, fills the synaptic vesicles of adrenergic nerve endings with an electron dense material. Cholinergic nerve endings possess synaptic vesicles which appear empty in ordinary electron micrographs. This reaction has been shown by Tranzer and Thoenen7 to be highly specific. After 5-hydroxydopamine was injected subconjunctivally and intraperitoneally into cynomolgus monkeys, the nerve terminals of the iris dilator and sphincter muscles and also in the trabecular meshwork were examined by electron microscopy. Some of the synaptic endings possess vesicles which appeared solid in all three tissues following this procedure. The proportion of adrenergic to cholinergic nerve terminals, determined in this manner, was three to one in the iris dilator and one-to-six in the iris sphincter muscle. Adrenergic and cholinergic nerve terminals in the trabecular meshwork are located mainly in the posterior part just anterior to the insertion of the longitudinal ciliary muscle. About one third of these nerve endings were adrenergic.

Key words: adrenergic nerve endings, cholinergic nerve endings, dense osmiophilic material, false sympathetic transmitter, 5-hydroxydopamine, iris dilator muscle, iris sphincter muscle, synaptic vesicles, trabecular meshwork.

It has been observed that the trabecular meshwork is transversed by many nerves.1-3 From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York.

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They have been shown to terminate, in some instances, in this tissue by electron microscopy,4-6 but to date these terminals could not be identified as adrenergic or cholinergic.

5-Hydroxydopamine which is a structural isomer of norepinephrine, a false or inactive sympathetic transmitter, is taken up and stored in the synaptic vesicles of adrenergic nerves. This uptake and storage of 5-hydroxydopamine produces ultrastructural changes in the synaptic vesicles characterized by the accumulation of a dense osmiophilic material. Tranzer and Thoenen,7 studying the sympathetically inner-
vated spleen capsule of cats, recognized this reaction as highly specific. Another type of nerve terminal was found in the iris and vas deferens of animals treated with 5-hydroxydopamine. They contained many small empty vesicles and an occasional large-cored vesicle. Using the acetylcholinesterase technique of Karnowsky, this type was shown to be cholinergic. 5-Hydroxydopamine, therefore, has been used successfully for the identification of adrenergic nerve terminals and, consequently, cholinergic nerve endings may be identified by the absence of this reaction. This technique was used in the present study to distinguish cholinergic from adrenergic nerve terminals in the iris dilator and sphincter muscle and also to determine the distribution of adrenergic and cholinergic nerve endings in the trabecular meshwork of cynomolgus monkey (Macaca irus).

Materials and methods

Two Cynomolgus monkeys (Macaca irus) weighing about two kilograms each were used in this study. Several lots of 100 mg. of 5-hydroxydopamine HCl (Aldrich Chemical Co.) were each dissolved in 1 ml. of previously boiled and cooled distilled water, nitrogenated, and put into 1 ml. syringes to avoid contamination with oxygen. Twenty-five and 75 mg. per kilogram of body weight of 5-hydroxydopamine HCl was injected four times in the anesthetized monkey both intraperitoneally and subconjunctivally. From one-third to one-sixth of the volume of the injection was given subconjunctivally, in several places, in one eye each time. The injections were given at 12-hour intervals and about 5 hours after the last injection both eyes were enucleated under ether anesthesia. After bisecting the eyes, the anterior portion was fixed in 3 per cent glutaraldehyde buffered with 0.1 M phosphate buffer at pH 7.4 for 2.5 hours and then in 1 per cent osmium tetroxide in the same buffer for 1.5 hours. The tissues were cut in several pieces during fixation and then dehydrated in a graded series of alcohol and embedded in Epon. Sections were cut with a Porter-Blum (MT-II) microtome and stained with Azure II for light microscopy. Thin sections obtained with a diamond knife and doubly stained with uranyl acetate and lead citrate were examined with a Siemens Elmiscope I.

Thick meridional section of the trabecular meshwork were obtained and sketches were made with a projection microscope. Serial thin sections of the same region were picked up on a single-hole copper grid with a supporting film of parlodion or on 100 mesh copper grids. Effort was made to locate all the nerve terminals in a single section on a single-hole copper grid and put their location on the sketch of the trabecular meshwork. Photographs were mainly taken of serial sections on 100 mesh copper grids.

Observations

Iris dilator muscle. The nerve terminals in this muscle in eyes treated by subconjunctival as well as by intraperitoneal injection of 5-hydroxydopamine were studied. Two types of nerve terminals were found depending on whether the synaptic vesicles appeared empty or filled. Some of the vesicles which appeared filled with an osmiophilic material ranged in diameter from 400 to 1,700 A. Some of these vesicles were only partially occupied by this dense material, which then had an eccentric position within the vesicle. Small empty vesicles 300 to 700 A in diameter were dispersed in these terminals in addition to mitochondria and microtubules. The dense content of the synaptic vesicle appears to be identical with the 5-hydroxydopamine-filled vesicles reported by Tranzer and Thoenen and, therefore, this type of terminal is thought to be adrenergic (Fig. 1). The terminals classified on the basis of their dense vesicles composed about 75 per cent of the terminals in the iris dilator muscle.

