Published in final edited form as:

J Neurochem. 2017 May ; 141(3): 436–448. doi:10.1111/jnc.13998.

The P2X7 receptor links mechanical strain to cytokine IL-6 upregulation and release in neurons and astrocytes

Wennan Lu¹, Farraj Albalawi^{1,2}, Jonathan M. Beckel^{1,5}, Jason C. Lim¹, Alan M. Laties³, and Claire H. Mitchell^{1,3,4}

¹Department of Anatomy and Cell Biology, University of Pennsylvania, Philadelphia, PA 19104

²Department of Orthodontics, University of Pennsylvania, Philadelphia, PA 19104

³Department of Ophthalmology, University of Pennsylvania, Philadelphia, PA 19104

⁴Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104

⁵Department of Pharmacology and Chemical Biology, University of Pittsburgh, PA 15261

Abstract

Background—Mechanical strain in neural tissues can lead to the upregulation and release of multiple cytokines including IL-6. In the retina, the mechanosensitive release of ATP can autostimulate P2X7 receptors on both retinal ganglion cell neurons and optic nerve head astrocytes. Here we asked whether the purinergic signaling contributed to the IL-6 response to increased intraocular pressure (IOP) *in vivo*, and stretch or swelling *in vitro*.

Methods—Rat and mouse eyes were exposed to non-ischemic elevations in IOP to 50–60 mmHg for 4 hrs. A PCR array was used to screen cytokine changes, with quantitative (q)PCR used to confirm mRNA elevations and immunoblots used for protein levels. P2X7 antagonist Brilliant Blue G (BBG) and agonist BzATP were injected intravitreally. ELISA was used to quantify IL-6 release from optic nerve head astrocytes or retinal ganglion cells. Receptor identity was confirmed pharmacologically and in P2X7^{-/-} mice

Results—Acute elevation of IOP altered retinal expression of multiple cytokine genes. Elevation of *IL-6* was greatest, with expression of *IL1m*, *IL24*, *Tnf*, *Csf1* and *Lif* also increased more than

---

Contact information: Dr. Claire H. Mitchell, Department of Anatomy and Cell Biology, University of Pennsylvania, 240 S. 40th St, Philadelphia, PA 19104-6030, Tel: 215-898-8994, FAX: 215-573-2324, chm@upenn.edu.

Declarations

Ethics Approval and Consent to Participate: All experiments approaches on rats and mice were approved by the Animal Care and Use Committee of the University of Pennsylvania protocol #803584.

Consent for publication: Not applicable.

Availability of data and materials: All readily reproducible materials described in the manuscript, including new software, databases and all relevant raw data will be freely available to any scientist wishing to use them.

Competing interest: The authors declare that they have no competing interests.

Funding: This work is supported by grants from the NIH EY015537 and EY013434 and core grant EY001583 (CHM). NIH DK106115 (JMB), Jody Sack Fund (WL). These funding bodies had no direct role in the design of the study and collection, analysis, and interpretation of data or in writing the manuscript.

Authors' Contribution: WL helped design the study, carried out most of the rat IOP experiments, performed analysis of molecular and protein data and immunohistochemistry. FA carried out experiments on elevation of IOP in mice and analyzing associated data. JMB was responsible for many of the *in vitro* experiments on rat optic nerve head astrocytes. JCL carried out experiments on isolated ganglion cells. CHM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

two-fold, while *Tnfsf11*, *Gdf9* and *Tnfsf4* were reduced. qPCR confirmed the rise in IL-6 and extracellular ATP marker *ENTPD1*, but not pro-apoptotic genes. Intravitreal injection of P2X7 receptor antagonist BBG prevented the pressure-dependent rise in *IL-6* mRNA and protein in the rat retina, while injection of P2X7 receptor agonist BzATP was sufficient to elevate *IL-6* expression. IOP elevation increased *IL-6* in wild type but not P2X7R knockout mice. Application of mechanical strain to isolated optic nerve head astrocytes increased *IL-6* levels. This response was mimicked by agonist BzATP, but blocked by antagonists BBG and A839977. Stretch or BzATP led to IL-6 release from both astrocytes and isolated retinal ganglion cells.

Conclusions—The mechanosensitive upregulation and release of cytokine IL-6 from the retina involves the P2X7 receptor, with both astrocytes and neurons contributing to the response.

Graphical abstract

Upregulation of IL-6 by mechanical strain involves pannexin-mediated ATP release and autostimulation of P2X7 receptors. Increased expression of IL-6 mRNA and protein in the retina following transient increase in intraocular pressure in vivo, and in stretched/swollen optic nerve head astrocytes was blocked by P2X7R antagonists. Stretch or P2X7R stimulation raised calcium and released IL-6 from astrocytes and retinal ganglion cell neurons.

Keywords

Cytokines; IL-6; mechanical strain; P2X7; astrocytes; neurons; retina; stretch; ATP release; IOP; glaucoma; TBI

Background

Mechanical strain to neurological tissues frequently leads to both inflammatory and protective responses (Corps *et al.* 2015). The cytokine interleukin 6 (IL-6) is of particular relevance as it can mediate pathological or protective actions in neural systems depending on context (Erta *et al.* 2012). IL-6 can lead to neuroinflammation after traumatic brain injury (TBI) and cerebrospinal fluid levels of IL-6 correlate with pathological progression after TBI (Yang *et al.* 2013, Kumar *et al.* 2015). However, IL-6 can also induce neurogenesis and

protect neural cells after damage (Erta et al. 2012, Penkowa *et al.* 2003). A better understanding of the pathways linking mechanical strain to IL-6 may help determine the mechanism for the shift of IL-6 from detrimental to protective actions.

The purinergic system has been implicated in regulation of IL-6 in several cell types including fibroblasts (Inoue *et al.* 2007), skeletal muscle cells (Bustamante *et al.* 2014), macrophages (Hanley *et al.* 2004) and microglia (Shieh *et al.* 2014). Purinergic signaling is particularly sensitive to mechanical strain, with ATP release accompanying increases in shear stress, stretch, and swelling (Praetorius & Leipziger 2009, Corriden & Insel 2010). In neural tissue, ATP can be released through pannexin hemichannels in response to mechanical strain (Iglesias *et al.* 2009, Xia *et al.* 2012). The release of ATP and stimulation of the P2X7 receptor is closely linked with inflammatory responses in non-neural cell types (Gombault *et al.* 2012), leading to inflammasome activation and IL-1 β release (Franceschini *et al.* 2015, Ferrari *et al.* 2006). Of particular relevance is the priming and release of IL-6 in microglial cells in response to stimulation of the P2X7 receptor (Shieh et al. 2014).

The retina provides an ideal model with which to examine the relationship between strain, purines and IL-6 in neural tissue. Mechanical strain is experienced by neurons and glial cells in the retina when the intraocular pressure (IOP) rises during glaucoma (Sigal & Ethier 2009, Downs 2015). Retinal ganglion cells are the most susceptible to neuropathological changes and death in response to elevated IOP, while the focal point for mechanical strain is the optic nerve head, with optic nerve head astrocytes identified as a critical intermediary (Downs *et al.* 2008, Hernandez 2000).

Perturbed purinergic signaling is implicated in response to glaucoma and elevated IOP. For example, human patients with both acute and chronic glaucoma have elevated levels of extracellular ATP in ocular fluids (Zhang *et al.* 2007, Li *et al.* 2011). Primate, rat and mouse models of sustained IOP elevation show elevated extracellular ATP (Lu *et al.* 2015). These models also demonstrated increased expression of the ectoATPase NTPDase1, previously identified to act as a marker for sustained elevation of extracellular ATP (Lu *et al.* 2007). The pressure-dependent ATP release from retina is inhibited by blockers of pannexin hemichannels and not linked to lactate dehydrogenase, suggesting it is a physiological response (Reigada *et al.* 2008). Both optic nerve head astrocytes (Beckel *et al.* 2014) and retinal ganglion cells (Xia et al. 2012) release ATP through pannexin hemichannels when subjected to mechanical strain. This released ATP can autostimulate the P2X7 receptor in both cell types.

