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Autonomic Nerves Containing Substance P in the Aqueous Outflow Channels and Scleral Spur of the Guinea Pig

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Purpose: To study the innervation of the aqueous outflow channels and scleral spur by autonomic nerves containing substance P.

Methods: The experiments were conducted on guinea pigs. Immunohistochemical techniques and capsaicin-ablation of the sensory nerves were used to investigate nerves containing substance P at the light and electron microscopic level.

Results: Nerves containing substance P were observed in the aqueous outflow channels and scleral spur regions. The fine structures of these nerves had a similar pattern in those regions, and the labeled elements had abundant small vesicles, a few large vesicles, and numerous neurotubuli. Following capsaicin treatment, these nerves remained intact and no degenerated substance P-like immunoreactive nerves were found.

Conclusions: Nerves containing substance P are most likely of autonomic origin in view of their ultrastructural features. These nerves innervate the aqueous outflow channels and scleral spur, and are probably important for neurogenic influences on the intraocular pressure by the autonomic nervous system. **Jpn J Ophthalmol 1999;43:272–278** © 1999 Japanese Ophthalmological Society

Key Words: Aqueous outflow channels, capsaicin, guinea pig, immunohistochemistry, substance P.

Introduction

Aqueous outflow channels and the scleral spur play important roles in the regulation of intraocular pressure (IOP), and are known to have varied peptidergic innervation, which have received special attention.[1] It is believed that the neuropeptides play an important role as neuroregulators of aqueous outflow regulation at these sites.

In the conventional outflow pathway, aqueous humor from the anterior chamber passes through the trabecular meshwork, crosses the inner wall of Schlemm's canal, and passes into the collectors channels, the intrascleral and episcleral venous plexuses.[2,3]

The scleral spur region should also be considered because it plays a part in outflow regulation.[4,5] The human scleral spur contains elastic tissue, similar to sclera, so that changes in the IOP might affect these tissues.[5]

There has been recent interest in neuromodulation by substance P (SP) in the adrenal gland from the works of Neri et al[6] and Livett et al,[7] who suggested that SP regulated stress-induced catecholamine secretion and the ACTH-stimulated release of aldosterone.

Kondo[8] classified autonomic efferent nerves into two groups: "adrenopeptidergic" and "cholinopeptidergic." From the results of an earlier study,[9] we suggested that SP of autonomic origin serves as a neurotransmitter or neuromodulator for the regulation of IOP in the guinea pig trabecular meshwork.

Because little information is available about the neuronal regulation of IOP,[10,11] such problems should be studied in more detail. In the present study, we

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have studied the innervation in a wider range of tissues, and have determined the nonsensory origin of the substance P-like immunoreactive (SP-LI) nerves inside the aqueous outflow channels and scleral spur using ultrastructural immunohistochemistry and capsaicin treatment.

Materials and Methods

All animal experiments were performed in conformity with the recommendations of the ARVO Resolution on the Use of Animals in Research.

Tissue Preparation

Five male Hartley guinea pigs weighing 300–400 g were used for the study. Sodium pentobarbital (50 mg/kg) and sodium heparin (1,000 units/kg) were injected intraperitoneally to anesthetize the animals.

Animals were perfused through the thoracic aorta with 0.1 mmol/L phosphate-buffered saline (PBS) (pH 7.4) followed by Zamboni's fixative solution. The eyeballs were enucleated, the anterior segment of the eye was removed and further fixed in the same solution for 6 hours at 4°C. After immersion in 0.1 mmol/L PBS containing 20% sucrose overnight at 4°C, the specimens were frozen and cut into 20-μm sections in a cryostat (CM-1800, Leica, Heidelberg, Germany). The sections were mounted on albumin-coated slides and dried at room temperature.

The immunostaining procedure was performed, not on free-floating sections, but on sections mounted on a slide. After immunostaining, a capsule containing epoxy resin was inverted over the stained sections mounted on the slides and a layer of epoxy resin was laid over, covering the capsule and section. Finally, the sections embedded in the epoxy resin were separated from the slides by heating the slides. After treatment with 1% hydrogen peroxide in methanol for 30 minutes, they were rinsed with 0.1 mmol/L PBS.

