The Structure of the Trabecular Meshwork, Its Connections to the Ciliary Muscle, and the Effect of Pilocarpine on Outflow Facility in Mice

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PURPOSE. To determine the connections between the ciliary muscle (CM), trabecular meshwork (TM), and Schlemm's canal (SC) and their innervations that allows CM contraction (by pilocarpine) to influence conventional outflow in mice.

METHODS. Sequential sections and whole mounts of murine corneoscleral angles were stained for elastin, α -smooth muscle actin (α SMA), vesicular acetylcholine transporter (VACHT), neuronal nitric oxide synthase (nNOS), vasoactive intestinal peptide (VIP), and tyrosine hydroxylase (TH). Elastic (EL) fibers between the CM, TM, and SC were examined in ultrathin, sequential sections from different planes. The effect of pilocarpine (100 μ M) on conventional outflow facility was measured by perfusion of enucleated mouse eyes.

RESULTS. The mouse TM contains a three-dimensional (3D) net of EL fibers connecting the inner wall of SC to the cornea anteriorly, the ciliary body (CB) internally and the choroid and CM posteriorly. The CM bifurcates near the posterior TM, extending outer tendons to the juxtacanalicular tissue and inner wall of SC and internal connections to the lamellated TM and CB. Ciliary muscle and lamellated TM cells stain with α SMA and are innervated by VACHT-containing nerve fibers, without TH, VIP, or nNOS. Pilocarpine doubled outflow facility.

CONCLUSIONS. Mouse eyes resemble primate eyes not only by their well developed SC and TM, but also by their 3D EL net tethering together the TM and SC inner wall and by the tendinous insertion of the CM into this net. The increase in outflow facility following cholinergic stimulation in mice, as in primates, supports using mice for studies of aqueous humor dynamics and glaucoma.

Keywords: trabecular meshwork, ciliary muscle, glaucoma, aqueous humor, pilocarpine

In the eyes of primates that have a well-developed accommodative system, contraction of the ciliary muscle (CM) increases outflow facility.^{1,2} Ciliary muscle tension is transmitted via elastic (EL) tendons that merge with the core of the corneoscleral lamellae or with the cribriform EL plexus that underlies the inner wall of Schlemm's canal (SC) in primates.^{3,4} The connections to the cribriform plexus provide a mechanism by which CM tone may directly affect outflow facility by maintaining patency of a collapsible SC and by modulating tension on the inner wall and juxtacanalicular tissue (JCT), where the bulk of outflow resistance is likely generated.^{5,6} An important consideration is whether similar anatomical and functional connections between the CM and SC exist within the trabecular meshwork (TM) of nonprimate animal models typically used for aqueous outflow and glaucoma studies.

The mouse is rapidly becoming an accepted model for studies of aqueous humor outflow and IOP regulation. Mice, like primates, have a continuous SC with a lamellated, albeit thinner, TM having 3 to 4 trabecular lamellae⁷ compared with nearly 20 in human.⁸ Mice are also relatively inexpensive and highly amenable to genetic manipulation, allowing detailed

studies of outflow physiology.^{9–19} Like humans, mice do not appear to exhibit the time-dependent increase in outflow facility known as “washout”¹⁵ that is observed in porcine,²⁰ bovine,²¹ and even living monkeys.²² Because several murine models of ocular hypertension or outflow obstruction have been developed,^{12,23–26} there are opportunities to exploit the mouse model to understand basic mechanisms of IOP regulation as relevant for glaucoma. Because mice do not accommodate, the CM is small and poorly developed^{7,27} relative to primates.²⁸ However, pilocarpine lowers IOP in living mice^{29–31} suggesting that despite its smaller anatomy, the murine CM may indeed be capable of physiological outflow regulation.

In this study, we examined sequential histologic and ultrathin sections to investigate the three-dimensional (3D) structure of the TM, CM, and EL fiber net in mouse eyes. To examine the functional role of this EL fiber net, we investigated the innervation of the outflow tissues and in enucleated mouse eyes measured the effect of pilocarpine on conventional outflow facility.