Alterations in cytokine IL-6 have also been recognized as an important response to elevated IOP. Levels of IL-6 have been detected in the aqueous humor of patients with chronic glaucoma (Zenkel *et al.* 2010, Chen *et al.* 1999). In the hypertonic saline model of chronic IOP elevation, *IL-6* was the most upregulated gene in the optic nerve head tissue (Johnson *et al.* 2011), while *IL-6* was also elevated following transient elevation of IOP (Cepurna *et al.* 2008). Several observations suggest IL-6 confers protection to retinal ganglion cells; exposure of isolated ganglion cells to high hydrostatic pressure *in vitro* led to apoptotic death that was attenuated by addition of recombinant IL-6 (Sappington *et al.* 2006), and IL-6 increased both the number and the length of neurites sprouting from isolated retinal ganglion

cells (Chidlow *et al.* 2012). While these observations suggest IL-6 has an important role in the response to increased pressure, the signaling mechanisms linking the mechanical strain to the IL-6 response are largely unknown.

Given the link between mechanical strain, ATP release and P2X7 receptor autostimulation in the retina, the connection between the purinergic signaling and IL-6 activation, and evidence implicating IL-6 in glaucoma, this study was based on the hypothesis that mechanosensitive stimulation of the P2X7 receptor was involved in the IL-6 response to elevated IOP in the retina. To distinguish between responses due to elevated IOP and those due to cell death, an *in vivo* model of acute but non-ischemic IOP elevation was employed as studies indicate it is generally not lethal to retinal neurons (Abbott *et al.* 2014, Crowston *et al.* 2015). Isolated optic nerve head astrocytes and retinal ganglion cells were also utilized to investigate the response in more mechanistic detail *in vitro*.

Methods

Animals

All experiments protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The P2X7 knockout (P2X7^{-/-}) mice originally generated from Pfizer (B6.129P2-P2rx7tm1Gab/J), along with age matched 9 month-old C57Bl6J wild type controls were obtained from Jackson Laboratories (Bar Harbor, ME). Sprague-Dawley and Long-Evans rats were obtained from Harlan Laboratories (Fredrick, MD). Mice and rats of both sexes were utilized.

Model of moderate temporally-controlled IOP elevation

Acute elevation of IOP experiments were performed using adult Sprague-Dawley rats based on the Control Elevation of IOP (CEI) protocol developed by John Morrison and colleagues (Morrison *et al.* 2010, Morrison *et al.* 2014). Adult rats were given a prior dose of 2 mg/kg meloxicam and then deeply anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Proparacaine (1%) was added to the ocular surface and one drop of Tropicamide (1%) was administered into each eye for pupil dilation. Once anesthesia had taken effect, one eye was cannulated with a 27 gauge shielded wing needle (Becton Dickinson, NJ) inserted into the anterior chamber and connected to a 20 ml syringe filled with sterile phosphate buffered saline (PBS). IOP was increased to 50 mmHg by positioning the syringe at the appropriate height (68cm H₂O) while the contralateral eye without cannulation served as a normotensive control. During the initial development of the model, IOP was calibrated with a TonoLab tonometer (Colonial Medical Supply, VT) at the beginning and end of the elevation of the reservoir. As IOP was found to be remarkably consistent both throughout the 4 hrs of elevation and between animals, it was usually just measured at the end of the 4 hr period during experiments to avoid excessive force on the needle tip inside the eye. The retina was carefully observed under an operating microscope to ensure that blood flow through the retinal vessels was maintained. After 4 hrs IOP elevation, pressure was returned to normal, the needle was removed and 0.3–1% gentamycin ointment or erythromycin (0.5%) was applied to the cornea. Animals were sacrificed 20 hrs

(i.e. 1 day) or 5 days later and the retina, including the optic nerve head material, was dissected.

Experiments were also performed on mice using procedures similar to those used for rat with parallels to those described by Crowston and colleagues (Crowston et al. 2015). Mice were given a prior dose of meloxicam and then anesthetized with 1.5% isoflurane. IOP was increased to 50–60 mmHg for 4 hours. Mice were sacrificed immediately after the pressure was returned to baseline, or 20 hrs later. The contralateral eye without cannulation served as a normotensive control.

PCR Array

Expression of mRNA for 84 rat interferons, cytokines and interleukins in the retina was determined using the Rat Common Cytokine RT2 Profiler™ PCR Array (#PARN-021A, SABiosciences Corp., Frederick, MD). Samples were processed according to the manufacturer's protocol. In brief, total RNA was isolated from the control and pressurized retinas using Trizol and RNeasy mini kit (Qiagen, Inc.), and RNA was quantified from optical density and purity determined (Nanodrop, Thermo Scientific, Inc.). Total RNA (1 µg) was reverse transcribed using genomic DNA elimination and RT² First Strand kit (#C-03, SABiosciences Inc.). Comparison of the relative expression of cytokine genes were performed using the PCR array on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Lactate dehydrogenase A, Ribosomal genes *L13A*, hypoxanthine phosphoribosyltransferase 1 (*HPI*), and beta actin (*Actb*) were used as housekeeping genes and were all stable in retina from eyes with control and elevated IOP. Data were analyzed with the SABiosciences Web-Based PCR Array Data Analysis, where *p* values were calculated based on a Student's *t*-test of the replicate 2^{-Ct} values for each gene in the control group and experimental groups.

Quantitative PCR

RNA was processed as above. Quantitative PCR (qPCR) was carried out using Power SYBR Green master mix with primer pair sequences shown in Table 1, using the 7300 Real-Time PCR System. Data were analyzed using the delta-delta CT approach, with results expressed as fold change in gene expression in eyes with elevated IOP versus control samples (2^{-Ct}) using an unpaired *t*-test as described recently (Lu *et al.* 2015).

Intravitreal injection

Intravitreal injections were performed as described (Hu et al., 2010) under a dissecting microscope with a micropipette connected to a microsyringe (Drummond Scientific Co., Broomall, PA). The glass pipette filled with drug was passed through the superior nasal region of sclera into the vitreous cavity at a point approximately 1 mm from the limbus. The total volume injected was 5 µl over a 30 sec time period. P2X7 receptor antagonist Brilliant Blue G (BBG, 0.8%) was dissolved in sterile saline and injected 1–3 days before IOP elevation. To examine the effects of P2X7 stimulation, Long Evans rats were injected with either 2 µl 250 µM P2X7 receptor agonist Bz-ATP or sterile saline. Rats were sacrificed and the retina dissected, with total RNA isolated from the retina and processed as described above.

Immunoblots

Immunoblots were processed as described (Guha et al., 2013). In brief, whole retinas were washed twice with cold PBS and lysed in RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, protease inhibitor cocktail (Complete; Roche Diagnostics, Germany), 1% Triton X-100, 0.1% SDS, and 10% glycerol. Samples were sonicated and cleared by centrifugation (10,000g) for 10 min at 4°C, with protein concentrations determined using a BCA Protein Assay (Pierce/ThermoFisher). Protein was separated using conventional SDS-PAGE, and processed using standard immunoblot protocols (Lu et al. 2015). Blots were incubated with a monoclonal antibody to rat IL-6 overnight at 4°C (1:1000 R&D Systems, # MAB5061), followed by incubation with anti-mouse IgG conjugated to horseradish peroxidase (1:5000; Amersham Biosciences Corp., Arlington Heights, IL) at room temperature for 1 hr. and developed by chemiluminescence detection (ECL detection system; Amersham Biosciences Corp.). The ImageQuant LAS 4100 imager and Image Quant software (Both GE Healthcare Lifesciences) were used to detect and quantify the intensity of the specific bands. Western blots were performed 3–4 times each.

