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TRPV4 and TRPC1 channels mediate the response to tensile strain in mouse Müller cells

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Abstract

Müller glia, a pillar of metabolic, volume regulatory and immune/inflammatory signaling in the mammalian retina, are among the earliest responders to mechanical stressors in the eye. Ocular trauma, edema, detachment and glaucoma evokes early inflammatory activation of Müller cells yet the identity of their mechanotransducers and their downstream signaling mechanisms remain unknown. Here, we investigate expression of genes that encode putative stretch-activated calcium channels (SACs) in mouse Müller cells together with their response to dynamical tensile loading in cells loaded with a calcium indicator dye. Transcript levels in purified glia were *Trpc1>Piezo1>Trpv2>Trpv4>>Trpv1>Trpa1*. Cyclic radial deformation of matrix -coated substrates produced dose-dependent increases in $[Ca^{2+}]_i$ that were suppressed by the TRPV4 channel antagonist HC-067047 and by ablation of the *Trpv4* gene. In addition, stretch-evoked calcium responses were reduced by knockdown and pharmacological inhibition of TRPC1 channels whereas the TRPV2 inhibitor tranilast had no effect. These data demonstrate that Müller cells are intrinsically mechanosensitive, with the response to tensile loading mediated through synergistic activation of TRPV4 and TRPC1 channels. Coupling between mechanical stress and Müller Ca^{2+} homeostasis has treatment implications, since many neuronal injury paradigms in the retina involve calcium dysregulation associated with inflammatory and immune signaling.

Graphical Abstract

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Author contributions

M.L., A.O.J. and D.K. designed research; A.O.J., M.L., and C.R. performed research; A.O.J., M.L., C.R. and D.K. analyzed data; A.O.J., M.L., and D.K. wrote the paper.

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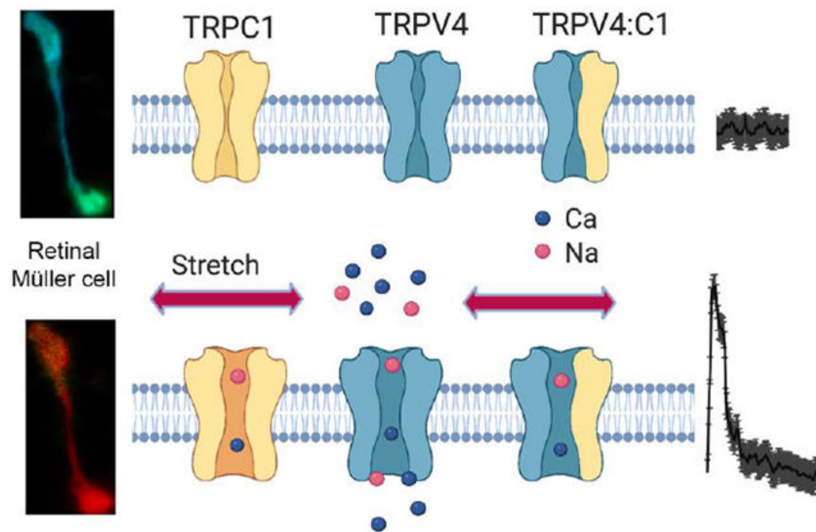

Keywords

Calcium; Stretch-activated channels; Mechanosensitivity; Glaucoma; Glia

1. Introduction

The vertebrate eye continually experiences various types of mechanical force impelled by eyeball growth and intraocular pressure (IOP) (Reichenbach et al., 1991; Kuhrt et al., 2012; Krizaj, 2019). Multiaxial distension, compressive, shear, osmotic and/or tractional forces are transformed by retinal cells into biological responses ("mechanotransduction") that control eye development and function. Under pathological conditions - chronic IOP elevations, diabetic cell swelling/edema, vitreous detachment, retinoschisis, abnormal ocular elongation and proliferative vitreoretinopathy - retinal circuits undergo architectural remodeling that, without exception, involves reactive gliosis (Rousseau and Sabel, 2001; Neelam et al., 2012; Sigal et al., 2014; Quigley et al., 2015). Inflammatory activation of retinal glia in response to aging and mechanical stress shares many key features with their brain counterparts (Hall et al., 2020; Shibasaki, 2020). Treatment of mechanically induced CNS inflammation and injury has been hampered by the poor understanding of glial mechanotransduction.

Müller cells constitute a major source of retinal osmoregulatory, nutritional, mechanical and signaling support (Reichenbach and Bringmann, 2010; MacDonald et al., 2015). This radial glia continually experience traction through contacts to vitreous fibers, lateral tension due to effects of IOP, changes in cell volume due to osmogradients, and serve as an important buffer for mechanical impact (Schubert 1989, Lu et al., 2013; Reichenbach and Bringmann, 2010; MacDonald et al., 2015). Like brain astrocytes, Müller cells respond to virtually every type of mechanical stress with stereotyped upregulation of GFAP/vimentin and increased release of proinflammatory, immune and angiogenic factors (Woldemussie et al., 2004; Lebrun-Julien et al., 2009; Wang et al., 2010; Huang et al., 2010; Križaj et al., 2014). This suggests that they express transducer molecules, which may be coupled to immune and inflammatory pathways that control gene expression, hypertrophy, ECM

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secretion, inflammatory signaling and tissue stiffness (Ryskamp et al., 2015; Barnes et al., 2017). Early electrophysiological and calcium imaging experiments showed that Müller cells respond to applied pressure steps and indentation with inward cation currents and $Ca^{2\pm}$ waves (Newman 2001; Lindqvist et al., 2010; Ryskamp et al., 2014; Agte et al., 2017; Toft-Bertelsen et al., 2019) that may sculpt excitatory and inhibitory signaling in adjacent neurons, astrocytes and endothelial cells (Newman, 2003; Metea and Newman, 2006). Analogy with cortical astrocytes (Sachs, 2010) suggests potential activation of calcium-permeable stretch-activated cation channels (SAC) in which open probability increases in response to mechanical membrane deformation by compression, tension and/or shear but involvement of SACs has been challenged by lack of effect of the nonselective Piezo1 antagonist GsMTx4 (Agte et al., 2017).