TABLE. Specific Antibodies Used for Immunofluorescence Microscopy

Protein	Antibody	Company, Clone/Catalog Number	Dilution in PBS
α -SMA	Rabbit monoclonal antibody	Epitomics (Burlingame, CA, USA); EPR5368	1:500
α -SMA-Cy3	Mouse monoclonal antibody	Sigma-Aldrich (St. Louis, MO, USA); 1A4	1:300
nNOS/NOS type I	Mouse monoclonal antibody	BD Biosciences (San Jose, CA, USA); 16/nNOS/NOS Type 1	1:200
TH	Rabbit polyclonal antibody	Millipore (Billerica, MA, USA); AB152	1:400
VIP	Rabbit polyclonal antibody	Sigma-Aldrich; V3508	1:700
VACHT	Goat polyclonal antibody	BD Biosciences; 556337	1:700

MATERIALS AND METHODS

All experiments were done in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under UK Home Office Project License 70/7306. All mice were 10 to 14 weeks of age at the time of the experiment. Morphology was examined in C57BL/6 and BALB/c (albino) mice, where BALB/c were used because the heavy pigmentation in C57BL/6 complicated immunohistochemistry especially for whole-mount preparations.

Immunofluorescence Staining

Twenty whole globes from 10 adult mice from two inbred strains (five C57BL/6 and five BALB/c) were enucleated and fixed in 4% paraformaldehyde (Carl Roth, Karlsruhe, Germany) for 4 hours, washed in PBS, and embedded in paraffin. Sagittal sections (5 μ m) were cut and stained as described below. To investigate regional differences, an additional 12 eyes from three C57BL/6 and three BALB/c mice were marked by placing a small suture through the superior or temporal quadrants. For each pair of eyes, one eye was cut along the nasal-temporal axis, while the contralateral eye was cut along the superior-inferior axis. For whole-mount preparations, eight additional eyes from two C57BL/6 and two BALB/c mice were bisected sagittally under a stereomicroscope, the lens carefully removed, and the iris lifted to visualize and cut the pectinate ligaments near their insertion at the cornea. The iris and ciliary processes were carefully separated from the CM and outer TM, and placed on a slide with the inner lamellated TM facing the microscope objective. The remaining specimen contained the JCT, outer lamellated TM and most of the CM, and was placed on a slide with the JCT facing the microscope objective. For depigmentation, whole mounts were bleached with 1% hydrogen peroxide for 4 to 6 hours.

The specimens were incubated in BLOTTO's Blocking Buffer (Thermo Scientific, Waltham, MA, USA) at room temperature for 1 hour to reduce nonspecific background staining. Following three washes in Tris-buffered PBS, specimens were incubated with the primary antibody (Table) at 4°C overnight, washed three times with PBS, and incubated with a secondary antibody (Alexa 488 or 555) for 2 hours. For double immunolabeling, specimens were washed three times with PBS, incubated with the second primary antibody (Table) at 4°C overnight, washed three times with PBS and incubated with secondary antibody (Alexa 555 or 488) for 2 hours. The slides were examined with a Keyence Biorevo BZ9000 microscope (Keyence, Neu-Isenburg, Germany).

Antibody against α -smooth muscle actin (α SMA) was used to visualize contractile cells, vesicular acetylcholine transporter (VACHT) to visualize cholinergic nerves, tyrosine hydroxylase (TH) to visualize sympathetic nerves, neuronal nitric oxide synthase (nNOS), and vasoactive intestinal peptide (VIP) to visualize parasympathetic fibers of the facial nerve. Antibody details are given in the Table.

Resorcin-Fuchsin Staining for Elastic Fibers

Sagittal sections and oblique/tangential sections were prepared and stained in an additional 12 paraffin embedded eyes from three C57BL/6 and three BALB/c mice. Oblique/tangential sections were parallel to the outer or inner walls of SC and the TM, and were examined to visualize the EL fiber structure of the TM and its interconnections to the CB, the anterior fixation of the TM at the cornea, and posterior insertion of the TM at the sclera. To investigate the choroidal EL fibers and their insertion into the sclera and TM as well as the outer EL CM tendons and their connection to the TM, whole mounts were prepared from another eight eyes from two C57BL/6 and two BALB/c mice. For these whole mounts, the globes were bisected midsagittally and the lens removed. The entire iris, CB, and most of the CM were removed, leaving the outer TM, the outer portion of the choroidal EL net, and portions of the CM tips that connected to the scleral spur (SS; if present), sclera, or outer TM. Whole mounts were examined en face with the choroidal EL net facing the microscope objective. To investigate the connections between the EL CM tendons and the TM EL fiber net, whole mounts from an additional four BALB/c mice were prepared in the same way as described above without removing the CB or CM so as to leave intact the entire TM. The whole mounts (~2 mm² in area) were fixed to a freezing block at the sclera, and sequentially cryosectioned through the TM and CM along a plane tangent to the scleral surface.