Optic nerve head astrocytes

Primary rat optic nerve head astrocyte cultures were grown as described (Beckel *et al.* 2014) based upon a protocol modified from Mandal et. al. (Mandal et. al., 2009). The optic nerve head tissue proximal to the sclera in rat pups up to postnatal day 5 was digested for 1 hr in 0.25% trypsin. Cells were grown in medium comprised of Dulbecco's minimal essential medium/F12, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 25ng/ml epidermal growth factor (EGF) and used up to passage 5. Cell identification was performed with GFAP immunostaining as described (Beckel et al., 2014). For stretch experiments, astrocytes were seeded on a silicon substrate (Silastic, Specialty Manufacturing, Saginaw, MI), bathed in isotonic solution (in mM; 105 NaCl, 5 KCl, 4 NaHEPES, 6 HEPES acid, 1.3 CaCl₂, 5 glucose, 5 NaHCO₃, 60 mannitol and 0.25 MgCl₂ pH 7.4). Cells were subjected to a 5% equibiaxial strain at 0.3 Hz for 2 min using a specially designed pneumatic piston as described (Winston *et al.* 1989, Beckel et al. 2014). Cells were exposed to 30% hypotonic solution (isotonic solution diluted with dH₂O) for swelling experiments with Brilliant Blue G (BBG, Sigma Corp.), A839977 (Tocris/BioTechne) or BzATP (Sigma Corp.) for 4 hrs at 37°C before RNA was extracted as detailed above. Samples of the extracellular media were taken before and after stretch or BzATP and stored at –80°C. The release of IL-6 from astrocytes was then measured by Rat IL-6 Quantikine® ELISA kit (#R6000B, R&D systems) following manufacturer's instructions, with data acquired using a 96-well plate reader SpectraMax M5 (Molecular Devices).

IL-6 release from isolated retinal ganglion cells

Isolation of retinal ganglion cells was performed using the immunopanning procedure as described (Xia et al. 2012, Zhang *et al.* 2010). Isolated RGCs were seeded onto 0.1% poly-L-lysine (Peptides International) and 1 µg/mL laminin coated coverslips or elastic silicone sheeting in stretch chambers and cultured at 37°C with 5% CO₂. Attached cells were bathed in 750µl of isotonic solution including 100 µM of the ectoATPase inhibitor βγ methylene ATP, and stretched by application of 20 mmHg of pressure resulting in a 4.1% deformation

strain (see (Xia et al. 2012) for detail). Pressure inside the stretch chamber was increased to 20 mmHg for 4 min, returned to 0 mmHg for 1 min and the cycle repeated three times for a total duration of 15 min. Immediately following stretch, a 250 μ L sample of the extracellular solution was collected from the center of the stretch chamber. Stretch did not induce release of lactose dehydrogenase. IL-6 levels were determined with the rat antibody cytokine array following manufacturer's instructions (R&D Systems), as described in detail in a recent publication (Lim *et al.* 2016). In brief, IL-6 levels were quantified by incubating the membrane in Streptavidin-HRP followed by chemiluminescent detection reagents (GE Healthcare). The production of light corresponding to levels of bound cytokine was determined with ImageQuant LAS4000 and the intensity of each spot was measured using ImageQuant TL analysis software (all GE Healthcare). Results represent 4 independent trials of stretch or BzATP experiments, each performed in duplicate.

Data analysis and study design

Data are reported as mean $\pm$ standard error of the mean. Statistical analysis used a 1-way ANOVA with appropriate post-hoc test, or a paired Student's t- test when comparing eyes from the same animal. Results with $p < 0.05$ were considered significant. When data were not distributed normally, analysis on ranks was performed. All statistical analysis was performed using SigmaStat software (Systat Software Inc.). The number of experimental repeats was determined in part by sample size calculations and power analysis. Data within two standard deviations of the mean was included unless accompanied by signs of animal distress or unexpected deviation. Analysis was performed in a masked fashion where appropriate.

Results

Pressure-dependent elevation in message for IL-6

Initial experiments to screen for cytokine pathways activated *in vivo* by transient elevations in IOP were determined using a cytokine PCR Array. IOP in one eye of a rat was raised to 50 mmHg for 4 hrs. Although this is considerably above the baseline IOP levels of 12.8 mmHg in the conscious Sprague-Dawley rat (Cabrera *et al.* 1999), this increase did not prevent blood flow through the retinal vessels. Similar transient rises in IOP have been found to induce minimal permanent damage (Crowston et al. 2015, Abbott et al. 2014, Zhi *et al.* 2012). qPCR analysis indicated no rise in pro-apoptotic genes, although expression of the early stress-response ATF3 was increased, consistent with findings in the hypertonic saline model (Guo *et al.* 2011) (Fig. S1).

To obtain an objective measure of the cytokine response to transient pressure elevation, retinal gene levels were examined 20 hrs after IOP returned to baseline using a cytokine PCR array. Analysis showed 9 genes with a >2 -fold change in expression levels between the pressurized and control rat retina (Fig. 1A, B). *IL-6* showed the greatest rise, with a 29- fold increase. *IL1m*, *IL24*, *Tnf*, *Csf1* and *Lif* were also elevated more than two-fold. Three genes, *Tnfsf11*, *Gdf9* and *Tnfsf4*, were down-regulated more than two-fold.

Given that *IL-6* was the gene altered most using the cytokine gene array, results were confirmed using traditional qPCR. *IL-6* was elevated 16.9 fold in eyes with increased IOP as

compared with contralateral eyes (Fig. 1C). This substantial increase measured using qPCR strongly supported the result from the PCR array suggesting that expression of *IL-6* was increased in retinas exposed to transient elevations in IOP.

Purines and IL-6 expression in vivo

As purinergic signaling has been repeatedly implicated in retinal cells exposed to elevated IOP (Reigada et al. 2008, Sanderson *et al.* 2014), expression of gene *ENTPD1* was examined. *ENTPD1* codes for the ectoATPase NTPDase1, which was previously identified as a possible marker for a sustained rises in extracellular ATP, with increased levels of the gene *ENTPD1* and protein for NTPDase1 triggered by sustained exposure to ATP (Lu et al. 2007). Levels of NTPDase1 were elevated in parallel to extracellular ATP concentrations in rat, mouse and primate models of chronic IOP elevation (Lu et al. 2015). In material from rat retinas obtained both 1 and 5 days after transient IOP elevation, *ENTPD1* was upregulated (Fig. 2A), suggesting levels of extracellular ATP were elevated. after moderate IOP elevation.

A considerable body of past work implicates autostimulation of the P2X7 receptor following the mechanosensitive release of ATP in the retina (Beckel et al. 2014, Zhang *et al.* 2006, Xia et al. 2012, Reigada et al. 2008), and recent work demonstrates P2X7 receptor stimulation leads to IL-3 responses in isolated retinal ganglion cells (Lim et al. 2016). As stimulation of P2X7 receptors by ATP has been associated with the upregulation of IL-6 in microglia cells (Shieh et al. 2014), the role of the P2X7 receptor in mediating the pressure-dependent rise in *IL-6* was examined.

Initial involvement of the P2X7 receptor was determined using antagonist Brilliant Blue G. While BBG can act at other P2X receptors (Bo et al. 2003), it is well tolerated in the eye (Totan *et al.* 2014). In addition, the blue color of the compound enabled the retinal distribution of the antagonist to be more accurately determined (Fig. 2B); material from the targeted retina was preferentially analyzed. The pressure-dependent increase in *IL-6* mRNA was blocked by intravitreal injection of 0.8% BBG 1–3 days before the IOP rise (Fig. 2C). Levels were compared to the rise seen in pressurized eyes injected with only saline, to control for any injection artifact. Immunoblots confirmed that IL-6 protein was also increased in the retina following a rise in pressure (Fig. 2D). Changes in protein level paralleled those of mRNA, with IOP rise leading to an increase in IL-6 protein that was prevented by BBG (Fig. 2E).

To determine whether stimulation of the P2X7 receptor was sufficient to trigger upregulation of *IL-6*, agonist BzATP was injected intravitreally (2 μ l, 250 μ M) with sterile saline injected into the contralateral eye and levels of *IL-6* mRNA present in the retina 24 hrs later were determined. Retinal *IL-6* expression was increased 4-fold by the P2X7 receptor agonist BzATP in the absence of any changes in IOP (Fig. 2F).

Involvement of purines in the response to elevated IOP was further probed by examining expression of certain receptors. Receptor genes *P2RX7* and *ADORA3*, coding for the adenosine A3 receptor, were elevated in many retinas examined after 1 day, but considerable variation meant the rises were not significant (Fig. S2). Genes *P2RX4* and *P2RY6* for

purinergic receptors were increased 1 day, but not 5 days after IOP elevation. While the precise contribution of these receptors remains to be determined, their increased expression is consistent for mechanosensitive purinergic signaling.

