Published in final edited form as:
Curr Eye Res. 2014 February ; 39(2): 105–119. doi:10.3109/02713683.2013.836541.

From Mechanosensitivity to Inflammatory Responses: New Players in the Pathology of Glaucoma

David Križaj[1,2,3], **Daniel A. Ryskamp**[1,3], **Ning Tian**[1,3], **Gülgün Tezel**[4], **Claire H. Mitchell**[5,6], **Vladlen Z. Slepak**[7], and **Valery I. Shestopalov**[8,9,10]

[1]Department of Ophthalmology and Visual Sciences, University of Utah School of Medicine, Salt Lake City, UT, USA

[2]Department of Physiology, University of Utah School of Medicine, Salt Lake City, UT, USA

[3]Program in Neuroscience, University of Utah School of Medicine, Salt Lake City, UT, USA

[4]Department of Ophthalmology & Visual Sciences, University of Louisville, Louisville, KY, USA

[5]Department of Anatomy and Cell Biology, University of Pennsylvania, Philadelphia, PA, USA

[6]Department of Physiology, University of Pennsylvania, Philadelphia, PA, USA

[7]Department of Molecular Pharmacology, University of Miami Miller school of Medicine, Miami, FL, USA

[8]Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami Miller school of Medicine, Miami, FL, USA

[9]Department of Ophthalmology, Cell Biology and Anatomy, University of Miami Miller school of Medicine, Miami, FL, USA

[10]Vavilov Institute for General Genetics, Moscow, Russia

Abstract

Purpose of the study—Many blinding diseases of the inner retina are associated with degeneration and loss of retinal ganglion cells (RGCs). Recent evidence implicates several new signaling mechanisms as causal agents associated with RGC injury and remodeling of the optic nerve head. Ion channels such as Transient receptor potential vanilloid isoform 4 (TRPV4), pannexin-1 (Panx1) and P2X7 receptor are localized to RGCs and act as potential sensors and effectors of mechanical strain, ischemia and inflammatory responses. Under normal conditions, TRPV4 may function as an osmosensor and a polymodal molecular integrator of diverse mechanical and chemical stimuli, whereas P2X7R and Panx1 respond to stretch- and/or swelling-induced adenosine triphosphate release from neurons and glia. Ca^{2+} influx, induced by stimulation of mechanosensitive ion channels in glaucoma, is proposed to influence dendritic and axonal remodeling that may lead to RGC death while (at least initially) sparing other classes of retinal neuron. The secondary phase of the retinal glaucoma response is associated with microglial activation and an inflammatory response involving Toll-like receptors (TLRs), cluster of differentiation 3 (CD3) immune recognition molecules associated with the T-cell antigen receptor, complement molecules and cell type-specific release of neuroactive cytokines such as tumor

Correspondence: David Krizaj, Department of Ophthalmology & Visual Sciences, Moran Eye Center, University of Utah School of Medicine, Salt Lake City, UT 84132, USA. david.krizaj@hsc.utah.edu.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). The retinal response to mechanical stress thus involves a diversity of signaling pathways that sense and transduce mechanical strain and orchestrate both protective and destructive secondary responses.

Conclusions—Mechanistic understanding of the interaction between pressure-dependent and independent pathways is only beginning to emerge. This review focuses on the molecular basis of mechanical strain transduction as a primary mechanism that can damage RGCs. The damage occurs through Ca^{2+}-dependent cellular remodeling and is associated with parallel activation of secondary ischemic and inflammatory signaling pathways. Molecules that mediate these mechanosensory and immune responses represent plausible targets for protecting ganglion cells in glaucoma, optic neuritis and retinal ischemia.

Keywords

ATP; calcium; cytokines; glaucoma; glia; inflammation; mechanosensation; retinal ganglion cells

INTRODUCTION

The spatiotemporal properties of retinal ganglion cell (RGC) action potentials within the axons of the optic nerve represent the entire visual output projected from the eye to the brain. Loss of RGCs therefore culminates in vision loss in debilitating blinding diseases such as ischemia, diabetic retinopathy and glaucoma. [1] While the biological mechanisms that compromise RGC viability in retinal disease are currently under intense experimental scrutiny, potentially useful insights into the disease etiology might be obtained from the observation that RGC degeneration may, at least initially, occur without injury to other classes of retinal neurons.[1,2] Many studies have investigated the anatomical and molecular mechanisms that could account for the selective vulnerability of RGCs in glaucoma and diabetes. It has been suggested that RGCs are uniquely vulnerable to disruptions in energy supply[3] mitochondrial function[4] and axonal transport[5] due to the need to support metabolically expensive long-distance axons. This need is tended through continuous supply of glucose, oxygen and signaling molecules across the blood–retina barrier (BRB), which in turn requires intact function of pericytes, astroglia, microglia and Müller cell endfeet that interface between the vascular endothelium and RGC somata/axons. Astroglia contribute to the metabolic homeostasis of RGCs through glucose/lactate transport mediated by monocarboxylate transporters (MCTs), glucose transporters (SLC2A), glutamate transporters, Gamma-aminobutyric acid (GABA) transporters and the proposed glutamate-glutamine shuttle driven by the bidirectional System N (SN1) transporter.[6–8] While blood vessels and glia maintain the ocular immune privilege by shielding the retina from systemic inflammation, glaucomatous RGC dysfunction might also involve the breakdown of the glial-vascular-immune interface, resulting in increased vascular permeability, hypoxia/ischemia, release of free radicals, cytokines, eicosanoids and growth factors and access of auto-immune molecules.[5,9] Importantly, RGCs are uniquely susceptible to trauma and biomechanical strain, leading to their selective loss in several debilitating blinding diseases.[9,10]

