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P2x7 Receptor Stimulation And Elevated Intraocular Pressure Rapidly Alter Microglia Morphology, State Markers, And Cytokine Release

Keith Campagno
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Abstract

The P2X7 receptor (P2X7R) is an ionotropic receptor that responds to high concentrations of extracellular adenosine triphosphate (ATP) leading to NF-κB-mediated inflammatory responses. P2X7 receptors are important regulators of microglial cells, the innate immune effector cells of the central nervous system (CNS). P2X7R stimulation and microglial morphological and molecular changes have been implicated in loss of neurons accompanying elevation of intraocular pressure (IOP) and associated ATP release. However, the mechanisms linking these processes remain unclear. Recent developments implicating astrocyte inflammation as the intermediaries between microglia inflammation and neural loss further complicate the relationship. We sought to interrogate the consequences of P2X7R stimulation to microglia morphology, the molecular alterations to microglia and astrocytes, in the retina, and the P2X7R role in loss of neurons from transient elevation of IOP. We administered P2X7R agonist BzATP to mice retinae to examine microglia morphological differences and inflammatory gene expression differences. This work was supported with in vitro exposure of cultured microglia to P2X7R stimulation. Finally, we used a model of elevated IOP to determine the effects on microglia and astrocytes. We found that retinal exposure to BzATP led to process retraction and elevation of Iba1 in microglia, as well as elevation of both pro-inflammatory (Tnfa, Nos2) and anti-inflammatory (Arg1, Chil3) markers. Transient elevation of IOP induced similar microglial process retraction and retinal gene expression, and these morphological and molecular differences were dampened in retinae derived from P2X7-/- mice. BzATP-injected retinae upregulated genes associated with induction of astrocyte neurotoxic inflammation. Finally, loss of neural populations in retinae subjected to elevated IOP was reduced in retinae from P2X7-/- mice. Furthermore, as P2X7R affects multiple metabolic processes, we sought to review literature to examine the intersection between microglial P2X7R stimulation and microglia phagocytosis or autophagy. We outlined P2X7R-mediated reduction of phagocytosis, transient induction of autophagy, and disruption of microglia lysosomes with longer exposure, and demonstrated a role for lysosomal leakage in cytokine release. These studies highlight the diverse consequences of microglial P2X7R stimulation, and indicate that stimulation plays a key role in retinal degeneration accompanying elevated IOP.

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P2X7 RECEPTOR STIMULATION AND ELEVATED INTRAOCULAR PRESSURE RAPIDLY ALTER MICROGLIA MORPHOLOGY, STATE MARKERS, AND CYTOKINE RELEASE

Keith Campagno

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in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2021

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Jorge Alvarez, Ph.D., Assistant Professor, Pathology

This thesis work is dedicated to my parents.

One night my father was helping me install a water heater. I was learning soldering from him. One hour drive to my place, two hours of work. On his way home, my sister called with a medical emergency and needed a babysitter. My father drove clear across South Jersey and stayed with them until the next morning just to make sure her kids were alright that night.

If he can work hard, so can I.

The following story is likely an amalgamation. One day my mother received her scores back from her New York statewide Regents exams. She did well on all exams, and they needed to confirm with the state that her score in French was perfect. Some unspecified time later, the Guidance Counselor sat her down and told her she can be anything she wants to be: secretary, schoolteacher, or librarian.

I have been angry ever since.

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When I entered this PhD program, I wanted to learn how to think like a scientist. With the years of continued engagement, challenge, and support of Dr. Claire Mitchell, I am proud to say that I have achieved that goal. She has always supported my endeavors, inside and outside of the lab, and pushed me to grow beyond my comfort zone. And beyond that, she was always willing to help me work through a problem, whether that be an experimental setback, or to structure a manuscript. But more than anything, she encouraged me to be proud of my work as a scientist, when all too often my natural skepticism took hold and I saw only gaps in knowledge. For that, I thank her.

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The Mitchell Lab was a wonderful and supportive environment to work in. I would like to thank all of the members who I have asked for help, to teach me a technique, or to ask for suggestions. I started in the lab with Dr. Nestor Más Gómez and Dr. Farraj Albalawi, both of whom helped mentor me. I work now with Dr. Lily See and Dr. Puttipong Sripinun, and we collaborate on experiments, and offer each other assistance (too often its them helping me and not vice versa). Collaborating with Dr. Assraa Hassan Jassim allowed me to plan experiments through Covid, to gain a different perspective in retinal research, and to facilitate writing a review. Furthermore, two undergraduate students were instrumental to the development of the data in this project. Aurora Cenaj and Huen-Yee Tso are, quite honestly, two of the most competent undergraduates that I have ever met. Together they ran too many qPCR plates.

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ABSTRACT

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Keith Campagno

Claire Mitchell

The P2X7 receptor (P2X7R) is an ionotropic receptor that responds to high concentrations of extracellular adenosine triphosphate (ATP) leading to NF-κB-mediated inflammatory responses. P2X7 receptors are important regulators of microglial cells, the innate immune effector cells of the central nervous system (CNS). P2X7R stimulation and microglial morphological and molecular changes have been implicated in loss of neurons accompanying elevation of intraocular pressure (IOP) and associated ATP release. However, the mechanisms linking these processes remain unclear. Recent developments implicating astrocyte inflammation as the intermediaries between microglia inflammation and neural loss further complicate the relationship. We sought to interrogate the consequences of P2X7R stimulation to microglia morphology, the molecular alterations to microglia and astrocytes, in the retina, and the P2X7R role in loss of neurons from transient elevation of IOP. We administered P2X7R agonist BzATP to mice retinae to examine microglia morphological differences and inflammatory gene expression differences. This work was supported with *in vitro* exposure of cultured microglia to P2X7R stimulation. Finally, we used a model of elevated IOP to determine the effects on microglia and astrocytes. We found that retinal exposure to BzATP led to process retraction and elevation of Iba1 in microglia, as well as elevation of both pro-inflammatory (*Tnfa*, *Nos2*) and anti-inflammatory (*Arg1, Chil3*) markers. Transient elevation of IOP induced similar microglial process retraction and retinal gene expression, and these morphological and molecular differences were dampened in retinae derived from $P2X7^{-/-}$ mice. BzATP-injected retinae upregulated genes associated with induction of astrocyte neurotoxic inflammation. Finally, loss of neural populations in retinae subjected to elevated IOP was reduced

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CHAPTER 1: INTRODUCTION

1.1 What is 'neuroinflammation'?

Inflammation is the process by which immune cells respond to harmful stimuli, such as damaged tissue or pathogens. Within the Central Nervous System (CNS), neuroinflammation is mediated by glial populations that communicate with direct interaction, chemokines, cytokines, and reactive oxygen species (ROS) as a protective mechanism to eliminate pathogens, repair tissue and restore homeostasis[437]. The context and duration of the neuroinflammatory processes determine if the outcomes are beneficial or detrimental, but are currently appreciated as encompassing a large role in neurodegenerative conditions[92,149,437]. Amelioration of neurodegenerative conditions requires close study of the cell origin, effectors, and receptors that contribute to neural pathogenic neuroinflammation.

1.2 The P2X7 receptor: diverse functions and inflammatory consequences

In 1972, adenosine triphosphate was proposed to be more than a mere intracellular energy molecule, but an important regulator of neurotransmission in nerve stimulation of gut and bladder smooth muscles[49] Furthermore, ATP was found to be released with neural stimulation in the peripheral and Central Nervous Systems (CNS)[49]. Since then, identification of receptors that are stimulated with purines has led to a deeper understanding of inflammation arising from danger signals and tissue injury. These receptors are classified into P1 receptors, which respond to adenosine, and P2 receptors, which are stimulated with adenosine diphosphate (ADP) or ATP. P2 receptors themselves are subdivided into ionotropic P2X receptors, which bind ATP, and P2Y metabotropic receptors, which respond to ATP and ADP[234].

The P2X7 receptor (P2X7R) is a member of the P2X ionotropic purinergic receptor family that is permeable to Na^+ and Ca^{2+} influx, and K^+ efflux. Seven mammalian receptors of the P2X family have been discovered to date, with P2X7 being the newest discovery[395]. P2X7R is a homo-trimeric receptor[413] that alters conformation to open at high (0.1 to 5 mM) concentrations of

ATP[107,395]. Each P2X7 subunit consists of an extracellular loop that participates in agonist and antagonist binding, and cytoplasmic N- and C-terminal domains[148,184,273]. Interestingly, P2X7R does not desensitize after binding due to a C-cys anchor binding to the inner plasma membrane via palmitoyl groups[273].

Experimental evidence has indicated that P2X7R has three major physiological states, which are dependent upon concentration of ATP and length of stimulation[90,110,184] (Figure 1.1). In the closed state, unstimulated by ATP, P2X7R can act as a scavenger receptor that can phagocytose bacteria and apoptotic cell material[139-141]. This property of P2X7R is of particular interest in the CNS, as P2X7R-mediated scavenger receptor activity is inhibited when exposed to the serum fraction that contains zinc- or copper-glycoproteins[137]. Upon exposure to sufficient concentrations of ATP, phagocytic capacity is inhibited[139], and the trimeric subunits rotate to open and allow ion passage. Elimination of phagocytic capacity after exposure to ATP is likely due to dissociation from the P2X7 receptor from cytoskeletal component non-muscle myosin heavy chain IIA with roughly the same kinetic timing as channel opening[138]. Channel opening and ion passage is associated with the most studied aspects of P2X7R signaling, with numerous downstream effectors driven by direct interactions[210], by K^+ efflux[294], or Ca^{2+} influx[69], activating metabolic pathways, autophagy, death pathways, and especially inflammation[314].