Pressure-dependent upregulation of IL-6 absent in P2X7 knockout mice

Further confirmation of the role of the P2X7 receptor in the pressure-dependent rise in *IL-6* was provided with P2X7 knockout mice. Elevating the IOP of wild-type C57Bl6J mouse eyes to 60 mmHg for 4 hrs led to a rise in *IL-6* levels analogous to that observed in the rat eye (Fig. 2G). In mice missing the P2X7 gene, however, this rise in IOP did not significantly change *IL-6* levels (Fig. 2H). This supported the pharmacological identification, while also demonstrating the response occurred in multiple species.

IL-6 upregulation and release from optic nerve head astrocytes

In vitro experiments from isolated cells were pursued to enable identification of specific cell types and better control of pharmacological manipulation. Optic nerve head astrocytes undergo multiple changes in response to the mechanical strain in glaucoma (Hernandez, 2000). As we have previously found that stretch of these astrocytes leads to the release of ATP through pannexin hemichannels and subsequent autostimulation of P2X7 receptors (Beckel et al. 2014), the mechanosensitive response of *IL-6* in these astrocytes and the contribution of the P2X7 receptor was examined.

Isolated rat optic nerve head astrocytes expressed GFAP, confirming the identity of the cultured cells (Fig. 3A). Astrocytes were plated on a silicone substrate and subjected to a 5% equilateral strain at 0.3 Hz for 4 hrs, followed by a 20 hr break before RNA was extracted to increase parallels to *in vivo* experiments. Levels of *IL-6* mRNA were increased 2-fold in stretched astrocytes as compared to controls (Fig. 3B). Unstretched astrocytes exposed to 50 μ M BzATP for 4 hrs also demonstrated a 2-fold rise in *IL-6*, suggesting the P2X7 receptor was sufficient to trigger the rise in *IL-6* mRNA expression (Fig. 3C) as found *in vivo*. An analogous rise in *IL-6* was produced by swelling astrocytes with a 30% hypotonic solution for 4 hrs (Fig. 3D); this rise in *IL-6* mRNA was prevented by P2X7 receptor antagonists BBG and A839977 (Fig. 3D).

To confirm the contribution of the P2X7 receptor, the rise in *IL-6* expression in optic nerve head astrocytes isolated from C57Bl6J mice and P2X7 knockout mice was compared. Swelling cells from wild type mice induced a significant increase in *IL-6* expression (Fig. 3E). In contrast, astrocytes isolated from P2X7^{-/-} mice showed a drop in the *IL-6* expression with swelling.

IL-6 released from optic nerve head astrocytes

While the ability of P2X7 receptors to trigger the upregulation of *IL-6* mRNA *in vivo* and *in vitro* implied an increased involvement of the cytokine, the ability of the receptor to trigger release of IL-6 was also tested. Measurement of IL-6 levels in the bath surrounding the astrocytes using an ELISA assay demonstrated that the cytokine was released into the bath after stretch (Fig. 3F). Exposure of astrocytes to agonist BzATP also led to a substantial release of IL-6 (Fig. 3G). Cytokine release in many cell types is mediated by increases in

intracellular calcium; for example, the release of IL-6 from spinal cord astrocytes is calcium dependent (Codeluppi *et al.* 2014). To confirm optic nerve head astrocytes experience a rise in calcium upon swelling, levels were monitored with indicator Fura-2. The rise in calcium was rapid and reversible (Fig. 3H). To determine whether this response was dependent upon autostimulation of the P2X7 receptor, the ability of BBG to antagonize this rise was examined. Pretreatment of astrocytes with blocker BBG eliminated the rise in calcium, implicating autostimulation of the P2X7 receptor, and consistent with a role for calcium in the release.

IL-6 released from isolated retinal ganglion cells

Although the above experiments clearly indicate that mechanical strain and stimulation of the P2X7 receptor can lead to release of IL-6 from optic nerve head astrocytes, immunostaining indicated that retinal ganglion cells expressed high levels of IL-6 (Fig. 4A). The staining pattern was particulate, consistent with IL-6 stored in vesicles. As such, the ability of retinal ganglion cells to release IL-6 was tested. As ganglion cells *in situ* are intertwined with various other cell types, a two-step immunopanning procedure was used to isolate retinal ganglion cells (Fig. 4B); previous analysis indicates that >98% of cells obtained in this way are ganglion cells (Zhang *et al.* 2006). The purified cells were plated on a silicone substrate and, once attached, a 4.1% deformation strain was applied to stretch the cells for 4 min. Cells were then returned to baseline for 1 min, with the stretch cycle repeated 2 more times. There was a significant increase in extracellular levels of IL-6 released into the bath after this stretch period (Fig. 4C). Analogous trials indicate that stimulation of the P2X7 receptor with BzATP also released IL-6 from isolated retinal ganglion cells (Fig. 4D). Attempts to process RNA from these isolated ganglion cells were unsuccessful, precluding examination of *IL-6* expression. However, application of BzATP led to a rapid increase in intracellular calcium in isolated retinal ganglion cells (Fig. 4E); the response was rapid, reversible and repeatable.

Discussion

The signaling pathways linking mechanical strain to inflammation play an important role in the cellular response to stress. The current study implicates the P2X7 receptor for extracellular ATP in the mechanosensitive upregulation of cytokine IL-6 in the retina. *In vivo* data demonstrate *IL-6* mRNA was substantially upregulated after a transient elevation of IOP in the rat retina, with the P2X7 receptor antagonist BBG preventing the upregulation of both *IL-6* mRNA and IL-6 protein in retinal tissue. The transient rise in IOP increased *IL-6* expression in the retina of wild type mice but not in P2X7 knockout mice, further implicating the P2X7 receptor and demonstrating the effect was not species dependent. In isolated optic nerve head astrocytes, *IL-6* expression was increased by stretch, swelling and directly by the P2X7 agonist BzATP. The swelling- induced rise in *IL-6* in astrocytes was prevented by two different P2X7 antagonists. In addition, both astrocytes and retinal ganglion cell released IL-6 in response to agonist BzATP or to mild stretch. Together these data identify a role for the P2X7 receptor in the mechanosensitive IL-6 response of neurons and astrocytes in the retina.

Signaling pathways linking mechanical strain to IL-6

The intracellular signaling pathways linking mechanical strain to the IL-6 response can be at least partially described by integrating previous findings with the results of the current study (see also the Graphical Abstract). Increased pressure in the whole retina, or mechanical strain to either optic nerve head astrocytes or retinal ganglion cells leads to ATP release through pannexin hemi-channels (Reigada et al. 2008, Xia et al. 2012, Beckel et al. 2014). Release from astrocytes is partially dependent on Rho kinase, consistent with a mechanosensor like TRPV4 as in other ocular cells (Shahidullah et al. 2012, Jo et al. 2015). In both astrocytes and retinal ganglion cells, the released ATP autostimulated P2X7 receptors on the same cell type.

The present study clearly implicates the P2X7 receptor in the IL-6 response to mechanical strain. The P2X7 antagonist BBG prevented the rise in *IL-6* expression *in vivo*, while BBG and a second antagonist A839977 prevented the rise in astrocytes. In addition, agonist BzATP emulated the effects of mechanical strain both *in vivo* and *in vitro*. Although BzATP and BBG can act at other P2 receptors (Bo et al. 2003, Wildman et al. 2003), A839977 is more selective (Honore et al. 2009). In addition, the reduced *IL-6* response in P2X7^{-/-} mice *in vivo*, and in optic nerve head astrocytes isolated from the P2X7^{-/-} mice, implicated the P2X7 receptor in linking the mechanical strain to the *IL-6* response. The retinal response resembles that in cultured microglia, where the P2X7 receptor triggers *IL-6* mRNA upregulation and release of the cytokine (Shieh et al. 2014).

