Published in final edited form as:

Ophthalmic Physiol Opt. 2016 January ; 36(1): 13–20. doi:10.1111/opo.12257.

Anteriorly located zonular fibres as a tool for fine regulation in accommodation

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Abstract

Purpose—To describe an anteriorly located system of zonular fibres that could be involved in fine-tuning of accommodation

Methods—Forty six human and 28 rhesus monkey eyes were dissected and special preparations were processed for scanning electron microscopy and reflected-light microscopy. Additional series of frontal and sagittal histological and ultrathin sections were analysed in respect to the origin and insertion of anteriorly located zonules. The presence of sensory terminals at the site of the originating zonules within the connective tissue of the ciliary body was studied by immunohistochemistry. For in-vivo visualization ultrasound biomicroscopy (UBM) was performed on 12 human subjects.

Results—Fine zonular fibres originated from the valleys and lateral walls of the most anterior pars plicata that covers the anterior and inner circular ciliary muscle portion. These most anterior zonules (MAZ) showed attachments either to the anterior or posterior tines or they inserted directly onto the surface of the lens. At the site of origin, the course of the MAZ merged into the connective tissue fibres connecting the adjacent pigmented epithelium to the ciliary muscle. Numerous afferent terminals directly at the site of this MAZ-origin were connected to the intrinsic nervous network of the ciliary muscle.

Conclusions—A newly described set of zonular fibres features the capabilities to register the tensions of the zonular fork and lens capsule. The close location and neural connection towards the circular ciliary muscle portion could provide the basis for stabilization and readjustment of focusing that serves fast and fine-tuned accommodation and disaccommodation.

Keywords

zonular fibres; accommodation; lens; ciliary body; ciliary muscle

Introduction

New sophisticated imaging methods such as high resolution real time video ultrasound biomicroscopy (UBM) and video endoscopy that enable close visualization of the inner eye structures in vivo, as well as detailed morphological studies based on transmission and scanning electron microscopy, have expanded and slightly modified our understanding of the underlying processes of accommodation. Three sets of zonular fibres can be structurally and functionally differentiated. The *posterior zonules* arise from the ora serrata region and cover the pars plana epithelium. They course forwards to the valleys and lateral walls of the posterior pars plicata. Here they are fixed within the zonular plexus or proceed as a second system of zonules, the *anterior zonules*. These zonules form the anterior and posterior zonular tines that are attached to the anterior and posterior surface of the lens capsule.¹ The third group of zonules are the *vitreal zonules*. These zonules comprise a group of anterior, intermediate and posterior vitreal zonules² that provide stabilization and fixation points of the vitreal membrane. The functional significance of the vitreal zonules and thus the involvement of the vitreal during accommodation and disaccommodation probably lies in the modification and smoothing of forward and backward movements of the lens.

There is still the question how fine regulation of focusing can be managed in a fast and constant way. The accommodative system in the primate eye has a much faster, smoother and less jittery focus and refocus capacity than any camera. Through all distances visual acuity is provided immediately without any sensation of blurring.

In a recent study³ we localised a dense nerve fibre plexus within the circular ciliary muscle portion and in the ground plate lying between the ciliary muscle and the anterior ciliary processes. In addition, mechanoreceptor-like endings were seen. Their location, however, is far anterior to the attachment and origin respectively of the posterior and anterior zonules at the zonular plexus within the posterior pars plicata. Yet, the presence of a dense network of nerve fibres and endings, as well as the special location next to the circular portion of the ciliary muscle might indicate an important function of this region with respect to accommodation.

Zonular fibres deriving from the anterior pars plicata have been described or visualised by some authors,^{4,5,6} but without the detailed description that is important to understand their functional significance.