A putative third function of P2X7R stimulation is a macropore opening. Exposure to ATP *in vitro* at higher concentrations or for prolonged time (on the order of seconds[184]) allows for permeation of large molecules of up to 900 Da. The mechanisms leading to pore formation remain unclear. Truncation of the unstructured C-terminus eliminated large molecule permeation while maintaining channel activity[2,395]. The subsequent cell death demonstrated with *in vitro* experimentation contributed to the labeling of P2X7R as a "death receptor"[395,420]. The labeling is questionable; it has since been demonstrated that P2X7R stimulation may promote proliferation[28,284], even in environments with elevated ATP levels, such as the tumor microenvironment[89,91] Additionally, widespread distribution of P2X7R indicates that cell death

should be much more common[89,91]. Furthermore, macropore opening has never been observed *in vivo*, suggesting that macropore opening may be an artifact of *in vitro* manipulation[91].

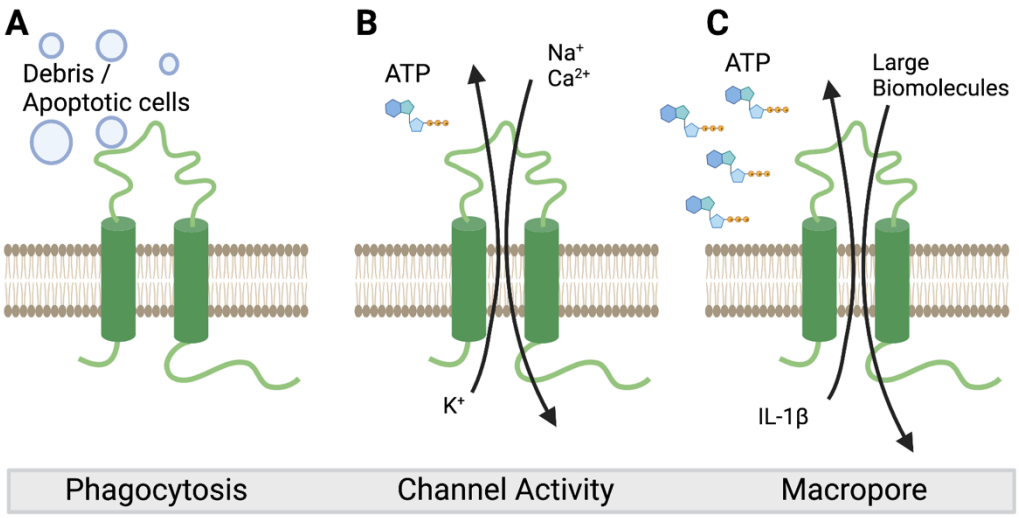


Figure 1.1. General functions of the P2X7 receptor. A Without stimulation by ATP, P2X7R functions as a scavenger receptor for cell debris or apoptotic cells. **B** Transient or low concentrations of ATP allow for channel opening and efflux of K+, and influx of Na+ and Ca+ to stimulate downstream processes. **C** Prolonged or high concentrations of ATP lead to putative opening of a macropore. This macropore allows permeation by large biomolecules such as YO-PRO or Ethidium Bromide, while efflux of cytokines such as IL-1β have been demonstrated.

The most widely recognized inflammatory pathway downstream of P2X7R signaling is the NOD-, LRR, and pyrin domain-containing protein 3 (NLRP3) Inflammasome. The NLRP3 inflammasome is a multimeric compound that acts as an intracellular sensor responding to a number of danger signals and producing cytokine Interleukin 1β (IL-1β)[129]. Canonical inflammasome activity is a two-step process. In the first step, dubbed "priming", pathogen- or damage-associated molecular patterns (PAMP and DAMPs, respectively) signal through Toll-Like Receptors (TLRs) leading to transcription factor NF-κB to translocate into the nucleus[153,282]. Translocation leads to upregulation of inflammasome components, including NLRP3, pro-caspase 1, and the pro-form of IL-1β[396]. In the second step, "activation", effectors cause assembly of NLRP3 with apoptosis-associate speck like protein containing caspase recruitment domain (ASC), and procaspase-1 (itself cleaved to its mature form). The inflammasome then cleaves pro-IL-1β into its

mature form for release through cleaved gasdermin D pore[396] . Assembly of the NLRP3 inflammasome activation commonly requires K^+ efflux[294], but reactive oxygen species (ROS)[17], Cathepsin B (CatB)[61] and direct P2X7R-NLRP3 interaction[114] have also been implicated. P2X7R is the most potent activator of the NLRP3 inflammasome[114,129]. P2X7R stimulation and downstream inflammatory processes are complicated that merit in-depth examination.

P2X7R stimulation of cytokines is not limited to the IL-1 family. P2X7R stimulation alone is sufficient to cause NF-κB translocation to the nucleus, and therefore act as its own "priming" agent[124,211] through direct interaction with myeloid differentiation primary-response protein 88 (MyD88), an adaptor protein for TLRs[242]. NF-κB signaling leads to transcription of a number of cytokines more than simply the IL-1 family, including IL-6, IL-12, and TNF-α[239] , and may regulate production nitric oxide via nitric oxide synthetase[118,297]. Interestingly, NF-κB signaling appears to be stimulus-specific, with DNA binding and gene expression (in epithelial cells)[38], or NF-κB monomer composition (in microglia cell lines)[106] differing based upon the stimulus. Taken all together, a full understanding of P2X7 receptor cytokine profile will elucidate specific inflammatory effectors.

The benefits of P2X7 inflammatory response against a pathogenic insult are well studied[359]. In contrast, the inflammatory response within the CNS generally contributes to progression of chronic neurodegenerative diseases[173]. However, the effects of P2X7R stimulation are context-dependent. P2X7R inhibition exacerbates negative effects in models of ischemia, epilepsy, and Amyotrophic Lateral Sclerosis, or results in conflicting effects in Alzheimer's disease (AD) progression that are likely dependent on the mouse model[194]. Differential effects of receptor antagonism can be attributed to the timing of intervention within the context of the disease, where early inhibition of P2X7R increased microglial and astrocyte reactivity, and reduced motor performance[10], while later inhibits. But additional considerations must be made to signaling in specific cell populations. P2X7R is widely expressed in many cell types, but at higher concentrations in microglia[189,395] where it is implicated in microglia neuroinflammatory processes[3]. P2X7R has demonstrated protective effects specific to retinal ganglion cell populations of the

eye[233,421]. A comprehensive understanding of P2X7R signaling need to incorporate time- and cell-specific outcomes into analyses.

1.3 Neuroinflammatory Glia in the CNS

Microglia. Microglia are the primary innate immune effector cells of the CNS. They comprise 10-15% of all tissue cells within the CNS compartments[220,283]. Unlike other neural cell populations which are derived from the neuroectoderm, microglia develop from erythromyeloid progenitor cells that migrate from the yolk sac early in development[198]. Once established, microglia adopt a ramified morphology and 'scan' the parenchymal environment with elongated processes[88,305], where they perform numerous functions to maintain homeostasis, including phagocytosis of apoptotic cells, synaptic pruning, and facilitation of communication between cells[224,397]. Microglia are long lived resident cells of the CNS, with region specific turnover between 8-41 months in mice[117,219,407], and up to an average of 4.2 years in humans[11,342]. In health, neurons themselves regulate microglia homeostasis with secretion of survival ligands such as interleukin-34 (IL-34)[134,311], or Transforming Growth Factor beta (TGF-β)[35,54], a Colony-Stimulating 1 Factor receptor (CSF1R) ligand[100], or immunoquiescence-promoting factors such as Cx3CL1[78] and CD200[44,313]. The phagocytic and neuroinflammatory functions that microglia perform are context specific. In maintaining synaptic plasticity or promoting tissue repair[259,361], they promote positive outcomes. But in cases of neurodegenerative conditions, they can contribute to ongoing pathology resolution, increased synaptic phagocytosis, or chronic inflammation[92,130].

Microglia are morphologically plastic cells. In an immunoquiescent state, microglia extend processes to synapses to surveil the environmental milieu[88,305,358]. Process ramification and surveillance in the resting state is maintained by tonic activation of THIK-1 channels[260], TAM receptor tyrosine kinases[113], and type-II myosin motors[276]. Upon exposure to PAMPs, DAMPs, or cytokines, microglia undergo several cell state changes[88,305,382], in a process referred to as 'activation' [151]. Activation is accompanied by a diverse number of morphological and molecular changes. Morphologically, microglia somas enlarge and processes retract and thicken, giving

microglia a 'bushy' appearance [88,382]. Molecular changes, including cytokine secretion and transcriptional changes follow morphological alteration[88,308]. Molecular alterations occurring late in the process of activation involve upregulation of Ionized calcium binding adaptor molecule 1 (Iba1)[176] and Cx3C chemokine receptor 1 (Cx3CR1) [64,133]. Both proteins are expressed on microglia and macrophage populations, and their upregulation are useful markers of inflammatory microglial activation. Interestingly, downregulation of microglia identity markers, which distinguish CNS-resident microglia from peripheral macrophage populations, are downregulated. P2Y12 is the major chemotactic receptor responsible for migration towards sites of ATP, and is downregulated in a timeframe long after its chemotactic role is fulfilled[154]. Transcriptional downregulation of identity marker TMEM119 occurs in inflammatory conditions[25]. Although these markers fulfill diverse roles in the physiology of the immune response, the assessment of these markers together provide a useful understanding of the inflammatory environment.

Microglial activation have traditionally been divided into several states, simplified into classical activation or M1 (M1), or alternative activation (M2)[77]. Classical activation is associated with neuroinflammatory process, and polarization with response to PAMPs and DAMPs. Classical activation effectors are varied, but include cytokines such as interferon gamma (IFNγ), which signals via the STAT1 pathway leading to transcription of IRFs factors. Lipopolysaccharides (LPS), which are bacterial components, lead to inflammation via signaling through Toll-Like Receptor 4 (TLR4), leading to NF-κB translocation into the nucleus and production of inflammatory molecules. Gene and protein upregulation include several inflammatory cytokines, such as Interleukin 1β (IL-1β), IL-6, Tumor Necrosis Factor (TNF), and redox molecules such as Nitrous Oxide (NO), with similar genetic markers identifying cell state (e.g. *Il1b, Il6, Tnfa, Nos2*). Alternative activation (M2) has traditionally been subdivided into subcategories itself. M2a alternative activation is entered with chemokines IL-4 or IL-13 through the STAT6 signaling, and is associated with tissue repair and regeneration. It is marked by canonical alternative activation markers Arginase 1 (Arg-1 with gene expression of *Arg1*), YM1 (*Chil3*), and Insulin Growth Factor 1 (IGF-1). M2b is associated with

immunomodulatory state with marked expression of IL-10 (*Il10*), while M2c is exemplified by acquired deactivation, with expression of Versican Proteoglycan (VCAN/*VCAN*).