Excessive mechanical stress compromises the viability of many sensory systems, including hearing, somatosensation and vision.[11,12] Glaucoma, the blinding disease most commonly associated with pathological mechanical stress in the eye, is a designation that covers many distinct eye diseases unified by anterior chamber dysfunction, optic neuropathy and glial activation. Its etiology is linked to many known risk factors that include mechanical, genetic (monogenic or polygenic), epigenetic and environmental factors, and possibly combinations thereof.[13] A major risk factor for developing primary open angle glaucoma (which accounts for the majority of glaucoma patients) is ocular hypertension[9,14] caused by increased production or decreased outflow of aqueous humor within the anterior chamber.[15] Positive

correlations between intraocular pressure (IOP) levels and RGC loss, and between duration of elevated IOP and RGC axon loss, have been reported for glaucomatous mice, rats, primates and humans.[9,14,16,17] Currently, pharmacological targeting of increased IOP represents by far the most common clinical treatment of glaucoma. Because the disease is too often identified by the time when axonal atrophy and somatic degeneration reach the irreversible terminal stage, there is an increasing interest in early diagnosis and neuroprotective strategies that will complement IOP reduction within the anterior eye.[18] Both require us to understand the mechanotransduction mechanism at the target (RGCs). As discussed below, application of pressure/stretch triggers influx of calcium ions into RGCs through several classes of putative mechanosensitive ion channels. Given that excessive calcium entry overloads cells with calcium and drives neuronal death in many neurodegenerative diseases in the retina and the brain,[19–21] mechanosensitive channels represent obvious neuroprotection targets in glaucoma.

Although pharmacological targeting of IOP-elevations represents by far the most common clinical treatment of glaucoma, recent studies suggest that the disease also involves pressure-independent mechanisms mediated by vascular, glial and immune cells. Reactive astroglia and microglia appear to regulate RGC survival through parallel and intersecting pathways that encompass elevated levels of the vasoconstrictor endothelin-1, inflammatory chemokines and cytokines (e.g. TNF-α, IL-1β and IL-18), ATP, eicosanoids and/or damage-associated molecular patterns (DAMPs) released by injured and dying RGCs, which in turn activate multiple RGC targets, including TNF-α, TGF-β, IL receptors, the inflammasome and T-cell antigen receptor (TCR)/major histocompatibility complex (MHC) immune complexes.[22–27] Secondary insults, triggered by molecules released from injured RGCs, together with cytokines released from reactive glia and immune molecules arriving from leaky blood vessels may further disrupt the blood retina barrier and facilitate additional infiltration of circulating immune cells,[28,29] thereby fueling RGC damage inflicted by the primary mechanical stress.[22,30,31] Obviously, diagnosis and treatment of glaucoma will need to simultaneously address the primary (pressure-related) and secondary (inflammatory and ischemic) symptoms as well as consider the possibility that glaucomatous injury is exacerbated through feedback interactions between primary and secondary pathways.

The aim of this mini-review is to describe recent developments in glaucoma research, focusing on genetic, physiological and pharmacological studies, many from the authors' laboratories. We introduce molecular mechanisms that underlie intrinsic RGC mechanosensation, showcase the intimate relationship between immune and inflammatory pathways in glaucoma and conclude by identifying a novel retinal immune recognition mechanism that might contribute to glaucomatous remodeling in the inner retina.

INTRAOCULAR PRESSURE AND GLAUCOMA

All cells and organisms live within mechanically active environments in which they must sense and adapt to physical forces such as hydrostatic pressure, osmotic swelling/shrinkage, shear flow and developmentally driven tissue stretch.[32,33] Cells in the eye are additionally exposed to intraocular pressure, the magnitude of which reflects the elasticity of ocular tissues and the balance between production and drainage of aqueous humor within the anterior chamber. Biomechanical strain, exerted by the IOP, was suggested to play a central role in the normal development of the vertebrate retina through scleral expansion and continuous stretching of the eye. Consistent with this view, IOP dissipation blocked ocular expansion even as the neural retina continued to grow.[34,35] Furthermore, Coulombre showed that IOP-deprived chick retinas are forced to increase their thickness, suggesting that mechanical stress is required for proper establishment of retinal circuits. Thus, as observed

in other tissues,[33,36] morphogenesis, migration, adhesion, osmoregulation and contractility of developing ocular cells are likely to be influenced by mechanical forces that include IOP.

Given that vision loss in animal glaucoma models and humans reflects the magnitude and time course of IOP elevations,[9,14,16,17,37] it would appear that the identification of mechanosensitive mechanisms within RGCs, retinal vasculature and glia should represent a priority target in glaucoma research. Potential candidate mechanisms might include pressure- and/or stretch-sensitive ion channels, enzymes, cytoskeletal proteins, extracellular matrix proteins or combinations thereof. Force-induced stretching of focal adhesion junctions could, for example, reveal intracellular binding sites for cytoskeletal proteins and influence activation of mechanosensitive ion channels.[38,39] Another possibility is that the pressure gradient across axons within the optic nerve mechanically and/or biochemically impairs axonal transport between the cell body and midbrain synapses, depriving RGCs of critical "neurotrophic factors".[40–44] The perfusion pressure difference between arm-measured blood pressure and IOP is a strong risk factor for incidence and progression of open angle glaucoma.[18] Variants of the "vascular hypothesis"[45,46] suggest that the primary defect in glaucoma is due to vasoconstriction and insufficient blood supply, caused by compromised arterial flow through the capillaries connected to the peripapillary choroid and the circle of Zinn–Haller. There is, however, little clear evidence that chronic IOP increases observed in most ocular hypertensive patients directly affect ocular blood flow. The biomechanics of this process, especially with respect to the late remodeling of the optic nerve head, has been reviewed elsewhere.[24,47]