Specimens were fixed in 4% paraformaldehyde (Carl Roth) for 4 hours and incubated in resorcin-fuchsin solution for 30 minutes. Specimens were washed three times with distilled water for 2 minutes each, incubated twice in 96% and once in 100% ethanol for 3 minutes each. To distinguish CM cells from their EL tendons and from the TM EL fiber net, tangential sections were additionally incubated in picric acid and thiazin red for 3 minutes to stain muscle cells yellow-brown so as to provide contrast against the purple resorcin-fuchsin staining of EL fibers. Slides were examined with a Keyence Biorevo BZ9000 microscope.

Electron Microscopy

Twenty whole globes from five BALB/c and five C57BL/6 mice were immersion fixed immediately after enucleation in Ito's fixative.³² A small incision was made in the cornea to promote fixative entry into the eye. All eyes were post fixed in OsO₄, dehydrated in a graded ethanol series, and whole eyes were embedded in Epon resin. Semithin sagittal sections (1- μ m thick) were cut with an ultramicrotome (Ultracut E; Reichert Jung, Vienna, Austria) and stained with toluidine blue. Histologic sections were viewed and photographed with a Biorevo BZ-9000 microscope. Sequential sagittal ultrathin sections of the TM were cut, stained with uranyl acetate and lead citrate, and viewed with an electron microscope (EM 902; Zeiss, Oberkochen, Germany). To obtain tangential sections parallel to the inner wall of SC, sequential semithin sections were cut through the sclera at the limbus until the outer wall

FIGURE 1. Conventional outflow pathway of the mouse eye, showing the TM, SC, and connections with the CM. (A) Sagittal semithin section stained with toluidine blue (C57BL/6 mouse). The CB apposes nearly two-thirds of the internal TM surface. Some of the TM lamellae attach to the pectinate ligament (PL) spanning between the peripheral cornea and iris root. (B) Histologic sagittal section showing α SMA labeling in the CM extending into the TM where there is intense labeling throughout the entire juxtacanalicular tissue and lamellated TM (C57BL/6 mouse). The internal and external branches of the CM are indicated. Red blood cells are visible within SC lumen, but are absent from the juxtacanalicular tissue and TM. (C) Whole mount of α SMA labeling showing the anterior-posterior extent of the compact CM from posterior tips (arrows) to the posterior margin of the TM, making occasional connections to the TM (arrowheads). Intense, but incomplete, α SMA staining is also present within the circularly arranged TM, which due to the unlabeled intertrabecular spaces can be distinguished based on its sponge-like appearance from the compact CM (BALB/c mouse). (D) Transmission electron micrograph near the posterior end of SC showing the branching of CM cells (arrow) that are surrounded by a continuous basement membrane. Cell processes connect CM cells to one another. Outer CM cells extend elastic (EL) fiber tendons (arrowhead) that make connections with the inner wall endothelium of SC, and the tendons and CM cells themselves connect to the EL fibers in the juxtacanalicular tissue. The CM is highly innervated by nerve fibers (N; C57BL/6 mouse).

of SC was reached, followed by sequential ultrathin sections until reaching the inner wall, JCT, or lamellated TM.

Perfusion of Enucleated Eyes

Thirteen eyes from seven C57BL/6J mice were enucleated within 15 minutes postmortem and perfused intracamerally via a 33-G needle connected to a computerized perfusion system²¹ optimized for mouse eyes.^{15,17} Experimental eyes were perfused with or without 100 μ M pilocarpine (Tocris Bioscience, MN, USA) in Dulbecco's PBS containing 5.5 mM glucose and 0.3% dimethyl sulfoxide (DMSO). Contralateral control eyes were perfused with Dulbecco's PBS containing 5.5 mM glucose and 0.3% DMSO without pilocarpine. Each eye was perfused at sequential pressure steps of 4, 8, 15, and 25 mm Hg, and conventional outflow facility (C) was calculated as the slope of the flow versus pressure relationship as previously

described.^{15,17} Statistical analysis to test the effect of pilocarpine was performed using the linear mixed model (IBM SPSS Statistics; IBM Corp., Armonk, NY, USA) that accounts for correlated errors between contralateral eyes while also allowing for the occasional unpaired eye (one eye was unpaired in this study due to perfusion failure), as previously described.¹⁹ Statistical significance was defined to be *P* less than or equal to 0.05.