While these divisions offer useful indications of physiological responses by microglia, developments since the implementation of these categorizations has pointed to the complexity of microglia responses to environmental stimuli[243,288,336]. Inflammatory activation into a mixed classical/alternative activation state has been found in several models[103,292,336]. Interestingly the prototypical pro- and anti-inflammatory markers inducible nitrous oxide synthetase (INOS/NOS-2) and Arg1, respectively, metabolize the same substrate, L-arginine[289], and have been utilized as prototypical pro- and anti-inflammatory markers. Elevation of arginine 1 had dampened the elevation of *Nos2* or release of redox species[57,192]. Microglia cell state is complex, and not permanent, and microglia can exhibit a mix of classical and alternative activation gene expression[288], and the balance of pro- and anti-inflammatory gene expression can be altered with external stimuli[125]. Close examination of gene expression remains a viable approach to understanding inflammatory effects.

Astrocytes. They constitute up to 40% of all glial populations in the parenchyma[158], yet care must be taken as they constitute a heterogeneous population[363]. Astrocytes maintain homeostasis by regulating extracellular glutamine, neurotransmitters, and ion concentrations[72,376]. As with microglia, positive or negative contributions to neurodegeneration are context-dependent. Recent work has pointed to astrocyte as contributors to neurotoxicity when activated by cytokines TNF-α, IL-1α, and C1q[145,231,232]. Modeled on traditional definitions of microglial cell state, these A1 neurotoxic are transcriptionally defined by gene expression from lipopolysaccharides (LPS) [444]. A1 astrocytes have been observed in post-mortem tissue from patients with Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and AD [231]. However, the mechanism of neurotoxicity remains unknown. Regardless, induction into A1 neuroinflammatory state reduced neural synapse formation using *in vitro* cocultures[231]. Upstream mediators leading to A1 astrocyte polarization are understudied, and remain an interesting avenue of research. Inhibition of A1 astrocyte conversion with exposure to a glucagon-like peptide-1 receptor (GLP1R) was

observed *in vivo* in a model of glaucoma[383], and *in vivo* and *in vitro* in models of exposure to PD protein alpha-synuclein[442]. As P2X7R stimulation induces secretion of TNF-α and IL-1α from microglia[155,370], microglial P2X7R presents a lucrative target for amelioration of A1-astrocyte response.

The definitions of A2 astrocytes are derived from gene expression[444] and expression of trophic factors[128]. A2 astrocyte functions have been identified primarily from analysis of ischemia[128], where A2 astrocytes are characterized by secretion of growth-promoting factors. Viral knockdown of protein deglycase DJ-1 after transient middle cerebral artery occlusion (tMCAO) reduced neuron survival, putatively via prevention of astrocyte Nfr2 translocation to the nucleus and subsequent reduction of glutathione secretion[321]. Astrocytes exposed to factors from bone-marrow replacement with Bone Marrow Stromal Cells (rBMSCs) then subsequently subjected to ischemia showed elevated expression of Brain-Derived Neurotrophic Factor (BDNF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF)[121], thus the insinuating that exposure to rBMSCs induce A2 reactive astrocytes[230].

As with microglia, clear delineations of “pro-inflammatory A1” versus “neuroprotective A2” are simplistic, but useful models of more complex phenomena[128,230]. RNA sequencing has identified five subpopulations of astrocytes within the CNS that were not regionally specific[186]. Furthermore, transcriptional diversity was observed even within layers of the cerebral cortex[18]. Astrocyte reactivity is currently being redefined to address pathology-specific cell ‘states’ but currently remains a tool to understand neuroinflammatory environment[101].

In the CNS, neurotoxic A1 astrocytes are induced with proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 1 alpha (IL-1α), and complement component C1q[231]. Specific origin of these factors has not been explored adequately, but microglia are hypothesized to be the likely candidates for expression of these factors[230]. Microglia exposed to LPS in co-culture models do express these factors, and subsequent exposure of cultured astrocytes to conditioned media containing the factors resulted in polarization towards an A1 phenotype[231]. Within the CNS, TNF-α[330], C1q[47], and IL-1α[15] are all primarily expressed or induced by microglia. However, ablation

of microglia failed to preserve loss of retinal neural populations after injury, as part of the work that popularized neurotoxic astrocyte mediated inflammation[231]. Although microglia likely play a large role in production of factors that induce neurotoxic astrocytes[232] origins of these cytokines should be confirmed. Moreover, the triggers which initiate the release of these components from microglia, and ultimately promote the astrocyte response, are unclear.

1.4 Inflammation in glaucoma and models of elevated retinal pressure

The retina is an "outpouching" of the CNS that functions to translate light entering the eye into neural signals[135]. It is organized into several discrete synaptic layers supported physically and metabolically by glial populations, with photoreceptors and epithelial cells comprising the outermost layer of the retina (Figure 1.2). Photoreceptors synapse on bipolar and horizontal cells at the Outer Plexiform Layer (OPL) to transmit light signals received on photoreceptors into neural signals. Bipolar cells act as interneurons, and synapse on Retinal Ganglion Cells (RGCs) at the Inner Plexiform Layer (IPL). Retinal ganglion cells (RGCs) are the primary neuronal populations of the retina. Their nuclei define the Retinal Ganglion Cell Layer (RGCL/GCL). With Müller Glia, RGC axons comprise the Nerve Fiber Layer (NFL) as axon tracts are directed to the optic nerve[166].
There exist three major populations of glial cells of the retina. Retinal astrocytes and Müller glia constitute macroglial populations, and microglia[339] (Figure 1.2). Retinal astrocytes are located at the NFL and the RGCL. Müller glia span the retina between the ONL and the NFL, and are the primary glial population of the retina[339]. Müller glia and astrocytes perform many of the same functions within the retina (with Müller glia acting as main homeostatic effectors), including recycling of neurotransmitters, ion buffering, and clearance of water that may accumulate with ion accumulation or temporary imbalances of vitreal fluid flow[339].

Microglia perform similar roles in the retina as the CNS by maintaining synaptic homeostasis, phagocytosing dead cells, and responding to environmental stimuli with inflammatory effectors In the healthy, mature retina, microglia are uniformly spaced. tiling the planes of the RGCL, IPL, and OPL[355]. Retinal microglia also exhibit slow turnover[257,310], a feature which may

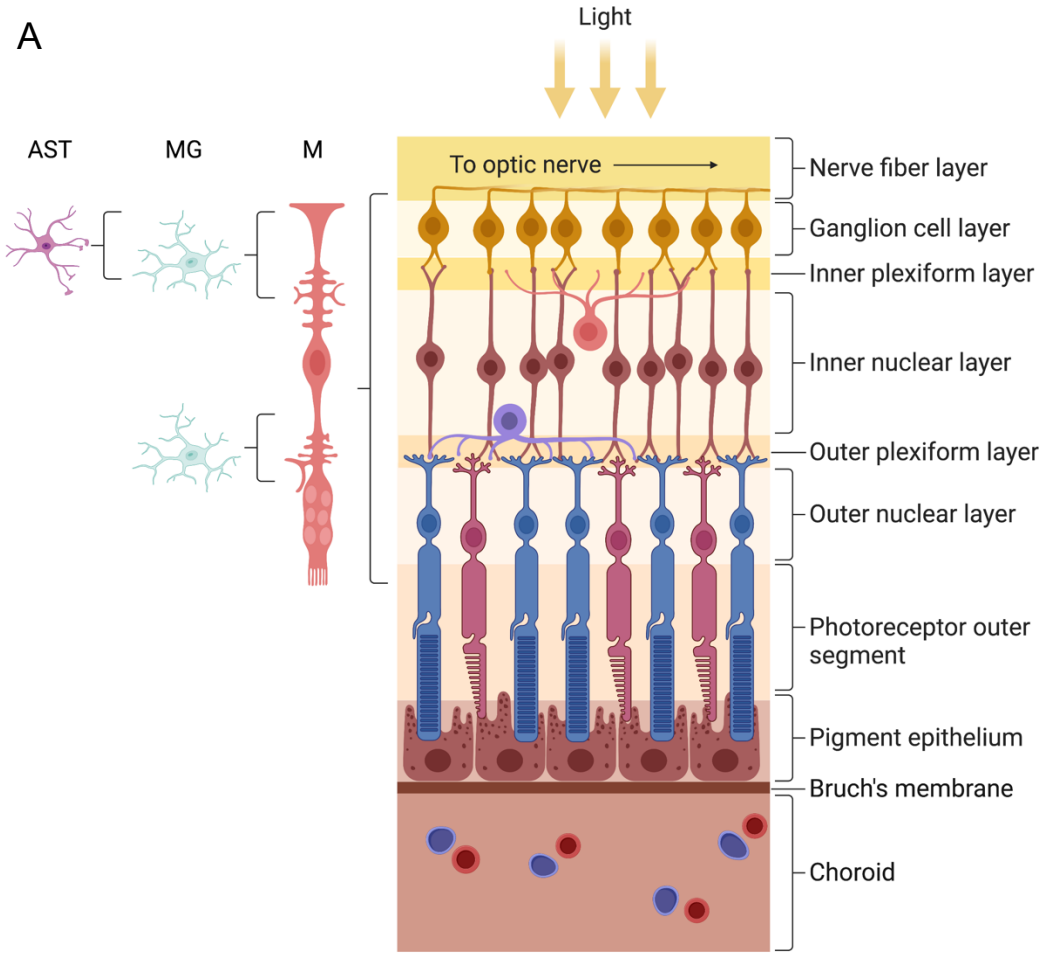


Figure 1.2. Structure of the retina. A Vertical diagram of a retina depicting layers. From the top. Left depicts the location of the major glial populations and the layers they reside in. AST: astrocyte, MG: microglia, M: Müller cell.

contribute to age-related inflammation[256]. Distribution in the RGCL, IPL, and OPL allow microglia dynamic contact with axonal, dendritic, and synaptic compartments of neurons[374]. microglia homeostatic function in the healthy retina is relatively understudied, but roles are similar to those in the CNS, in that they maintain synaptic health and phagocytose apoptotic cells[374]. Chronic ablation of microglia for thirty days led to altered responses to light and increased synaptic degeneration[425], confirming that microglia play an active role in healthy retinae.