Immunohistochemical Processing (Pre-embedding Method)

For immunohistochemistry, the specimens were treated with 10% normal goat serum for 30 minutes at room temperature and incubated with rabbit anti-SP serum (1:500; Chemicon International, Temecula, CA, USA) for 48 hours at 4°C. They were then incubated in peroxidase-labeled affinity-purified antibody to rabbit IgG serum (1:200; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) for 12 hours at 4°C and fixed in 1% glutaraldehyde in 0.1 mmol/L PBS for 20 minutes at 4°C.

The specimens were soaked in freshly prepared 0.02%, 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.05 mmol/L Tris-HCl buffer (pH 7.6) for 30 minutes. Then soaked in 0.02% DAB and 0.0025% H_2O_2 in Tris-HCl buffer (pH 7.6) for 5 minutes at room temperature, washed in 0.1 mmol/L PBS, and postfixed in 2% osmium tetroxide in 0.1 mmol/L PBS for 1 hour at room temperature. The sections were then dehydrated and finally embedded in epoxy resin and incubated. After incubation, the slides were separated from the sections embedded in epoxy resin by heating. Ultrathin sections and semithin sections (1 μm) were cut on a microtome (MT-2B; Ivan Sorvall, Norwalk, CT, USA). Ultrathin sections were stained with 1% uranyl acetate and lead citrate, and examined under a transmission electron microscope (H-500; Hitachi, Tokyo). Semithin sections were stained with Toluidine blue and examined under a light microscope (Nikon, Tokyo).

Capsaicin Treatment

Chemical ablation of sensory nerves was performed on seven male Hartley guinea pigs as described previously.[12] A total dose of 125 mg/kg capsaicin (Na-

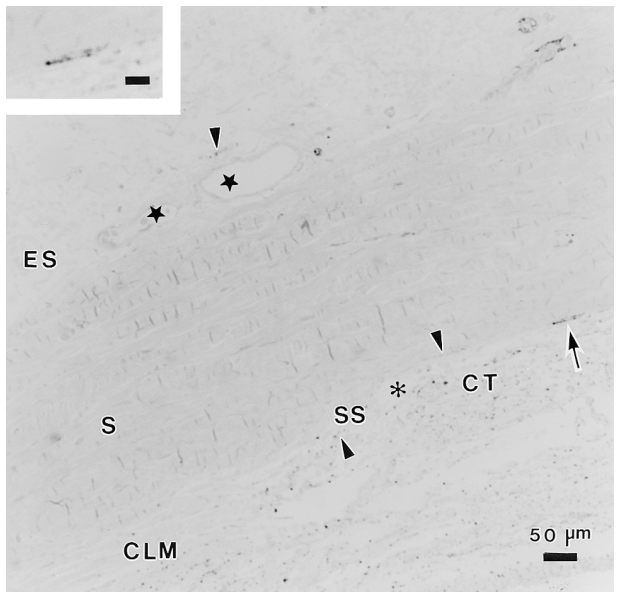

Figure 1. Light micrograph of substance P-like immunoreactive (SP-LI) innervation in outflow channels and scleral spur (SS). SP-LI nerves (arrowheads) are scattered throughout these sites. Inset: high magnification (Bar = 150 μm) of varicose axon (arrow). Asterisk: Schlemm's canal, stars: episcleral vessels, S: sclera, ES: episclera, CT: cribiform layer in trabecular meshwork, CLM: ciliary longitudinal muscle.

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calai Tesque, Kyoto) was injected subcutaneously over 2 days under sodium pentobarbital anesthesia 2 weeks before the immunohistochemical study. The elimination of the blink reflex, tested by gentle stimulation of the cornea with gauze with the animals restrained, was used to confirm the ablation of the sensory nerves by the capsaicin treatment. The tissues surrounding the Schlemm's canal were used as control.

To assess immunohistochemical specificity, another section of 20 μm was incubated in 0.1 mmol/L PBS to check intrinsic peroxidase, and was placed in rabbit normal serum with the same dilution (500×) as SP antiserum to check nonspecific reaction.