In this study we have examined the presence of anterior zonules and their connection to the nervous plexus in eyes of human donors and rhesus monkeys. The latter have been proven to be the best animal model for the investigation of human accommodation and presbyopia due to their similar morphology and age-related decline of accommodation.^{1, 7-12}

Material and Methods

Morphology in enucleated eyes

46 eyes from 23 human donors were obtained from the Department of Anatomy of the University Erlangen-Nürnberg and of the Donor Eye Bank of Nijmegen, Netherlands, after

appropriate consent and in accordance with the Declaration of Helsinki for research involving human tissue. The eyes were derived from donors aged 16 to 89 years without any history of ocular eye disease or intraocular lens replacement and had been enucleated after a post mortem period between 4 to 24 hours. Immediately after enucleation, anterior and posterior slits were made into the eye bulbs and the eyes were then placed in fixative (one eye in 4% PFA and one in Ito's solution) for at least 24 hours.

28 eyes of 14 rhesus monkeys (*Macaca mulatta*) of either sex, ranging in age from 3 to 14 years, were obtained from caged colonies of the Wisconsin National Primate Research Center (Madison, Wisconsin). The animals had been euthanised in conjunction with nonocular protocols. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were in accordance with institutionally approved animal protocols.

Before death, after ketamine induction (3–30 mg/kg IM) the animals were deeply anaesthetised with pentobarbital sodium (10–15 mg/kg IV, supplemented with 0.5–10 mg/kg IV, as needed) and then perfused transcardially with 1 litre of 0.1 M PBS (phosphate buffered saline) followed by paraformaldehyde (PFA) 4% for 10 to 15 minutes. After systemic perfusion fixation, the eyes were enucleated and slits were cut in the posterior sclera and in the anterior cornea to enhance the penetration of the fixative. The entire eyes were then placed in either PFA or Ito's fixative and sent to Erlangen.

Scanning electron microscopy (SEM)—18 human and 10 rhesus monkey eyes, which had been fixed in Ito's solution, were prepared for SEM: the eyes were placed in cacodylate buffer overnight, then cut into two halves. Small pie-shaped sectors containing the ciliary body, the adjacent cornea, sclera and lens equator were cut from all four quadrants of the anterior halves.

To reveal the architecture of the anterior zonular fibres several approaches were undertaken. Having carefully removed the overlying vitreous membrane, in some specimens the iris and the adjacent lying tips of the ciliary processes were excised to get better insight into the area of the valleys and adjacent lateral walls of the processes. In other specimens different multiple sagittal sections through the ciliary processes towards the valleys were examined. All specimens were post-fixed in 1% osmium tetroxide for 5 hours, dehydrated and subjected to critical-point drying. After coating with gold the specimens were viewed from different planes, either sagittally or from an intraocular en face perspective, using a SEM (Stereoscan; Cambridge Instrument Co., Ltd., Cambridge, UK).

Macroscopic investigation—To investigate the zonules under various conditions of strain, PFA-fixed anterior eye segments of 10 human and 8 rhesus monkey eyes containing ciliary body, lens and zonules were rinsed overnight in PBS. Some specimens were bleached with several incubations in hydrogen peroxide until the pigment was sufficiently removed, and were then rinsed again in PBS. The specimen were then placed under a dissecting microscope (leica-microsystems.com/products/stereo-microscopes-microscopes/macrosopes/details/product/leica-z6-apo-a/) and stained with a few drops of Weigert's stain for elastic fibres including fibrillin. After an incubation time of one to two minutes, the

specimens were rinsed in PBS and checked for sufficient labelling of the zonular fibres. As the vitreous body and vitreous zonules were also stained they could be carefully removed from the posterior lens capsule and ciliary body by fine scissors under direct observation. To obtain better visualization of the anterior zonules, cornea and iris were carefully removed in some specimens and at places the tips of the most anterior ciliary processes were also cut away. Other specimens were cut into small specimens, containing two ciliary processes and a valley. Gentle pulling of single zonular strands allowed the observation of their movement and exact insertion. Photos were taken with a Leica DFC 495 camera ([leica-microsystems.com/products/microscope-cameras/industry/details/product/leica-dfc495/](https://www.leica-microsystems.com/products/microscope-cameras/industry/details/product/leica-dfc495/)).

