Retinal Glial (Müller) Cells: Sensing and Responding to Tissue Stretch

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PURPOSE. To test whether Müller glial cells sense, and respond to, mechanical tension in the retina.

METHODS. A device was designed to stretch the retina at right angles to its surface, across retinal layers. Pieces of retina were mounted between two hollow tubes, and uniaxial force was applied to the tissue using a micrometer-stepping motor. Müller cells were selectively stained with the fluorescent, calcium-sensitive dye X-Rhod-1 and were monitored in real time during retinal stretch in vitro. Immunohistochemistry was used to study protein levels and activation of intracellular pathways in stretched retinas.

RESULTS. Müller cells responded acutely with transient increases in fluorescence during stretch, indicative of increased intracellular calcium levels. All the Müller cells elongated uniformly, and there was no apparent difference between retinal layers in resistance against mechanical deformation. After stretch, Müller cells showed fast activation of extracellular signal-regulated kinase (after 15 minutes), upregulation of transcription factor c-Fos (after 1 hour), and basic fibroblast growth factor (after 3 hours). No changes in intermediate filament protein expression were observed in Müller cells up to 3 hours after stretch.

CONCLUSIONS. A novel technique was developed for real-time monitoring of Müller cells during retinal stretch, which allowed the identification of Müller cells as a mechanoresponsive cell type. Mechanical stress triggers molecular responses in Müller cells that could prevent retinal damage. (Invest Ophthalmol Vis Sci. 2010;51:1683–1690) DOI:10.1167/iovs.09-4159

The retina is a thin lamella on the inside of the eye that is responsible for photodetection. According to its ontogenetic origin from the distal part of the ocular vesicle, it is continuous with the underlying retinal pigment epithelium (RPE) at its peripheral (blind) margin. However, most of the retinal tissue is only loosely adherent to the RPE and is kept in place mainly by intraocular pressure through the vitreous body and by a (relatively) negative pressure in the subretinal space resulting from water efflux through the RPE.1

The retina may be injured by tractional forces in a variety of conditions. For example, if degenerative axial myopia results from excessive ocular growth, the retina becomes overstretched, which causes tractional retinal detachment or splitting of retinal layers (retinoschisis).2,3 The retina may be injured by tractional forces in proliferative diabetic retinopathy or in X-linked juvenile retinoschisis and even during “normal” aging, when the shrinking vitreous body may pull on the retina, leading to retinal detachment, retinoschisis, or both.4,5 Furthermore, traumatic events may cause axial stretch of the retina,6 and the diurnal thickness oscillations of the choroid may induce physiological axial stretch,7 though this remains to be studied in detail.

Under all physiological (and many pathologic) conditions, the structural integrity of the retina is strikingly well maintained if one considers its fragility.8 This study was conducted to determine whether there are cells in the retina that are sensitive to mechanical stress and that respond to mechanical stress by cellular reactions to promote the maintenance of structural integrity.

The retina is built of several cellular layers, constituted by several types of neurons and glial cells. Whereas most retinal cells, including all classes of retinal neurons, are confined to one or a few layers, the Müller glial cells span the entire retinal thickness.9 Their somata are localized to the inner nuclear layer (INL), and have two emanating radially oriented stem processes, one abutting the inner (vitread) surface by an end foot and the other reaching the outer limiting membrane and then extending microvilli into the subretinal space. These stem processes give rise to side branches that ensheath neuronal synapses in the inner plexiform layer (IPL) and outer plexiform layer (OPL) and neuronal somata in the nuclear layers. Because of their unique morphology, Müller cells may sense even minute changes in retinal structure because of the mechanical stretch of their long processes or side branches. Furthermore, Müller cells were shown to be mechanically softer than their neuronal counterparts,10 and it is likely that they constitute soft embedding and structural protection for retinal neurons. Additionally, Müller cells provide neurons with neurotrophic factors,11 possibly also after mechanical stress. Müller cells would be particularly well suited to play mechanosensing and mecanoprotective roles given that they are in contact with virtually every neuron in the retina. To test this hypothesis, we designed an in vitro system to stretch the retina across its cellular layers. Using this system, we monitored immediate Ca2+ responses of Müller cells to retinal stretch and delayed (molecular-) biochemical responses after stretch. This in vitro system may reproduce events occurring in the in vivo retina.
MATERIALS AND METHODS