While the use of agonists, antagonists and knockout mice together imply the P2X7 receptor makes a substantial contribution to the mechanosensitive IL-6 response, a contribution from other P2 receptors cannot be ruled out in the present study, and other P2 receptors have been linked to IL-6 (Kawano et al. 2015, Inoue et al. 2007, Shigemoto-Mogami et al. 2001). A study of the same P2X7^{-/-} mice used here found that while most of the peritoneal rise in IL-6 accompanying ATP injection was eliminated in the knockout mice, the residual response may have reflected action of additional receptors, with P2Y receptors suggested as a possible source (Solle et al. 2001). The increased expression of the P2Y6 receptor in retinas exposed to transient pressure elevation in Figure S2 is interesting, but as the agonist for this receptor is UDP, and ATP itself has little affinity, activation of this receptor by ATP released after elevated pressure is likely to be complex (Communi et al. 1996; Satrawaha et al. 2011). It is also not clear whether the response is direct or reflects a secondary response to IL-1 β release, as IL-1 β can lead to upregulation of *IL-6* expression (Cadman et al. 1994). Experiments are currently underway to determine whether stimulation of the P2X7 receptor leads to IL-1 β release.

In addition to the upregulation of *IL-6* message and protein levels, mechanical strain and the P2X7 receptor also triggered a rapid release of IL-6 from astrocytes and retinal ganglion cells. The P2X7 receptor is a ligand gated non-selective cation channel, and its stimulation raises intracellular calcium in both astrocytes and retinal ganglion cells (Xia et al. 2012, Beckel et al. 2014). The vesicular release of IL-6 from spinal cord is calcium dependent (Codeluppi et al. 2014), and the time course of the IL-6 release above implies the signaling mechanisms are distinct from those involved in transcriptional upregulation. While the

increased expression of *IL-6* would provide more IL-6 for release upon later stimulation, this complex positive feedback pathway was not investigated in the present study.

Separating mechanical strain from cell death and the P2X7 receptor

The data here indicate that P2X7 receptor was involved in the increase in *IL-6* after a transient non-ischemic elevation in IOP. We used this model because it was reported to induce little cell death (Morrison et al. 2010, Morrison et al. 2014, Abbott et al. 2014, Crowston et al. 2015), and enabled us to distinguish between responses resulting from mechanical strain and those due to cell death; the lack of response in genes *ANAX3*, *BAX* or *CCND1*, associated with apoptosis or extreme stress, suggest this distinction was largely achieved. In a variant of the rat model in which IOP was raised to 50 mmHg for 8 h, there was no substantial RGC loss or decreases in axon transport (Abbott et al. 2014). Elevation of mouse IOP to 50 mmHg for 30 min led to a transient reduction in the photopic negative response (PhNR), attributed largely to retinal ganglion cell function, although the number of ganglion cells was not reduced when examined 7 days later (Chrysostomou & Crowston 2013, Crowston et al. 2015). Presumably the maintenance of retinal blood flow prevents the retinal ganglion cells loss associated with more ischemic models (Zhi et al. 2012). Overall this suggests that the robust IL-6 response, and the stimulation of the P2X7 receptor which precedes it, are distinct from cell death.

Relevant cell types

Our *in vivo* experiments identified elevated *IL-6* mRNA and IL-6 protein using material from the entire retina. The optic nerve head is the focus of the mechanical forces induced upon elevation of IOP (Downs 2015), and the *in vitro* experiments clearly demonstrate a rise in *IL-6* expression in optic nerve head astrocytes, consistent with previous findings of a large rise in optic nerve head *IL-6* in response to IOP elevation (Johnson et al. 2011). However, the optic nerve head tissue is a minor component of the retina and it is likely that other cell types contribute to the rise in *IL-6* expression found in the whole tissue. While the restricted levels of cell material in panned retinal ganglion cells precluded reliable molecular analysis of *IL-6* levels in this study, the cells are also likely to contribute. The increased expression of *IL-6* 1 day after IOP elevation using the laser photocoagulation model co-localized with amyloid precursor protein, a marker of fast axonal transport, and suggested the axonal transport of IL-6 synthesized in retinal ganglion cells was impeded with increased IOP (Chidlow et al. 2012). This may relate to a more recent study in which IL-6 increased with age in the proximal optic nerve of DBA mice, and correlated with the loss of axonal transport (Wilson *et al.* 2015). The predicted involvement of microglial cells here is complex; cultured retinal microglia released IL-6 when subjected to hydrostatic pressure increase (Sappington et al. 2006), while activated microglial cells were observed *in vivo* only one week after elevation of IOP but not at earlier time points (Kezic *et al.* 2013). Future experiments are needed to understand the role of microglial cells given their responsiveness to extracellular ATP (Franke *et al.* 2007).

Physiological implications

While the results from the present study clearly demonstrate a role for the P2X7 receptor in the upregulation and release of IL-6, the physiological implications will depend upon the

cell types involved, the conditions that lead to the response, and whether the resulting IL-6 mediates protective or detrimental effects. IL-6 signaling is complex; although IL-6 is traditionally described as a “pro-inflammatory” cytokine, it can be both protective and pathological in neural tissues (Spooren *et al.* 2011). Expression of IL-6 in cortical astrocytes confers protection from focal injury in neural tissue (Penkowa *et al.* 2003). In the retina, several groups have identified protective actions by IL-6 and suggested it is an early protective response. The death of retinal ganglion cells following increased hydrostatic pressure was prevented by IL-6 (Sappington *et al.* 2006), and IL-6 enhanced neurogenesis in retinal ganglion cells (Chidlow *et al.* 2012). If IL-6 represents an early response to protect neurons, then the present study suggests that the mechanosensitive release of ATP through pannexin hemichannels and autostimulation of P2X7 receptors that lead to the increased IL-6 response may also be protective, at least in young healthy tissue. This would add to the increasing recognition of the P2X7 receptor as more than just a “death receptor” in neural tissues.

Conclusion

In conclusion, this study demonstrates a role for the P2X7 receptor in linking mechanical strain to upregulation and release of cytokine IL-6 in the retina. Involvement of the P2X7 receptor was demonstrated both *in vivo* and *in vitro* in astrocytes and neurons. As IL-6 has many protective effects in the retina, this study may consequently identify a beneficial role of the P2X7 receptor in neural tissues. Given the emerging relationship between cytokines and mechanical strain in traumatic brain injury (TBI), this study suggests further investigation of the P2X7 receptor in TBI is warranted.

Portions of this work have appeared in abstract form (Lu *et al.* 2013, Lu *et al.* 2011, Lim *et al.* 2011)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Jonathan Huwe for help with qPCR experiments.

List of Abbreviations

ATP	adenosine triphosphate
BBG	Coomassie Brilliant Blue G
BzATP	(4-benzoyl-benzoyl)-ATP
ELISA	enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP1	hypoxanthine phosphoribosyltransferase 1
HRP	horseradish peroxidase
IL-1β	interleukin 1 beta
IL-6	Interleukin 6
IOP	intraocular pressure
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NTPDase1	Ectonucleoside triphosphate diphosphohydrolase-1
PBS	phosphate buffered saline
qPCR	quantitative PCR
RIPA	Radioimmunoprecipitation assay buffer
RGC	retinal ganglion cell
SDS	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
TBI	traumatic brain injury

References

- Abbott CJ, Choe TE, Lusardi TA, Burgoyne CF, Wang L, Fortune B. Evaluation of retinal nerve fiber layer thickness and axonal transport 1 and 2 weeks after 8 hours of acute intraocular pressure elevation in rats. *Invest Ophthalmol Vis Sci*. 2014; 55:674–687. [PubMed: 24398096]
- Beckel JM, Argall AJ, Lim JC, et al. Mechanosensitive release of ATP through pannexin channels and mechanosensitive upregulation of pannexin channels in optic nerve head astrocytes: a mechanism for purinergic involvement in chronic strain. *Glia*. 2014; 62:1486–1501. [PubMed: 24839011]
- Bo X, Jiang LH, Wilson HL, Kim M, Burnstock G, Surprenant A, North RA. Pharmacological and biophysical properties of the human P2X5 receptor. *Mol Pharmacol*. 2003; 63:1407–1416. [PubMed: 12761352]
- Bustamante M, Fernandez-Verdejo R, Jaimovich E, Buvinic S. Electrical stimulation induces IL-6 in skeletal muscle through extracellular ATP by activating Ca(2+) signals and an IL-6 autocrine loop. *Am J Physiol Endocrinol Metab*. 2014; 306:E869–882. [PubMed: 24518675]
- Cabrera CL, Wagner LA, Schork MA, Bohr DF, Cohan BE. Intraocular pressure measurement in the conscious rat. *Acta Ophthalmol Scand*. 1999; 77:33–36. [PubMed: 10071145]
- Cadman ED, Naugles DD, Lee CM. cAMP is not involved in interleukin-1-induced interleukin-6 release from human astrocytoma cells. *Neurosci Lett*. 1994; 178:251–254. [PubMed: 7529912]
- Cepurna WO, Guo Y, Doser TA, Dyck JA, Johnson EC, Morrison JC. An interval of controlled intraocular pressure elevation alters optic nerve head gene expression without compromising retinal perfusion. *Invest Ophthalmol Vis Sci*. 2008; 49 ARVO E-Abstract 3695.
- Chen KH, Wu CC, Roy S, Lee SM, Liu JH. Increased interleukin-6 in aqueous humor of neovascular glaucoma. *Invest Ophthalmol Vis Sci*. 1999; 40:2627–2632. [PubMed: 10509659]