RESULTS

Ciliary Muscle and Its Relationship to the Trabecular Meshwork

In mice, SC is well developed, typically consisting of a single lumen, with the CB and associated ciliary processes typically apposing approximately two-thirds of the internal TM surface

FIGURE 2. Regional differences in the conventional outflow pathway of the mouse eye. (A) Sagittal semithin section stained for toluidine blue (BALB/c, temporal quadrant). Schlemm's Canal and outer TM are expanded, and the CM is relatively thick compared with Figure 1A. The posterior muscle tips (*arrow*) extend into the choroid underlying the peripheral retina (R), which is artificially damaged in the section. (B) Histologic sagittal section showing α SMA labeling in the small CM extending along the internal face of the lamellated TM (C57BL/6, nasal quadrant). In contrast to Figure 1B, the α SMA-positive region of the TM does not cover the entire extent of SC, and there is no staining of the outer TM or juxtacanalicular tissue in this section (*arrowhead*). (C) Whole mount of the CM and TM showing that where the anterior ciliary artery (CA) passes through the sclera into the uvea, there is no α SMA labeling in the CM region (BALB/c, superior quadrant). The TM shows nonuniform α SMA staining. (D) Electron micrograph of a sagittal section through the posterior end of SC and TM in a region near a CA (C57BL/6, superior quadrant). In the area where in other parts of the circumference CM cells are typically present (cf. Fig. 1D), CM cells are absent and there are only connective tissue beams (*arrows*) continuous with the central core of the TM lamellae.

(Fig. 1A). The small CM consists of nearly parallel fiber bundles extending anteriorly from the choroid to reach the posterior TM and SC. α -Smooth muscle actin reactivity reveals that the CM bifurcates near the posterior TM into internal and external fiber bundles (Fig. 1B). The external CM fibers terminate in the sclera or SS (see below) near the posterior margin of SC or extend to the JCT region underlying the inner wall of SC. The internal CM fiber bundle extends into the lamellated trabeculae that are continuous with the connective tissue of the CB stroma.

The cells that cover the lamellated trabecular beams show an intense, but nonuniform, labeling for α SMA (Fig. 1B), and α SMA-labeled TM cells are not easily distinguished from CM cells within individual sagittal sections. In whole mounts, however, α SMA labeling within the TM shows a predominately circumferential orientation with a sponge-like appearance due to the intertrabecular spaces (Fig. 1C). In contrast, the α SMA labeling of the CM forms a compact continuous structure on its

anterior end, whereas its posterior end forms pointed tips terminating in the choroid (Fig. 1C). At the ultrastructural level, the CM fibers consist of branching smooth muscle cells with nearly parallel myofibrils attached via adhesion plaques to the cell membrane (Fig. 1D). Ciliary muscle cells are highly innervated and are surrounded by a complete basement membrane, and CM cells are connected to one another by cell processes (Fig. 1D). Ciliary muscle cells contact the EL fibers present within the connective tissue of the muscle and within the JCT (Fig. 1D). Elastic fiber tendons extending from CM cells also contact the EL fibers of the JCT and the inner wall endothelium of SC (Fig. 1D).

There are regional differences in the structure of the anterior CM and the extent of α SMA labeling in the TM. The thickness of the anterior CM varies along the circumference (cf. Figs. 2A, 1A), but is often thinner in the nasal quadrant (Fig. 2B). In some locations, α SMA labeling extends anteriorly past