To address the mechanism of mechanically induced calcium signaling in Müller cells glia, we simulated *in vivo* tensile stress (e.g., MacDonald et al., 2015; Fortune, 2019) with a bioreactor that was designed to mimic physiologically relevant mechanical strains. Its advantage is that it generates precisely calibrated stimuli while obviating the effects of purinergic, glutamatergic and/or adenosinergic inputs from tissue-adjacent neurons, astrocytes, and vasculature. Optical imaging of stretch-induced calcium responses in wild type and KO Müller cells in combination with molecular assays allowed us to define the identity of endogenous molecular transducer of mechanical stretch. Mechanical forces applied across the ECM induced immediate increases in $[Ca^{2+}]_i$ that were dominated by contribution from TRPV4 (Transient receptor potential vanilloid type 4) channels, with partial contribution from TRPC1 (Transient receptor potential canonical type 1) channels. These findings identify potential mediators of gliotic and inflammatory phenotypes in mechanically induced retinal and brain injury.

2. Materials and Methods

2.1. Animals

Animal handling, anesthetic procedures and experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committees at the University of Utah. Mouse strains were C57BL/6J, global *Trpv*$^{-/-}$ nulls with excised exon-encoding transmembrane domains 5 and 6 (Liedtke and Friedman et al., 2003), and *Trpc1*$^{-/-}$ knockout mice developed by Dr. Lutz Birnbaumer (NIEHS) (Liu et al., 2007) and characterized previously in Müller glia (Molnar et al., 2016). The animals were maintained in a pathogen-free facility with a 12-hour light/dark cycle and *ad libitum* access to food and water. Cells gathered from male and female mice (P30 – P120) showed no obvious differences in responsiveness to stretch.

2.2. Reagents

The TRPV4 antagonist HC-067047 (HC-06) was purchased from Cayman Chemical (Ann Arbor, MI), Orai inhibitor GSK-7975A from Aobious (Gloucester, MA) and the TRPV2 antagonist tranilast from Hello Bio (Princeton, NJ). Salts and other reagents including the agonist GSK1016790A (GSK101) were obtained from Sigma-Aldrich (St. Louis, MO) or

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VWR (Radnor, PA). GSK101, SKF96365, tranilast and HC-06 (all 1 mM) stock aliquots were prepared in DMSO and subsequently diluted into working saline concentrations (25 nM, GSK101; 10 μM, SKF96365; 5 μM, HC-06; 10 μM, tranilast).

2.3. Magnetic-activated cell sorting (MACS)

Retinas were incubated in an enzyme solution containing 16 U/ml papain (Worthington, Lakewood, NJ), 0.2 mg/ml L-cysteine (Sigma-Aldrich, St. Louis, MO) and 50 U/ml DNase I recombinant (Roche, Indianapolis, IN) for 45 min at 37 °C with gentle agitation and triturated with D-PBS solution containing 1.5 mg/ml BSA, 1.5 mg/ml Trypsin inhibitor, pH: 7.4, to yield a single cell suspension that was passed through a 70 μm pre-separation filter and centrifuged. The cell pellet was re-suspended and incubated in 0.5 % BSA solution containing anti-CD29 (Clone Ha2/5; 1:10; BD Biosciences, Chicago, IL) for 15 min at 4 °C. After additional washing and centrifugation, cells were re-suspended in the presence of anti-biotin microbeads (1:10; Miltenyi Biotec, San Diego, CA) and incubated for 15 min at 4 °C. After washing, Müller cells were separated using cell columns from Miltenyi Biotec. Magneto-separated cells were used for quantitative RT-PCR analysis.

2.4. Semiquantitative Real-Time PCR

Total RNA was isolated with the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Waltham, MA) as described (Phuong et al., 2017; Lakk et al., 2018). 50 nanogram of total RNA was used for reverse transcription. The cDNA samples were pre-amplified using AdvAnced™ PreAmp Supermix (Bio-Rad, Hercules, CA). First-strand cDNA synthesis and PCR amplification of cDNA were performed using qScript™ XLT cDNA SuperMix cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). The PCR products were run on 2% agarose gels and visualized by ethidium bromide staining, along with a 100-bp DNA ladder (Thermo Fisher, Carlsbad, CA). SYBR Green based real-time PCR was performed with Apex qPCR Master Mix (Genesee Scientific, San Diego, CA) and experiments performed in triplicate (at N > 4). The comparative C_T method (C_T) was used to measure relative gene expression where the fold enrichment was calculated as: $2^{-[\ CT\ (sample) -\ CT\ (callbrator)]}$ after normalization. *Gapdh* was utilized as endogenous control to normalize fluorescence signals. The primer sequences and expected product sizes are given in Table 1.

2.5. Müller cell dissociation

Müller cell -enriched primary cultures from adult mouse retinas were prepared as described (Molnar et al., 2016; Lakk et al., 2017). Following enucleation, retinas were dissected in ice cold L15 medium (Life Technologies, Carlsbad, CA) containing 11 mg/ml L15 powder, 20 mM D-glucose, 10 mM Na-HEPES, 2 mM Na-pyruvate, 0.3 mM Na-ascorbate, and 1 mM glutathione. Extracellular matrix (ECM) was digested by incubating the retinas in papain (7 U/ml; Worthington, Lakewood, NJ) at RT. Retinas were diced into ~500 μm pieces, triturated and plated on coverslips pretreated with Concanavalin A (1 mg/ml). Acutely dissociated Müller cells maintain their distinctive polarized morphology (Fig. 2A; Molnar et al., 2016; Toft-Bertelsen et al., 2019).