- Chidlow G, Wood JP, Ebner A, Casson RJ. Interleukin-6 is an efficacious marker of axonal transport disruption during experimental glaucoma and stimulates neuritogenesis in cultured retinal ganglion cells. *Neurobiol Dis*. 2012; 48:568–581. [PubMed: 22884876]
- Chrysostomou V, Crowston JG. The photopic negative response of the mouse electroretinogram: reduction by acute elevation of intraocular pressure. *Invest Ophthalmol Vis Sci*. 2013; 54:4691–4697. [PubMed: 23766473]
- Codeluppi S, Fernandez-Zafra T, Sandor K, et al. Interleukin-6 secretion by astrocytes is dynamically regulated by PI3K-mTOR-calcium signaling. *PLoS One*. 2014; 9:e92649. [PubMed: 24667246]
- Communi D, Parmentier M, Boeynaems JM. Cloning, functional expression and tissue distribution of the human P2Y6 receptor. *Biochem Biophys Res Commun*. 1996; 222:303–308. [PubMed: 8670200]
- Corps KN, Roth TL, McGavern DB. Inflammation and neuroprotection in traumatic brain injury. *JAMA Neurol*. 2015; 72:355–362. [PubMed: 25599342]
- Corriden R, Insel PA. Basal release of ATP: an autocrine-paracrine mechanism for cell regulation. *Sci Signal*. 2010; 3:re1. [PubMed: 20068232]
- Crowston JG, Kong YX, Trounce IA, Dang TM, Fahy ET, Bui BV, Morrison JC, Chrysostomou V. An acute intraocular pressure challenge to assess retinal ganglion cell injury and recovery in the mouse. *Exp Eye Res*. 2015; 141:3–8. [PubMed: 25753840]
- Downs JC. Optic nerve head biomechanics in aging and disease. *Exp Eye Res*. 2015; 133:19–29. [PubMed: 25819451]
- Downs JC, Roberts MD, Burgoyne CF. Mechanical environment of the optic nerve head in glaucoma. *Optom Vis Sci*. 2008; 85:425–435. [PubMed: 18521012]
- Erta M, Quintana A, Hidalgo J. Interleukin-6, a major cytokine in the central nervous system. *Int J Biol Sci*. 2012; 8:1254–1266. [PubMed: 23136554]
- Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, Di Virgilio F. The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol*. 2006; 176:3877–3883. [PubMed: 16547218]
- Franceschini A, Capece M, Chiozzi P, Falzoni S, Sanz JM, Sarti AC, Bonora M, Pinton P, Di Virgilio F. The P2X7 receptor directly interacts with the NLRP3 inflammasome scaffold protein. *FASEB J*. 2015; 29:2450–2461. [PubMed: 25690658]
- Franke H, Schepper C, Illes P, Krügel U. Involvement of P2X and P2Y receptors in microglial activation in vivo. *Purinergic Signal*. 2007; 3:435–445. [PubMed: 18404456]
- Gombault A, Baron L, Couillin I. ATP release and purinergic signaling in NLRP3 inflammasome activation. *Front Immunol*. 2012; 3:414. [PubMed: 23316199]
- Guo Y, Johnson EC, Cepurna WO, Dyck JA, Doser T, Morrison JC. Early gene expression changes in the retinal ganglion cell layer of a rat glaucoma model. *Invest Ophthalmol Vis Sci*. 2011; 52:1460–1473. [PubMed: 21051717]
- Guha S, Baltazar GC, Coffey EE, et al. Lysosomal alkalinization, lipid oxidation, impaired autophagy and reduced phagosome clearance triggered by P2X7 receptor activation in retinal pigmented epithelial cells. *FASEB J*. 2013; 27:4500–4509. [PubMed: 23964074]
- Hanley PJ, Musset B, Renigunta V, Limberg SH, Dalpke AH, Sus R, Heeg KM, Preisig-Muller R, Daut J. Extracellular ATP induces oscillations of intracellular Ca²⁺ and membrane potential and promotes transcription of IL-6 in macrophages. *Proc Natl Acad Sci U S A*. 2004; 101:9479–9484. [PubMed: 15194822]
- Hernandez MR. The optic nerve head in glaucoma: role of astrocytes in tissue remodeling. *Prog Retin Eye Res*. 2000; 19:297–321. [PubMed: 10749379]
- Honore P, Donnelly-Roberts D, Namovic M, et al. The antihyperalgesic activity of a selective P2X7 receptor antagonist, A-839977, is lost in IL-1 α knockout mice. *Behav Brain Res*. 2009; 204:77–81. [PubMed: 19464323]
- Hu H, Lu W, Zhang M, et al. Stimulation of the P2X7 receptor kills rat retinal ganglion cells in vivo. *Exp Eye Res*. 2010; 91:425–432. [PubMed: 20599962]
- Iglesias R, Dahl G, Qiu F, Spray DC, Scemes E. Pannexin 1: the molecular substrate of astrocyte “hemichannels”. *J Neurosci*. 2009; 29:7092–7097. [PubMed: 19474335]