FIGURE 3. Elastic fiber net within the conventional outflow pathway of mouse eyes. (A) Histologic sagittal section stained with resorcin-fuchsin for elastin (BALB/c, nasal quadrant). The scleral sulcus is surrounded by stained EL fibers (purple) along the outer wall of SC and in the TM. In this nasal quadrant, there is a well-developed SS containing numerous EL fibers. Purple-stained tendons of the outer CM insert into the SS (arrow). At the anterior surface of the CB stroma, the TM lamellae are cut in a somewhat oblique plane showing EL fibers (arrowheads) extending outwardly from the CB to outer TM. The EL fiber staining is continuous between the anterior TM, pectinate ligament and peripheral cornea (CO). (B) Oblique histologic section through the CB and TM stained with resorcin-fuchsin showing a dense net of EL fibers stretching between the CB and inner wall of SC (C57BL/6 mouse). Arrows indicate nuclei of SC endothelial cells along the dense net of EL fibers stretching between the CB and inner wall of SC. (C) Whole mount of TM, choroid, and remnants of CM muscle tips (dark areas) stained with resorcin-fuchsin showing longitudinal EL fibers of the choroid and tendons of the CM extending into and merging with the circumferential EL fibers of the TM (BALB/c mouse). Arrows indicate EL fibers and tendons bending into the EL net of the TM. (D) Tangential section through the TM and CM stained with resorcin-fuchsin and picric acid (to stain CM fibers yellow) showing EL tendons (arrows) extending anteriorly from the CM to bend and insert into the EL fiber net of the TM (arrowheads) or into the EL fibers of the CO (C57BL/6 mouse). S, Sclera.

SC (Fig. 1B) and may be continuous with the α SMA labeling of the iris dilator muscle; while in other parts of the circumference, α SMA labeling terminates midway along the anterior-posterior extent of the TM (Fig. 2B). Pronounced regional differences are also seen in α SMA labeling in the JCT, with some areas showing nearly complete α SMA labeling of JCT cells (Fig. 1B), and other regions showing little or no α SMA labeling in the JCT. Lack of α SMA labeling in the JCT is often seen in the nasal quadrant (Fig. 2B), where a SS is typically present (see below). In the superior and inferior quadrants, where the anterior ciliary arteries pass through the CM

region,³³ α SMA-positive CM cells are largely absent (Fig. 2C). Ultrastructural investigation reveals that where the CM cells are absent, connective tissue beams tether the TM to the choroidal connective tissue (Fig. 2D).

Structure of the Elastic Fiber Net

In mice, like in primates, the scleral sulcus contains more EL fibers than the sclera proper, with the most intense staining for EL fibers underneath the outer wall of SC (Fig. 3A). In the nasal quadrant, there tends to be a well-developed SS that stains

FIGURE 4. Ultrastructural images of the connections between the EL fiber net and inner wall endothelium of SC and ciliary muscle (CM). (A) Sagittal section through the posterior juxtacanalicular tissue showing that the anterior tips of the CM cells, identified by their complete basement membrane, form fine fibrillar tendons (arrows) that connect to the TM cells in the outer juxtacanalicular tissue, identified by their incomplete basement membrane (C57BL/6 mouse). (B) Sagittal section through the posterior juxtacanalicular tissue and SC showing CM cells, extending toward the inner wall endothelium of the posterior SC (BALB/c mouse). *Inset:* higher magnification of the CM cell forming a tendon (arrow) that extends toward a juxtacanalicular cell that exhibits significant nuclear deformation, likely resulting from the contractile force of the CM cell. (C) Tangential section through the inner wall endothelium of SC and juxtacanalicular tissue showing numerous EL fibers that make direct connections to SC cells (BALB/c mouse). (D) Tangential section through the inner wall at higher magnification showing direct connections between EL fibers and SC cells (arrowhead) or their basement membrane (arrow; BALB/c mouse).

intensely for EL fibers (Fig. 3A) and is nearly absent elsewhere. Intense staining for EL fibers is also seen in the TM, at the transition between the TM and CM and, in the nasal quadrant, between the TM and SS. Some EL tendons of the CM merge with the EL fibers of the SS, and the EL fibers of the TM extend from the SS to the anterior termination of the TM at the peripheral cornea and pectinate ligaments (Fig. 3A).

Internally, numerous EL fibers extend from the CB stroma adjacent to the ciliary epithelium and pass outwardly to connect to the TM and the endothelial lining of SC (Fig. 3A, 3B). These outward-directed fibers interweave with anterior-posterior directed fibers in the TM that extend from the choroid and CM toward the cornea and pectinate ligaments. These interweaving fibers create a dense EL fiber net that tethers

FIGURE 5. Whole mounts of the CM and TM of BALB/c mice analyzed with antibodies against α SMA (red) and vesicular acetylcholine transporter (VChT) or TH (green). (A) There are several VChT immunoreactive (IR) nerve fibers and terminals along the CM (arrows). (B) Numerous VChT-IR nerves and terminals (arrows) are also present in the TM. (C) A large TH-IR nerve bundle (arrow) passes through the CM, but there are no reactive nerve varicosities along either CM or TM cells. Single varicosities are observed along small vessels in the CM. (D) The same whole mount as shown in (C), but focused deeper into the episclera, where the episcleral vessels (arrows) are surrounded by TH-IR nerve varicosities.

together the inner wall, TM, and CB (Fig. 3B). The longitudinally oriented EL fiber net of the choroid and the EL tendons of the outer CM make 90° bends and merge with the EL fiber net of the TM (Fig. 3C). Elastic fiber tendons extending from CM cells branch and join the EL fiber net in the TM or extend anteriorly to merge with the EL fibers of the peripheral cornea (Fig. 3D).