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2.6. Mechanical Stimulation

2.6.1. Cyclic Stretch.—Primary Müller cells were plated onto flexible silicon membranes coated with type I/IV collagen or Concanavalin-A (0.5 mg/ml) and placed into chambers. Cyclic radial stretch was imposed by the Flexcell-5000 Tension device (Flexcell International Corporation, Hillsborough, NC), a computer-controlled bioreactor that utilizes vacuum to strain cells adherent to the ECM. Continuous mechanical stimulation was applied for 5 or 10 min for Ca^{2+} responses. The strain was delivered as a sinusoidal waveform with elongation from 0 to 15% and a frequency of 0.5 Hz. The stretch period was chosen to optimize capture of the stretch-evoked fluorescent response by accounting for the change in focal plane. Control cells were treated in the same manner under same conditions but without applied mechanical strain. All experiments were performed at room temperature (20 – 22°C).

2.6.2. Indentation.—Indentation responses were elicited with a glass pipette (tip diameter ~ 3 μm) positioned at ~30° angle and ~ 2 μm from the cell with a Sutter MPC-200 micromanipulator, as described in Yarishkin et al. (J Physiol. 2021). Cells were indented with a rapid manual step to the depth of ~ 60 nm for 1 sec.

2.7. Real-time Calcium Imaging.

Imaging experiments followed the protocols from Molnar et al. (2016) and Lakk et al. (2017). Acutely dissociated Müller cells were loaded with the ratiometric fluorescent dye fura-2 AM (Life Technologies, Carlsbad, CA) (K_d at RT = 225 nM) (5-10 μM) for 30 min and washed for 10-20 min in dye-free L-15. Under these experimental conditions, Müller cells maintain Ca^{2+} homeostasis and stimulus responsiveness for many hours (Molnar et al., 2016; Toft-Bertelsen et al., 2019). Epifluorescence images were acquired with inverted Nikon Ti or upright Nikon E600 FN microscopes with 20x (0.75 N.A. oil), 40x (1.3 N.A. oil & 0.8 N.A. water) and 60x (1.0 N.A. water) objectives. Trapping by de-esterification was assumed to accumulate the intracellular dye concentration to ~100 μM (Krizaj and Copenhagen, 1998). 340 nm and 380 nm excitation was delivered by an arc lamp (Lambda DG-4; Sutter Instruments, Novato, CA). Emissions at 510 nm were detected with 12-bit cameras (Delta Evolve or HQ2, Photometrics, Tucson, AZ) operated through NIS-Elements (Technical Instruments, San Francisco, CA). R/R (peak F^{340}/F^{380} ratio – baseline/baseline) was used to quantify the amplitude of Ca^{2+} signals in baseline-subtracted cells.

2.8. Data Analysis

Statistical analyses were performed with GraphPad Prism 6.0 and Origin Pro 8.5. TRPV2 inhibitor (tranilast) experiments represent averages from 4 retinas from two mice, the remainder of the results represent averages of Müller cell responses from at least three animals, with 2-5 slides/animal and 1-4 cells/slide. Means are shown ± SEM. Unless indicated otherwise, an unpaired t-test was used to compare two means and a one-way or two-way ANOVA along with the Tukey's test was used to compare three or more means. $p > 0.05$ = NS, $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = *** and $p < 0.0001$ = ****.

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3. Results

3.1. Müller cells express genes that encode putative mechanosensitive ion channels

RT-qPCR was employed to determine the identity of nonselective Ca^{2+}-permeable channels that mediate the Müller cell response to mechanical stretch, with mRNA expression normalized to *Trpv4* levels (e.g., Jo et al., 2015). The relative expression of putative mechanochannel genes was dominated by *Trpc1* mRNA, with the sequence *Trpc1>Piezol>Kcnk2>Trpv2>Trpv4>Piezo2>>Trpv6* and barely detectable levels of *Trpv1, Trpv3, Trpa1* and *Kcnk4* mRNAs (Fig. 1). Expression the *Kcnk2* gene that encodes the mechanosensitive K^+ channel TREK-1 was much more prominent than the expression of *Kcnk4* that encodes its cognate TRAAK. Thus, mouse Müller glia express multiple potential SACs that might be activated in parallel by mechanical stress.

3.2. Stretch regulates Müller cell Ca^{2+} homeostasis

Transduction of lateral strain was investigated in acutely dissociated Müller cells loaded with the Ca^{2+} indicator Fura-2 AM and plated onto flexible elastomeric membranes coated with collagen I/IV. Periodic calibrated membrane displacements/relaxations (0.5 Hz) mimicking *in situ* ECM deformations (Schmitt et al., 2012; were applied for 5 or 10 min; the strains were kept in physiological range (1 - 10% elongation) to suppress tachyphylaxis (response to the subsequent stretch stimulus) (Lindqvist et al., 2010) and reduce cell injury (e.g., Tschumperlin et al., 2000; Chierto et al., 2018). Calcium signals were not tracked during the transient loss of focus following stretch induction (red arrow in Fig. 1B). Figure 1 shows that controlled mechanical stimulation reliably elevates cytosolic $[Ca^{2+}]_i$ in cells with the polarized morphology of Müller glia. At 6% stretch, the calcium signal was increased by ~5 fold from the baseline of 0.216 ± 0.017 to 1.138 ± 0.068 (n = 22; P = 0.0000893). The response often appeared first in the endfoot compartment, was reversible (Fig. 2B) and dose-dependent (Fig. 2C). The post-peak relaxation to baseline presumably reflects endogenous Ca^{2+} clearance and sequestration mechanisms (Krizaj et al., 2004).

Osmotransduction in Müller cells requires activation of TRPV4, a polymodal nonselective cation channel that functions as a SAC in some but not every, cellular contexts (Ryskamp et al., 2014; Jo et al., 2015; Rocio Servin-Vences et al., 2017; Lapajne et al., 2020). To assess its role in stretch-evoked Ca^{2+} signaling in Müller glia, we stimulated the cells in the presence of the antagonist HC-06 applied at the concentration (5 μM) that obliterates the response to the agonist GSK1016790A (Ryskamp et al., 2014; Jo et al., 2015). HC-06 reduced the amplitude of stretch-evoked $[Ca^{2+}]_i$ increases by 53.24 ± 9.95 % (n = 15 cells; P < 0.000351). A comparable reduction in stretch responsiveness was observed in *Trpv4*-deficient Müller cells (54.76 %; n = 12; P < 0.000105) (Fig. 3).

