

# Immunohistochemical Characterization of Neurotransmitters in the Episcleral Circulation in Rats

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**PURPOSE.** Episcleral venous pressure (EVP) greatly influences steady-state IOP and recent evidence suggests a neuronal influence on EVP. Yet little is known about the innervation of the episcleral circulation and, more specifically, the neurotransmitters involved. We identify possible neurotransmitter candidates in the episcleral circulation of rats.

**METHODS.** Eight immersion-fixed rat eyes taken from four animals were cut into serial sections, followed by standard immunohistochemistry. Antibodies against choline acetyltransferase, dopamine- $\beta$ -hydroxylase, synaptophysin, PGP 9.5, VIP, neuronal nitric oxide synthase (nNOS), substance P, CGRP, and galanin were used. Additionally, colocalization experiments with smooth muscle actin and neurofilament (200 kDa) were performed.

**RESULTS.** In all specimens, the episcleral vessels showed immunoreactivity for smooth muscle actin and were reached by neurofilament (200 kDa)-positive structures. Furthermore, these structures colocalized with immunoreactivity for PGP 9.5, synaptophysin, choline acetyltransferase (ChAT), dopamine- $\beta$ -hydroxylase, VIP, CGRP, nNOS, substance P and galanin.

**CONCLUSIONS.** These findings indicate that there is neuronal input to the episcleral circulation. ChAT and VIP as well as dopamine- $\beta$ -hydroxylase suggest parasympathetic and sympathetic innervation. Further studies are needed on whether the positively-stained structures are of functional significance for the regulation of the episcleral venous pressure and thereby IOP.

**Keywords:** IOP, anatomy, innervation

IOP is the most important risk factor for glaucoma and lowering IOP is the mainstay of glaucoma treatment.<sup>1–3</sup> The Goldmann equation expresses steady state IOP as the relationship between aqueous humor production and outflow facility with episcleral venous pressure (EVP) as an additive factor. Thus, EVP has great impact on steady state IOP, as it must be overcome by any fluid to leave the eye.<sup>4,5</sup>

Despite its major influence on IOP, the physiology of EVP remains poorly understood. The episcleral circulation has numerous arteriovenous anastomoses that may provide the anatomic basis for active pressure regulation.<sup>6,7</sup> There also is evidence from in vivo studies demonstrating that EVP is actively regulated during changes in posture, as opposed to a passive behavior.<sup>8</sup> Furthermore, autonomic innervation of the episcleral circulation has been described for several animal models and also humans.<sup>7,9</sup> More recently, the functional role of the innervation was investigated further. The decrease in EVP in response to topical anesthesia suggests the presence of a neuronal tone<sup>10</sup> and electrical stimulation of a brainstem nucleus (salivatory superior nucleus) elicits increases in EVP.<sup>11</sup>

Thus, the current knowledge is that EVP is under neuronal control with a neuronal tone present. To be able to alter EVP, detailed knowledge of the neurotransmitters governing this neuronal tone is necessary. Investigation of the neuronal pathway through electrical stimulation is done most commonly

in rat models, as a detailed map of the entire brain with stereotactic coordinates is available.<sup>12</sup>

Currently, data on the rat episcleral circulation are limited, with only one study demonstrating the presence of neuropeptide Y and VIP, CGRP, and substance P.<sup>7</sup> Therefore, we investigated the presence of neurotransmitters in the rat episcleral circulation through immunohistochemistry.

## MATERIALS AND METHODS

All animal procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local governmental animal care committee. Sprague Dawley rats ( $n = 4$ ) were euthanized with an overdose of phenobarbital sodium (100 mg/kg intraperitoneal) and all eight eyes were enucleated. A small suture thread was attached cranially on each eye to allow for subsequent orientation on the slides. The eyes were submerged in PBS containing 4% paraformaldehyde (PFA) for 1 hour, rinsed in phosphate ( $\text{PO}_4$ ) for 48 hours at 4°C. They were further submerged in  $\text{PO}_4$  containing 15% sucrose for an additional 24 hours at 4°C and frozen at –80°C in liquid nitrogen-cooled methyl butane until further processed.