Glaucoma. Glaucoma is a progressive retinal degenerative disease that affected nearly 80 million people by 2020[375,428]. In the United States and Europe, the majority of cases consist of Primary Open Angle Glaucoma (POAG) whereby the drainage angle of the trabecular meshwork remains open[428]. POAG patients can suffer from irreversible vision loss, with some patients suffering complete blindness[428]. The major risk factors for onset of glaucoma are age, elevated intraocular pressure (IOP), and a thin cornea[334], with conventional definition of elevated IOP as being greater than 21 mm Hg[428]. IOP elevation is caused by increased production of aqueous humor or decreased outflow through the trabecular meshwork at the anterior chamber of the eye[380] (Figure 1.3). Onset of elevated IOP can occur years or decades prior to measurable loss of visual function[428], suggesting a link between pressure and retinal damage. Current treatments focus on lowering IOP with surgical intervention or therapeutic eye drops, but disease progression can continue even when patients maintain normal IOP[136,156,333]. The processes connecting elevated pressure at the anterior of the eye connecting to RGC damage at the posterior of the eye are unclear. Several pathogenic factors have been suggested to contribute to glaucomatic progression, such as elevated glutamate levels, ischemic events, oxidative stress, and immune activation[415], but further study is merited.

ATP has been found in the aqueous humor of patients suffering from Primary Acute Angle Closed Glaucoma (PAACG)[448] and Primary Chronic Angle Closed Glaucoma (PCACG)[223], in addition to POAG[332]. In the instances of PAACG and PCACG, elevated ATP levels correlated to elevation of IOP[223,448]. Increased ATP was detected in Rat, Mouse, and Primate eyes after elevation of IOP. Also, loss of RGCs observed in the episcleral vein scarring model of chronic IOP elevation in rats and the genetic mouse models[250]. Altogether, this indicates that purinergic signaling may link elevated IOP to RGC damage.

Measurement of the effects of mechanical strain in glaucoma are driven by a number of rodent model systems, with accompanying strengths and weaknesses. The most common genetic model in use is the DBA/2J inbred model, whereby onset of IOP elevations occur at 7-9 months resulting from iris atrophy[229] The DBA/2J is a useful model to study the effects of chronic elevated

IOP and subsequent loss of RGCs. However, IOP elevations were inconsistent and noninvasive tonometry measurements were complicated by corneal calcification[416]. Additionally, about 20% of the mice develop major complications, which result in high rate of omission from data analysis[416],

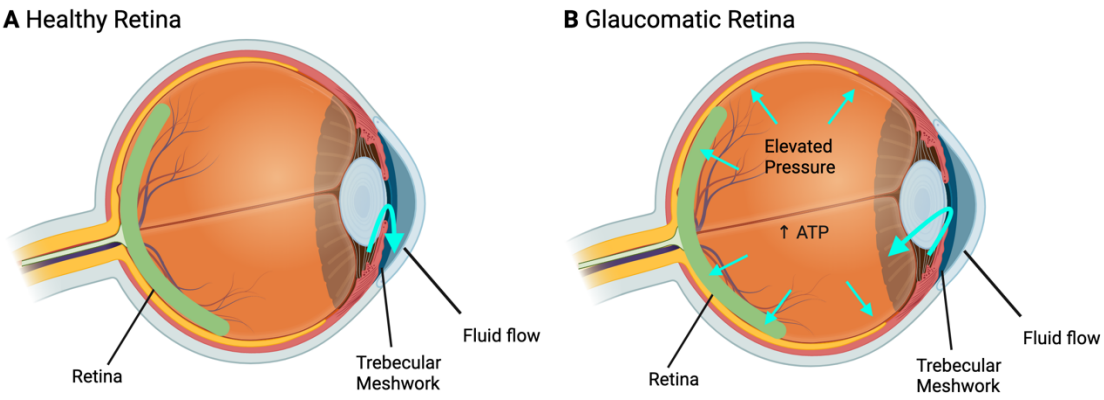


Figure 1.3. The eye in health and disease. A In a healthy eye, fluid from the anterior chamber flows out via the trabecular meshwork. **B** In a glaucomatic eye, fluid outflow via the trabecular meshwork is reduced, resulting in elevated pressure within the eye. The elevated mechanical strain leads to release of ATP from cells of the retina.

casting doubt on the utility of the model. In non-transgenic models, IOP elevations can be achieved for days to weeks through mechanical manipulation of outflow. Increasing outflow resistance through episcleral vein cauterization (EVC) or injection of hypertonic solution resulting in elevated IOP within a week and lasting up to several weeks, with moderate variability of IOP elevation among subjects[87,291,368]. Scleral damage is common with cauterization, complicating the phenotypic results[349]. Though less technically demanding than EVC, hypertonic saline injection requires weekly or biweekly injections to maintain elevated IOP[274]. Success at manipulating outflow with less injury has been found with injection of magnetic microbeads to block the trabecular meshwork[352,356]. As with hypertonic saline injection, a second injection is often required to sustain elevation of IOP after several weeks. Optic nerve crush (ONC) is an invasive model to replicate RGC loss without elevation of IOP[364]. Data presented in this dissertation relies upon cannulation of the anterior chamber of the eye as an acute model of elevated IOP. Within the model, the fluid pressure into the anterior of the eye is precisely controlled by a reservoir attached to the cannulating needle. Only this model of cannulation provided for precise control over the pressure and timing of

the IOP. Understanding the effects of elevated pressure on inflammation and RGCs is strengthened when data are supported in multiple models.

Glaucoma and P2X7R. The mechanical strain associated with elevated IOP is a likely candidate for extracellular ATP release [50,398]. ATP has been found to be released from a variety of cell types via sheer stress[50], distention of tissue[207], and cell swelling[280]. Mechanosensitive pannexin hemichannels are implicated in ATP release[20,341,433]. Likewise, the existence of ATP in glaucomatous patients suggests a role for purinergic receptors in progression of retinal damage from mechanical strain. As such, the P2X7 receptor represents an ideal target to study negative downstream effects from strain. In the DBA/2J genetic mouse model, elevated gene and protein expression of the P2X7 receptor was observed at 3 months (compared to C57Bl/6J counterparts), with elevation further increased at 15 months[218,323]. This mouse model additionally demonstrated reduced RGC synaptic organization and retinal function[323]. Topical allosteric P2X7R antagonist JNJ4795567 was chronically applied to this model, and led to preservation of RGC number and function at 38 weeks of age[346] Elevation of IOP in the rat EVC model of chronic ocular hypertension resulted in elevated Iba1 levels of microglia and moderate loss of RGCs within one week, which was inhibited by administration of P2X7 receptor orthosteric antagonist Brilliant Blue G (BBG)[94]. Similar observations were made elsewhere, with acute elevation of IOP in rats to ischemic levels. Elevated IOP led to loss of RGCs in rats, which was prevented by coadministration of orthosteric antagonists BBG and Oxidized ATP (OxATP) in a dose-dependent manner[392]. Additionally, direct administration of P2X7R-selective agonist BzATP to the retina has led resulted in the loss of RGCs in rats[94] and mice[168].

Interestingly, P2X7R stimulation in these models has demonstrated some benefits. Transient elevation of IOP reduces function of the subpopulation of RGCs that respond to decreases in light intensity (OFF-RGCs)[354,421]. However, retinae derived from $P2X7R^{-/-}$ subjected to elevated IOP demonstrated an additional reduction of light-evoked responses in RGCs that respond to *increases* in light intensity (ON-RGCs)[421]. Isolated primary mouse RGCs that were subjected to strain or stimulated with BzATP secreted Inteleukin-3 (IL-3) more than other

cytokines[233]. IL-3 promotes neuron survival [233,254,445]. Finally, Interleukin-6 (IL-6) was elevated in whole retinae after transient IOP elevation, as well as *in vitro* primary retinal astrocytes following application of mechanical strain or hypotonic solution, inhibited with BBG[6]. IL-6 frequently acts as a neuroprotective, especially when signaling via the membrane-bound form of the IL-6 receptor[348].

Glaucoma and Microglia. Mounting evidence supports a role for neuroinflammation in glaucoma[409]. In human patients, antibody titers against heat shock proteins (HSPs), myelin basic protein, GFAP, and vimentin were detected in the aqueous humor of the eye (reviewed in Tsai *et al.*, 2019[415]). Furthermore, elevated complement proteins were detected in the retinae and sera of POAG patients[34,410]. Immunohistochemical analysis of human glaucomatous patient samples has detected elevated expression of TNF-α, COX-2, and NOS2 colocalizing with microglia[440], as well as elevated GFAP immunoreacitvity[423]. Furthermore, human glaucomatous tissue revealed upregulation of Toll-like Receptors (TLRs) -2, -3, -4 in Iba1+ microglia and GFAP+ astrocytes/Müller cells[253]. TLRs are broad pathogen-sensing receptors that induce inflammatory responses[399]. The specific components of neuroinflammation remain to be studied.