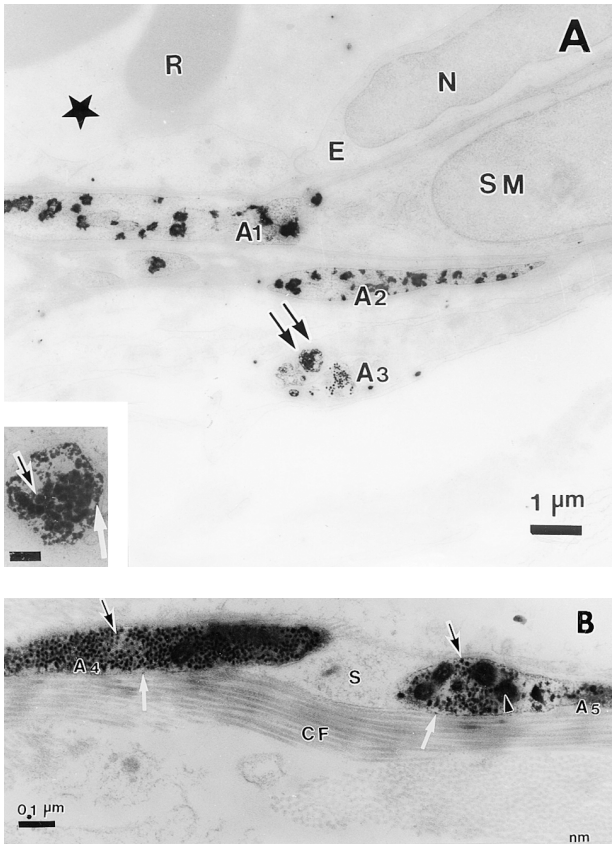

Figure 2. Electron micrographs of substance P-like immunoreactive (SP-LI) nerves. (**A**) SP-LI nerves (A1, A2, A3) are seen around episcleral vein (star). Inset: Higher magnification (Bar = 0.1 μm) of axon (double arrows). SP-LI materials are confined to numerous small vesicles (small arrow) and neurotubuli (white arrow). SP-LI nerves sectioned longitudinally (A1, A2) and transversely (A3). E: endothelial cells of episcleral vein, SM: smooth muscle cell, R: red blood cell, N: nucleus. (**B**) SP-LI nerves (A4 and A5) are seen in close proximity to collagen fiber networks (CF) in scleral spur. A4 packed with numerous small vesicles (small arrow) and some neurotubuli (white arrow). A5 contains numerous small vesicles (small arrow), several large vesicles (arrowhead), and numerous neurotubuli (white arrow). S: Schwann cell. Bar = 0.1 μm.

Results

Light Microscopic Findings After Capsaicin Treatment

In semithin sections, SP-LI nerves were observed as varicose or nonvaricose linear structures in the aqueous outflow channels and scleral spur. These varicosities had the typical appearance of autonomic nerves (Figure 1).

Ultrastructural Findings in Aqueous Outflow Channels and Scleral Spur

SP-LI nerves in and around the aqueous outflow channels and scleral spur regions are shown in Figure 2. Most of the SP-LI nerves were filled with many labeled small vesicles (30–60 nm), a few large vesicles (100–200 nm), and numerous neurotubuli. Some of the neurotubuli were seen in close proximity to the axolemma.

SP-LI nerves were located very close to an episcleral vascular channel (Figure 2A) and were in close contact with networks of collagen fibers (Figure 2B).

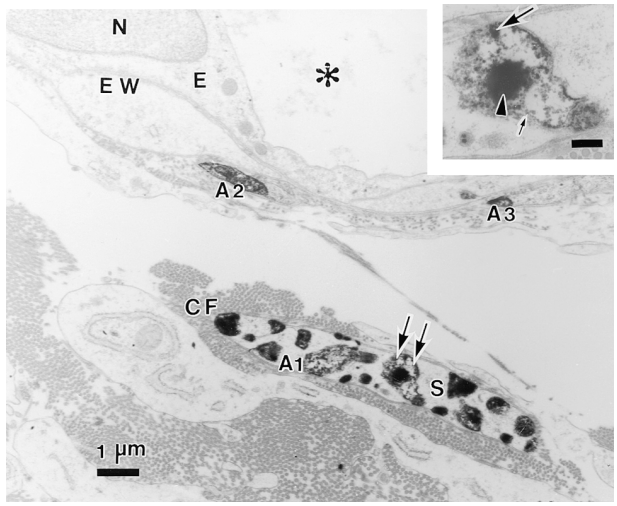

Figure 3. Electron micrograph of substance P-like immunoreactive (SP-LI) nerves following capsaicin treatment. SP-LI nerves (A1, A2, and A3) are found in close proximity to collagen fiber networks (CF) around the external wall (EW) of Schlemm's canal (asterisk). Inset: High magnification (Bar = 0.1 μm) of axon (double arrows). SP-LI materials confined to small vesicles (arrow), large vesicle (arrowhead), and numerous neurotubuli (small arrow). E: endothelial cell of Schlemm's canal, N: nucleus, S: Schwann cell.