Light microscopy and immunohistochemistry—To investigate the correlation between the stromal nerve terminals and origin of the anterior zonules the anterior halves from 9 human and 5 monkey eyes that had been fixed in PFA were dissected into four quadrants. From each quadrant small 3–5 mm wide wedge shaped specimens containing ciliary body, sclera, adjacent parts of the iris and cornea as well as lens equator were prepared and partly frozen in liquid nitrogen for the preparation of cryostat sections or embedded in paraffin wax. Serial sections were cut at 6 to 25 µm in sagittal, frontal and horizontal planes, mounted on 0.1% poly-L-lysine-coated slides and allowed to dry for several hours. After preincubation in 1% dry milk solution for 15 minutes, the sections were incubated with the primary antibody (mouse anti-fibrillin/quartett, 1:30; rabbit anti-human elastin/Millipore 1:50; rabbit anti-pan neurofilaments/Biotrend 1:400; mouse anti-synaptophysin/Dako 1:10; rabbit anti-calretinin/swant 1:2000) and appropriate Alexa Fluor 488- or Alexa Fluor 555-labelled immunoglobulin G secondary antibody (MobiTec, 1:300). For light microscopy paraffin sections were stained with Weigert's stain. All sections were viewed with a Keyence BZ-9000 ([keyence.de/products/microscope/fluorescence-microscope/bz-9000/models/bz-9000e/index.jsp](https://www.keyence.de/products/microscope/fluorescence-microscope/bz-9000/models/bz-9000e/index.jsp)).

Electron microscopy—For ultrastructural evaluation Ito-fixed specimens from 9 human and 5 monkey eyes were rinsed overnight in cacodylate buffer and cut in 2 to 3 mm wide segments containing parts of the lens, the ciliary body and zonular apparatus. The specimens were counterstained in uranyl citrate and embedded in Epon. Series of 1 to 2 µm thick sagittal and frontal sections starting from the root of the iris to the posterior regions of the pars plicata, were cut and stained with Richardson's stain. The semithin sections were viewed and corresponding ultrathin sections were cut to create complete series of sections. The ultrathin sections were viewed with a Zeiss EM 109 microscope ([zeiss.de/microscopy/de_de/home.html](https://www.zeiss.de/microscopy/de_de/home.html)) and photos were taken with a CCD camera TRS (Tröndle Restlichtverstärker Systeme, Moorenweis, Germany).

Ultrasound Biomicroscopy (UBM)

Material—Twelve human subjects (5 males and 7 females) ranging in age from 16 years to 65 years, with normal eyes were recruited, and informed consent was obtained. All subjects received a complete eye examination by an ophthalmologist as a prerequisite for enrolment. Preliminary data collection included refraction measurement, slit lamp biomicroscopy, direct ophthalmoscopy, and external and ocular motility examination. Subjects with any ocular

abnormalities or a refractive correction of greater than ± 2.0 dioptres were excluded from the study. This research adhered to the tenets of the Declaration of Helsinki.

Instrumentation—The UBM H (Model # 840; Paradigm-medical.com/ubms.html) was used to image the anterior portion of the ciliary body, vitreous zonule, and lens equator and the images were recorded to tape. The UBM-ER (Model # MHF-1 Ultraview System Model P60; Reichert.com or E-Technologies, Etechultrasound.net/) has lower resolution than the UBM-H, but it has a wider field of view (i.e., 13 mm) and, thus, was used to image the entire sagittal extent of the ciliary body, from the region of the ora serrata to the cornea, vitreous zonule, lens equator, and anterior and posterior lens surfaces. Some of the results from these 12 subjects were reported previously.¹³ The current study reports new data from the same eyes.