- Inoue K, Hosoi J, Denda M. Extracellular ATP has stimulatory effects on the expression and release of IL-6 via purinergic receptors in normal human epidermal keratinocytes. *J Invest Dermatol*. 2007; 127:362–371. [PubMed: 16946718]
- Jo AO, Ryskamp DA, Phuong TT, Verkman AS, Yarishkin O, MacAulay N, Krizaj D. TRPV4 and AQP4 channels synergistically regulate cell volume and calcium homeostasis in retinal Muller glia. *J Neurosci*. 2015; 35:13525–13537. [PubMed: 26424896]
- Johnson EC, Doser TA, Cepurna WO, Dyck JA, Jia L, Guo Y, Lambert WS, Morrison JC. Cell proliferation and interleukin-6-type cytokine signaling are implicated by gene expression responses in early optic nerve head injury in rat glaucoma. *Invest Ophthalmol Vis Sci*. 2011; 52:504–518. [PubMed: 20847120]
- Kawano A, Kadomatsu R, Ono M, Kojima S, Tsukimoto M, Sakamoto H. Autocrine regulation of UVA-induced IL-6 production via release of ATP and activation of P2Y receptors. *PLoS One*. 2015; 10:e0127919. [PubMed: 26030257]
- Kezic JM, Chrysostomou V, Trounce IA, McMenamin PG, Crowston JG. Effect of anterior chamber cannulation and acute IOP elevation on retinal macrophages in the adult mouse. *Invest Ophthalmol Vis Sci*. 2013; 54:3028–3036. [PubMed: 23572110]
- Kumar RG, Diamond ML, Boles JA, Berger RP, Tisherman SA, Kochanek PM, Wagner AK. Acute CSF interleukin-6 trajectories after TBI: associations with neuroinflammation, polytrauma, and outcome. *Brain Behav Immun*. 2015; 45:253–262. [PubMed: 25555531]
- Li A, Zhang X, Zheng D, Ge J, Laties AM, Mitchell CH. Sustained elevation of extracellular ATP in aqueous humor from humans with primary chronic angle-closure glaucoma. *Exp Eye Res*. 2011; 93:528–533. [PubMed: 21745471]
- Lim JC, Lu W, Beckel JM, Macarak EJ, Laties AM, Mitchell CH. Mechanosensitive release of cytokines and chemokines from isolated retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2011; 52:5458. ARVO-E-Abstract.
- Lim JC, Lu W, Beckel JM, Mitchell CH. Neuronal release of cytokine IL-3 triggered by mechanosensitive autostimulation of the P2X7 receptor is neuroprotective. *Front Cell Neurosci*. 2016; 10:270. [PubMed: 27932954]
- Lu W, Beckel J, Lim J, Zode G, Sheffield V, Laties A, Mitchell C. Elevation of IOP triggers responses from cytokines IL-6 and IL-1 β ; involvement of both optic nerve head astrocytes and retinal ganglion cells. *Invest Ophthalmol Vis Sci*. 2013; 54:784. ARVO E-Abstract.
- Lu W, Guo Y, Huwe J, Laties AM, Mitchell CH. Pressure, purines and cytokines; early response genes upregulated by short term IOP elevation. *Invest Ophthalmol Vis Sci*. 2011; 52:5345–5345. ARVO E-Abstract.
- Lu W, Hu H, Sevigny J, et al. Rat, mouse, and primate models of chronic glaucoma show sustained elevation of extracellular ATP and altered purinergic signaling in the posterior eye. *Invest Ophthalmol Vis Sci*. 2015; 56:3075–3083. [PubMed: 26024091]
- Lu W, Reigada D, Sevigny J, Mitchell CH. Stimulation of the P2Y1 receptor up-regulates nucleoside-triphosphate diphosphohydrolase-1 in human retinal pigment epithelial cells. *J Pharmacol Exp Ther*. 2007; 323:157–164. [PubMed: 17626796]
- Morrison JC, Cepurna WO, Doser TA, Dyck JA, Johnson EC. A short interval of controlled elevation of IOP (CEI) reproduces early chronic glaucoma model optic nerve head (ONH) gene expression responses. *Invest Ophthalmol Vis Sci*. 2010; 51:5216. ARVO E-Abstract.
- Morrison JC, Choe TE, Cepurna WO, Johnson EC. Optic nerve head (ONH) gene expression responses to elevated intraocular pressure (IOP), anesthesia and anterior chamber cannulation in the CEI (Controlled Elevation of IOP) model of IOP-induced optic nerve injury. *Invest Ophthalmol Vis Sci*. 2014; 55:2402. ARVO E-Abstract.
- Penkowa M, Giralt M, Lago N, Camats J, Carrasco J, Hernandez J, Molinero A, Campbell IL, Hidalgo J. Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. *Exp Neurol*. 2003; 181:130–148. [PubMed: 12781987]
- Praetorius HA, Leipziger J. ATP release from non-excitable cells. *Purinergic Signal*. 2009; 5:433–446. [PubMed: 19301146]

- Reigada D, Lu W, Zhang M, Mitchell CH. Elevated pressure triggers a physiological release of ATP from the retina: Possible role for pannexin hemichannels. *Neuroscience*. 2008; 157:396–404. [PubMed: 18822352]
- Sanderson J, Dartt DA, Trinkaus-Randall V, et al. Purines in the eye: recent evidence for the physiological and pathological role of purines in the RPE, retinal neurons, astrocytes, Muller cells, lens, trabecular meshwork, cornea and lacrimal gland. *Exp Eye Res*. 2014; 127:270–279. [PubMed: 25151301]
- Sappington RM, Chan M, Calkins DJ. Interleukin-6 protects retinal ganglion cells from pressure-induced death. *Invest Ophthalmol Vis Sci*. 2006; 47:2932–2942. [PubMed: 16799036]
- Satrawaha S, Wongkhantee S, Pavasant P, Sumrejkanchanakij P. Pressure induces interleukin-6 expression via the P2Y6 receptor in human dental pulp cells. *Arch Oral Biol*. 2011; 56:1230–1237. [PubMed: 21641579]
- Shahidullah M, Mandal A, Delamere NA. TRPV4 in porcine lens epithelium regulates hemichannel-mediated ATP release and Na-K-ATPase activity. *Am J Physiol Cell Physiol*. 2012; 302:C1751–1761. [PubMed: 22492652]
- Shieh CH, Heinrich A, Serchov T, van Calker D, Biber K. P2X7-dependent, but differentially regulated release of IL-6, CCL2, and TNF-alpha in cultured mouse microglia. *Glia*. 2014; 62:592–607. [PubMed: 24470356]
- Shigemoto-Mogami Y, Koizumi S, Tsuda M, Ohsawa K, Kohsaka S, Inoue K. Mechanisms underlying extracellular ATP-evoked interleukin-6 release in mouse microglial cell line, MG-5. *J Neurochem*. 2001; 78:1339–1349. [PubMed: 11579142]
- Sigal IA, Ethier CR. Biomechanics of the optic nerve head. *Exp Eye Res*. 2009; 88:799–807. [PubMed: 19217902]
- Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, Griffiths RJ, Gabel CA. Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem*. 2001; 276:125–132. [PubMed: 11016935]
- Spooen A, Kolmus K, Laureys G, Clinckers R, De Keyser J, Haegeman G, Gerlo S. Interleukin-6, a mental cytokine. *Brain Res Rev*. 2011; 67:157–183. [PubMed: 21238488]
- Totan Y, Guler E, Dervisogullari MS. Brilliant Blue G assisted epiretinal membrane surgery. *Sci Rep*. 2014; 4:3956. [PubMed: 24510005]
- Wildman SS, Unwin RJ, King BF. Extended pharmacological profiles of rat P2Y2 and rat P2Y4 receptors and their sensitivity to extracellular H⁺ and Zn²⁺ ions. *Br J Pharmacol*. 2003; 140:1177–1186. [PubMed: 14581177]
- Wilson GN, Inman DM, Dengler Crish CM, Smith MA, Crish SD. Early pro-inflammatory cytokine elevations in the DBA/2J mouse model of glaucoma. *J Neuroinflammation*. 2015; 12:176. [PubMed: 26376776]
- Winston FK, Macarak EJ, Gorfien SF, Thibault LE. A system to reproduce and quantify the biomechanical environment of the cell. *J Appl Physiol*. 1989; 67:397–405. [PubMed: 2668257]
- Xia J, Lim JC, Lu W, Beckel JM, Macarak EJ, Laties AM, Mitchell CH. Neurons respond directly to mechanical deformation with pannexin-mediated ATP release and autostimulation of P2X7 receptors. *J Physiol*. 2012; 590:2285–2304. [PubMed: 22411013]
- Yang SH, Gangidine M, Pritts TA, Goodman MD, Lentsch AB. Interleukin 6 mediates neuroinflammation and motor coordination deficits after mild traumatic brain injury and brief hypoxia in mice. *Shock*. 2013; 40:471–475. [PubMed: 24088994]
- Zenkel M, Lewczuk P, Junemann A, Kruse FE, Naumann GO, Schlotzer-Schrehardt U. Proinflammatory cytokines are involved in the initiation of the abnormal matrix process in pseudoexfoliation syndrome/glaucoma. *Am J Pathol*. 2010; 176:2868–2879. [PubMed: 20395431]
- Zhang M, Hu H, Zhang X, Lu W, Lim J, Eysteinson T, Jacobson KA, Laties AM, Mitchell CH. The A3 adenosine receptor attenuates the calcium rise triggered by NMDA receptors in retinal ganglion cells. *Neurochem Int*. 2010; 56:35–41. [PubMed: 19723551]
- Zhang X, Li A, Ge J, Reigada D, Laties AM, Mitchell CH. Acute increase of intraocular pressure releases ATP into the anterior chamber. *Exp Eye Res*. 2007; 85:637–643. [PubMed: 17822699]

- Zhang X, Zhang M, Laties AM, Mitchell CH. Balance of purines may determine life or death of retinal ganglion cells as A3 adenosine receptors prevent loss following P2X7 receptor stimulation. *J Neurochem.* 2006; 98:566–575. [PubMed: 16805847]
- Zhi Z, Cepurna WO, Johnson EC, Morrison JC, Wang RK. Impact of intraocular pressure on changes of blood flow in the retina, choroid, and optic nerve head in rats investigated by optical microangiography. *Biomed Opt Express.* 2012; 3:2220–2233. [PubMed: 23024915]

Figure 1. Changes in expression of cytokine genes 24 hours after moderate IOP elevation
Changes in cytokine gene expression 24 hrs after IOP was elevated to 50 mm Hg for 4 hrs (20 hrs after pressure returned to baseline). (A). Heat map of fold-changes in gene expression in pressurized eye compared to control. Red indicates upregulation while green symbolizes downregulation (B). Layout of 84 genes shown in heat map and fold changes relative to the control retina. Numbers are mean change in pressurized eye compared to control in 2 pairs. Genes were evaluated based on the criteria of at least a 2 fold up- or down-regulation compared to control. (C). Increased expression of *IL-6* in pressurized retinas confirmed using qPCR. N=9, p<0.001.