Animals

In the present study, guinea pig and rat retinas were used. To monitor calcium levels in Müller cells during retinal stretch, we used adult guinea pig (weight range, 300–800 g) retinas because their Müller cells are large and clearly visible in vital preparations. For immunohistochemical analysis of molecular responses after retinal stretch, we used retinas from adult Long-Evans rats (weight range, 250–350 g) because of the specificities of available antibodies. Twenty-nine guinea pigs and 21 rats were used for the experiments. Animals were provided by the Faculty of Medicine at the University of Leipzig and were housed in standard cages, fed ad libitum, and maintained in temperature-controlled rooms with a 12-hour light/12-hour dark cycles, with light intensity ranging from 8 to 24 lux. Animals were deeply anesthetized with urethane (2 g/kg intraperitoneally) before decapitation and enucleation of the eyes. Retinas were dissected and used immediately for stretch experiments. Experiments were carried out in accordance with European Community guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The local ethics committee for experimental animals scrutinized the procedures.

Custom-made Device to Stretch the Retina In Vitro

We designed a device to stretch the retina at right angles to its surface (Fig. 1). A rectangular Perspex chamber (17 × 12 × 2 cm) was filled with extracellular solution (ECS; 230-mL volume); it contained the retina, which was held in place by negative pressure applied through two hollow metallic tubes (3-mm outer diameter). One of the tubes was fixed to the chamber and the other one to a micrometer-stepping motor (Nippon, Otsu, Japan); their open ends were fitted to meet in the center of the chamber. For the experiments, a piece of retina was positioned between cellulose acetate filters (Sartorius AG, Göttingen, Germany) and was mounted between the ends of the tubes (Fig. 1). Negative pressure was provided by a pump (Ilmvac GmbH, Ilmenau, Germany) connected to the metal tubes by plastic tubing. This negative pressure served to attach the filters to the metal tubes and the retina to the filters. To avoid bulging of the filters, the ends of the metal tubes contained a thin wire cross. The same level of negative pressure was applied to both sides of the retina, and the attachment of the retina to the filters was controlled by the pore size of the filters. A pore size of 5 μm allowed adequate adhesion of the retina to the filters. The mechanical force necessary to stretch the retina was provided by the motion of the computer-controlled motor.