The sensitivity of Müller cells to swelling-induced stretch is an order of magnitude higher compared to retinal neurons (Toft-Bertelsen et al., 2019) and was suggested to play a role in swelling-induced cytokine release (Matsumoto et a., 2018). We therefore tested whether calcium responses observed with weak tensile inputs include a TRPV4 component. 1% stretch evokes small but detectable $[Ca^{2+}]_i$ elevations to 0.8843 ± 0.09, that were reduced by HC-06 to 0.5004, a 43% inhibition (N = 3, n = 14 - 24). *Trpv4*$^{-/-}$ cells showed a

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trend towards reduction when stimulated with 1% stretch but the response did not reach significance (P = 0.0667) (Supplementary Fig. 1).

In situ indentation of Müller endfeet evokes Ca^{2+} waves that propagate towards the cell body and the apical process and may contribute to glial communication with adjacent neurons and vasculature (Metea and Newman 2006; Ryskamp et al., 2014; Agte et al., 2017; Toft-Bertelsen et al., 2019). To determine whether TRPV4 plays a role in deformation-induced calcium signals, Müller endfeet were rapidly indented with a beveled pipette in the presence of the antagonist HC-06. Poking increased the ratiometric signal in wild type cells from 0.142 ± 0.03 to 1.685 ± 0.02 ($P < 0.005$), with endfoot responses typically larger compared to the cell body (SFig 2A). Stretch-evoked signals were insensitive to preincubation with GSK7975A, an antagonist of Orai channels that mediates store-operated Ca^{2+} entry in Müller glia (Molnar et al., 2016) but was ~50% inhibited by HC-06 (0.843 ± 0.081; n = 5; $P < 0.005$) and in KO cells (0.681 ± 0.044; $P < 0.005$) (Supplementary Fig. 2). Thus, glial TRPV4 channels may contribute to the sensing of physiological tissue deformations induced by tensile stretch and acute indentation.

3.3. TRPC1 partially mediates the stretch-evoked calcium response

The partial suppression of stretch-evoked responses in *Trpv4*$^{-/-}$ and HC-06-treated cells (Fig. 5) suggests activation of auxiliary transduction mechanisms. TRPC1 is a nonselective cation channel that contributes to Ca^{2+} homeostasis in Müller glia (Molnar et al., 2016) and has been implicated in mechanotransduction (Maroto et al., 2015). *Trpc1*$^{-/-}$ cells showed modest but significant reductions in the amplitude of stretch-evoked Ca^{2+} responses to 0.830 $\pm$ 0.017 (21.98 % reduction; n = 15; P = 0.0423). Stretching cells in the presence of the nonselective inhibitor SKF96365 likewise showed a comparable reduction in Ca^{2+} responses (25.29 % reduction; n = 8; P = 0.0393) Furthermore, stimulation of *Trpc1*$^{-/-}$ cells in the presence of HC-067 did produce a reduction additive compared to the inhibition/ablation of TRPV4 channels alone (0.407 ± 0.036; n = 11; P = 0.0208) whereas stretching *Trpc1*$^{-/-}$ cells in the presence of SKF96365 did not (P = 0.09657)(Fig. 5).

3.5. TRPV2 does not play a major role in the Müller stretch response

Given the relatively prominent *Trpv2* signal in mouse Müller cells and its proposed function as a neuronal stretch sensor (Shibasaki et al., 2010), we investigated the effect of its inhibitor tranilast on the stretch response. The antagonist did not reduce the amplitude of Ca^{2+} signals evoked by 8% stretch (P =0.358; n = 14) (Supplementary Fig. 3). These data suggest that TRPV2 does not contribute to the Ca^{2+}- stretch response despite the prominent expression of its mRNA.

4. Discussion

The objective of this study was to decipher the molecular mechanisms that mediate the intrinsic response of radial glia to mechanical strain. Our data showed that mouse Müller cells respond to physiological strains with dose-dependent $[Ca^{2+}]_i$ elevations that are mediated by TRPV4, TRPC1 and a yet-to-be-identified cytosolic Ca^{2+} influx pathway. These findings implicate polymodal nonselective TRP channels in induction of

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the inflammatory response and reactive gliosis in retinas subjected to mechanical stress, with potential roles in glaucoma and diabetes.

The degree and distribution of strains examined in our study matched the currently available data on the micromechanics of the inner plexiform layer, inner limiting layer and the glial lamina at the optic nerve head (Križaj et al., 2014; Korneva et al., 2020). At Young's modulus of ~0.5 kPa (Lu et al., 2013), even modest amount of stress (~200 Pa) at the inner limiting membrane would suffice to produce ~10% strain (e.g., Safa et al., 2021). Previous studies of the Müller stretch response utilized 15% - 20% radial stretch (Wang et al., 2013; Lindqvist et al., 2010) whereas we opted for 6% stretch to mimic the strains calculated for the neural retina (Sigal et al., 2005) and to minimize cell injury from Ca^{2+} overloads. Similar to the effects of *in situ* stretch (Lindqvist et al., 2010) and experimentally induced elongation of cultured trabecular meshwork cells, astrocytes, neurons, epithelial and endothelial cells under radial stretch (Vlahakis et al., 1999; Sachs, 2010; Ryskamp et al., 2016; Turovsky et al., 2020), we found that Müller glia respond to mild repetitive radial ECM strains ranging from 1 - 10% with dose-dependent $[Ca^{2+}]_i$ elevations. We identified TRPV4, a Ca^{2+}-permeable nonselective cation channel, as a central determinant of stretch-evoked Ca^{2+} signaling in Müller glia across the range of tensile stimuli (1 – 12%) employed in the study. Pharmacological inhibitors of TRPV4 and *Trpv4* gene deletion suppressed ~50% of the response induced by 6% stretch, with additional evidence suggesting potential involvement of TRPV4 channels at threshold (~1%) strains. These observations are in accord with the remarkable sensitivity for weak swelling stimuli exhibited by the TRPV4 protein variant expressed in Müller cells (Toft-Bertelsen et al., 2019). The properties of the pressure-activated nonselective cation current reported by Puro in intact human Müller cells (1991) are consistent with TRPV4 activity. Indeed, virtually all Müller cells in mouse, rat, pig and human preparations show TRPV4-ir and/or responsiveness to the agonist GSK1016790A and other channel effectors such as hypotonicity, arachidonic acid, eicosanoids and moderate temperature (Ryskamp et al., 2014; Jo et al., 2015; Lakk et al., 2017; Taylor et al., 2016; Matsumoto et al., 2018), in contrast to cortical astrocytes and satellite glia, which show limited TRPV4 expression (Shibasaki et al.; 2014; Rajasekhar et al., 2015; Pivonkova et al., 2018). TRPV4 is clearly a major Müller transducer of tensile strain, with the downstream calcium signal including contributions from Ca^{2+}-induced store release (Ryskamp et al., 2014), SOCE (Molnar et al., 2016) and/or Ca^{2+}-dependent purinergic-Pnx1 mechanisms (Agte et al., 2017; Toft-Bertelsen et al., 2019; Lapajne et al., 2020).