The eyes were mounted on cryostat plates (HM550; Microm, Waldorf, Germany) with tissue embedding medium, the tread



TABLE 1. Listing of All Antibodies Used in the Study

Antigen	Host	Dilution	Company
Neurofilament 200	Mouse	1:100	Sigma-Aldrich Corp., Vienna, Austria
Neurofilament 200	Chicken	1:25000	Abcam, Cambridge United Kingdom
Smooth muscle actin	Goat	1:1000	Abcam, Cambridge, United Kingdom
Smooth muscle actin	Mouse	1:2500	Sigma, Vienna, Austria
Lectin	Bandeirae	1:500	Sigma, Vienna, Austria
nNos	Rabbit	1:100	Axxora, Lausen, Switzerland
ChAT	Goat	1:1000	Chemicon, Nürnberg, Germany
Synaptophysin	Mouse	1:1000	Sigma, Vienna, Austria
CGRP	Rabbit	1:1000	Phoenix, Karlsruhe, Germany
Dopamin- $\beta$ -Hydroxylase (DBH)	Rabbit	1:1000	Biotrend, Köln, Germany
PGP 9.5 (human, rat, mouse)	Guinea pig	1:1000	Chemicon, Nürnberg, Germany
Galanin	Guinea pig	1:1000	Peninsula, San Carlos, USA
Substance P	Guinea pig	1:1000	Novus Biologicals, Wiesbaden, Germany
VIP	Guinea pig	1:1000	Progen, Heidelberg, Germany

marker orientation kept in place, then sliced into 16  $\mu$ m mounts that were collected on Poly-L-Lysin-coated slides and air dried for 1 hour at room temperature (RT). Slides were preincubated in Tris-buffered saline (TBS; Roth, Karlsruhe, Germany) containing 0.5% Triton X-100 (Merck, Darmstadt, Germany), 1% BSA (Sigma-Aldrich Corp., Vienna, Austria), and 5% donkey serum (Sigma-Aldrich Corp.) for 1 hour at RT. After being rinsed three times for 5 minutes, the slides were incubated with the primary antibody (Table 1) overnight at RT, then put in TBS containing 0.5% Triton X-100 and 1% BSA for 1 hour at RT and rinsed again in TBS three times for 5 minutes.

Additionally to one neurotransmitter or structure under investigation, each slide was incubated with antibodies against smooth muscle actin (or lectin) and neurofilament (200 kDa). All three primary antibodies were incubated at the same time with an appropriate selection of antibody hosts. For each antibody, its optimal concentration has been determined in preceding experiments.

The secondary antibodies were applied for 1 hour at RT and the slides were again rinsed and put in TBS 0.5% containing Triton X-100 and 1% BSA for 1 hour at RT.

Slides then were rinsed in TBS (0.05 M); three times for 5 minutes at RT while kept in dark and 4',6-diamidino-2 phenylindole dihydrochloride (DAPI, 1:2000, stock 1 mg/mL; VWR, Vienna, Austria) was applied for 1 hour then rinsed in 0.05M TBS again twice for 5 minutes. The slides were embedded in TBS: glycerol (1:1 at pH 8.6).

Negative controls were performed by omission of the primary antibodies during incubation and all slides showed absence of immunoreactivity.

The area of the limbal episcleral vessels (Fig. 1) was evaluated.<sup>7,13</sup> Criteria for a positive slide were a specific

structural immunoreactivity with all three antibodies and colocalization of neurofilament and the neurotransmitter under investigation at the vessel walls. The slides were examined using z-stacks (in z-increments of 1.6 $\mu$ m) to confirm colocalization of neuronal and vascular structures with the neurotransmitter under investigation.

To document immunohistochemistry, a confocal laser-scanning unit (Axio ObserverZ1 attached to LSM710;  $\times 20$  dry or  $\times 40$  and  $\times 60$  oil immersion objective lenses, with numeric apertures 0.8, 1.30, and 1.4, respectively; Zeiss, Göttingen, Germany) was used. Sections were imaged using the appropriate filter settings for Alexa 647 (647 nm excitation, coded white), Alexa555 (555 nm excitation, coded red), Alexa488 (488 nm excitation, coded green), and DAPI (405 nm excitation, coded blue).