Dye Loading and Calcium Imaging during Retinal Stretch

A 1 mM stock solution of the calcium indicator dye, X-Rhod-1, provided as a cell-permeant acetoxymethyl ester (Molecular Probes, Eugene, OR), was prepared in dimethylsulfoxide with 2% copolymer (Pluronic F-127; Sigma, St. Louis, MO). For dye loading of Müller cells, pieces of freshly dissected guinea pig retinas were incubated with a final concentration of 5 μM X-Rhod-1 for 45 minutes at room temperature (24°C) in ECS consisting of 110 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM Na₂HPO₄, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethyl-anesulfonic acid (HEPES), 11 mM glucose, and 25 mM NaHCO₃ adjusted to pH 7.4, with Tris-(hydroxymethyl)aminomethane (Tris-base) and bubbled with carbogen (95% O₂, 5% CO₂). X-Rhod-1 selectively stains the Müller cells,12 as do other calcium-sensitive fluorescent dyes.13 After staining, guinea pig retinas were put in ECS alone for 10 minutes so that any excess dye could be washed away. Small quadrants (approximately 5 × 5 mm) of retinas were cut such that they were larger than the open endings of the metallic tubes. A vertically mounted scalp blade was used to cut one side of the retina at right angles to its surface. The stretch device was mounted in a microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) with appropriate filter sets, and its chamber was filled with ECS in which the retinal stretch was performed. A retinal quadrant was mounted between two filter papers of the same size as the retinal quadrant and was positioned between the tubes in the device, with the retinal side cut parallel to the focal plane of the microscope. Negative pressure was applied to hold...
Figure 2. Retinal stretch triggers calcium increases in Müller glial cells. Müller cells in adult guinea pig retinas were stained with the fluorescent and calcium-sensitive dye X-Rhod-1. A piece of retina was cut at right angles to its surface, positioned between filters, and put between the metal tubes in the device under negative pressure. (A) Two images of the same section of a retina in cross-section. Left: unstretched, relaxed retina with weakly fluorescent Müller cells in the inner nuclear layer. Right: the retina was stretched to a 20% increase in retinal thickness using a speed of 2 μm/s, and an immediate increase of fluorescence intensity in Müller cells could be observed as shown. Fluorescence was localized to Müller cell somata, inner processes, and end feet (arrows) and in outer processes. Scale bar, 12 μm. (B) Quantification of intracellular calcium levels in Müller cell somata during retinal stretch. The changes in fluorescence over time were calculated relative to the fluorescence at time point 0 (ΔF/F0). Arrows: start of the stretch. Horizontal bars: time period during which the retina was kept in a stretched position; note that the fluorescence decreased during this time. The stretch was repeated using the same parameters, and a similar increase in fluorescence was observed but with lower amplitude. Data are from 35 Müller cells in a representative experiment, and values are given in mean ± SD. (C) Quantification of retinal thickness and lengths of Müller cell processes during stretch. Images captured during the experiments were used to measure the thickness of the retina and the lengths of individual Müller cell inner processes (cell body to inner end foot). Data are from three independent experiments, and values are given in mean ± SD. The ratio of inner process length to the entire retinal thickness remained constant during stretch, indicating that the Müller cell somata retained their relative position in the retina during stretch and that the inner and outer processes were stretched to a similar extent.

Results
Mechanosensitivity and Elongation of Müller Glial Cells during Retinal Stretch
To apply force across retinal layers, retinas were mounted between filter papers, and negative pressure was applied to the filters (Fig. 1). To avoid retinal detachment, the increase in retinal thickness was limited to 20%, and the stretch speed was adjusted accordingly. Of 74 stretched retinas, two-thirds were successful stretches, that is, they occurred without retinal detachment from the filters. Other materials tested for the attachment included adhesion barrier (Seprafilm; Genzyme, Cambridge, MA)16 instead of filters or the generation of a fibrin clot, but the use of negative pressure simply through a filter was easiest to manage. To avoid possible toxic effects, glue was not used.

Increases in intracellular calcium have been shown to indicate mechanosensitivity in different cell types.17,18 To investigate the calcium dynamics in Müller cells during retinal stretch, we used guinea pig retinas whose Müller cells have thick and clearly visible stem processes. During retinal stretch the X-Rhod-1 fluorescence in Müller cells increased, which indicated increased intracellular calcium levels (Fig. 2A). When retinas were kept in a stretched position, the fluorescence subsequently declined and reached basal levels after approximately 100 seconds (Fig. 2B). To determine whether the Müller cells
responded to repeated exposure, another stretch was performed using the same parameters. A similar increase in fluorescence was observed during the second stretch; however, its amplitude was lower than that of the first response. The increase in fluorescence was found most clearly in the cell bodies of Müller cells but occurred also in their end feet, inner processes, and outer processes (Fig. 2A).

To investigate whether the deformation caused by the applied mechanical stress was homogeneously distributed throughout all retinal layers, we compared the change in total retinal thickness during stretch with the changes in the measured lengths of inner and outer Müller cell processes (Fig. 2C). This analysis revealed that the ratio between the inner part of the Müller cells, defined as the length from the cell body to the inner end foot, and the entire retinal thickness was constant during stretch. Thus, the inner and outer parts of Müller cells were stretched equally, and their somata retained their relative position in the retina. These data suggest that there is no retinal layer of particular stretch resistance or compliance.