The observations that some of the earliest actions of increased IOP target the dendritic field size, the number of synapses as well as light-evoked responses of RGCs[48–53] suggested that the primary retinal pressure sensors may be RGCs themselves. Consistent with this view, pressure-induced reductions in axon thickness and deformation of the optic nerve head in primate glaucoma models appear later than abnormalities in the dendritic arbors.[54] How do RGCs sense mechanical stress? Ocular hypertension could affect the cells through compressive forces (force/cross sectional area) and/or tensile strain (local stretch of the tissue). A major role for compression is doubtful given that the neural retina is entirely enclosed within the eye. However, even in healthy eyes IOP-driven increases in ocular volume could exacerbate tensile forces that impinge on pre-stressed extracellular matrix, cytoskeleton and plasma membrane structures. The pressure–volume relationship described by Friedenwald's ocular rigidity coefficient ("resistance exerted against distending forces") is between 0.0126 mm Hg/μL and 0.0224 mm Hg/ μL.[55,56] According to Pallikaris et al.,[57], 20 mm Hg increase in IOP ought to increase the volume of the human eye by ~30 microliters whereas tonometric measurements from living eyes give a larger volume increment of ~45 microliters,[58] which leads to the prediction that a 20 mm Hg increase in IOP would expand the ocular volume by ~1%. Showing that retinal cells respond to 1% stretch would confirm that they are directly sensitive to the tensile forces driven by IOP changes that are commonly observed in glaucoma. Ocular rigidity is lower in glaucoma compared to healthy subjects.[59] Thus, an increase in IOP will provoke larger tensile stretch forces across membrane/matrix proteins in glaucomatous RGCs compared to healthy cells and should be more efficacious in crossing the thresholds of intrinsic mechanosensitive mechanisms. Mechanical forces and submicrometer displacements generated by IOP elevations are comparable to the measured free energies of known mechanosensitive channels.[60,61]

MECHANOSENSATION, TRPV4 SIGNALING AND RGC NEURODEGENERATION

The long-standing question in glaucoma research has been whether RGCs are themselves capable of transducing mechanical stimuli generated by physiological changes in IOP amplitude. *In vitro*, *in vivo* and preclinical evidence published in recent years shows that RGCs are themselves highly sensitive to mechanical forces.[9,62–67] RGC viability has been shown to be affected by physical compression, tensile stretch, prolonged swelling and IOP elevations, which, in intact preparations, were able to induce changes in the molecular composition and synaptic organization within hours to weeks.[63,68–70]

The recently identified Transient Receptor Potential (TRP) and Piezo channels represent obvious candidates for retinal IOP transducers. While little is known about the Piezo family, the seven subfamilies of the TRP superfamily – so named after their *Drosophila* homolog, which plays a key role in phototransduction – are crucial for the perception of sensory information in vertebrates and invertebrates.[11] Most TRP isoforms are nonselective cation channels that are permeable to Ca^{2+}, therefore their activation serves as suitable trigger for many different types of intracellular signaling events. Members of four TRP subfamilies, specifically of the vanilloid (TRPV), ankyrin (TRPA), polycystin (TRPP), and canonical (TRPC) families are relevant to mechanosensation. These channels are only weakly sensitive to depolarization but open in response to a wide variety of mechanical, osmotic, chemical and thermal stimuli.[22] RGCs express mechanosensitive TRPC1, 3-, 6-, 7- and TRPV1- and 4-channel isoforms.[66,71–73] TRPV4 is a particularly attractive candidate as a glaucoma mechanosensor because, while strongly expressed in RGCs, it is excluded from other types of retinal neuron.[66] Selective TRPV4 agonists, such as 4α-PDD and GSK1016790A, induce calcium influx into RGCs and increase the rate of spontaneous RGC firing, whereas excessive TRPV4 stimulation induces RGC apoptosis but spares other retinal neurons.[66,74] Mechanosensitive TRPV4-mediated responses could account for the increased excitability and reduced RGC survival induced by experimental elevation of IOP or membrane stretch.[66] The precise mechanism through which membrane tension activates RGC TRPV4 channels is unclear. The mutually not incompatible mechanisms include direct activation by lipid stretch,[75] phospholipase A2 or through mechano-chemical feedback involving β1 integrins and/or focal adhesion kinases.[76,77]

It remains to be determined whether excessive calcium influx through TRPV4 channels contributes to calcium dysregulation that has been linked to the pathogenesis of glaucoma in animal studies and clinical trials.[23,78,79] Interestingly, the risk for developing the disease in humans is increased by taking high daily doses of calcium supplements[80] or by not taking calcium channel blockers.[78] At the very least, calcium ions are going to play a central role in cytoskeletal reorganization that underpins dendritic/axonal remodeling in glaucoma. According to the model shown in Figure 1, local Ca^{2+} influx driven by excessive TRPV4 activation contributes to increased baseline $[Ca^{2+}]_i$ levels and RGC hyperexcitability. This leads to activation of Ca^{2+}-dependent genes belonging to NFAT (nuclear factor of activated T-cells), c-fos, DREAM (*DRE antagonistic modulator protein*) and/or CREB (cAMP response element-binding protein) families and triggers Ca^{2+}-dependent catabolic enzymes such as calcineurin and calpains, as well as the cytoskeletal remodeling pathways involving actin and/or microtubular assemblies.[8,23,81–86] Calcium levels also modulate the opening probability of purinergic channels and pannexin hemichannels and could affect their responsiveness to mechanical stimuli. Consistent with this, TRPV4-mediated Ca^{2+} entry was shown to regulate ATP release, which, *via* P2X7 receptors, could exacerbate mechanically-induced cell injury and facilitate release cytoactive molecules such as endothelin and/or TNF-α.[87,88]

The emergence of new models of mechanical gating[33,89] suggests that force transduction cannot be disentangled from intracellular biochemistry. Hence, while the direct mechanosensory function of TRPV4 figures most prominently, the polymodal features of TRPV4 activation, such as sensitivity to swelling and inflammatory agents[75,76] place the channel squarely within the crossroads of mechanosensing, inflammatory signaling and anatomical remodeling. Inflammatory mediators would exacerbate RGC injury, induced by mechanically generated TRP-mediated Ca^{2+} overload (Figures 1 and 2). According to this view, TRPV4 signaling represents an epicenter that links primary, pressure-induced RGC damage to secondary pathophysiological mechanisms mediated by glialvascular inputs (delineated below). Pannexin channels link the mechanosensitive release of ATP to P2X7 receptor-mediated death of RGCs.