## RESULTS

Throughout the slides we found a consistent picture of nervous structures (neurofilament positive, green) around vessels (smooth muscle actin or lectin positive, red).

Additional neurotransmitter and structures tested are contained in Table 2 (see also Figs. 2–4).

## DISCUSSION

The purpose of our study was to identify possible neurotransmitters in the episcleral circulation in rats.

The episcleral circulation receives autonomic innervation in many species (humans, monkeys, rabbits).<sup>7,9</sup> The superior salivatory nucleus has been identified as one parasympathetic

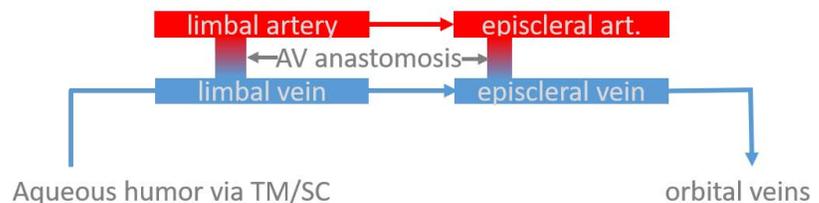
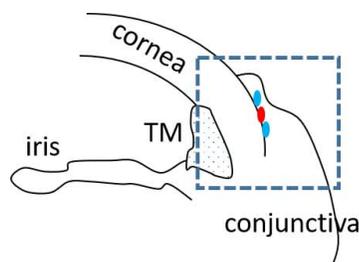
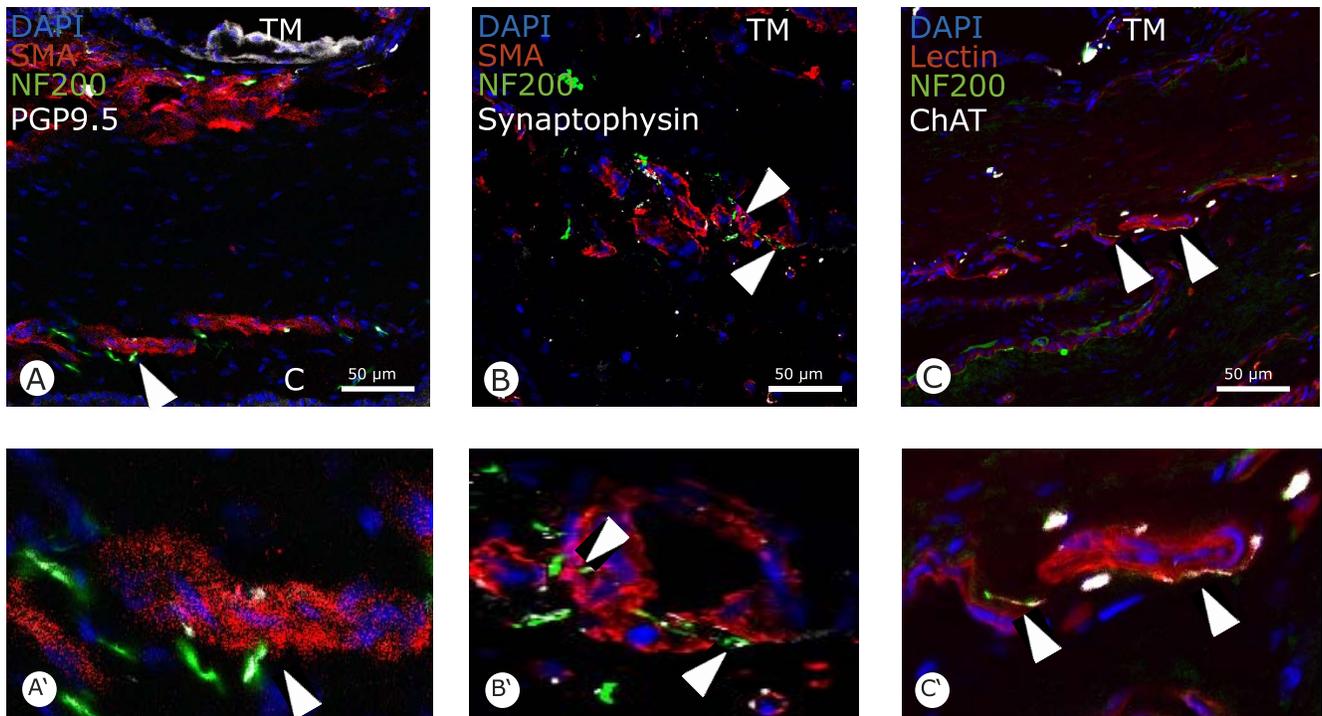
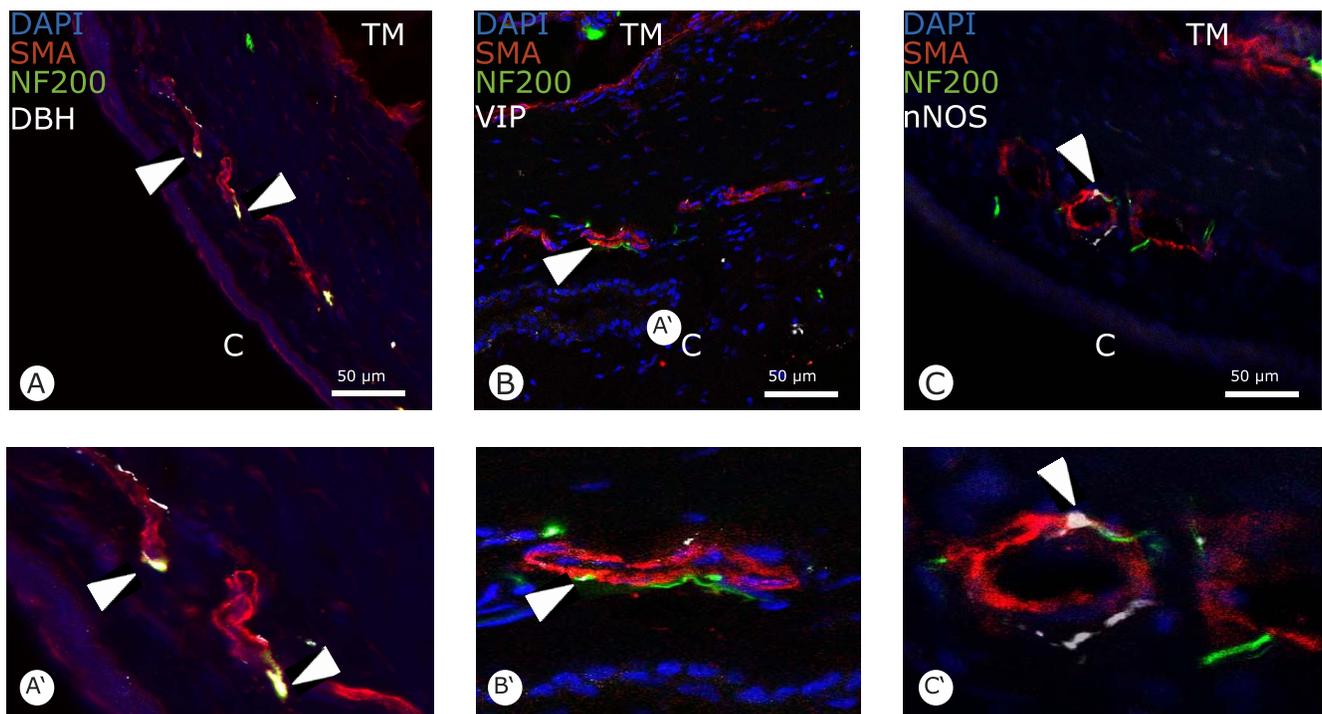