Pressure clamp, indentation and ECM stretch experiments in *in vitro* overexpression systems, cells and organs (Alessandri-Haber et al., 2004; Lechner et al., 2011; Ryskamp et al., 2016; Shibasaki, 2020) implicated TRPV4 in stretch responsiveness in some (Loukin et al., 2010; Yarishkin et al., 2021) but not all (Servin-Vences et al., 2017; Lapajne et al., 2020; Sianati et al., 2021; Nikolaev et al., 2019) preparations. Thus, TRPV4 mediates ECM stretch-induced $[Ca^{2+}]_i$ signaling in urothelial, pulmonary epithelial, endothelial and trabecular meshwork cells (Mochizuki et al., 2009; Potla et al., 2020; Ryskamp et al., 2016) with potential roles in nociception (Alessandri-Haber et al., 2004), volume regulation (Toft-Bertelsen et al., 2017) and inflammation (Balakrishna et al., 2014) but, despite prominent mRNA/protein expression, appears to be stretch-insensitive in corneal epithelial cells and chondrocytes (Servin-Vences et al., 2017; Lapajne et al., 2020). We propose that its stretch-

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sensitivity reflects cell-type specific context such as tethering to ECM, β1-integrins, lipids and/or modulatory signaling mechanisms (e.g., Src kinases, phospholipase A2, PIP3 kinase, Rho/Rac1 pathway; Potla et al., 2020; White et al., 2016; Toft-Bertelsen et al, 2019; McCray et al., 2021; Lakk and Krizaj, 2021; Lakk et al., 2021).

Our finding that TRPV4 inhibition/deletion does not abolish the stretch response in Müller glia demonstrates that, as reported for trabecular meshwork, urothelial and pulmonary epithelial cells (Ryskamp et al., 2016; Mochizuki et al., 2009; Pairet et al., 2018), the cells' mechanosensitivity reflects synergistic activation of multiple SACs. Given the prominent *Trpc1* expression in purified cells (Fig. 1), *Trpc1* mRNA in the inner nuclear layer (Gilliam and Wensel, 2011; Molnar et al., 2012), TRPC1 functions in Müller cell Ca^{2+} homeostasis (Molnar et al., 2016) and TRPC1 mechanosignaling in overexpression systems (Maroto et al., 2005), neurons (Garrison et al., 2012; Kerstein et al., 2013), kidney (Fabian et al., 2012), endothelial (Berrout et al., 2012), epithelial cells (Li et al., 2019), myocytes (Stiber et al., 2008; Burks et al., 2019), and possibly amphibian photoreceptors (Szikra et al., 2008; 2009; Bocchero et al., 2020), we considered the possibility that Müller cell TRPC1 might contribute to the stretch response. Consistent with this, *Trpc1*$^{-/-}$ and SKF96365-treated glia showed modest yet consistent (~25%) reductions in the amplitude of the stretch response. Because TRPC1 homotetramers are not believed to form functional channels and mechanosensitive currents are unaffected in some overexpression (Gottlieb et al., 2007) and gene knockout (Dietrich et al., 2007) studies, we propose that the TRPC1-dependence of the Müller stretch response reflects heteromerization with TRPV4 (Du et al., 2014) or TRPC3-5 subunits (Molnar et al., 2016; Zergane et al., 2021; Strubing et al., 2001). The former possibility is supported by the linearity of the TRPV4 I-V relationship in the presence of extracellular Ca^{2+} (Ma et al., 2011; Ryskamp et al., 2014) and the absence of additional reduction of the stretch response in *Trpv4*$^{-/-}$ cells treated with SKF96365 (Figure 4). TRPC1 and the C-terminal tail of TRPV4 could interact through the Stromal Interaction Molecule 1 (STIM1), a Ca^{2+} influx regulator that regulates SOCE in Müller cells (Shin et al., 2015; Molnar et al., 2016).

The expression pattern of genes that encode nonselective Ca^{2+}-permeable cation channels in mouse Müller cells (*Trpc1>Piezo1>Trek1>Trpv2>Trpv4*) differs from retinal microglia (*Trpv2>Trpv1>Trpv3>Trpv4*; Redmon et al., 2021) and astrocytes (*Trpc1>>Trpm7>Trpv2>Piezo1*; Choi et al., 2015), and suggests that retinal glial subtypes which show vast differences in connectivity and function may also be differentially susceptible to different mechanical, lipid and inflammatory stressors. mRNA profiling of purified Müller glia showed moderate *Trpv2* expression. Because TRPV2 may contribute to mechanical signaling in myocytes, neurons and HEK293 overexpressors (Muraki et al., 2003; Shibasaki et al., 2010), we tested the stretch responsiveness of Müller cells in the presence of tranilast, which did not affect the amplitude or time course of the calcium response. GsMtx4, a nonselective inhibitor of Piezo channels suppressed stretch-induced ET-1 secretion in rat astrocytes (Ostrow et al., 2011) and poke-evoked Ca^{2+} signaling in trabecular meshwork cells (Yarishkin et al., 2021), did not reduce mechanically induced Ca^{2+} signals in guinea pig Müller glia (Agte et al., 2017). Its transcript expression levels, however, point at Piezo1 as a potential candidate for mediating the residual signals in strained mouse *Trpv4*$^{-/-}$ cells. Many recent reports noted that nonexcitable cells express

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multiple mechanosensors that respond to different modalities of mechanical stress and may be coupled to separate intracellular signaling pathways (Servin-Vences et al., 2017; Lapajne et al., 2020; Yarishkin et al., 2021; Desplat et al., 2021; Nakazawa et al., 2021).