Results

The results in human and rhesus monkey eyes were in general the same and are therefore described together.

Origin of most anteriorly located zonules (MAZ)

In SEM preparations the presence of anteriorly located zonules was best seen in specimens in which the tips of the ciliary processes had been cut away and that were viewed facing the ciliary processes from the anterior (iridal) side. These zonules often took their origin in the very anterior portion of the pars plicata near the transition into the iris (Fig. 1). Here within the valleys and in the depths of the valleys, these most anterior zonules (MAZ) were located in the middle between two processes. They divided into bundles that became attached via smaller radially diverging fibrils onto the bases of the valleys or onto the basolateral walls of their bordering ciliary processes. Additional bundles of zonular fibrils were found in the vicinity of the MAZ that traversed the valleys at right-angles to the two opposing ciliary processes where they also attached to the basolateral walls of the ciliary processes and/or the adjacent base of the valley, thus stabilising the configuration of the valley (Fig. 1).

Conventional electron microscopy of this region revealed that the microfibrils of the MAZ were merged with the internal limitans membrane of the underlying nonpigmented ciliary epithelium (NPE). At these places the membrane was thickened and multilayered (Fig. 2).

Series of light microscopic sections cut frontally and stained for fibrillin or Weigert's stain (Fig.3) showed that at MAZ origin there were also structural peculiarities in the adjacent pigmented epithelium (PE) and connective tissue of the ground plate. Here PE-cellular indentations or protrusions into the underlying stromal tissue were seen that appeared as rounded or irregularly shaped elongations of cellular processes. They were especially prominent in older eyes (Fig. 3).

Within the connective tissue of the ground plate, thin bundles of collagenous fibres were characteristically arranged in continuation of the MAZ-course (Fig. 3). Thus the orientation of the zonules was reflected in the underlying collagen bundles that were attached to the

basement membrane of the pigmented epithelium and anchored within the deeper-lying connective tissue surrounding the adjacent circular portion of the ciliary muscle.

In this location, the collagen fibres were in contact with numerous nervous structures that became evident in sections stained for neurofilaments and synaptophysin. These endings also showed a very strong and abundant labelling for the calcium-binding protein calretinin. The calretinin-positive nerve fibres and terminals covered the basal surfaces of the pigmented epithelium just underneath the attachment of the MAZ at the overlying NPE. Here they formed basket like structures surrounding the basement membrane of the PE (Fig. 4). Ultrathin sections revealed that in this location nerve fibres and terminals had contact to mechanoreceptor-like structures that were closely attached to the PE (Fig. 2). The nerve fibres were also connected to calretinin-labelled Ruffini-like endings within the adjacent-lying stroma and to the deeper-lying prominent nervous net that covers the inner portions of the ciliary muscle (Fig. 4, 5).³

These nerve fibres, terminals and ganglion cells were furthermore in contact with the intrinsic nervous network of the inner portions of the ciliary muscle described previously³ (Fig. 5).

Our present investigations show that all these aggregations of nerve fibres and terminals were indeed mainly confined to the region of the MAZ. They were neither seen in the posteriorly-lying regions of the ciliary processes nor in the area of the zonular plexus.

Insertion of the MAZ to the anterior and posterior tines and lens

From their origin at the base of the valleys, the MAZ emerged between the processes and then either joined the thick bundle of the anterior tines close to the ciliary processes (Fig. 6A), or followed the anterior tines and joined them next to their insertion at the anterior surface of the lens (Fig. 6B).

Some MAZ took a posterior course and attached to the posterior tines (Fig. 7).

At places there were also MAZ that did not join the tines but took a separate course before they tethered to the anterior or posterior lens surface (Fig. 8).

Ultrasound Biomicroscopy (UBM)

Ultrasound biomicroscopy (UBM) at 50 MHz could visualise the presence of anterior located zonules (Fig. 9). The UBM probe was oriented at an angle to keep the ciliary muscle and vitreous zonule in a fairly horizontal plain within the image.