As the primary innate immune cells of the retina, microglia likely contribute to disease progression[427]. Morphological alterations in microglia in concert with activation peaked at 3 months of age, prior to loss of RGCs the DBA/2J mouse model of chronic elevated IOP [42]. Microglia activation at 6 weeks preceding loss of RGCs at 15 weeks was observed in rats whose IOP was elevated using chondroitin sulfate (CS)[37]. In rats where IOP was elevated with ECV, whole retinae subjected to elevated IOP expressed increased content of oxidation proteins NOS2 and NADPH oxidase-4 (NOX4), and increased translocation of NF-κB p65 subunit into the nuclear fraction[171]. In a mouse model of elevated IOP that was induced by laser irradiation, expression of protein TNF-α was upregulated[296]. Neutralization of TNF-α with antibodies or genetic knockout mouse models reduced loss of RGCs and oligodendrocytes at the optic nerve head. A study in rats utilizing episcleral vein blockage to elevate IOP determined that TNF-α was expressed only in microglia and Müller cells[84] Interestingly, loss of RGCs was also reduced in mice where complement receptor 3 (CR3, previously Mac-1) was deleted[296], pointing to the role of complement factors in loss of

RGCs. Cytokine elevation is an early event in progression of pathology in the DBA/2J model. Retinae were found to have significant upregulation of inflammatory mediators such as IL-1β, IL-6, and TNF-α prior to 5 months[431]. However, anti-inflammatory mediators IL-10 and IL-5 were also detected[431], indicating that the contributions that inflammation makes to RGC loss may be a balance, rather than a binary switch. An understanding of compensatory neuroprotective mechanisms will elucidate specific mechanisms leading to RGC death[324].

Activation itself may be a contributing factor, possibly through increased phagocytosis[45]. Deprived of immunoquiescent signaling via Cx3CR1, microglia were found to phagocytose healthy RGCs and at-risk photoreceptors in a mouse model of Retinitis Pigmentosa[443]. Stimulation of microglial immunoquiescent receptor Cx3CR1 has been demonstrated to reduce microglial activation[422,443]. Deletion of the receptor resulted in a greater loss of RGCs after transient elevation of IOP[422], and increased cytokine secretion and phagocytosis of photoreceptor material in the Rd1 mouse model of Retinitis Pigmentosa[443]. Inhibition of activation with antibiotic minocycline after elevation of IOP[422] or in the DBA/2J chronic IOP model[40] reduced RGC loss as well, indicating that activation-mediated phagocytosis plays a role.

Microglia depletion in models of elevated IOP can offer insights regarding the role of microglia in disease progression. However, data were found in only one study utilizing the ONC model. Microglia depletion using PLX5622 had little effect on neural degeneration or axonal regeneration[160]. In other retinal disease models, microglia depletion has demonstrated diverse outcomes, but roughly falling in line with putative neuroinflammatory or phagocytic roles that microglia inhabit. An alteration of inflammatory marker expression (but not a reduction), as well as loss of RGCs was observed two months after depletion of microglia with subsequent optic burn, with evidence pointing to inflammatory peripheral monocyte engraftment and adoption of microglia morphology[318,319]. Similar migration and engraftment was observed two months after acute elevation of IOP without depletion as well[318]. Ablation of microglia in the retinal vein occlusion model of retinal ischemia reduced inflammation and preserved RGCs[187]. In the *Rho*^{P23H} model of retinitis pigmentosa, where retinal degeneration occurs from excessive light damage (LD), microglia

depletion via diphtheria toxin (DT) resulted in accumulation in photoreceptor debris and worse retinal damage than their non-depleted counterparts[311]. A comprehensive analysis of early microglial signaling in elevation of IOP still needs to be determined.

Glaucoma, astrocytes, and Müller glia. Onset of POAG is associated with polymorphisms of *TNF*[43] and *IL1A*[285]. Human patient samples revealed upregulated protein expression of TNF[441] and C1q[381], all associated with astrocyte neurotoxicity[231]. In a model of chronic elevated IOP induced with bead blockage of aqueous outflow, genes associated with neurotoxic A1 astrocytes were detected[145,383]. Furthermore, gene expression elevation was found specific to macroglial populations in the retina[383]. In both cases, inflammatory gene upregulation was not observed in retinae from mice where *Tnfa*, *Il1a*, and *C1qa* were ablated[145,383]. Together this indicates the primary inflammatory role that macroglia play in loss of RGCs from elevated IOP. Reactive GFAP+ astrocytes/Múller glia mediate neuroprotective roles after elevation of IOP. Genetic ablation of astrocyte transcription factor signal transducer and activator of transcription 3 (STAT3) which activates downstream of ligands such as interferons, epidermal growth factor, and IL-6[161] resulted in an attenuation of astrocyte activation, greater loss of RGCs and greater visual dysfunction after several models of elevated IOP[393].

Indicators of reduced of oxygenation in cells, a process termed hypoxia, colocalize with Müller glia 4 weeks after elevation of IOP with the bead model, likely reducing glial antioxidant support of RGCs [180] likely reducing glial antioxidant and neuroprotective factors[182]. Müller glia additionally contribute to production of excitotoxic factors[449]. Astrocytes in mechanical strain models proliferated near the optic nerve in the DBA/2J model[79], as well as upregulated components in the NLRP3 inflammasome following transient IOP elevation[6]. The contributions of macroglial populations to progression of damage from elevated IOP are complex, and merit close study.

1.5 Phagocytosis and Autophagy

P2X7Rs make a critical contribution to the clearance functions of microglia, in addition to their effects on inflammatory signals in the cells. Data are emerging that P2X7R influences major

microglial functions associated with clearance of extracellular debris. P2X7R influences phagocytosis both by acting as a scavenger receptor, and influencing phagocytic receptor expression via inflammatory processes. Additionally P2X7R-mediated promotion or inhibition of autophagy is time- and context-specific, while autophagy regulates P2X7R inflammation mechanisms[60,314].

The process of microglial phagocytosis can be categorized into discrete steps[328]. First, pruned synapses, apoptotic cells, or debris are recognized and bind to phagocytic receptors on the surface of the host cell[109]. Microglia express a diverse profile of phagocytic receptors, such as pattern recognition receptors (PRRS), like TLRs or Scavenger Receptors, or opsonic receptors, such as CD45 or CD11b. Second, cytoskeletal actin rearrangement promotes formation of the phagocytic cup for engulfment of the target. Third, the material is shuttled via the ESCRT system through the phagosome, which matures into a late endosome, then fuses with the lysosome (forming a phagolysosome) for degradation. Finally, degraded material is recycled, while content influences cell response through gene transcription. Additionally, material that comprised the endosome is recycled back to the plasma membrane fuses with the *trans*-Golgi network (TGN)[377].

In contrast to processes that degrade extracellular components, the process of autophagy degrades intracellular components to maintain internal homeostasis and for purposes of recycling. The most studied autophagic process, whereby cargo is taken into vesicles away from the lysosome, is known as macroautophagy (herein: autophagy). The process of autophagy is complex and multifactorial, usually broken into several steps[317,327]. Initiation of autophagy usually begins with inhibition of Mechanistic Target of Rapamycin Complex 1 (mTORC1)[235], which disinhibits formation of Autophagy-related (Atg) 1/13/17/31/29 kinase complex (in yeast) or the Unc-51-like (ULK1) kinase complex (in humans). Subsequently, recruitment of the beclin-1/vacuolar protein sorting 34 (BECN-1/Vps34) complex to catalyze the formation phosphatidyl-inositol-3-phosphate (PI3P) participates in ‘nucleation’ of phagophore formation[306]. Following initiation, in ‘elongation’, the double-membraned structure involves the lipidation of microtubule-associated light chain 3 (LC3) with phosphatidylethanolamine (PE)[317], a transition denoted by the change from LC3-I to

LC3-II[206]. Thus the autophagosome is formed. Next, the autophagosome is merges with a lysosome to create the autolysosome[317]. with GTPase Ras-related protein RAB7[177], SNAP Receptor (SNARE) proteins[175], and lysosome ion channel signaling[270,271] all implicated in fusion. Autophagy is regulated by energy and nutrient sensing. mTORC1 is inhibited in an environment of low amino acids, thereby initiating autophagy[452]. Additionally, a combination of low intracellular ATP and high intracellular adenosine monophosphate (AMP) activate AMP-activated protein kinase (AMPK), which inhibits mTORC1[317].

Disruptions in autophagy and phagocytic play a role in microglia signaling in neurodegenerative conditions[188,327,328,377]. In aging, myelin[350] and lipid droplets[266] accumulate in microglia and contribute to expression of Major Histocompatibility Complex II (MHCII) proteins[350], and reactive oxygen species (ROS) generation[58], respectively. While the influence of autophagic/phagocytic pathways is unclear in age, autophagic machinery is known to participate in phagocytosis and degradation of materials in proteinopathies[327]. with reduction of uptake observed *in vitro* In mouse models of Parkinson's disease inclusions of alpha-synuclein (α-syn)[405], parenchymal deposition of which is elevated by deletion of *Atg7* and influenced by lipid metabolism[436]. Incomplete phagocytosis of amyloid-beta (Aβ) deposits contribute to "microglia exhaustion" and release of neuroinflammatory factors[388,389]. Microglia from human AD patients show reduced beclin 1 protein levels, with knockdown of the gene *in vitro* or in hippocampal brain slices revealing reduced Aβ uptake and recycling of phagocytic receptor Trem2 protein[252].

P2X7R influences over autophagy are diverse, with autophagic induction with transient stimulation, while evidence points to P2X7R negatively regulating autophagy with chronic stimulation. Within cultured microglia, P2X7R stimulation has demonstrated to elevate ratios of LC3-II compared to LC3-I, likely through induction of autophagy[103,402]. Yet transient stimulation has also demonstrated aberrant endolysosomal secretion of lysosome-derived degradative enzyme cathepsin D (CatD) into the extracellular space[401,402], while prolonged stimulation leads to elevation of pH_{Lys} in primary microglia[402] and microglial cell lines[365]. In astrocytes, P2X7R deletion led to upregulation of heat shock protein B1 (HSPB1) and induction of autophagy via mTOR-independent

pathways[203]. In models of osteoarthritis, P2X7R stimulation led to induction of autophagy factors Beclin-1 and LC3B within weeks, whereas prolonged stimulation led to upregulation of mTOR and inhibition of autophagy, eventually leading to cell death[226]. It is likely that P2X7R stimulation affects autophagy in a time-dependent and cell-specific manner.