MECHANOSENSITIVE RELEASE OF ATP VIA PANNEXINS AND AUTOSTIMULATION OF P2X7R RECEPTORS ON RGCs

In the eye, the mechanical strains resulting from increased IOP are also associated with ATP release. This ATP can mediate physiological and pathological responses through binding to purinergic P2 receptors, the ligand-gated ion channels activated by ATP.[90–92] Several isoforms of ionotropic P2X receptors, including P2X3-5 and P2X7 were reported to be expressed in RGCs.[93] Increased concentrations of extracellular ATP are present in the aqueous humor of human patients with acute[94] or chronic[95] glaucoma. Extracellular ATP is also elevated in the retina following acute elevations in IOP from rat and bovine retina,[68,96] and preliminary data suggest a prolonged increase in retinal ATP occurs in primate and rat models of chronic glaucoma.[97,98] Because extracellular ATP is rapidly degraded in the central nervous system (CNS) by ecto-ATPases, such sustained increase is the evidence of a prolonged release from stressed neural cells. While the ATP released in response to mechanical strain can act at multiple receptors, the P2X7 receptor is of particular interest given its ability to initiate both inflammatory responses and neuronal death.[99,100] Since RGCs express P2X7Rs, stimulation with its agonist, 2′(3′)-O-(4-Benzoylbenzoyl) adenosine-5′-triphosphate (BzATP), elevates intracellular Ca^{2+} and kills RGCS *in vitro*.[101] BzATP also kills RGCs *in vivo*; this death is inhibited by P2X7R blockers MRS 2540 and Brilliant blue G.[102] This suggests that the mechanosensitive release of ATP accompanying elevation of IOP may influence ganglion cell health in acute and chronic glaucoma.[103,104]

Given the pathological effects of excess extracellular ATP on ganglion cells, the cellular source of this released ATP and the signaling pathways leading to this release are of interest. Although Müller cells release ATP into the region surrounding RGCs upon mechanical stimulation, its rapid dephosphorylation into adenosine may limit the concentrations reaching RGC membranes.[105,106] In a healthy retina with little mechanically-evoked ATP release, ATP dephosphorylation regulates P2X7 receptor activity, because P2X7R requires relatively high concentrations of ATP for activation.[107] In disease, sufficient ATP to activate the P2X7 receptors may come from the efflux of ATP through channels in close proximity to the P2X7 receptor on the membrane, with local concentrations high enough to autostimulate the receptors. The pannexin-1 (Panx1) channel, which can be recruited and directly activated by P2X7R, has been recently implicated in this role.[67,108–110]

Pannexins are membrane channels with a high single channel conductance of 500 pS that are permeable to molecules over 1 kDa.[111] Unlike connexin gap junction proteins, pannexin channels are not coupled to partners in adjacent cells but instead act as pores connecting the cell interior to extracellular space; opening of the pore is tightly regulated to maintain cellular integrity.[109] Pannexins are widely distributed and have important implications for inflammation, as discussed below. However, pannexins have two characteristics essential to the current context; they are highly permeable to ATP and open upon application of

mechanical strain to the membrane.[67] Whether pannexins are themselves the primary mechanosensor or activated by other upstream mechanosensitive sensors through Rho kinase,[112] their ability to release ATP in proximity to P2X7 receptors upon stretch of the membrane implicates them in connecting elevated IOP with activation of the p2X7 receptors.

This pannexin/P2X7R system was recently found to translate mechanical strain into receptor activation in ganglion cells.[113] RGCs strained by stretching on a silicone substrate, or swollen with hypotonic solution, released ATP. This release was inhibited by pannexin blockers carbenoxolone, probenecid and inhibitory peptide, [10] Panx, implicating the pannexin channel in the efflux. Importantly, this mechanosensitive release came from isolated immunopanned cells, identifying RGCs themselves as a cellular source of releasable ATP. Whole cell ion currents activated by swelling were reduced by pannexin channel blockers by removal of extracellular ATP with apyrase or by P2X7R blockers A438079, AZ10606120 and zinc. Together, these observations strongly support a model whereby the mechanosensitive release of ATP through pannexin channels on RGCs autostimulates P2X7 receptors on the cells.

The consequences of this mechanosensitive auto-stimulation are likely to be more complex than originally thought. Although stimulation of the P2X7R is widely associated with cell death, the expression of pannexins[114] and P2X7 receptors[115,116] in healthy adult RGCs suggests that death is not a necessarily consequence of receptor stimulation. However, the massive ATP release that accompanies excessive mechanical strain may push the system into a pathological state. The location of the pannexin/P2X7 receptors may also influence the function of this mechanosensitive ATP release and autostimulation. According to immunohistochemical analysis, Panx1 and P2X7 proteins are expressed on both the soma and neurites of isolated ganglion cells.[113] As much of the mechanical strain in glaucoma occurs at or near the optic nerve head,[117] expression of this mechanosensitive signaling pair along neurites suggests the system may translate strain in the optic nerve head to local damage in ganglion cell axons.

THE ROLE OF PANNEXIN1-ACTIVATED PATHWAYS IN RGC INJURY

The Panx1 protein forms large non-selective membrane channels and is implicated in paracrine signaling and regulation of the inflammasome. Compared to connexin hemichannels, Panx1 channels are less sensitive to extracellular Ca^{2+}, and open when intra-cellular Ca^{2+} increases, suggesting them to serve as an additional pathway for Ca^{2+} influx across membrane in pathological conditions.[118–120] Importantly, Panx1 membrane channels have superior permeability to ATP, which prompted referring to them as “the ATP channels” suitable for paracrine signaling in astrocytes, neurons and other cell types.[88] As described above, pannexins are implicated in massive ATP release by glial and blood endothelial cells is typically observed in various pathologies.[89–92] Moreover, Panx1 channels were shown to regulate the inflammasome.[121,122] The Panx1-mediated pathway activates faster in pathogenic conditions, i.e. after stress, injury and cytokine exposure, when cells were shown to decrease the number of gap junctions in the plasma membrane in favor of hemichannels made of either connexins or pannexins.[123–125] Mechanistically, the abnormal opening of Panx1 channels is facilitated by a combination of pathogenic stimuli typically released after stress or injuries, including mechanical stress, extracellular K+, ATP, glutamate, cytokines and Zn^{2+} and proteolysis by active caspase-3.[109,126–129]