Another type of nerve terminal was found which contained many empty vesicles 400 to 700 A in diameter and an occasional large cored vesicle, 1,000 to 1,500 A in diameter. The latter does not seem to increase in density after treatment with 5-hydroxydopamine. Mitochondria and microtubules were also found in these terminals. They were thought to be cholinergic in nature (Fig. 1).

Both types of terminals often lay in close apposition, separated from one another only by an intercellular space of about 200 A. Many of them were found at a considerable distance from a smooth muscle cell from which they were separated by a gap of more than 1,000 A in which two layers of basement membrane appeared between the two cells, one close to the nerve terminals and the other close to the smooth muscle cell. Only a small number of these terminals were located very close to an adjacent smooth muscle cell without an intervening basement membrane. Several axons were observed proximal to their terminals. These axons contained synaptic vesicles characteristic of either adrenergic or cholinergic nerves. The axons were more or less completely enclosed by a Schwann cell process. Where two axons lay near each other they were separated by an intercellular space of about 200 A.
Identification of adrenergic and cholinergic nerve endings

Fig. 1. An electron micrograph showing nerve terminals in the iris dilator muscle of the eye treated with subconjunctival and intraperitoneal injection of 5-hydroxydopamine. Note the dense osmiophilic material that represents 5-hydroxydopamine storage in the synaptic vesicles of adrenergic terminals (Ad). Small empty vesicles and an occasional large cored vesicle (arrow) in cholinergic terminals (Ch) remain unchanged. Two types of terminals are in close apposition (*), separated from one another only by an intercellular space of 200 Å. SM, smooth muscle. ×15,600.

The nerve terminals in the dilator muscle of the eye which did not receive a subconjunctival injection but which were exposed to 5-hydroxydopamine injected by the intraperitoneal route only, contained a large number of small empty vesicles 400 to 800 Å in diameter and some larger cored vesicles 900 to 1,400 Å in diameter. Occasionally, a small cored vesicle could be seen. These cores were small, vague, and eccentrically located. The dense material occurring in the solid vesicles of adrenergic terminals treated by both intraperitoneal and subconjunctival injections of 5-hydroxydopamine was never observed.

Iris sphincter muscle. The adrenergic and cholinergic nerve terminals in this muscle were similar in structure to those observed in the dilator muscle when treated by both subconjunctival and intraperitoneal injections of 5-hydroxydopamine. Both types of terminals were occasionally in close apposition to each other, separated by the usual intercellular gap of about 200 Å. Approximately 15 per cent of the terminals were of the adrenergic type as shown by the solid osmiophilic contents of their synaptic vesicles (Fig. 2). Many nerve terminals were located between the smooth muscle cells and separated from them by a wide space of more than 1,000 Å, containing either a single or double layer of basement membrane material. The cytoplasm of the smooth muscle cell near the nerve terminals held many pynocytotic vesicles and showed much smooth-surfaced endoplasmic reticulum.

The trabecular meshwork. The two types of nerve endings similar to those observed in the dilator and sphincter muscles were found in the trabecular meshwork of eyes treated by both intraperitoneal and subconjunctival injections of 5-hydroxydopamine. Those which we must suppose are adrenergic contained many solid synaptic vesicles of 400 to 1,700 Å in diameter plus a few small empty vesicles (Fig. 3). Another type of terminal, probably cholinergic, contained only small empty vesicles 400 to 800 Å in diameter and an occasional larger cored vesicle 1,100 to 1,500 Å in diameter (Fig. 4).

A small number of synaptic vesicles were found...
Fig. 2. An electron micrograph of iris sphincter muscle of the eye treated with subconjunctival and intraperitoneal injection of 5-hydroxydopamine. Both the adrenergic terminals (Ad) with solid synaptic vesicles and the cholinergic terminals (Ch) with small empty vesicles are seen in the intercellular space between smooth muscle cells (SM). A single or double layer of basement membrane lies between the terminals and the adjacent smooth muscles (SM), except in the place (*) where a cholinergic terminal is in close contact with a small muscle cell. ×17,400.

in nerve axons, proximal to their terminal, and wrapped partly with Schwann cell processes. Other axons, both myelinated and unmyelinated, occurred which did not contain any synaptic vesicles but only neurofilaments and neurotubules plus a few mitochondria (Fig. 5).

The axons and their nerve terminals, located in a trabecular sheet or the scleral spur, were separated from the adjacent endothelial or smooth muscle cell by a gap of more than 1,000 A. Basement membrane material was present in this space. The nerve endings with solid synaptic vesicles, which we interpret as adrenergic nerve terminals, comprised approximately one third of those found in the trabecular meshwork. As shown in the schema in Fig. 6, both the adrenergic and cholinergic nerve terminals are located mainly in the posterior part of the trabecular meshwork and anterior to the insertion of the longitudinal ciliary muscle.