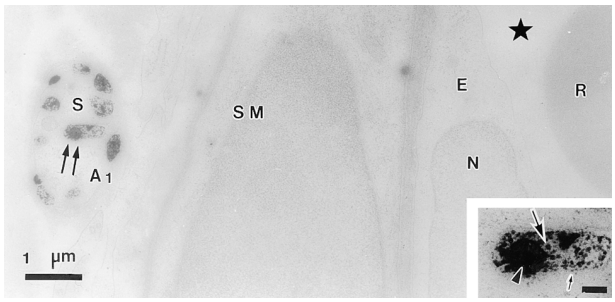

Figure 4. Electron micrograph of substance P-like immunoreactive (SP-LI) nerves following capsaicin treatment. SP-LI nerve bundle (A1) is seen in close proximity to episcleral vein (star). Inset: High magnification (Bar = 0.1 μm) of axon (double arrows). SP-LI materials are confined to small vesicles (arrow), large vesicle (arrowhead), and numerous neurotubuli (small arrow). N: nucleus, E: endothelial cell of episcleral vein, SM: smooth muscle cell, S: Schwann cell. R: red blood cell.

Findings in Aqueous Outflow Channels and Scleral Spur After Capsaicin Treatment

SP-LI nerves remained intact in the aqueous outflow channels and scleral spurs sites following capsaicin treatment (Figures 3–5). Most of the SP-LI nerves possessed a greater proportion of small vesicles (30–60 nm), a few large vesicles (100–200 nm), and numerous neurotubuli. These SP-LI nerves in treated specimens were very similar to those in non-capsaicin-treated specimens. Most of these labeled nerves were unmyelinated and measured <1 μm in diameter. No degenerated SP-LI nerves were found, but some degenerated, nonimmunoreactive nerves that contained large vacuoles were observed (Figures 6 and 7).

Findings in Control Sections

No immunostaining of nerve elements was observed when the SP antiserum was replaced by rab-

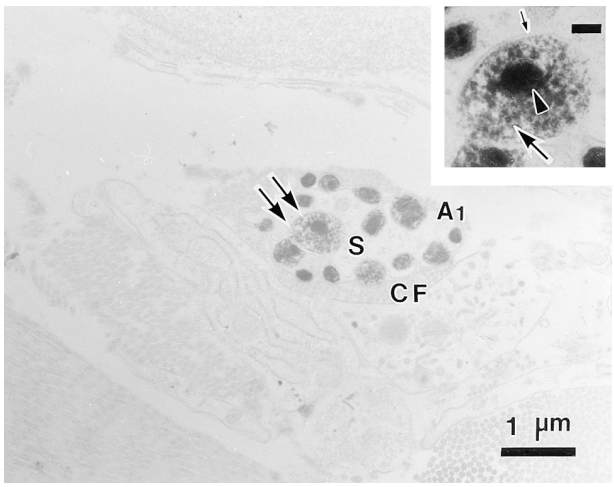

Figure 5. Substance P-like immunoreactive (SP-LI) nerve bundle (A1) is seen in close proximity to collagen fiber networks (CF) in scleral spur. Inset: High magnification (Bar = 0.1 μm) of axon (double arrows). SP-LI materials are confined to numerous small vesicles (arrow), a large vesicle (arrowhead), and numerous neurotubuli (small arrow).