**Activation of MAPK Pathway in Müller Cells after Retinal Stretch**

The mitogen-activated protein kinase (MAPK) pathway is a major signaling pathway also shown to exist in Müller cells.19,20 The MAPK pathway is triggered in cells subjected to tensile stress.21 To investigate whether activation occurred in Müller cells because of mechanical stress we analyzed ERK, a protein downstream in the MAPK cascade activated by phosphorylation.22 Using an antibody that detects both phosphorylated ERK1 and ERK2 (p44 and p42 MAPK), we found ERK activation 15 minutes after stretch mainly in Müller cells (Fig. 3B). The signal remained at approximately the same level for at least 60 minutes (Fig. 3C). Immunoreactivity (IR) for phosphorylated ERK (P-ERK) was localized mainly to Müller cell somata but was also visible in Müller cell end feet and inner processes (Fig. 3B). In unstretched control retinas, we observed a basal ERK phosphorylation that was significantly lower than in the stretched retinas, mainly in Müller cell end feet in the ganglion cell layer (GCL; Fig. 3A). Similar basal ERK phosphorylation was seen in untreated control retinas in situ (data not shown).

We investigated the total level of ERK (including the inactive form) using an antibody toward ERK1 and ERK2 and found light labeling mainly in the inner retina in the INL, including Müller cell somata, the inner plexiform layer (IPL), and the GCL (Fig. 3E). In the outer nuclear layer (ONL), which contains the photoreceptor cell somata, total ERK levels were comparatively lower. The strength and pattern of total ERK labeling were similar in control retinas in situ and in unstretched and stretched retinas, and there was no increase in expression during the time points analyzed after stretch. It was also not possible to clearly detect an intracellular relocalization of total ERK protein after stretch. No IR was detected in the negative controls using secondary antibody only (Fig. 3G).

**Stretch-Dependent Synthesis of Transcription Factor c-Fos and of Basic Fibroblast Growth Factor in Müller Cells**

To investigate the effects of retinal stretch on gene expression, we analyzed IRs for transcription factor c-Fos, bFGF, and the cytoskeletal proteins vimentin and GFAP. c-Fos is considered an immediate early transcription factor23 and, therefore, hypothetically is an early marker for stretch effects on gene transcription. c-Fos IR in the unstretched retinas could not be clearly detected (Fig. 4A), as in unstretched control retinas in situ (data not shown). Retinas were analyzed 15, 30, 60, and 120 minutes after stretch. Increased c-Fos IR was first seen 60 minutes after stretch (Fig. 4B) and remained at this level until 120 minutes after stretch (data not shown). Strong c-Fos IR was identified in a population of retinal cells in the INL (Fig. 4B), but weak IR also occurred in cells in the GCL. Detailed analysis
using an antibody toward the Müller cell-selective marker CRALBP and Hoechst stain revealed that the c-Fos IR was localized and restricted to Müller cell nuclei (Figs. 4C–F).

In the unstretched retina, bFGF showed a low but clear expression by cells in the INL and weakly in cells in the GCL (Fig. 5A). Similar expression was found in untreated control retinas in situ (data not shown). Analysis at 15, 30, 60, 120, and 180 minutes after stretch revealed that bFGF IR had significantly increased in INL cells after 180 minutes (Fig. 5B). Using the CRALBP stain, we found a specific localization of bFGF to Müller cells (Figs. 5C–E).

We also analyzed the expression of GFAP and found no significant expression in the unstretched retina, confirming earlier data24; there was no increase in GFAP expression up to 180 minutes after stretch, as analyzed by immunohistochemistry. Vimentin was expressed in the retina by Müller cells, as shown previously,25 but again there were no changes of the expression levels up to 180 minutes after stretch.