Ultrastructural images show the EL fiber net in the TM and EL tendons of the anterior CM cells that make extensive connections to the cells of the JCT (Figs. 4A, 4B) and inner wall of SC (Figs. 4C, 4D). The EL fibers appear osmiophilic with interrupted elastin surrounded by a sheath of fine fibrillar material. Whereas, the innermost portion of the EL net is contained within the cores of the trabecular lamellae surrounded by TM cells, the outermost portion of the EL net in the JCT is exposed without a continuous layer of surrounding cells. Adhesions to the EL net occur through direct connections to the inner wall cells (Figs. 4C, 4D). The JCT and SC cells that are connected to CM tendons often appear highly contorted with cell and nuclear processes extending toward the CM fiber, suggesting that the CM cells are capable of exerting significant tensional force on the JCT and inner wall (Fig. 4B).

Parasympathetic and Sympathetic Innervation of the Mouse Outflow Pathway

Whole-mounts analyzed with antibodies against VChT reveal parasympathetic VChT-labeled fine nerve fibers and terminals

surrounding the CM bundles (Fig. 5A). Vesicular acetylcholine transporter-labeled fibers and terminals are also present in the TM (Fig. 5B). In contrast, TH reactivity for sympathetic nerves reveal only large nerve fibers passing through the muscle area without varicosities at the muscle cells but only at the vasculature between muscle fibers (Fig. 5C). Tyrosine hydroxylase-labeled varicosities are absent from the TM, however extensive TH-labeled varicosities are observed along the episcleral vessels (Fig. 5D). The CM and TM do not show labeling for nNOS nor VIP, however nNOS-labeled fibers and terminals are seen surrounding the episcleral vessels (not shown), similar to that seen for TH. All described morphological features are present in BALB/c and C57BL/6 mice with no prominent differences between the two strains.

Effects of Pilocarpine on Outflow Facility

To examine the functional significance of the connections between the CM and TM, we perfused enucleated C57BL/6J mouse eyes with or without 100 μ M pilocarpine. At each perfusion pressure, pilocarpine significantly increases the flow rate (Fig. 6). Conventional outflow facility (C), defined as the slope of the flow-versus-pressure relationship, increases nearly 2-fold in response to pilocarpine from 6.9 ± 2.5 to 13.1 ± 3.9 nL/min/mm Hg (mean $\pm$ SD; $P = 0.003$). In contrast, there is no change in the zero-pressure intercept, often taken to represent unconventional or uveoscleral outflow, with or

FIGURE 6. The flow rate (Q) measured at each perfusion pressure (P) for enucleated mouse eyes with or without 100 μ M pilocarpine (Pilo). Pilocarpine significantly increases Q for each P and increases 2-fold the slope of the Q versus P relationship, representing a 2-fold increase in conventional outflow facility (C57BL/6 mice).

without pilocarpine (-26.2 ± 26.7 vs. -10.3 ± 19.7 nL/min; $P = 0.21$).

DISCUSSION

The mouse TM contains a net of EL fibers that spans the scleral sulcus and tethers together the CM, choroid, TM, and inner wall of SC (Fig. 7). This EL net is similar to that found in primates,^{4,34} and in mice as in primates, the sheath surrounding the EL fiber net makes extensive connections to cells of the JCT and SC or to their basement membranes.³⁵ A similar EL net is also present in bovine³⁶ and porcine³⁷ eyes that possess a reticular rather than lamellated TM and a discontinuous angular aqueous plexus (AAP) rather than a continuous SC, and the EL net attaches directly to the AAP endothelium.^{36,37} The EL net of the TM represents a tensionally-integrated system that presumably functions across species to tether the TM and inner wall to surrounding tissues so as to oppose IOP-induced collapse of outflow pathways that would otherwise compromise outflow function.