Within microglia, autophagy plays a large role in degrading P2X7R-mediated inflammatory components. Elevated autophagy is associated with decreased NLRP3 inflammasome function *in vivo*[63] and *in vitro*[63,68,150,438] in microglia. Similarly, components of the NLRP3 inflammasome colocalize ASC[369] or IL-1β[152] with autophagic or lysosomal markers, indicative of autophagic-mediated degradation. Administration of rapamycin to microglia reduced IL-1β protein and Iba1 immunoreactivity (emblematic of activation) that was brought on in a model of chronic migraine[183]. A more granular understanding of the P2X7R intracellular crosstalk among phagocytosis, autophagy, and lysosome signaling will offer new insight into influencing inflammation. Furthermore, the influence of inflammatory mechanisms on autophagy are unclear. The role of P2X7R and associated inflammatory mechanisms and the complex crosstalk with the microglial endolysosomal system is explored in Chapter 4.

1.6 Dissertation goals

The research described in this dissertation is focused on the role of the P2X7 receptor in microglial activation and microglia inflammatory effects. Work presented here utilized *in vitro* cell culture manipulations and *in vivo* retinal injections and a model of mechanical strain, whereby intraocular pressure was exogenously increased. Since the contributions that microglial activation and P2X7R-mediated inflammation make to loss of retinal ganglion cell populations remain understudied, a comprehensive understanding of P2X7R effects can lead to more specific therapies to inhibit negative effects arising from P2X7R signaling, while preserving any positive effects. Additionally, a specific understanding of P2X7R effects on microglial phagocytosis and autophagy had not been completed. As such, the goals of this dissertation were to 1) Determine the detailed morphological and molecular effects of direct stimulation of P2X7R on microglial cells

in vitro and *in vivo*, and explore the receptor contribution upon IOP elevation; 2) explore the role of P2X7R in microglial/astrocyte activation pathways and 3) describe the roles of P2X7R in autophagy and phagocytosis by microglial cells. We find that P2X7R acts as a key regulator or microglial activation, inflammation, phagocytosis, and astrocyte reactivity.

CHAPTER 2: RAPID MORPHOLOGIC CHANGES TO MICROGLIAL CELLS AND UPREGULATION OF MIXED MICROGLIAL ACTIVATION STATE MARKERS INDUCED BY P2X7 RECEPTOR STIMULATION AND INCREASED INTRAOCULAR PRESSURE *

2.1 Abstract*

The identification of endogenous signals that lead to microglial activation is a key step in understanding neuroinflammatory cascades. As ATP release accompanies mechanical strain to neural tissue, and as the P2X7 receptor (P2X7R) for ATP is expressed on microglial cells, we examined the morphological and molecular consequences of P2X7 receptor stimulation in vivo and in vitro and investigated the contribution of the P2X7 receptor in a model of increased intraocular pressure (IOP). In vivo experiments involved intravitreal injections and both transient and sustained elevation of IOP. In vitro experiments were performed on isolated mouse retinal and brain microglial cells. Morphological changes were quantified in vivo using Sholl analysis. Expression of mRNA for M1 and M2-like genes was determined with qPCR. The luciferin/luciferase assay quantified retinal ATP release while fura-2 indicated cytoplasmic calcium. Microglial migration was monitored with a Boyden chamber. Sholl analysis of Iba1-stained cells showed retraction of microglial ramifications one day after injection of P2X7R agonist BzATP into mouse retinae. Mean branch length of ramifications also decreased, while cell body size and expression of *Nos2*, *Tnfa*, *Arg1*, and *Chil3* mRNA increased. BzATP induced similar morphological changes in ex vivo tissue isolated from Cx3CR1$^{+/GFP}$ mice, suggesting recruitment of external cells was unnecessary. Primary microglial cultures expressed P2X7R; elevation of cellular Ca2+ by BzATP and block by antagonist A839977 provided functional confirmation. BzATP induced process retraction and cell body enlargement within minutes in isolated microglial cells, and increased *Nos2* and *Arg1*. While ATP increased

* Some text and figures taken or modified from Campagno KE, Lu W, Jassim AH, Albalawi F, Cenaj A, Tso HY, Clark SP, Sripinun P, Gómez NM, Mitchell CH. Rapid morphologic changes to microglial cells and upregulation of mixed microglial activation state markers induced by P2X7 receptor stimulation and increased intraocular pressure. (2021) *J Neuroinflammation* **18**(1):217.

microglial migration, this required P2Y12R, not P2X7R. Transient elevation of IOP increased retinal ATP release and microglial process retraction, cell body enlargement and gene upregulation paralleling changes with BzATP injection. Pressure-dependent changes were reduced in P2X7$^{-/-}$ mice. Death of retinal ganglion cells accompanied increased IOP in C57Bl/6J, but not P2X7$^{-/-}$ mice, and neuronal loss showed some association to microglial activation. P2X7R stimulation induced rapid morphological activation of microglial cells, including process retraction and cell body enlargement, and upregulation of markers linked to both M1 and M2-type activation. Parallel responses accompanied IOP elevation, suggesting ATP release and P2X7R stimulation influence the early microglial response to rising pressure.

2.2 Introduction

The contribution of microglial cells to neural damage is complex, and untangling interactions between stimuli, cell types, and signaling cascades requires a detailed analysis [55]. Microglial cells are resident immune cells in neural tissue responsible for synaptic maintenance and the innate immune response to injury or microbial infiltration [76,351]. While considered largely beneficial in their immunoquiescent M0 state [397], microglial dysregulation is implicated in several neuroinflammatory pathologies [42,53,269], including diseases that involve mechanical strain to neural tissue, such as traumatic brain injury, cerebral edema and glaucoma [93,122,170,335]. Identifying signals that link mechanical strain to microglial activation could help reduce the destructive neuroinflammation associated with these disorders.

The release of ATP is a widespread response to mechanical strain found throughout the body, including in neural tissue [20,81,426]. The released ATP stimulates ionotropic P2X or metabotropic P2Y receptors, and can act at adenosine receptors after dephosphorylation [417]. Inflammatory responses are most frequently associated with the P2X7 receptor, and stimulation by ATP can lead to activation of the NLRP3 inflammasome and release of various cytokines linked to neural inflammation [3,359]. P2X7 receptors are emerging as important regulators of microglial cells,

with *in vitro* experiments in murine microglial cells showing that receptor stimulation leads to release of the IL-1 family cytokines, caspase 1 cleavage and altered phagocytosis [155,179].

The mechanical strain accompanying the elevation of intraocular pressure (IOP) is associated with both ATP release [223,250,344], and microglial activation [41,181,441]. The P2X7 receptor has been implicated in increased staining for microglial markers CD68 [392] or Iba1 [94] following IOP elevation, but details of the molecular and morphological changes induced in microglial cells by P2X7 receptor stimulation following IOP elevation, and the timing of such responses, remain unclear. In this regard, the particular type of activation state induced in microglial cells by P2X7 receptor stimulation is of particular relevance, as the classical activation state (M1) is traditionally defined by release of proinflammatory cytokines or neurotoxic effectors that exacerbate neurotoxicity [33,67,146], while the alternative activation states (M2) promote phagocytosis and waste clearance, neurogenesis, axon remodeling, or remyelination after an injury [119,169,241]. As microglial activation states are now recognized to be more fluid and less binary than suggested by the traditional M1 and M2 classifications [312,336], a more nuanced understanding of how stimuli modify the morphological and molecular response of microglial cells is needed.

This study provides a detailed analysis of the molecular and morphologic consequences of P2X7 receptor stimulation in microglia cells *in vitro* and *in vivo*. The ability of receptor stimulation to trigger process retraction and migration was probed, and effects on markers for M1 and M2 activation states were examined. Receptor stimulation was examined in a more relevant pathophysiological context by elevating IOP. The relationship between IOP elevation, microglial activation and death of retinal ganglion cells was also investigated. The findings confirm a central role for the P2X7 receptor in the early activation of microglial cells following pressure elevation.

2.3 Materials and Methods

Animal care and use: All procedures were performed in strict accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals" and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) in protocol

#803584. All animals were housed in temperature-controlled rooms on a 12:12 light:dark cycle with food and water *ad libitum*. Mice were obtained from Jackson Laboratories (Bar Harbor, ME). B6.129P2(Cg)-CX3CR1$^{tm1Litt/J}$ mice were bred with C57BL/6J mice for pups that were heterozygous for GFP expression on the CX3CR1 promoter (CX3CR1$^{+/GFP}$). Though microglia derived from CX3CR1$^{+/GFP}$ mice have an altered transcriptome [147] and measured physiological responses [221], differences likely represent a more aged phenotype [147], and not a physiologically irrelevant response.

Intravitreal injections Intravitreal injections were performed as previously described [168]. Briefly, after mice were anesthetized with 1.5% isofluorane, 1.5 µl Sterile Balanced Saline solution or 250 µM Benzoylbenzoyl-ATP (BzATP; Sigma Aldrich) was injected into the superior nasal region of the vitreous cavity approximately 0.5 mm from the limbus with a micropipette attached to a microsyringe (Drummond Scientific Co.). Animals were excluded if the lens was damaged. Differences in gene expression between saline-injected and naïve retinae from litter-controlled mates were not significant.