**DISCUSSION**

Here we demonstrate the successful use of an in vitro system to apply tractional force to the living retina. This system allowed monitoring of Müller cell responses during retinal stretch in real time. We found that Müller glial cells, which are the only cells spanning the entire retinal tissue, were sensitive to mechanically enforced changes in the retinal structure. They responded to retinal stretch both with fast changes, as evidenced by transient intracellular calcium increases, and with slower changes in protein expression. Earlier it had been shown that mechanical stimulation of astrocytes using a pipette elicits Ca2+ responses in neighboring Müller cells,26 but, to our knowledge, this is the first study reporting a mechanosensory function of Müller cells in the retina.
Application of Mechanical Stress across the Retinal Layers

Under both physiological and pathologic conditions, the retina is subjected to mechanical stress. This stress may occur tangentially to its surface (e.g., during postsynaptic expansion of the retinal surface area) or perpendicularly to it (e.g., because of shrinkage of the vitreous body or retinoschisis). Müller cells have both radial stem processes and tangential side branches, and we subjected the retina to radial (axial) stretch because this direction corresponds to the main axis of Müller cells. This likely caused much less mechanical stimulation of cells with tangentially oriented cell processes, including astrocytes (the only other type of macroglia in the rat retina), horizontal, amacrine, and ganglion cells. To achieve this stretch, we developed a device to apply mechanical force orthogonal to the retinal surface. A retinal piece was mounted between filters using negative pressure to create attachment, and a stepping motor provided the force to stretch it. This system proved to be useful to monitor Müller cells during a moderate stretch of the retina and to collect the tissue for later immunohistochemical analysis of gene/protein expression. Using this method, retinas could be reliably deformed up to 120% of their original thickness without causing splitting of the tissue in a certain layer.

Comparative Biomechanical Stress Resistance of Retinal Layers and Cells

It has been concluded from artifacts during histologic preparation of retinal tissue and from clinical observations in human retinas with retinoschisis that the plexiform layers may have particularly low stretch resistance and that Müller cell stem processes may play a role in holding retinal layers together. In this context the cytoskeletal components expressed by Müller cells, such as intermediate filaments consisting of vimentin and GFAP, have attracted interest. When vimentin and GFAP were knocked out in mice, the inner retina was more susceptible to mechanical stress. Hypothetically, Müller cells might upregulate vimentin or GFAP in response to mechanical stress to increase resistance against mechanical disruption. In our short-term study, we did not observe increased vimentin or GFAP expression in Müller cells. Still we cannot exclude that GFAP expression might have increased later given that it appears not to be a particularly fast event.

When analyzing the elongation of Müller cell inner and outer processes during retinal stretch, we found that both were elongated to a similar extent in guinea pig retinas. It cannot be excluded that the virtual compliance of Müller cell processes against stretch is dependent on their local retinal microenvironment, but the data are compatible with the view that inner and outer processes do not differ in strength or stiffness. This is surprising because there is a difference in cytoskeletal components between inner and outer processes; the inner processes are rich in intermediate filaments, and the outer processes are rich in microtubules. However, this view is in accordance with a recent study in which it was shown by atomic force microscopy that the inner and outer processes, measured in individual isolated Müller cells, were equal in stiffness.

Mechanosensitivity by Müller Cells: Intracellular Pathways

We found robust increases of intracellular calcium levels in Müller cells during retinal stretch. Calcium increases indicate mechanosensitivity in various cell types such as inner ear hair cells and endothelial cells, and during stretch-induced injury in astroglia and neurons. The calcium increases in Müller cells observed in our experiments were likely early responses to mechanically induced structural changes in the retinal architecture. Because the guinea pig retina lacks astrocytes, signaling from astrocytes to Müller cells cannot be involved. The mechanism behind these calcium increases must be investigated in further detail, but the kinetics of the calcium transients may indicate that they were receptor mediated, for example by the activation of ATP (P2Y) receptors in Müller cells. ATP is released from mammalian cells in a mechanosensitive manner. Alternatively, Müller cells experience mechanosensitive channel opening after membrane stretch, thereby facilitating calcium influx. Although only the Müller cells span the entire thickness of the stretched tissue, many neuronal (particularly bipolar) cell processes were certainly stretched as well. It cannot be completely excluded that stretched neurons contributed to the observed Müller cell reactions. For example, the stretch could have triggered the release of growth factors from neurons that, in turn, activated receptor tyrosine kinases on Müller cells, leading to a release of calcium from intracellular stores via PLCγ. In vital retinal tissue, Müller cell physiology is strongly affected by retinal neurons, complicating a detailed analysis.