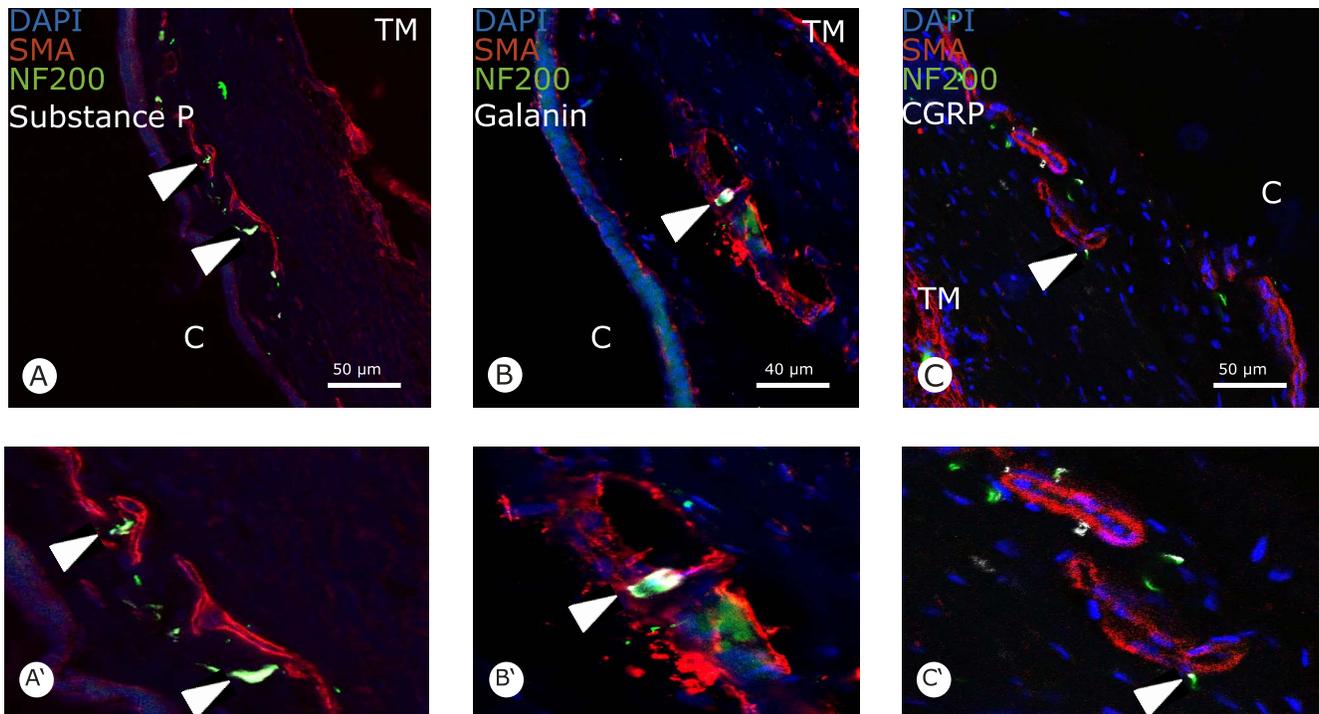
FIGURE 1. *Left*: Schematic drawing of the region under investigation. Sagittal sections of the rat eyes were used after removal of the lens. Depending on the magnification, some images contain sections of the trabecular meshwork as well. In all images, the orientation of the conjunctiva and trabecular meshwork is marked with C and TM, respectively. *Right*: schematic drawing of the subsequent vascular beds in the rat episcleral circulation (redrawn after Ref. 7 and Ref. 13).



**FIGURE 2.** Immunohistochemistry results for PGP 9.5 (A), synaptophysin (B) and ChAT (C). In all images the same color-coding was applied (top left corner of the image). The white arrowhead shows the overlap between the neurotransmitter under investigation (white, magnified image of [A'-C'] displayed in the lower row) and the neurofilament positive structures (NF200, green) in close proximity to the vessel wall (SMA or Lectin, red). The orientation of the images (not the structures) is indicated with C and TM respectively. Figure 1 provides a description of the morphology.