Retinal imaging: Dissected retinae were nicked to preserve orientation after sacrificed and enucleation. Retinae were fixed in 4% paraformaldehyde for 20 min, incubated with 0.1% Triton X-100 (Sigma-Aldrich) in SuperBlock buffer (ThermoFisher) for 30 min at 25°C, then 10% goat serum in SuperBlock for 1 hr. Retinal whole mounts or sections were incubated with primary antibodies 18-48 hrs at 4°C, with secondary antibodies for 60 min at 25°C, then mounted with SlowFade Gold anti-fade mounting medium (Molecular Probes). Antibodies are listed in Table 2.1. For *ex vivo* experiments, CX3CR1$^{+/GFP}$ mice were euthanized by CO_2 and enucleated eyes placed into isotonic solution (consisting of (in mM) 105 NaCl, 5 KCl, 6 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES) acid, 4 Na 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 $NaHCO_3$, 60 mannitol, 5 glucose, 0.5 $MgCl_2$ and 1.3 $CaCl_2$, pH 7.4) with or without BzATP and imaged on a Nikon A1R microscope of the University of Pennsylvania Live Cell Imaging Core. Retinal whole mounts were dissected as previously described [168]. Following exposure to BzATP or isotonic solution for 2 hrs

Table 2.1: Antibodies used for immunofluorescent staining

Antibody	Source	Identifier	Dilution	Use
Primary Antibodies				
Anti-Brn-3a	Santa Cruz	sc-31984	1:250	Whole mounts
Anti-Iba1	Chemicon	MAB360	1:500	Immunocytochemistry
Anti-GFAP	Wako	019-19741	1:500	Whole mounts, Cryosections, Immunocytochemistry
Anti-Iba1	Abcam	AB48004	1:200	Immunocytochemistry
Anti-P2X7 Receptor	Alomone	APR-008	1:200	Immunocytochemistry
Anti-Synaptophysin	Thermo Fisher	MA5-14532	1:250	Immunocytochemistry
Secondary Antibodies				
Anti-Mouse Alexa Fluor 488	Thermo Fisher	A21202	1:500	Whole mounts
Anti-Rabbit Alexa Fluor 488	Thermo Fisher	A21206	1:500	Immunocytochemistry
Anti-Mouse Alexa Fluor 488	Thermo Fisher	A11001	1:500	Immunocytochemistry
Anti-Goat Alexa Fluor 488	Thermo Fisher	A11055	1:500	Immunocytochemistry
Anti-Rabbit Alexa Fluor 546	Thermo Fisher	A11035	1:500	Immunocytochemistry
Anti-Goat Alexa Fluor 555	Thermo Fisher	A21432	1:500	Immunocytochemistry
Anti-Rabbit Alexa Fluor 555	Thermo Fisher	A31572	1:500	Whole mounts, Immunocytochemistry
Anti-Goat Alexa Fluor 555	Thermo Fisher	A10042	1:500	Immunocytochemistry

at 37° C, retinae were fixed with 4% PFA for 15 minutes at 25°C and mounted using SlowFade Gold medium (ThermoFisher).

Image analysis: Z-stacks were acquired from retinal whole mounts with a TCS SP8 confocal microscope (Leica). Iba1+ cells of the Retinal Ganglion Cell (RGC) and Inner Plexiform (IPL) layers were counted using FIJI [362] and randomized (www.randomizer.org). Fluorescence intensity was determined in a 5 µM radius of the nucleus center. Sholl analysis/summed branch length was performed outside of this radius using the FIJI Simple Neurite Tracer (SNT) plugin [245].

Observer quantification of morphological alterations: Retinal whole mount images were acquired from the central, middle, and peripheral regions of the retina as defined above using a Nikon Eclipse microscope (Nikon), with each region taken at the superior, nasal, inferior, and temporal quadrants. De-identified images were scored individually based upon morphology. Smaller cell body size and elongated, thin processes received a score of 1, larger body size and short, thick processes received a score of 3 (Fig. S2a). Observer scores of BzATP-exposed retinae correlated to Iba1-

intensity of the soma region (Fig. S2b), and there was close agreement among observers, with the standard deviation of the scores between observers being less than 15% of the mean across all regions, validating the approach.

Quantitative PCR: Retinae or isolated microglia were homogenized using TRIzol (Invitrogen). RNA was purified with an RNeasy mini kit (Qiagen, Inc.) and converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems); qPCR was performed with Power or PowerUp SYBR green (Applied Biosystems) on the 7300 or Quant Studio 3 Real-Time PCR systems (Applied Biosystems) using standard annealing and elongation protocols, with data analyzed using the delta-delta CT approach as described [247]. Primers are listed in Table 2.2.

Table 2.2: Primers used for qPCR

Gene name	Genbank accession	Primer (F: 5'-3'; R: 3'-5')	Size (bp)
Nos2	NM_010927.4	F: CCCTTCAATGGTTGGTACATGG R: ACATTGATCTCCGTGACAGCC	158
Tnfa	NM_013693.3	F: AAATGGCCTCCCTCTCATCAG R: GTCACTCGAATTTTGAGAAGATGATC	73
Arg1	NM_007482.3	F: ACAAGACAGGGCTCCTTTCAG R: GGCTTATGGTTACCCTCCCG	148
Chil3	NM_009892.3	F:AGAAGGGAGTTTCAAACCTGGT R: GTCTTGCTCATGTGTGTAAGTGA	109
GAPDH	NM_017008	F: TCACCACCATGGAGAAGGC R: GCTAAGCAGTTGGTGGTGCA	169

Microglial cell cultures: Primary retinal microglia were isolated from mouse pups of both sexes P12-P20 using standard methods [258,347]. Primary brain microglia were also isolated [227]. In both cases, mixed cell cultures were grown in media consisting of High Glucose DMEM (HG-DMEM; Invitrogen) with 10% Fetal Bovine Serum (FBS; Sigma-Aldrich), 1% Penicillin/Streptomycin (Pen/Strep; Gibco), 1% GlutaMAX (Gibco), and 1x MEM nonessential amino acids (Sigma-Aldrich). Upon confluence, microglia were "shaken off" manually and plated in dishes coated with 0.1% Poly-L-Lysine (PLL; Peptides International, 0.01%) and Collagen IV (Corning, 4 µg/ml) in HG-DMEM with 5% FBS, plus 10% of media previously exposed to the mixed cell culture for 5-7 days. Media was changed to DMEM with 5% FBS, 1% Pen/Strep, 1% GlutaMAX, and 1x MEM nonessential amino acids 24 hours prior to experimentation.

Time-lapse of morphological alterations: Retinal mouse microglia were plated as above and incubated in Mg^{2+}-free isotonic solution with 10 μM A839977 (Tocris) or DMSO as solvent control. Phase contrast images were taken every 12s or 15s using a Keyence BZ-X700 Series All-in-One Fluorescence Microscope (Keyence Corporation). BzATP (Sigma-Aldrich) was added in the presence of A839977 or vehicle. Representative video was derived using "Focus tracking" function, where time-lapse video is comprised of best-focused images derived from a panel of 7 images spaced 0.7 μm apart at each time point. FIJI [362] was used to modify intensity, with parallel processing for all time-lapsed sequences.

Immunocytochemistry: Isolated retinal microglial cells were mounted on 12mm glass coverslips coated with PLL and collagen as above. Cells were fixed in 4% paraformaldehyde for 10 min at 37°C, washed in PBS with 1% Tween 20 (Bio-Rad), permeabilized with 0.1% Triton-X 100 for 15 min (Sigma-Aldrich) then blocked with 20% Superblock (Thermo Fisher) plus 10% goat or donkey serum. Primary and Secondary antibodies used are listed in Supplementary Table 1. After incubation in Hoechst (Cell Signaling, 1 μg/ml) for 10 min, coverslips were washed and mounted using SlowFade Gold (Thermo Fisher). Imaging was performed using a Nikon Eclipse microscope (Nikon) with NIS Elements Imaging software (Nikon v. 4.60). ImageJ was used in parallel processing to modify intensity, and merge pseudocolored images. Retinal cryosections were blocked with 1% Triton X-100, 0.5% Bovine Serum Albumin (BSA, Sigma-Aldrich), 0.9% Sodium Chloride (Thermo Fisher), and 5% donkey serum (DKS; Jackson ImmunoResearch) in 1% Phosphate-Buffered Saline (PBS-T-BSA), quenched using 0.3%, H_2O_2, then incubated with primary antibody overnight at 4°C, rinsed, then blocked in PBS-T-BSA. Secondary antibody incubated for 2 hrs followed by application of 4′,6-diamidino-2-phenylindole (DAPI; 1:2000). Sections were mounted using Fluoromount-G (Southern Biotech). Images were acquired of from retinal cryosections, using a Nikon Eclipse microscope (Nikon, USA) and NIS Elements Imaging software (Nikon v. 4.60). All cells of the RGC and IPL layers were utilized for soma Iba1-intensity measurements.

Ca^{+2} imaging: Microglia were plated on 25 mm coverslips coated with PLL (0.01%) and Collagen IV (4 µg/ml) and loaded with 10µM Fura-2 AM (Thermo Fisher) with 0.02% Pluronic F-127 (Thermo Fisher) for 45 min at 37°C. Cells were washed, mounted in a perfusion chamber, and perfused with isotonic solution without Mg^{2+}. Ratiometric measurements were performed using a 40x objective on a Nikon Diaphot microscope (Nikon) by alternating excitation between 340nm and 380nm wavelengths and quantifying emission ≥512 nm with a charge-coupled device camera (All Photon Technologies International) as described [131]. Data were expressed as the ratio of light excited at 340nm to 380nm, $F_{340/380}$, due to complexity of calibration. Statistical comparisons were made by averaging the peak value ± two measurements for all BzATP conditions and the average of the final five measurements for the baseline conditions, including isotonic and A839966 in isotonic solution.

Microglia migration: Mouse retinal microglia were grown to 80% confluence, detached, resuspended in media outlined above to a concentration of 50,000 cells in 390 µl. After incubation in inhibitors or vehicle for 1 hr, Hoechst (1 µg/ml) was added to cells for 10 minutes. The cell suspension was added to the top wells of a chemotaxis chamber (Neuro Probe) separated from a solution of 1 mM ATP (Sigma-Aldrich) in media by a 10 µm pore filter (Neuro Probe). Cells were allowed to migrate for 3 hrs, after which unmigrated cells were removed from the top of the filter. Filters were washed in Phosphate Buffered Saline, fixed in 4% PFA, then imaged using the Fluoroskan fluorometer (Thermo Fisher) at 340ex/527em. Background fluorescence was subtracted and data normalized to control. Fluorescence measurements were validated by correlating fluorescence to labeled cell counts from images acquired of the underside of the pore filter.

Vitreal ATP measurement: Given the difficulties in sampling the small extracellular spaces in the retina without touching cells to trigger mechanosensitive ATP release or rupturing cells to trigger cytoplasmic ATP release, levels in the posterior vitreous were determined. ATP concentration was determined by enucleation after IOP returned to baseline and then fast-freezing eyes in dry ice.