Recent studies utilizing gene knockdown and knockout mouse models suggested that Panx1 plays a key role in ischemic death of multiple types of neurons.[130,131] Several reports showed that over-stimulation of the Panx1 channels facilitate neuronal loss in models of

stroke, glaucoma, retinal ischemia, spreading depression and enteric colitis.[99,130–135] Because RGCs express high levels of Panx1 and are extremely susceptible to ischemic injury, we tested the hypothesis that activation of Panx1 directly facilitates rapid and selective loss of RGC neurons in ischemia. Our data generated using the Panx1 knockout mice, which are significantly protected from ischemic injury, showed that two distinct neurotoxic processes are mediated by these channels in ischemic conditions.[130]

As revealed by dye transfer and calcium imaging assays, one mechanism involves permeation of RGC plasma membranes. This causes an imbalance of small molecules, and, in particular, an influx of Ca^{2+} and the efflux of ATP.[130,132,136] Ca^{2+} overload, which activates Ca^{2+}-dependent proteases and facilitates apoptosis, can occur directly *via* Ca^{2+}-insensitive Panx1 channels (Figures 1 and 2). In addition, an opening of Panx1 channels can occur *via* the P2X7R-dependent mechanism in response to several external and internal stimuli of physiological or pathological nature, which prompted researchers to name P2X7R-Panx1 a “death complex”.[99,120,137–139] It is plausible that prolonged opening of Panx1 can be triggered by a combination of pathological factors such as increases in extracellular concentration of known agonists including ATP, K^+, Zn^{2+}, glutamate and pro-inflammatory cytokines. [67,109,124,128,140] Such a combination is common in retinal or brain ischemia (stroke) and other CNS injuries.[99,130,131]

The second process that is interrupted in the Panx1 knockout is the activation of caspase-1 and inflammasome-mediated production of IL-1β and IL-18.[84–86] The inflammasome is a macromolecular complex, first characterized in macrophages[108,141] and, more recently, in glia and neurons.[122] The well-known pathway for transcriptional activation of IL-1β and IL-18 genes involves stimulation of TNF and Toll-like receptors (TLRs),[28,142–146] which causes the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)-dependent gene transcription (Figures 2 and 3). Thus, production of these ILs depends on two independent pathways: (1) transcriptional activation *via* the NF-κB pathway and (2) proteolytic processing of IL precursors by the inflammasome.

How does Panx1 contribute to inflammasome activation? One feasible mechanism is a direct interaction with the inflammasome complex that facilitates proteolytic processing of caspase-1 and results in Il-1β release, as reported by Pelegrin and Surprenant.[141] The second mechanism of Panx1-mediated inflammasome activation involves transcriptional activation of IL-1β, since the large pore of the Panx1 channel can provide a gateway for the entry of pro-inflammatory molecules into the cell, leading to stimulation of intracellular membrane receptors such as TLR3.[86,108,141] TLR3, as well as surface receptors TLR4 and TNFR, cause transcriptional activation of NF-κB (Figure 2) and have been recently implicated in several neurodegenerations, including glaucoma.[147–149] In a similar fashion, the activation Panx1 and, subsequently, the inflammasome can be triggered *via* stimulation of P2X7 receptors by extracellular ATP.[150,151] It was demonstrated that Panx1 is essential for P2X7R-induced proteolytic cleavage of caspase-1 and subsequent IL-1β maturation/release, which can be blocked by pharmacological blockade of the Panx1 channels with small interfering RNA, mimetic peptide or carbenoxolone.[108,121,151,152] Likewise, genetic ablation of Panx1 resulted in a robust neuroprotection in mouse models of enteric colitis and traumatic brain injury.[39,43] Importantly, a study of neuron-specific Panx1 knockout mice demonstrated that Panx1-mediated neurotoxicity is facilitated by the endogenous, neuronal inflammasome.[84,122] Neuronal types expressing high levels of Panx1, such as RGCs, are vulnerable to Panx1-mediated death in response to certain pathological and pro-inflammatory stimuli. Opening of Panx1 channels was shown to be independent of TLR activation.[86] Combined with our own results,[130] this finding allowed us to propose a model where Panx1 acts in parallel, not downstream of TLRs. This model implies synergy between the MyD88-NF-κB pathway and Panx1-mediated processes for IL-1β processing and

secretion. Indeed, cytokine maturation appears to be a crucial step in the neurotoxic pro-inflammatory program that is activated in injured CNS *via* the MyD88-NF-κB pathway.[122,130,153] Consistent with our hypothesis, the extent of neuroprotection in Panx1 knockout mice is similar to that observed in the knockouts of caspase-1, P2X7 receptor, TNF receptors 1/2, TLR3/4 and conditional knockouts of NF-κB.[130,142,154–158] In a similar fashion, pharmacological blockade of P2X7R, NALP1/ 3 or ASC subunits of inflammasome showed robust neuroprotection in various CNS injuries,[84,85,151,159] a strong evidence for neurotoxic effects of inflammasome activation.