Total ERK was found mainly in the inner retina. Within the INL it may be ascribed to the bipolar, horizontal, and amacrine neurons in addition to Müller cells. The pattern and strength of ERK expression was unchanged a few hours after stretch, though we cannot exclude a long-term regulated expression of ERK, as observed in an in vivo model. ERK was activated in astroglia after mechanical injury in vitro and in vivo. After retinal stretch, we found fast phosphorylation and activation of ERK localized mainly to Müller cell bodies (including the nuclei), inner processes, and end feet. Activated ERK, translocated to the cell nucleus, generally plays a role in gene transcription, and P-ERK found there probably performed this function. P-ERK localized to inner processes and end feet of Müller cells have also been seen after retinal detachment and uveitis and may be a sensitive indicator for abnormal mechanical stress in the retina.

c-Fos is a transcription factor rapidly induced in cells by different stimuli, and it accumulates predominantly in the cell nucleus. In the retina, c-Fos increased after penetrating focal injury and retinal detachment. In our model, after retinal stretch, we found an increase of c-Fos exclusively in Müller cell nuclei concomitant with ERK activation, indicating an early altered gene transcription in this cell type.

It can be anticipated that retinal stretch caused some degree of injury to the retina. bFGF is a factor reported to increase after mechanical penetration of the retina and bFGF derived from Müller cells has been implicated in the protection of retinal neurons, including photoreceptors. In addition, during the induction of eye enlargement by form deprivation, endogenously produced bFGF could limit malignant ocular elongation and stretch-induced damage to the retina.

In the unstretched retina, we found low levels of bFGF expression in Müller cells. Three hours after stretch, however, a significant increase of bFGF in Müller cells was observed. This is, to our knowledge, the first report showing that Müller cells respond with a fast increase in bFGF expression to retinal stretch. This reaction could be part of an injury-induced system aiming to limit damage to retinal cells after acute stretch. Müller cells are in direct physical contact with virtually every neuron in the retina, and bFGF released from Müller cells could activate fibroblast growth factor receptors on nearby neurons in a direct mode of action.

Our experiments were carried out on retinas from either rat or guinea pig. We think it is reasonable to assume that the
observed Müller cell reactions to mechanical stress are general phenomena in mammalian retinas for the following reasons: rat Müller cells responded to stimulation (including mechanical stress) by calcium increases\(^{60–62}\) similarly to guinea pig Müller cells\(^2,63\); Müller cells of all mammalian species studied thus far display similar reactions to stimulation, including the production and release of neuroactive or neuroprotective substances\(^9\); and mechanical stress is a general phenomenon occurring in all mammalian retinas.

CONCLUSIONS
To summarize, we have developed a novel method to apply mechanical stress perpendicularly to the retinal surfaces in vitro. This technique allowed the detection of Müller cells as mechanosensitive. Overall, the stretch-induced changes noted in Müller cells could not be observed in other retinal cells. The results may predict that when the retina is stretched in vivo, stretch-dependent responses are elicited in Müller cells. However, stretch during development or pathology is likely to be much slower than in our experiments. It is unknown whether the changes we found would occur during much slower stretch.

Further applications for our device may be to apply pressure to the retina and to study the effects on retinal cells. This could contribute to a deeper understanding of the development of glaucoma. An attached force sensor could be used to grade the amount of pressure or stretch applied.

We believe that the device we have generated, and the results obtained with it, will further elevate our understanding of retinal biomechanics and Müller cell physiology.

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