The sensitivity of TRPV4 to modest strains suggests the channel could contribute to reactive gliosis that precedes neuronal injury in hypertensive eyes (Woldemussie et al., 2004; Inman and Horner, 2007). Hypertensive mice show increased amplitude of astroglial TRPV4 currents (Diaz et al 2018), with acute decreases in cerebral perfusion producing TRPV4-dependent elevations in $[Ca^{2+}]i$ (Kim et al., 2015; Marina et al., 2020). Consistent with this, intravitreal injection of GSK1016790A induced Müller reactivity in animal glaucoma models (Ryskamp et al., 2014), and TRPV4 blockers and gene knockdown were able to suppress agonist-, swelling- and detachment-induced gliosis in mouse and porcine Müller glia (Ryskamp et al., 2014; Taylor et al., 2016; Matsumoto et al., 2018). TRPV4 agonists promote activation of the MAPK/ pathway, cytokine release and pathological swelling in edematous and inflamed retina and brain (Pairet et al., 2018; Matsumoto et al., 2018; Hoshi et al., 2018; Orduna-Rios et al., 2019) whereas TRPV4 inhibition and deletion of the *Trpv4* gene suppress the release of proinflammatory cytokines/angiogenic factors (MCP-1, VEGF, TNFα) (Taylor et al., 2016; Matsumoto et al., 2018). We therefore predict that IOP- and stretch-induced expression of c-fos, MAPK/ERK1/2, Jak-STAT and Epha2/Nrn1 pathways in proinflammatory and proangiogenic Müller glia (Lindqvist et al., 2010; Wang et al., 2013) is downstream from TRPV4 and potentially TRPC1, channels. Other manifestations of excessive TRPV4 activation include rapid loss of endothelial barrier permeability in retinal microvasculature (Phuong et al, 2017), ventilator-induced epithelial injury & immune activation (Pairet et al., 2018), and pathological swelling of the CNS in diabetic and ischemic tissues (Orduna-Rios et al., 2019; Pivonkova et al., 2018). Consistent with roles in mechanically induced immune and inflammatory signaling, TRPV4 gene knockdown lowered alleviated inflammatory lung injury, inflammation-associated neuropathic sensitization, temporomandibular joint pain, pancreatic inflammation, and CNS injury in Alzheimer's disease (Alessandri-Haber et al., 2004; Ceppa et al., 2010; Chen et al., 2013; Balakrishna et al., 2014; Bai and Lipski 2014; Li et al., 2019). Interestingly, however, normotensive *Trpv4*$^{-/-}$ retinas also show mild gliosis (Ryskamp et al., 2014). It is thus possible that moderate and constitutive TRPV4 activity may be required to maintain an anti-inflammatory homeostatic state.

In conclusion, this study supports the view that TRPV4 and possibly TRPC1 function as stretch sensors that integrate transmission of mechanical forces across radial glia. We propose that the channels serve as initiators of regenerative calcium signaling in Müller glia exposed to mechanical stress (Zahs and Newman, 1997; Metea and Newman, 2006; Ryskamp et al., 2014). Future work will show how TRPV4 interacts with integrin-based focal adhesions that transfer forces across discrete cytoskeletal elements and/or how different SAC combinations, potentially involving Piezo1 channels, optimize the responsiveness of different glial populations within the retina to tugging, lateral stretch, compression, volume expansion and shear. Areas under higher local strain, for example, may promote local inflammatory responses that trigger axonal dysfunction and permeability changes at the neurovascular unit but may expand in the presence of generalized stress to

drive acute and chronic remodeling of retinal architecture and function (Lu et al., 2013; Fortune 2019; Inman and Horner, 2007; Sun et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium concentration
ECM	extracellular matrix
IOP	intraocular pressure
ECM	extracellular matrix
GFAP	glial fibrillary acidic protein
HC-06	HC067047
KO	knockout
MAPK	mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
qRT-PCR	quantitative real-time polymerase chain reaction
SAC	stretch-activated channel
STIM1	(Stromal Interaction Molecule 1)
TNFα	tumor necrosis factor-alpha
TRPC1	transient receptor potential canonical isoform 1 channel
TRPV4	transient receptor potential vanilloid isoform 4 channel
VEGF	vascular endothelial growth factor

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Highlights

- Müller cells, the principal class of glia in the vertebrate retina are known to respond to increases in intraocular pressure, traumatic injury, and pathological osmogradients with inflammatory activation.
- Stretch-activated nonselective TRPV4 and TRPC1 cation channels mediate calcium signaling in response to physiological strains.
- TRPV2 antagonists do not affect stretch-evoked $[Ca^{2+}]i$ signals despite prominent *Trpv2* expression.