TNFR and TLR Signaling Promote Inflammation through NF-κB, Driving RGC Degeneration

Increased glial production of TNF-α in the glaucomatous human retina and optic nerve has been implicated in RGC death and inflammatory processes through the TNFR signaling.[28,160–162] High-throughput characterization of the retinal proteome has recently indicated a prominent up-regulation of TNFR-mediated apoptosis pathway and inflammation signaling in human glaucoma.[161] Retinal proteins exhibiting increased expression in glaucoma have included TNF-α, TNFR1 and various downstream adaptor/ interacting proteins, such as TNFR1-associated death domain protein (TRADD) and the members of the TNFR-associated factor (TRAF) family, and protein kinases involved in TNF-α/TNFR1 signaling. Proteomics data support that a complex crosstalk relationship between multiple signaling pathways determines diverse bioactivities of TNF-α.[28] Besides the proteolytic caspase cascade, co-activation of calpain-mediated pathways, mitochondrial dysfunction and endoplasmic reticulum stress may reinforce each other during RGC apoptosis in glaucoma. Regarding TNFR-mediated inflammation signaling, proteomics analysis of the glaucomatous human retina has produced data supporting NF-κB activation, JAK/STAT signaling and inflammasome assembly.[161] Proteomics analysis of RGC and astrocyte samples has also showed cell-specific regulation of TNF-α signaling in experimental glaucoma, such as caspase activation leading to apoptosis in RGCs, but NF-κB activation promoting cell survival and inflammation in astrocytes.[162] In addition, the type of receptor preferentially used is important in determining the outcomes of TNF-α signaling. This multifunctional cytokine can bind two different receptors of the TNFR superfamily, TNFR1 (p55) and TNFR2 (p75). TNFR1 appears to be the primary receptor for both neurodegenerative and inflammatory consequences of TNF-α signaling in glaucoma.[161] This is because a death domain present on the intracellular region of TNFR1, but not present in TNFR2, leads to apoptotic cell death, while signaling through TNFR2 leads primarily to cell proliferation. TNFR1 is also the primary signaling receptor responsible for the majority of TNF-α-mediated inflammatory responses, particularly those mediated by the soluble TNF-α required for inflammation.[28,163]

The glaucomatous human retina also exhibits up-regulation of TLR signaling[149] (Figures 2 and 3). Innate immune activity in the CNS can be triggered by numerous pathways after recognition of invading pathogens or tissue stress/injury by pattern recognition receptors,[164] which include TLRs and nucleotide-binding oligomerization domain-like receptors (NLRs). Although TLRs are membrane-spanning receptors, NLRs are cytoplasmic sensors that form a platform for the assembly of the inflammasome, a multiprotein complex that processes pro-ILs into their mature forms *via* proteolytic cleavage by caspase-1,[165] as is evident in glaucoma.[161,162] TLRs recognize a wide variety of pathogen-associated molecular patterns and also the DAMPs expressed during tissue stress or injury.[166] Recent proteomics studies of human glaucoma and animal models have revealed that glial cells, including both microglia and astroglia, are the main cell types that express a repertoire of TLRs, as well as several inflammasome-related molecules.[149,162] In addition, there is *in vitro* evidence indicating that glaucomatous stress-related intrinsic ligands, such as heat shock proteins (HSPs) and oxidation products, can activate glial TLRs and stimulate T cells.[149] After

recognizing specific molecular patterns, TLRs recruit adaptor proteins, such as MyD88, and activate NF-κB (Figure 2), a major transcription factor for the expression of pro-inflammatory cytokines.[167] Proteomic data from human glaucoma and animal models, along with the findings of *in vitro* treatment experiments, support the notion that the glial TLR signaling initiated by glaucomatous stress-related ligands includes MyD88-dependent pathways.[149]

NF-κB activation after binding TNFRs and TLRs triggers the transcriptional activation of pro-ILs that are processed into their active forms by the inflammasome.[165] Glaucomatous retinal proteome exhibits increased glial expression of specific kinases involved in the NF-κB activation pathway, such as receptor-interacting serine-threonine kinase (RIPK), NF-κB-inducing kinase (NIK), and inhibitory kappa B (IκB) kinases (IκKs), including a master regulator, IκKgamma (NF-κB essential modulator), and phosphorylation of NF-κB subunits, NF-κB1-p105/p50 and p65.[161,162] Although NF-κB regulates neuronal survival programs, including in the retina and optic nerve,[168] this transcription factor is a master regulator of the inflammatory responses leading to secondary neurodegenerative processes.[167,169] The NF-κB pathway may similarly play a major role in regulation of glia-driven pro-inflammatory processes during glaucomatous neurodegeneration.[162] As implicated in other neurodegenerative diseases,[170] TNF-α/TNFR signaling, TLR signaling and the inflammasome together exhibit the potent inflammatory capacity with beneficial and detrimental outcomes in glaucoma. NF-κB, as being a common player of inflammation through TNFR or TLR signaling, appears to be a promising treatment target to provide immunomodulation in glaucoma[171] and deserves further studies.

Function and Possible Mechanisms of Activation of Immune Molecules in the Retina

Recent studies demonstrated that genes typically associated with the immune system, such as those in the MHC and complements, are expressed by neurons in various regions of the CNS, including retina, and may play important roles in synapse formation during normal development and pathogenesis in CNS diseases.[25,172–177] Consistent with this notion, genetic deletion or mutation of a number of MHC class I genes (such as a MHCI co-subunit β2-microglobulin or a key component of MHCI receptor complex CD3ζ), complements or complement receptors result in the failure of development of the eye-specific segregation of RGC axonal projections to the dLGN.[25,174,178,179] On the other hand, over-expression of MHCI molecules caused effects on the retinogeniculate projections opposite to that of MHCI or complement mutations.[180] Furthermore, the expression of complements is up-regulated in glaucomatous retinas,[181] and over-expression of MHCI molecules significantly enhanced the recovery of locomotor abilities after spinal cord injury.[181]