Figure 2. Involvement of ATP and P2X7 receptor in *IL-6* elevation *in vivo*

(A). Expression of ectoATPase gene *ENTPD1* was elevated 1 day after increase in IOP to 50 mmHg for 4 hrs (Pressure, * $p=0.033$, $N=10$). *ENTPD1* remained elevated 5 days after the procedure (* $p=0.004$, $N=8$). (B). The distribution of P2X7 antagonist Brilliant Blue G (BBG) in the retina 1 day after intravitreal injection. The staining pattern suggests distribution of BBG through the vitreal cavity to the retina was restricted. A similar staining pattern remained in retinas examined 6 days after injection. (C). The pressure-dependent rise in *IL-6* mRNA was substantially decreased following injection of BBG. Data are expressed as relative gene expression in the pressurized vs non-pressurized retina for eyes injected with 0.8% BBG or saline 1–3 days before the moderate elevation of IOP to 50 mmHg for 4 hrs. $N=6-9$. * $p<0.004$ saline pressurized vs. non-pressurized; * $p<0.013$ saline pressurized vs. BBG pressurized. (D). Representative immunoblots from whole retina lysates probed for *IL-6* (22 kDa) and housekeeping protein GAPDH (GAP, 37 kDa). Expression of *IL-6* is greater in the eye subject to the moderate IOP increase (Pr) treated with saline as compared to the contralateral non-pressurized control eye, but this pressure-dependent increase is reduced after injection with BBG. (E). Summary of relative protein expression from experiments illustrated in C quantified with densitometry; $N=4-5$. * $p<0.001$ saline pressurized vs. non-pressurized; * $p<0.035$ saline pressurized vs. BBG pressurized. (F). P2X7R agonist BzATP was sufficient to increase levels of *IL-6* mRNA in the retina 1 day after intravitreally injection (250 μ M, 2 μ l per eye), $N=5$, * $p=0.021$. (G). In wild type C57Bl6J mice, transient elevation of IOP to 60 mmHg for 4 hrs (Pressure) raised retina

levels of *IL-6* mRNA. N=7, *p<0.001. (H). In P2X7 knockout mice, the same elevation in IOP did not significantly increase levels of *IL-6*. N=6.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. *IL-6* response in astrocytes

(A). Cultured rat optic nerve head astrocytes stained for GFAP (green) and DAPI (blue). Bar=20 μ m. (B). Increased expression of *IL-6* mRNA in stretched astrocytes; cells were subject to a 5% equilateral strain at 0.3 Hz for 4 hrs, followed by a 20 hr break before extraction of RNA. N=8–9, * p =0.011. (C). *IL-6* expression was increased in astrocytes exposed to 50 μ M BzATP for 4 hrs. N=5, * p =0.008. (D). Expression of *IL-6* was also increased in cells exposed to moderate swelling induced by 30% hypotonicity for 4 hrs. However, this rise in expression was inhibited by P2X7R antagonists BBG (50 μ M) or A839977 (A83; 10 μ M). Cells were pretreated with antagonists in isotonic solution for 1 hr. before swelling. N=4 * p <0.001 Swell vs. Control, * p <0.001 Swell vs. swell+BBG,

* $p < 0.001$ Swell vs. swell+A839977. (E). Cell swelling in 30% hypotonic solution induced rise in *IL-6* mRNA was observed in optic nerve head astrocytes from C57Bl6J mice (N=6, * $p = 0.006$), but swelling in astrocytes isolated from P2X7^{-/-} mice actually reduced *IL-6* expression ($p = 0.043$, N=6). (F) The concentration of IL-6 in the bath surrounding astrocytes was higher after exposing cells to stretch (* $p = 0.036$, N=7). (G). Levels of IL-6 in the bath were also increased after exposure of astrocytes to 50 μ M BzATP for 30 min (* $p = 0.011$, N=6, paired t-test for F and G). (H) Swelling of astrocytes by hypotonic solution rapidly raised intracellular calcium, as indicated by the ratio of light excited at 340 nm vs 380 nm in cells loaded with indicator Fura-2. In the presence of 100 μ M BBG, no rise in cell calcium was observed. Symbols represent mean $\pm$ SEM, N=16.

Figure 4. IL-6 release from isolated retinal ganglion cells

(A). Retinal section stained for IL-6 indicating expression of the cytokine in the ganglion cell layer (GCL). IPL: inner plexiform layer, OPL: outer plexiform layer. Bar = 50 μ m. (B). Retinal ganglion cells isolated with the two-step immunopanning procedure. The comparison of the phase contrast image (top) with the fluorescence image (ex 360nm) indicates the cells are labeled with Fluorogold (FG) transported from the superior colliculus, confirming their ganglion cell identity. Cells used for cytokine measurements were unlabeled and plated at a much higher density. (C). Application of a 4.1% deformation strain to stretch isolated RGCs attached to a silicone substrate increased the level of IL-6 detected in the bath. N=8, *p=0.022. (D). Levels of IL-6 in the bath surrounding isolated RGCs increased in cells exposed to 50 μ M of P2X7R agonist BzATP. N=8, *p=0.006 paired t-test for C and D. (E) Application of 50 μ M BzATP for 15 sec led to a repeatable and reversible

rise in intracellular calcium in isolated retinal ganglion cells. Each bar represents the time of BzATP application.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Primers used for quantitative real time PCR

Gene Name	GenBank Accession	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Size(bp)
Anxa3	NM_012823	ATCCGAAAGCAATCAAAGG	CCATGACATGCTCAAAGTGG	174
Bax	NM_017059	TGCCAGCAAACCTGGTGCT	ACCCAACCACCCTGGTCTT	129
Cfos	NM_022197	CCTGTGAGCAGTCAGAGAAGG	CGGAAGAGGTGAGGACTGG	194
CyclinD1	NM_171992	CCCACGATTTTCATCGAACACT	GATCATCCGCAAACATGCA	77
ATF3	NM_012912	CGAAGACTGGAGCAAAATGATG	CAGGTTAGCAAAATCCTCAAACAC	123
IL-6	NM_012589	CTCCGCAAGAGACTTCCAG	GGTCTGTTGTGGGTGGTATC	119
P2X4R	NM_031594	GCAAGACGTTCTTCCACCCTATACA	TCCATACGCTCACAAGTATAAGCC	137
P2X5R	NM_080780	GACATCCAGGAGACACTTAGCTTCG	CAGCAAGAGCTGAAGTGCACAAGTC	230
P2X7R	NM_019256	TAATGCCTCAGCCTAGTGCCTTTGG	CTGCTGCTCCAGAGGGCTCAAGTTC	107
P2Y1R	NM_012800	GCAGCTTCCACTGCCAAAGGCTAAT	ATTGTAAAGCTTCAAGATCTGGCAG	172
P2Y2R	NM_017255	AGCAGCTCAGTCAGGTGTCAGTTCA	TCAGGTGGCGTTGCCTTAGATACGA	214
P2Y4R	NM_031680	ATAGCTGTCTTGATCCAGTGCTCTA	AGCAGCAGGGTTACAATCGATCTCC	215
P2Y6R	NM_057124	TAGGTCCTGGAATAGCACTGCAAAT	AAAGTCTTGGCAAATGGATGGGAAT	171
A1AR	NM_017155	AGCCTGGATGTCTTCTTGTATGGA	TAGACATAGGGACCTCCTTGAGAAC	121
A3AR	NM_012896	GAGCTTCTCTCATTCAATTCTGTGG	CCTAGGGATCCTTCAACGCAGGTTC	183
GAPDH	NM_017008	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC	195