In contrast to bovine and porcine, the CM in mice forms tendons that extend anteriorly and bend to insert into the EL net of the lamellated TM or to make direct contact with JCT or SC cells. Tension generated by the CM is thus transmitted directly to the TM EL net, and thereby to SC endothelium and JCT. This connection between CM tendons and the TM EL net in mice most resembles that in primates, where it is well established that CM contraction can expand the TM and JCT to increase outflow facility.³ In contrast, in porcine and bovine the CM is located far posteriorly and has no direct contacts to the TM EL net.^{36–38} Thus, the integration of the CM tendons into the TM EL net allows the potential for active regulation of outflow by the CM in mice and primates.

In mice, the anterior insertion of the TM with its pectinate ligaments is wider than the posterior insertion at the small CM, so that the TM in mice gains a fan-like appearance that can open anteriorly. This is in contrast with the TM in primates where the TM-fan is fixed anteriorly at the cornea and opens posteriorly. Regardless of differences in anterior versus posterior TM fixation, CM contraction appears to open the TM in both species³¹ to presumably increase outflow facility. In fact, our physiological data reveal that pilocarpine increases outflow facility in enucleated mouse eyes, consistent with the

FIGURE 7. A schematic illustration of the architecture of the conventional outflow pathway in mouse eyes, showing the relationship between the CM, TM, and inner wall (IW) of SC. The CM bifurcates into internal and external branches. An EL fiber net is present within the TM and juxtacanalicular tissue, and tendons of the external CM and the EL fibers of the choroid bend to insert into the juxtacanalicular EL net or connect directly to the IW endothelium. The internal CM makes connections to the lamellated TM that is continuous with the CB stroma. Elastic fibers bridge between the lamellated TM and juxtacanalicular tissue, and α SMA-labeled cells (pink) are present within the lamellated TM and occasionally in the juxtacanalicular tissue.

pilocarpine-induced decrease in IOP observed in living mice.^{29,30} Unfortunately, the opening of the TM and/or anterior chamber angle could not be reliably measured by histology in the present study because displacement of the large lens during sectioning may affect the dimensions of the outflow pathway. These problems may be overcome by *in vivo* studies where either the eye is fixed via perfusion through the heart or by direct imaging of the anterior chamber angle in living mice exposed to pilocarpine. In our accompanying paper, Li et al.³¹ demonstrate that pilocarpine increases outflow facility in living mice with a concomitant widening of SC and opening of the TM-fan. The magnitude of the pilocarpine-induced facility increase in mice (2-fold) is consistent with the 2- to 3-fold facility increase reported in vervet¹ and owl monkeys.³⁹

The effect of pilocarpine on outflow facility is consistent with the dense cholinergic innervation of the CM in mice. In mice, as in primates,⁴⁰ there are no TH-labeled varicosities in the CM, suggesting the absence of sympathetic innervation. Presumably in mice, like in primates, there is no muscular antagonist to CM contraction, but rather the relaxed CM is pulled backward by the recoil of the choroidal EL net and the posterior EL tendons of the CM that were expanded during contraction of the CM.

The TM in mice, like in primates, contain VACHT-immunoreactive (IR) nerve terminals⁴⁰ and α SMA-IR cells.^{41–43} In mice, like in primates,⁴³ α SMA-IR cells are found in all layers of the TM, whereas in bovine eyes α SMA-IR cells are restricted to the elongated cells located between the CM and TM.⁴⁴ *In vitro* perfusion of bovine anterior segments suggest that cholinergic stimulation induces contraction of α SMA-IR cells in the TM and decreases outflow facility.⁴⁵ On the other hand, in mice, like in primates, pilocarpine applied to the entire eye increases outflow facility, indicating that the effect of CM contraction on widening of the outflow pathways is stronger than the occluding effect of pilocarpine on TM cell contraction. We

therefore suggest that the cholinergic innervation of the TM in both mice and primates, in addition to modulation of TM outflow, is important for strengthening the TM during CM contraction to provide a firm anterior CM attachment.

In summary, mouse eyes resemble primate eyes not only by having a continuous SC and lamellated TM, but also by having a 3D EL net that tethers together the TM and SC inner wall with CM tendons that insert into this net. The innervation of the TM is also similar between mice and primates, and cholinergic stimulation increases outflow facility similarly in both species. The similarities suggest that the mouse may be a good model for studying aqueous humor outflow as occurs in primates and as relevant for glaucoma.

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