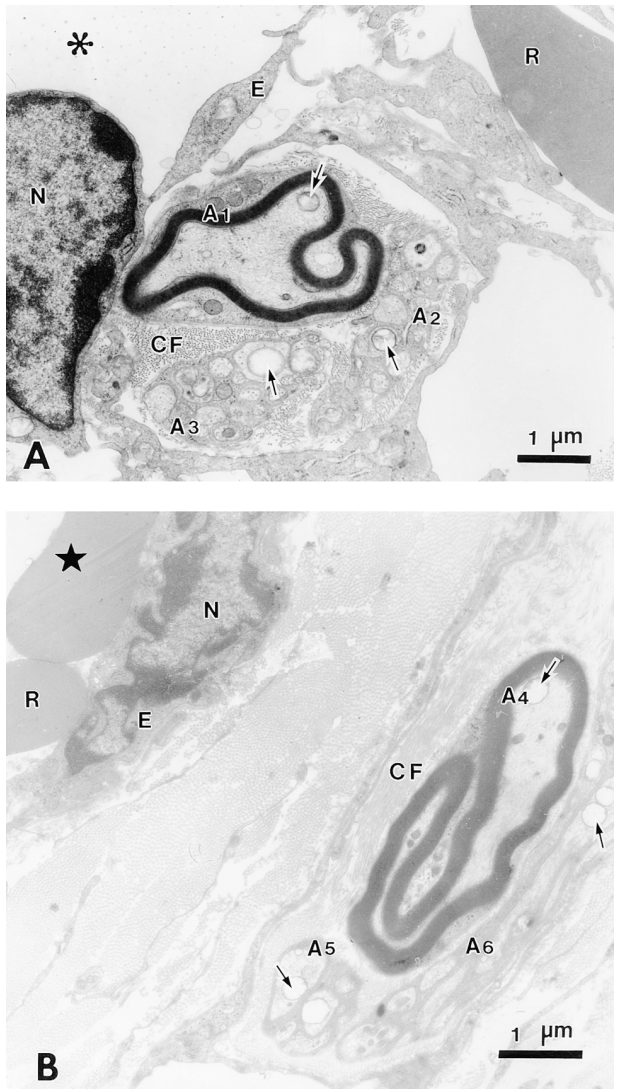

Figure 6. Degenerated nonimmunoreactive bundles (A1, A2, A3, A4, A5, and A6) are found in lateral wall of Schlemm's canal (A) and episcleral region (B). Large vacuoles (arrows) occur within axons. E: endothelial cells of Schlemm's canal (asterisk) or episcleral vein (star), N: nucleus, CF: collagen fiber networks, R: red blood cell.

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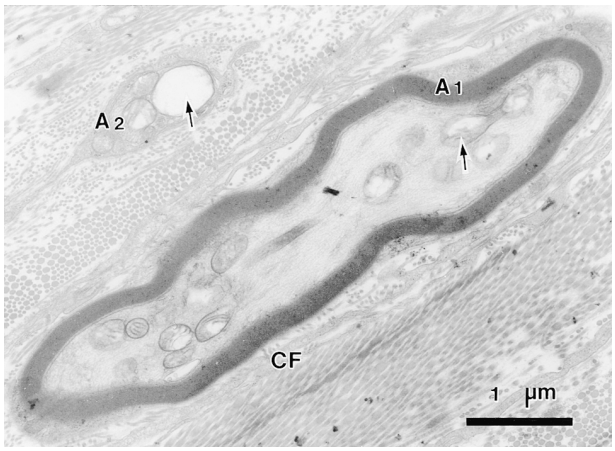

Figure 7. Degenerated nonimmunoreactive nerves (A1 and A2) are seen in close proximity to collagen fiber networks (CF) in scleral spur. Large vacuoles (arrows) are seen in axons.

bit normal serum (Figure 8A) or 0.1 mmol/L PBS as controls (Figure 8B).

Discussion

We have demonstrated the existence of SP-LI nerve supply to the aqueous outflow channels and scleral spur by immunohistochemical techniques, and shown that these nerves remained intact following capsaicin treatment for chemical ablation of sensory nerves. No degenerated SP-LI nerves were found following capsaicin treatment. It is possible that sensory SP-LI are not present at these sites. After capsaicin treatment, the blink reflex was lost within 1 day. Games et al[12] have reported that as soon as 15 minutes after the intraventricular injection of capsaicin, rats were insensitive to a noxious chemical stimulus applied to the cornea.

The varicose appearance of some of the SP-LI nerves is the typical appearance of autonomic nerves at the light microscopic level. In addition, the fine structure of the SP-LI nerves had a similar pattern in these regions, consisting of labeled nerve elements with abundant small vesicles, a few large vesicles, and numerous neurotubuli. These nerves did not show the characteristics of sensory nerves, which have a high content of mitochondria and glycogen particles.[13] Thus, they should most probably be regarded as autonomic nerves. The accumulation of reaction products of SP-LI might be due to proliferation of antibody. In the present experiment, we studied the SP-LI innervation in a wider range of tissues, and found that the fine structure of SP-LI nerves had a