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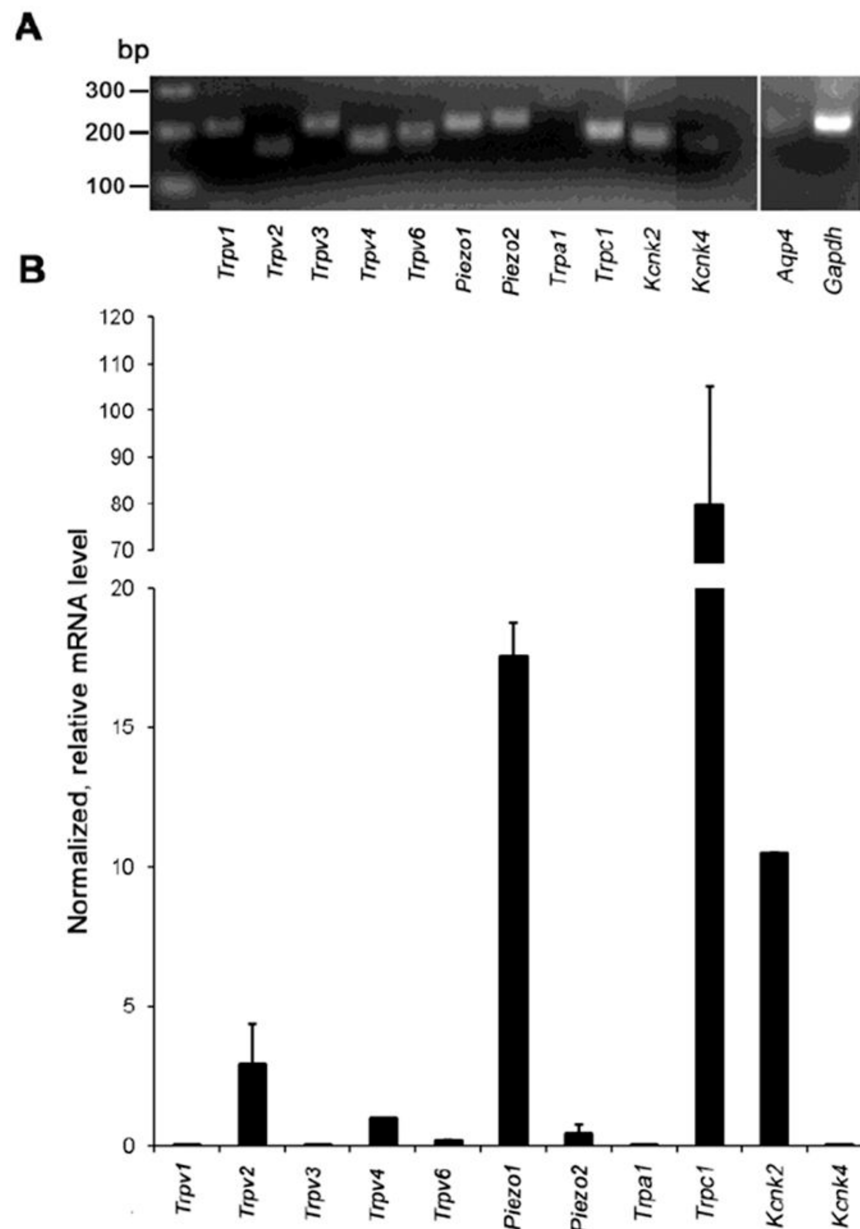

Figure 1. Müller cells express mechanosensitive ion channels.

(A) End-point PCR products with sequence primers from putative mechanosensors. *Aqp4* (aquaporin 4) mRNA served as a standard phenotype marker. *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) mRNA served as a loading control. (B) Semiquantitative RT-PCR. The relative abundance of putative mechanosensors normalized with respect to *Trpv4* expression (n = 3).

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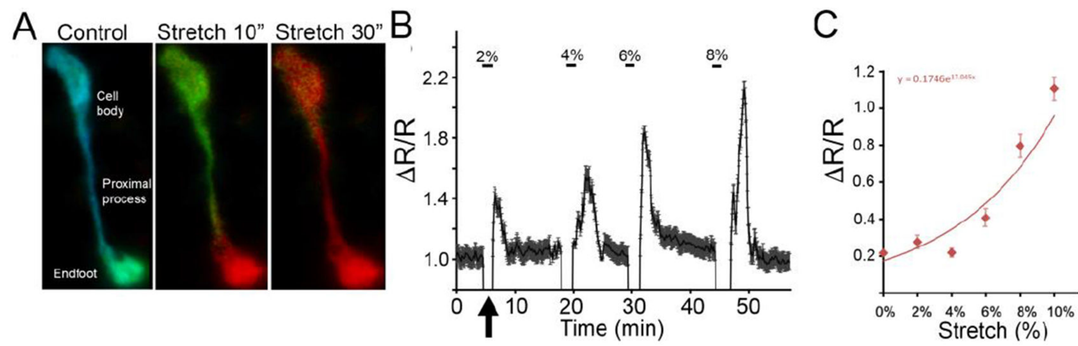

Figure 2. Mechanical strain evokes $[Ca^{2+}]_i$ responses in Müller cells.

Calcium imaging, Fura-2 AM-loaded, acutely dissociated cells were cultured on silicon membranes and exposed to cyclic stretch (0.5 Hz, 2 - 8%). (A) 6% stretch-evokes $[Ca^{2+}]_i$ increase initially in the endfoot compartment (10 sec following refocusing) and the entire cell (30 sec following the refocusing). (B & C) The amplitude of stretch-evokes $[Ca^{2+}]_i$ signals was proportional to the applied strain. Acquisition was paused during stretch application (arrow) due to focus loss.

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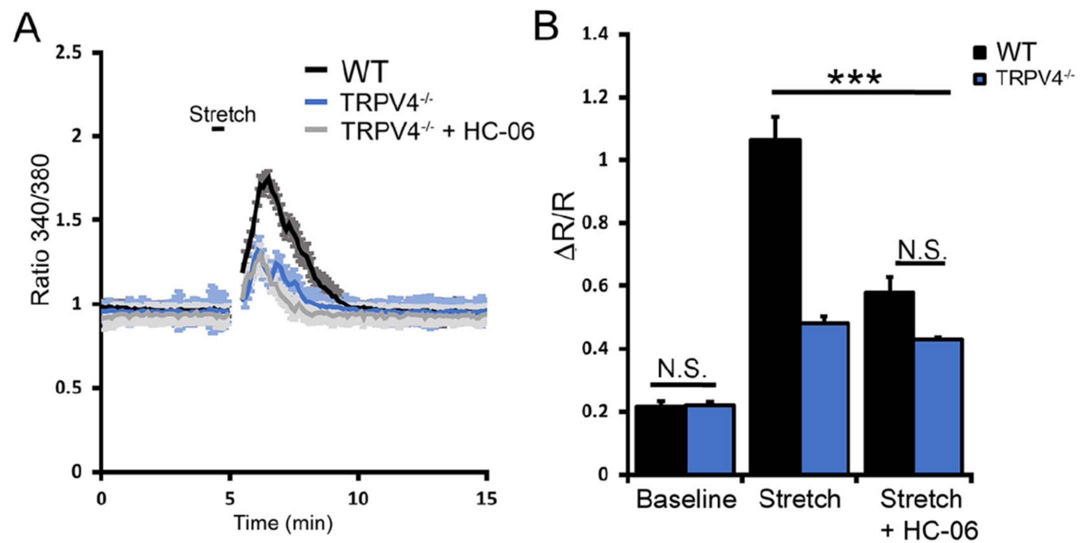