The precise molecular mechanisms of how MHCI molecules and complement cascade expressed by CNS neurons are activated, and how they regulate the normal development and pathogenesis of CNS diseases are unclear. It was suggested that the expression and activity of MHCI molecules and complement cascade are regulated by neuronal activity. Consistently, retinal activity was found to regulate the level of mRNA of MHCI molecules in the dorsal lateral geniculate nucleus (dLGN).[173] In retina, the effects of CD3ζ seem to be cell-type and neurotransmitter-specific. Xu et al.,[25] reported that CD3ζ is specifically expressed by RGCs and mice with genetic mutation of CD3ζ exhibited a selective reduction of glutamate receptor-mediated synaptic transmission of RGCs. It was also postulated that activation of immune molecules in neurons could produce similar intracellular signals as those generated in immune cells but with different ultimate effects, such as altering synaptic development, strength, neuronal morphology or circuit properties downstream of synaptic activity[25,182,183] (Figure 3). In the immune system, activation of CD3ζ triggers several downstream cascades, including a Ras-MAPK pathway and actin-based cytoskeleton

reorganization, which regulates immune cell polarization, migration and dendritic growth.[184–186] It has been shown that most components of these cascades are expressed in the CNS and implicated in activity-dependent synaptic plasticity.[182,187] In addition, direct activation of CD3ζ on hippocampal neurons affects cell morphology by promoting dendritic pruning through a tyrosine-based phosphorylation signaling motif common to the immune system.[172] Furthermore, neuronal activity in the retina is also suggested to regulate the complement-dependent activation of the resident immune cells in retina, microglia, which in turn regulates the developmental remodeling of RGC axonal projection during normal development through a process similar to "phagocytosis".[178] Recent studies also implied that retinal microglia might play an important role in RGC death in glaucomatous retinal degeneration.[188–190] These observations strongly support the possibility that the immune molecules and cells could regulate the neuronal structure and function through mechanisms similar to those in the immune system.

CONCLUDING REMARKS

Analysis of signaling pathways associated with RGC injury points at intracellular involvement of ubiquitous messenger molecules such as Ca^{2+} and ATP, which could participate in intrinsic mechanosensation and drive feedback pathways associated with secondary glial and vascular mechanisms. Because $[Ca^{2+}]_i$ maintenance is critically important for the regulation of excitability, cytoskeletal integrity, metabolism and synaptic function, disruption of Ca^{2+} homeostasis would ultimately lead to anatomical and physiological remodeling observed in glaucoma. Recent evidence suggests that such Ca^{2+} overloads in RGCs could be mediated by TRPV4, P2X7R and/or pannexin channels. The effect of mechanical stress on resident mechanosensitive channels has to be placed within the larger context encompassing secondary inflammatory and immune responses driven by feedback loops between injured RGCs, astrocytes, microglia and the vascular endothelium. Inflammatory cytokines and complement molecules released from glial and endothelial cells could drive the plasma membrane P2X7R-Panx1 complex as well as complex arrays of immune/ inflammatory signaling molecules that might include TNF-α, IL-1β, Toll-like and T-cell receptors. The ensuing reconfiguration of intracellular signals is proposed to involve the NF-κB pathway and activation of the inflammasome complex.

Current glaucoma treatments are limited to minimization of mechanical impact mediated by elevated IOP and lack tools that would protect RGCs by targeting the mechanosensing mechanisms and/or secondary inflammatory/immune pathways within the retina. Thus, development of novel neuroprotective treatments depends on our ability to characterize the force transduction mechanisms that mediate retinal IOP sensing (TRPV4, Panx1 and P2X7R discussed here) together with the role of secondary interactions between RGCs and the surrounding vascular endothelial cells, pericytes, astrocytes, Müller cells and microglia.

Acknowledgments

Supported by NIH grants T32DC008553 (DAR), EY14232, EY021517 (VIS), EY013434, EY015537 (CHM), EY13870, EY022076 (DK), EY018666 and GM060019 (VZS), EY013813 and EY017131 (GT), Center Grants P30 EY014801 (VIS) and P30 EY14800 (DK), Department of Defense (VIS, DK), Russian Federal Special Program Grant 2012-1.5-12-000-1002-018 (V.I.S.), University of Utah Translational Seed grant (DK), State of Utah TCIP (DK), Foundation Fighting Blindness (DK) and unrestricted grants from RPB to Bascom Palmer Eye Institute (Miami) and Moran Eye Institute (Salt Lake City). The authors would like to thank Dr. Harilaos Ginis (University of Crete, Greece) for helpful comments.

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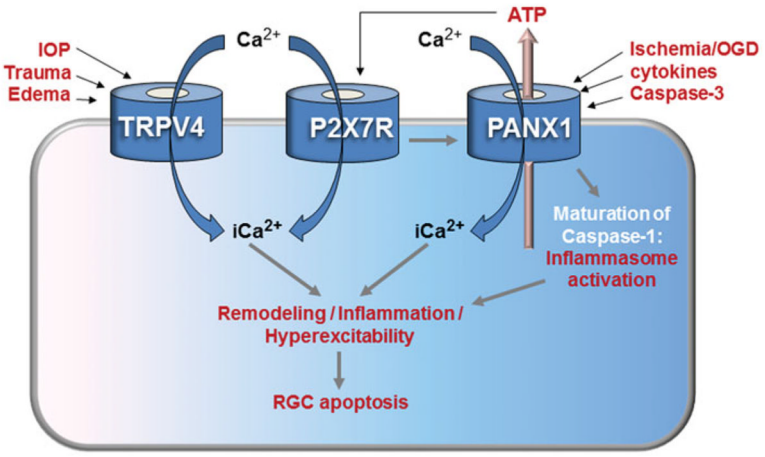

FIGURE 1.

Proposed model for RGC mechanotransduction. Pressure-induced membrane stretch activates plasma membrane TRPV4 channels leading to calcium entry, activation of Panx1 and ATP release. This leads to secondary activation of P2X channels and P2Y receptors on neurons and glial cells. Calcium dysregulation may then trigger dendritic and axonal remodeling, inflammation, glial reactivity, RGC hyperexcitability, and eventually, apoptosis.