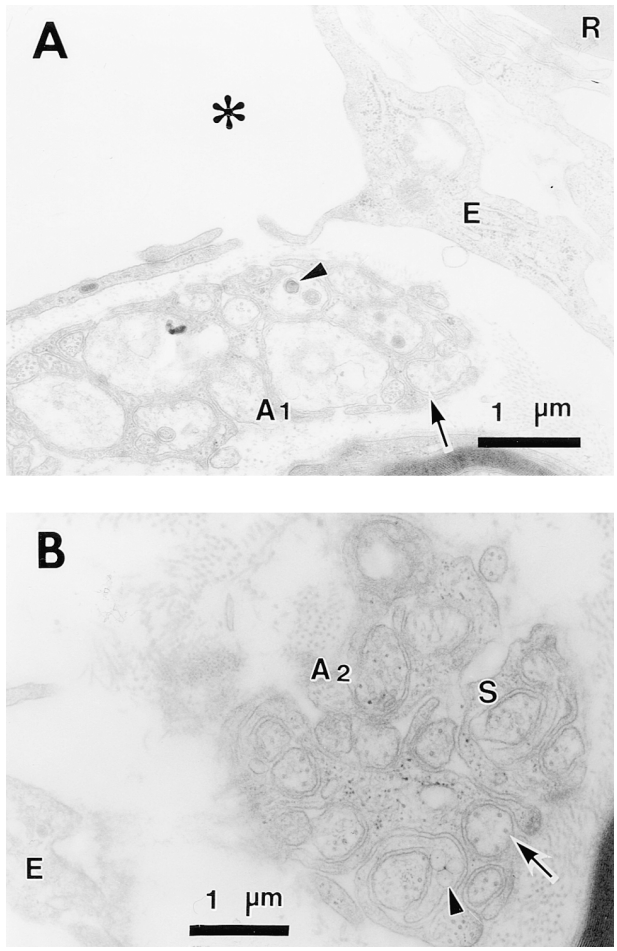

Figure 8. No immunostaining of nerve bundles (A1 and A2) is observed around Schlemm's canal (asterisk) when SP antiserum is replaced by phosphate-buffered saline to check for intrinsic peroxidase (A), and by rabbit normal serum to check nonspecific reaction (B). Arrowheads: large vesicles, arrows: small vesicles, S: Schwann cell, R: red blood cell, E: endothelial cells of Schlemm's canal.

pattern similar to the trabecular meshwork region, as reported in our previous study.[9]

Stone et al[14,15] have demonstrated that the SP-LI nerves of the outflow channels have the typical appearance of autonomic nerves with characteristic varicosities, but they did not provide more detailed light microscopic information. In the ophthalmic field, there is still little information on the detection of sensory peptides in autonomic neurons,[9,16–18] and the ultrastructure of SP-LI nerves has not been described at these sites.

Sympathetic and parasympathetic terminals have been found in the aqueous outflow channels and scleral spur region of the monkey.[4,19–21] Also, auto-

nomic nerves have been shown to be involved in aqueous outflow regulation in various ways.[13,22,23]

Mittag[22] has demonstrated that catecholamines affect function in all structures involved in aqueous humor dynamics. Macri and Cevario[23] have studied the parasympathetic involvement in the aqueous outflow by ciliary ganglion stimulation. The facility of outflow via the trabecular pathway increases with increasing episcleral venous pressure, presumably from the dilation of Schlemm's canal and the opening of its collapsed segments.[24] We have found SP-LI nerves in close contact with Schlemm's canal. α-Smooth muscle actin is distributed in the external wall of Schlemm's canal, surrounding the collector channels, and these tissues have contractile properties.[25] SP-LI nerves were in close contact with the collagen fiber networks around the external wall of Schlemm's canal.

The episcleral vasculature has typical arteriovenous anastomoses with rich innervation; the episcleral venous pressure and the IOP may be influenced by these anastomoses.[26] SP-LI nerves were seen in the vicinity of the vascular channels, which have autonomic nervous control. The ciliary longitudinal muscle is involved in IOP regulation.[1,3,27] The ciliary longitudinal muscle tendons penetrate into the scleral spur[11] and are connected by a delicate network of elastic-like fibers.[2] From a histological viewpoint, there is some speculation whether the scleral spur is related to the control of aqueous humor outflow.

The SP-LI nerve innervation of the aqueous outflow channels and the scleral spur region, which we have documented, is probably relevant to the neurogenic influences on aqueous outflow.

It is concluded that the release of neurotransmitters in the autonomic nervous system, such as SP, probably contributes to the regulation of IOP.

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