**FIGURE 3.** Immunohistochemistry results for DBH (A), VIP (B), and nNOS (C). In all images the same color-coding was applied (top left corner of the image). The white arrowhead shows the overlap between the neurotransmitter under investigation (white, magnified image of [A'-C'] displayed in the lower row) and the neurofilament positive structures (NF200, green) in close proximity to the vessel wall (SMA, red). The orientation of the images (not the structures) is indicated with C and TM respectively. Figure 1 provides a description of the morphology.



**FIGURE 4.** Immunohistochemistry results for Substance P (A), Galanin (B), and CGRP (C). In all images the same color-coding was applied (top left corner of the image). The white arrowhead shows the overlap between the neurotransmitter under investigation (white, magnified image of [A'–C] displayed in the lower row) and the neurofilament positive structures (NF200, green) in close proximity to the vessel wall (SMA, red). The orientation of the images (not the structures) is indicated with C and TM, respectively. Figure 1 provides a description of the morphology.

central nervous system (CNS) origin of this innervation pathway in rats (Fitzgerald ME, personal communication) and functional data could confirm the ability of this pathway to alter EVP.<sup>11</sup> The decrease in episcleral venous pressure after application of topical anesthesia (using proparacaine) suggests the presence of a neuronal control of the vascular tone.<sup>10</sup> Furthermore, the subsequent orbital veins downstream of the episcleral circulation exhibit considerably lower pressure values of approximately 2 mmHg in rabbits.<sup>14</sup> While the origin of this pressure drop is unknown, this evidence from the literature indicates IOP lowering potential by blocking the neuronal tone in the episcleral circulation.

The positive staining for CGRP, neuronal nitric oxide synthase (nNOS), VIP, and substance P is in accordance with previously reported data. Selbach et al.<sup>7</sup> demonstrated the presence of these neuropeptides in the arteriovenous (AV) anastomosis and the limbal arcades of the episcleral circulation in rats and rabbits,<sup>7</sup> while the presence of choline acetyl transferase (ChAT), dopamine  $\beta$  hydroxylase, PGP 9.5, and galanin have not been reported to the best of the authors' knowledge.

Thus, the novel finding of our study is the morphologic proof of the autonomic innervation of the episcleral circulation.

A broad panel of antibodies was used to search for candidate substances for neurotransmitters in the episcleral circulation for further functional experiments. The morphology of the episcleral circulation has been described in detail by Selbach et al.<sup>7</sup> and it was not the scope of our study to repeat this work.

The physiologic function of the AV anastomosis is only partly known, with the effect of constriction and dilation on IOP well demonstrated.<sup>15</sup> Even though it is tempting to speculate on further functions based on the neurotransmitters found in our study, appropriate in vivo experiments are

needed. Some neurotransmitters have well-established functions in other organs and are discussed below.

A puzzling morphologic feature is the existence of two different types of AV anastomoses, one in the limbal vasculature and one in the more posterior episcleral vasculature (Fig. 1). While in vivo observations of the latter show an effect on IOP during diameter changes,<sup>15</sup> for the former no such data exist.

Substance P and CGRP have been identified in the episcleral circulation and their vasoactive properties are well documented.<sup>16,17</sup> A cautious interpretation of the morphology–function relationship is necessary, however. Results from the mesenteric artery, for example, show no effect of substance P antagonists in neuronal stimulation experiments despite Substance P presence in immunohistochemistry.<sup>18</sup>

The nitric oxide system needs thorough discussion as well. nNOS has been found in the nerve fiber ending at the arteriovenous anastomosis of the episcleral circulation of rats before.<sup>19</sup> Zamora and Kiel<sup>20</sup> demonstrated an increase in EVP after the application of the NO donor nitroprusside and a decrease after NOS inhibition in a rabbit model without neuronal stimulation.<sup>20</sup> The effect on neuronal control, however, might be more complex. A recent study investigating the neuronal control of the retinal circulation suggests that NO is not the sole neurotransmitter, as NOS inhibition diminished but did not abolish the stimulation effect.<sup>21</sup> A likely candidate as an additional neurotransmitter is vasoactive intestinal peptide (VIP) as numerous immunohistochemistry studies have resulted in congruent staining of those two neurotransmitters.