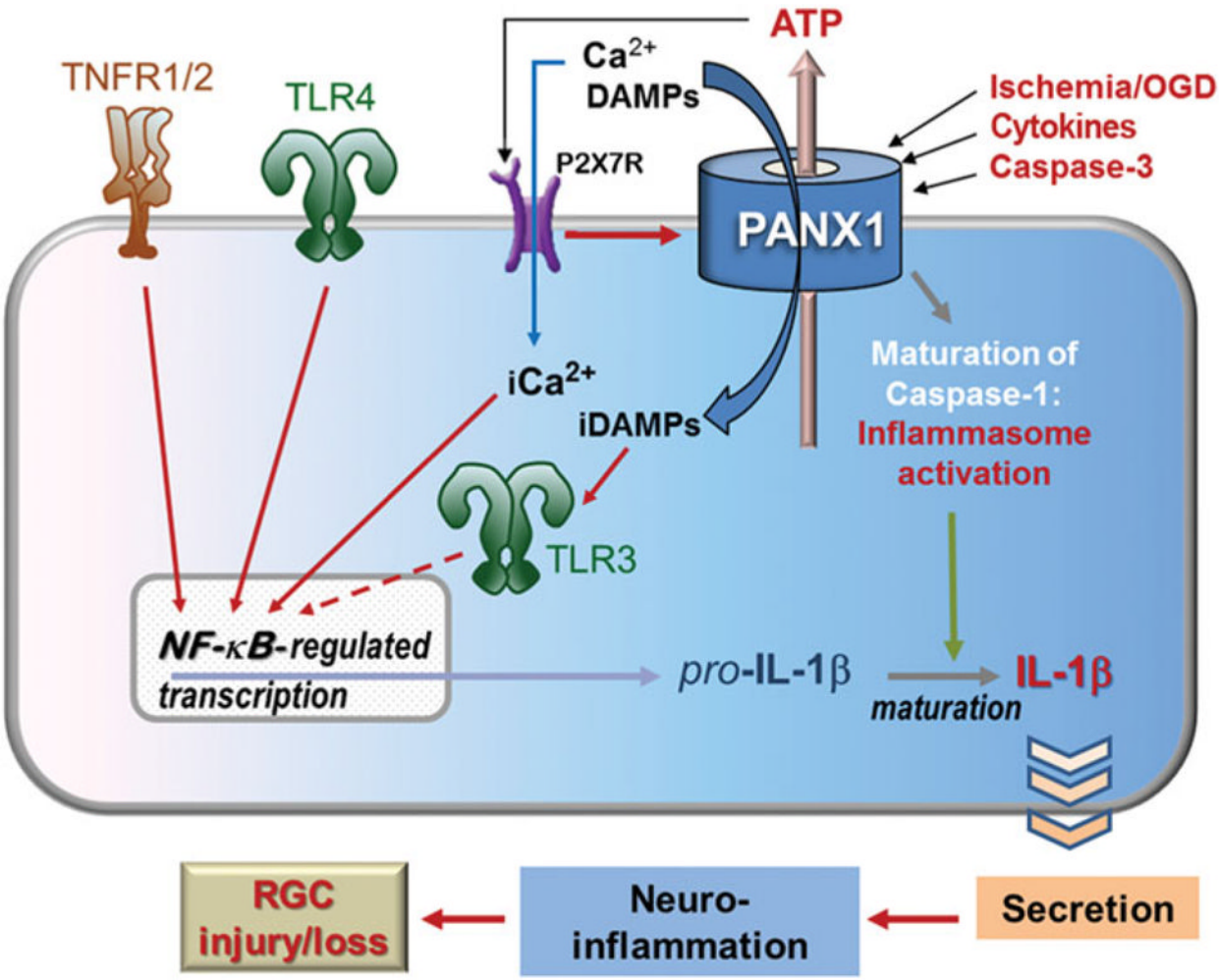

FIGURE 2.

Schematic diagram of signaling mediated by surface receptors and Panx1 in the injured retina. Although signaling by TNFRs, TLR4 (signals through MyD88 and/or TRIF) and Ca^{2+} can feed directly into NF-κB activation, TLR3 signaling *via* TRIF first results in the transcription of type 1 interferon genes. These can also promote NF-κB-regulated transcription (dashed arrow). Ultimately, the maturation of pro-IL-1β into IL-1β ***via*** inflammasome pathways perpeturates inflammation and worsens the glaucomatous damage.

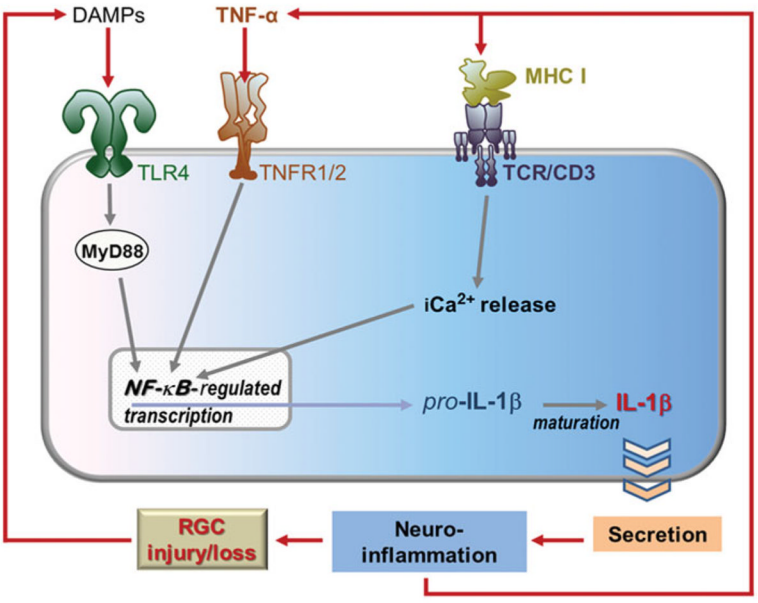

FIGURE 3.

Retinal signaling and cellular remodeling mediated by TLR, TNF and immune molecules involves neuronal-glial circuits. The activation of TLR4, TNFRs and TCRs can promote NF-κB-regulated transcription, generating pro-IL-1β, which is then processed into IL-1β and secreted. This drives glial reactivity and furthers inflammation (e.g. the release of more TNF-α), thus perpetuating the inflammatory cycle. As RGCs are damaged and killed, they may release DAMPS (e.g. certain HSPs) that activate innate immune receptors such as TLR4 and thereby worsen glaucoma. The activation of TCR/CD3 may be involved in glaucomatous dendritic remodeling, which may disturb normal circuit functions and degrade the fidelity of visual processing.