Discussion

Far anteriorly located zonules (MAZ) stand out from the remaining anterior zonules as their point of fixation at the ciliary body lies adjacent and inwards to the circular inner muscle portion that defines shape and inner edge of the ciliary muscle during accommodation. This portion of the ciliary muscle houses an intrinsic net of nitrergic nerve cells¹⁴ that is able to relax ciliary muscle cells, supporting disaccommodation and likely mediating fast

fluctuations. Sudden relaxation of the ciliary muscle's contraction state lead to a sudden flaccidity of the anterior and posterior tines that require readjustment and stabilising zonules for a well-regulated focusing process.¹⁵

The stromal layer of the ciliary body adjacent to the anchoring region of the MAZ at the ciliary epithelium is characterised by an abundance of calretinin-positive nerve endings and mechanoreceptors, both presumably representing afferent nerve endings. They are especially numerous surrounding protrusions of the PE that are connected to bundles of collagen fibres showing the same course as the overlying MAZ. This location of the endings alludes to a proprioception of tensional forces. The PE invaginations into the stromal layer become more prominent with age presumably due to these tensional forces over the years. Similar PE protrusions have been described at the insertion of the zonular plexus¹⁶ where they also increase with age.¹⁷ As the MAZ are attached to the anterior and posterior tines as well as to the lens capsule they can also measure tensional changes deriving of these structures. Hiraoka *et al.*¹⁸ lamented about the lack of innervation within the lens itself for understanding the capability of a precise lenticular configuration focusing near or far objects. The presence of afferent terminals at the MAZ that are also connected to the intrinsic nervous system of the inner ciliary muscle and beyond to the autonomic ganglia outside the eye might create a system for fast response to the requirements of instantaneous refocusing.

Our findings redirect our conception of a purely mechanical suspensory apparatus of the lens to a very complex fine regulatory system, that by means of its tensile perception and further sensory and motor processing is able to generate the fast, fine and smooth alterations required for continuous, sharp focusing.

Acknowledgments

The authors thank Johannes Rohen for invaluable help in preparing the specimens for SEM investigations and Elke Kretzschmar, Hong Nguyen, Gerti Link and Marco Gösswein for their expert technical assistance.

This work was funded in part by NEI grants RO1 EY10213, R21 EY018370-01A2, and R21 EY018370-01A2S1 to PLK; the Ocular Physiology Research & Education Foundation; the Wisconsin National Primate Research Center, University of Wisconsin Madison base grant # 5P51 RR 000167; the Core Grant for Vision Research grant # P30 EY016665; Research to Prevent Blindness unrestricted Departmental Challenge Grant.

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Figure 1.

Scanning electron micrograph of a ciliary body specimen from a 24-year old human donor eye. A valley between two ciliary processes anteriorly at the transition to the iris (I) is shown with a zonule (arrow) that takes its origin from the depth of the valley. The zonule belongs to the most anterior zonules (MAZ) that are attached to the base and basolateral walls of the valley by radially diverging fibrils. Separate transversely coursing fibrils (asterisks) are seen that support and stabilise the valley in this region.

Figure 2.

Ultrathin section cut frontally through the valley from the anterior pars plicata of a 5-year old rhesus monkey. At the base of the valley the internal limiting membrane is thickened (arrowheads) in the area where the most anterior zonules (MAZ) are attached to the underlying nonpigmented epithelium (NPE). Just underneath the pigmented epithelium (PE) there is a receptor-like structure (asterisk) with numerous mitochondria and lamellar vesicles (inset) that is surrounded by adjacent nerve fibres (N) and terminals (T).

Figure 3.