Figure 3. TRPV4 mediates a major component of the strain-induced $[Ca^{2+}]_i$ response.

(A & B) The amplitude of calcium signal evoked by 6% strain was decreased in cells with ablated *Trpv4* gene (blue traces) and in cells pretreated with the TRPV4 antagonist HC-06. Stretching cells in the presence of HC-06 (gray trace in Fig. 2A) did not produce additional decreases in the response amplitude. ***, $p < 0.005$, N.S., not significant.

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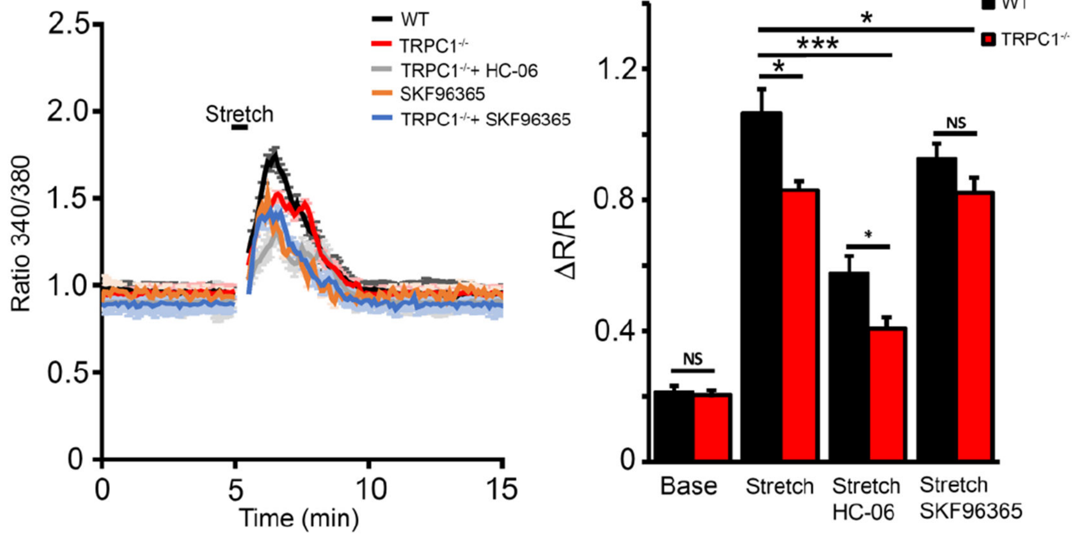

Figure 4. TRPC1 contributes to the stretch response.

(A) The amplitude of calcium signal evoked by 6% strain is decreased in cells with an ablated *Trpc1* gene (red trace) and in cells treated with the nonselective TRPC1 inhibitor SKF293365 (orange trace). (B) Summary (n = 10-25 cells per experiment). The amplitudes and fractions of stretch-evoked $[Ca^{2+}]_i$ increases are comparable between wild type controls and *Trpc1*$^{-/-}$ cells. *, p<0.05; ***, p < 0.001, N.S., not significant.

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Table 1.

Primer sequences used for PCR analysis

Name	Forward primer	Reverse primer	NCBI reference number
TRPV1	AGGGTGGATGAGGTGAACTGGACT	GCTGGGTGCTATGCCYATCTCG	NM_001001445.2
TRPV2	GTTGGCCTACGTCCTCCTCACCTA	TGCACCACCAGTAACCATTCTCC	NM_011706.2
TRPV3	CTGACCTTCGTCCTCCTCCTCAAC	CAGCCGGAAGTCCTCATCTGCTA	NM_145099.3
TRPV4	TCCTGAGGCCGAGAAGTACA	TCCCCCTCAAACAGATTGGC	NM_022017.3
TRPV6	GACTCTGTGGTCCGTGCCTCA	CAGTGTTCTCCATCCGTCGTCTG	NM_022413.4
Piezo 1	TGGTGGCCATCCTTACAC	GGTACAGCCACTTGATGAGG	NM_001357349.1
Piezo 2	AAACCAACATTCCCCTTCAT	CTGGAGCAGTCAGGTTGTTT	NM_001039485.4
TRPA1	GCAGTGGCAATGTGGAGCAATAG	GCCAAAAGCCAGTAGGAGGAAGAT	NM_177781.5
TRPC1	AGCCTCAGACATTCCAGGTTT	AACATTTTGCACTGACGGGC	NM_011643.4
TRPM4	CCATCGGCTGCACCTCTACCTCT	GGGCCCCAGCTGCTTGTTCAC	NM_175130.4
TREK1	AGTCCAGTCCCTGCATCTAGC	ATCACGTAAGCCCAAGCCTC	NM_001159850.1
TASK1	CCTTCTACTTCGCCATCACC	CAGCAGGTACCTCACGAAG	NM_010608.3
TRAAK	GCAGGCTCAGAAGAAAATGG	CAAGCTGATGAGTGGTTGCT	NM_008431.3
AQP4	AGCAATTGGATTTTCCGTTG	TGAGCTCCACATCAGGACAG	NM_001317729.1
GAPDH	GGTTGTCTCCTGCGACTTCA	TAGGGCCTCTCTTGCTCAGT	NM_001289726.1