Discussion

Adrenergic nerve terminals contained three kinds of synaptic vesicles: small empty vesicles, small cored vesicles, and large cored vesicles. Of these three only the small cored vesicles are characteristic of adrenergic terminals and are, therefore, of importance in differentiating them from cholinergic terminals, however, it is often difficult to get good preservation of these small cored vesicles. The "core" is easily dissolved or washed out during processing and the vesicles appear to be small, empty, and similar in structure to those observed in cholinergic terminals.

5-Hydroxydopamine, a false or inactive sympathetic transmitter, seems to resolve this problem. The three varieties of synaptic vesicles, in adrenergic terminals, take up the false transmitter and appear solid after treatment with this drug. The vesicles then appear to be filled with a dense osmiophilic material and range in diameter between 400 and 1,700 A. Although some small empty vesicles remain, at least half of the vesicles stored a dense
Fig. 3. An electron micrograph showing an adrenergic terminal in the posterior trabecular meshwork of the eye treated with subconjunctival and intraperitoneal injection of 5-hydroxydopamine. Adrenergic terminal (Ad) contains a lot of solid synaptic vesicles of 400 to 1,700 Å in diameter. En, endothelium; BM, basement membrane. ×15,600.

Fig. 4. An electron micrograph showing cholinergic terminals (Ch) in the posterior trabecular meshwork of the eye treated with subconjunctival and intraperitoneal injection of 5-hydroxydopamine. Cholinergic terminals (Ch) contain small empty vesicles and an occasional large cored vesicle (arrow) both of which remain unchanged. One of the terminals appears to be in close association with the endothelium (En). ITS, intertrabecular space; CM, ciliary muscle. ×15,600.
material and there was no difficulty in identifying adrenergic terminals. The uptake of 5-hydroxydopamine has been studied in the vas deferens of the mouse. It was found that 80 to 90 per cent of the synaptic vesicles were filled when more than 100 mg. per kilogram of the 5-hydroxydopamine was administered.  

Another type of nerve terminal, containing only small empty vesicles and occasional large cored vesicles has been found in the iris and vas deferens of animals treated with 5-hydroxydopamine. Experiments utilizing the acetylcholinesterase technique of Karnowsky have shown this type of nerve terminal to be cholinergic.  

The synaptic vesicles of the cholinergic terminal remain unchanged after treatment with epinephrine (noradrenaline) as well as with 5-hydroxydopamine. The present study suggests that both the iris dilator and sphincter muscles have a dual innervation, adrenergic and cholinergic, and confirms light microscopic studies with histofluorometric methods and acetylcholinesterase techniques. In the iris dilator muscle of cynomolgus monkey, 75 per cent of the nerve terminals have been found to be adrenergic and 25 per cent cholinergic. In contrast, 85 per cent of the nerve terminals in the sphincter muscle were cholinergic and 15 per cent were adrenergic. A similar proportion of nerve terminals have been demonstrated in the iris dilator and sphincter muscle of guinea pigs.

It is interesting that adrenergic and cholinergic axons or terminals are often closely adjacent to one another, separated by an intercellular space of about 200 Å. Membrane thickening has been observed between these nerve terminals and support the hypothesis that the two nerve terminals influence one another in addition to the...
Identification of adrenergic and cholinergic nerve endings

Fig. 6. A sketch of the trabecular region showing the location of all the nerve terminals observed. Both adrenergic (solid circles) and cholinergic terminals (open circles) are mainly located at the posterior part of the trabecular meshwork just anterior to the insertion of the longitudinal ciliary muscle. About one-third are adrenergic. The identification of two types of nerve terminals depends on whether the synaptic vesicles appear empty or filled after the treatment with 5-hydroxydopamine. Area studied with electron microscopy is enclosed by dotted lines.

As mentioned, adrenergic and cholinergic nerve terminals are located in the posterior portion of the trabecular meshwork anterior to the longitudinal ciliary muscle. About one-third of them is adrenergic. Degeneration of nerve fibers in the trabecular region has been reported in open-angle glaucoma and in the aging process of the human. Although these facts tempt us to consider the function of these nerves in the regulation of aqueous outflow, many problems remain to be solved before this conclusion can be accepted.

It is well known that topical epinephrine has been used in the treatment of glaucoma. Epinephrine seems to increase the outflow facility as well as decrease the rate of aqueous secretion. Chemical sympathetomy, by the use of 6-hydroxydopa-
mine, has recently been proposed as a therapeutic measure. This causes release of norepinephrine at the adrenergic terminal, at first, and later a hypersensitivity of the effector organ to small amounts of norepinephrine. The site of epinephrine or norepinephrine action to improve the outflow facility is not known. It is interesting that the effect of these drugs appears to be more prominent in glaucomatous eyes, in which the adrenergic nerves of the inner ocular tissues are supposed to be destroyed.

REFERENCES
3. Laties, A. M., and Jacobowitz, D.: A comparative study of the autonomic innervation...