Frontal section of the anterior ciliary body of a 62-year old human donor eye. An anterior zonular fibre bundle (MAZ) from the anterior pars plicata is seen that emerges from the valley between the walls of two bordering ciliary processes. Near the site of the attachment at the nonpigmented epithelium (NPE), the MAZ split into two thinner branches that are attached to the basolateral walls of the ciliary processes. In this region the underlying pigmented epithelium (PE) shows characteristic invaginations into the underlying stroma (arrowheads). Note that the course of the MAZ is reflected in the course of the collagen fibres within the underlying connective tissue (arrows). These collagen fibres are connected to both the basement membrane of the PE within the MAZ-origin site and to the connective tissue surrounding the circular ciliary muscle (CM).

Figure 4.

Paraffin section cut frontally through the anterior pars plicata of a 48-year old human donor eye at the region of the most anterior zonules (MAZ) and immunolabelled for calretinin. Intense labelling is seen within the connective tissue underneath the valley. The tangentially sectioned areas of the pigmented epithelium (PE) are covered by a net of calretinin-IR nerve fibres and boutons (arrows) and at places there are also adjacent lying Ruffini-like endings (arrowheads). These nerve fibres are continuous with the calretinin labelled net of the deeper layers surrounding the ciliary muscle (CM).

Figure 5.

Paraffin section cut frontally through the anterior pars plicata of a 48-year old human donor eye and immunolabelled for calretinin. Calretinin stained nerve fibres and terminals from the region underneath the ciliary epithelium (CE) of a valley are connected to the intensely labelled nervous network of the groundplate (arrow) that surrounds the circular ciliary muscle portion, and further to the labelled ganglia (arrowheads) and their connections within the ciliary muscle (CM).

Figure 6.

A, B Scanning electron micrograph of the anterior ciliary processes (CP), the anterior tines (a) and the lens (L) seen from anteriorly (38-year old donor eye). The tips of the processes and the iris had been carefully removed. The thick bundles of the anterior tines (a) are seen coursing anteriorly between the ciliary processes to their insertion at the anterior surface of the lens.

A: From the bases of the valleys between the anterior ciliary processes, two most anterior zonules (MAZ, arrows) emerge from the valleys (asterisks) and attach to the tines at the level of the processes (arrows).

B: A lower magnification shows that there are also MAZ that follow the tines up to their insertion at the lens surface.

Figure 7. Scanning electron micrograph of a sagittally cut specimen of the ciliary body, zonules and lens (L) of a 4-year old rhesus monkey eye. The anterior (a) and posterior (p) tines are attached to the zonular plexus (arrowhead) posteriorly at ciliary processes. A more anteriorly originating zonule (MAZ, arrow) is attached to the posterior tines. Note the different fixation of the MAZ origin (asterisk) and zonular plexus (arrowhead) in relation to the ciliary muscle.

Figure 8.

Sagittally cut specimen of the ciliary body, zonules and lens (L) of a 72-year old donor eye stained with Weigert's stain after bleaching with hydrogen peroxide. The anterior tines (asterisk) have been elevated and the posterior tines have been partly removed. From their fixation at the zonular plexus (arrowhead) the anterior tines course anteriorly and are attached to the anterior surface of the lens. A separate bundle of the most anterior zonules (MAZ, arrow) is fixed at the anterior surface of the lens.

Figure 9.

Ultrasound biomicroscopic image (UBM) of a 21-year old human subject. The ciliary body with cornea (C), sclera (SC), ciliary muscle (CM), iris (I) and lens capsule (LC) is seen. Note the separate bundle of most anterior zonules (MAZ, arrow) that can be differentiated from the anterior tines (arrowhead). Following the course of the MAZ and anterior tines towards the ciliary body, their different point of fixation at the ciliary body becomes obvious (x: fixation of MAZ, y: fixation of the anterior zonules at the zonular plexus). The vitreous zonules (VZ) that insert also at the zonular plexus (y) are also visible. The bundle of the MAZ is shown at higher magnification (inset) with two zonular bundles (a, b) inserting onto the lens capsule and one bundle (c) attaching to the anterior tines (arrowhead).