VIP is expressed in cholinergic parasympathetic neurons and has been described previously as having vasodilating properties.<sup>22</sup> VIP immunoreactivity has been demonstrated around the episcleral vasculature in rats<sup>7</sup> and was found again

TABLE 2. Neurotransmitter Results

Antibody Against	Results
nNOS	nNOS positive structures overlap with NF200 immunoreactivity and run through the SMA positive vascular structure (Fig. 3C).
ChAT	Fine NF200 positive structures run along the SMA positive vascular structures, with colocalized ChAT immunoreactivity. (Fig. 2C)
Synaptophysin	NF200 positive structures run across the SMA positive vessel wall. Dotted synaptophysin immunoreactivity can be seen along the nerves in the vessel wall. (Fig. 2B)
CGRP	CGRP immunoreactivity colocalizes with NF200 reactivity adjacent to the SMA positive vessel wall. (Fig. 4C)
Dopamine- $\beta$ -Hydroxylase (DBH)	DBH and NF200 colocalize adjacent to the SMA positive vessel wall. (Fig. 3A)
PGP 9.5	PGP 9.5 positive immunoreactivity overlaps with NF200 immunoreactivity in close proximity and partial overlap with SMA positive vascular structures. (Fig. 2A)
galanin	A GAL and NF200 positive perikaryon was detected in close proximity to the vessel wall. (Fig. 4B)
Substance P	Multiple NF200 positive structures approach the vessel wall and colocalize with SP (Fig. 4A)
VIP	Fine nervous structures (NF200 pos.) run in parallel to the vessel wall with colocalized VIP immunoreactivity. (Fig. 3B)

in this study with colocalization in neuronal structures (neurofilament positive).

PGP 9.5 (UCHL1) is a protein involved in generating the ubiquitin monomer and is highly conserved among species.<sup>23</sup> It is considered to be highly specific for neuronal tissues in the peripheral and central nervous system. In our study, PGP 9.5 was found in structures colocalizing with neurofilament (200 kDa).

ChAT is expressed in cholinergic neurons. Its presence in the episcleral circulation in rats suggests parasympathetic innervation, as has been demonstrated for humans and monkeys. Furthermore, the concomitant immunoreactivity for VIP and ChAT also is indicative of parasympathetic innervation. However, any assumptions on a possible functional significance should be made with caution, as the parasympathetic innervation might have different purposes than episcleral pressure regulation. In the rabbit choroid, for example, muscarinic blockade did not alter the blood flow response to parasympathetic stimulation (facial nerve) despite ChAT positive fibers in the choroid.<sup>24,25</sup>

Dopamine  $\beta$  hydroxylase is the last enzyme in noradrenaline synthesis and was used as a marker for sympathetic innervation.<sup>26</sup> Positive immunoreactivity indicates a sympathetic innervation of the episcleral structures, which is in accordance with data for humans and monkeys. For the functional significance of the sympathetic innervation, there is more evidence than for the other candidate substances. Kawarai and Koss<sup>27</sup> and Koss<sup>28</sup> demonstrated that the sympathetic innervation of the choroid in rats elicits vasocon-

striction in the choroid. For the episcleral circulation there is evidence for a vasoconstriction (and drop in IOP) through epinephrine, but this study did not address the innervation.<sup>29</sup>

Galanin has not been described in the episcleral circulation in the current literature. A recent study, however, reported galanin staining through all layers of the conjunctiva next to the limbus (in humans).<sup>30</sup> While galanin has a plethora of biological functions,<sup>31</sup> its possible vasoactive properties are relevant to our study.<sup>32</sup>

In conclusion, our study adds knowledge about neurotransmitters in the episcleral circulation in rats. Some of the candidate substances detected have been previously examined in other studies and are in accordance with our findings. Especially ChAT and DBH in combination with VIP and substance P suggest parasympathetic and sympathetic innervation, which has not been reported for rats. Furthermore, galanin-positive staining is a novel finding in the episcleral circulation across all species investigated in the current literature. Additional studies investigating the physiology and pharmacology of these transmitters are needed to clarify their role in EVP regulation.

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