Eyes were later dissected over dry ice, and vitreal samples were collected by chipping away frozen samples; this prevented intracellular ATP from the cut tissue edges from seeping into the vitreous and contaminating the sample [250]. ATP levels were measured using the luciferin/luciferase assay (Sigma-Aldrich) as described [21].

Intraocular pressure elevation. A transient controlled elevation of IOP (CEI) procedure was produced in adult mice of both sexes using a modification of the approaches of Morrison [408] and Crowston [83] as previously described [6] to raise IOP to ~ 60 mmHg for 4 hours. Sustained elevation of IOP was also induced using the microbead injection method as described [180].

Transient elevation of IOP: Mice were deeply anesthetized with 1.5% isoflurane after receiving 2 mg/kg meloxicam. Proparacaine (0.5%) and tropicamide (0.5-1%) were administered and one eye was cannulated with a 30- gauge needle attached to polyethylene tubing (PE 50; Becton Dickinson) inserted into the anterior chamber, connected to a 20 ml syringe filled with sterile PBS. IOP was increased to 57.0 ± 0.4 mmHg by elevating the reservoir to the appropriate height; blood flow through the retina was maintained throughout to avoid acute ischemia, although some reduction in blood flow is likely. After 4 hrs, IOP was returned to baseline, the needle removed, and 0.5% erythromycin was applied to the cornea. The contralateral eye without cannulation served as a normotensive control. Retinal tissues were isolated 22-24 hrs after elevation of IOP.

Sustained elevation of IOP: Sustained elevation of IOP was induced using the microbead injection method [352]. Mice were anesthetized in 2.5% isoflurane, and 2 µL of magnetic microbeads (COMPEL COOH-Modified 8-µm diameter; Bangs Laboratories) was injected into the anterior chamber of the eye using a glass-pulled micropipette connected to a manual microsyringe pump (World Precision Instruments) as described [180]. A neodymium magnet was used to draw the magnetic beads into the iridocorneal angle, blocking aqueous humor outflow through the trabecular meshwork and elevating IOP [352]. Both eyes were injected with beads to eliminate the confounding factor of contralateral eye effects on glial activation [345]. Separate mice injected with saline served as controls. Minimum damage to ocular structures was observed. Ten IOP measurements per eye using a TonoLab tonometer (Colonial Medical Supply) were averaged; a baseline measurement

was taken before bead injection, then weekly measurements after bead injection. The IOP integral (mm Hg-days exposure over baseline) was calculated to quantify cumulative IOP elevation [180]. Fixed retinae were cryoprotected in 30% sucrose (Sigma Aldrich) and 0.02% sodium azide (Sigma Aldrich) in 0.1M PBS and embedded in optimal cutting temperature medium. Sagittal sections at 10 to 15 μm using a Leica cryostat were obtained. Six to ten representative slides (three-four sections/slide) were imaged using a Nikon Eclipse microscope (Nikon). Mean intensity of Iba1 in microglia soma were analyzed using four sections per slide, and ten slides per retina using FIJI [362]. Data was derived from the central regions of the retina, consistent with above methods of intensity measurements.

Data analysis: Statistical analysis was performed using GraphPad Prism software version 9.0.0 (Graphpad Software). Normality of data was tested using the Shapiro-Wilk test. Significant differences between two unrelated groups were assessed by unpaired Student's t-test; paired Student's t-tests employed when making comparisons between eyes of the same mouse. For comparisons among three or more groups, one-way analysis of variance (ANOVA) with Dunnet's multiple comparisons (MC) test was used. For comparisons among groups with two variables, two-way ANOVA followed by Tukey's multiple comparison's test was applied. For quantification of calcium imaging and sustained IOP measurements one-way ANOVA with repeated measures with Sidak's test for multiple comparisons was used. For Sholl analysis, two-way ANOVA with repeated measures (RM) with Dunnet's test for MC was used. Results returning $p<0.05$ were considered significant. Data is represented as mean ± standard deviation.

2.4 Results

P2X7 receptor stimulation leads to morphologic and molecular activation of microglia in vivo.

The response of mouse retinal microglial cells to P2X7 receptor agonist BzATP was investigated *in vivo* to determine if receptor stimulation was sufficient to evoke morphologic changes. BzATP (250 μM) or saline control was injected intravitreally into eyes of C57Bl/6J mice, and retinae removed after 24 hours; this dosage and time course were chosen based on previous

trials [168,247]. Treatment with BzATP led to elevated Iba1 staining and retracted microglial processes, consistent with microglial activation (Fig. 2.1a, b) [6,7]. While some degree of increased staining for Iba1 was found throughout the retinal layers (Fig. 2.1c, d), analysis was focused on the retinal ganglion cell and inner plexiform layers as the response there was more consistent.

To quantify the extent of process retraction induced by P2X7 receptor stimulation, images were traced to produce binary outputs, and Sholl analysis was performed (Fig. 2.1e, Supplemental Figure S2.1a) [290]. Retinal BzATP exposure led to a significant reduction in microglial process length and complexity compared to saline controls (Fig. 2.1f). Peak intersection distance (Fig S2.1b) and longest intersection distance (Fig. S2.1c) were both reduced upon exposure to BzATP. Furthermore, the cumulative length of all branches was reduced by BzATP (Fig 2.1g), representing approximately a 10% reduction in summed process length (Fig. S2.1d). Iba1 intensity was measured in a defined area (5 μm) encircling the microglial soma in randomly selected microglia from saline- or BzATP-injected eyes (Fig. 2.1h, i). Exposure to BzATP led to a significant elevation of Iba1 immunostaining intensity when compared to control counterparts (Fig. 2.1j), reflecting a combination of increased soma size and Iba1 expression. Analysis of individual images supported microglial activation in retinae exposed to BzATP (Fig. S2.1e-i). Sholl analysis and soma staining was supported by evaluation of Iba1 intensity and process retraction by masked observers, with consistent signs of activation across all quadrants in retinae exposed to BzATP (Fig. 2.1k, S2.2a-d).

qPCR was used to evaluate the molecular changes accompanying P2X7 receptor stimulation. Genes associated with classical M1 activation, *Nos2* and *Tnfa*, and genes associated with the alternative M2 activation state, *Arg1* and *Chil3*, were elevated in retinae 24 hours after *in vivo* exposure to BzATP (Fig. 2.1l). Together, the morphological and molecular changes are consistent with microglial activation after injection of agonist BzATP.

The response of microglial cells in isolated *ex vivo* retinal whole mounts to agonist BzATP was examined to determine if resident microglia were sufficient for the observed increase in staining and alterations in morphology following P2X7 receptor stimulation. Retinal whole mounts derived

from heterogeneous mice with a fluorescent tag attached to microglia/macrophage receptor Cx3CR1$^{+/GFP}$ were used to provide an additional method to track changes in microglial morphology. Exposure to BzATP led to a considerable increase in fluorescence, with morphological changes resembling those observed after BzATP injection *in vivo* (Fig. 2.2). The increased signal was most noticeable around the optic nerve head (Fig. 2.2a, b), with prominent cell bodies apparent. Exposure to BzATP also increased the signal throughout the central (Fig. 2.2c, d) and peripheral retina, and across retinal layers (Fig. 2.2e, f). This *ex vivo* response in isolated retina suggests that microglia normally resident within the retina are capable of responding to P2X7 receptor stimulation, although it cannot rule out recruitment of additional monocytes following exposure *in vivo*.

P2X7 receptor stimulation leads to morphologic and molecular activation of isolated microglial cells

Isolated microglia cells were examined to determine whether stimulation of the P2X7 receptor could induce effects on microglial cells directly. The relative staining for Iba1 and astrocyte marker GFAP, and neural marker synaptophysin suggested preparations contained >95% microglial cells (Fig. S2.3a, b). Similar analysis confirmed identity of brain microglia as shown in more detail recently [60]. To support the microglial identity of the cells, the ability of lipopolysaccharides (LPS) and interleukin-4 (IL-4) to induce expression of activation state markers was determined using qPCR, as these two agonists are traditionally associated with classical and alternative activation states, respectively [67,243]. 4-hour stimulation of isolated retinal microglial cells with LPS (10 ng/ml) increased expression of *Nos2* and *Tnf*, while stimulation with IL-4 (10 ng/ml) increased expression of markers for the alternative activation state such as *Chil3* and *Arg1* (Fig. S3c). These responses resemble those recently found for isolated brain microglia [60] and suggest cells cultured under these conditions responded as predicted for microglial cells.

Immunocytochemical staining indicated Iba1-positive isolated retinal microglial cells expressed the P2X7 receptor (Fig. 2.3a). Functional expression of the P2X7 receptor was assessed by examining levels of cytoplasmic Ca^{2+} with the ratiometric indicator Fura-2. A one-minute addition of BzATP raised cytoplasmic Ca^{2+} in the microglial cells (Fig. 2.3b, c); the response was rapid, with

most cells showing a response within 20 sec. The response was also reversible upon wash-out of BzATP, and repeatable upon reapplication; these characteristics are consistent with an ionotropic channel with little inactivation like the P2X7 receptor as observed previously [142,367], and resembled the response reported recently in isolated brain microglia [60]. The P2X7 receptor-specific inhibitor A839977 [164] significantly reduced the Ca^{2+} rise triggered by BzATP, with the robust response to BzATP after removal of the A839977 confirming this decrease (Fig 2.3b, c). These responses support the presence of functional P2X7 receptors on these isolated cells, while the responses to IL-4 and LPS (Fig. S2.3c) supports their characterization as microglial cells.

The effect of BzATP on morphology of isolated microglia was examined to determine whether changes observed *in vivo* could be replicated in the absence of other cell types. BzATP triggered a retraction of microglial processes and a rounding of the cell body in greater than 75% of observed cells (Fig. 2.4a). This response was rapid, starting less than 7 minutes after BzATP application; an additional movie files show this in more detail (Fig. S2.4). The effect of BzATP on microglial morphology was greatly reduced in the presence of inhibitor A839977, supporting action of BzATP at the P2X7 receptor. This suggests that stimulation of the P2X7 receptor acting directly on microglial cells was sufficient to trigger the rapid morphological changes seen *in vivo*.

Stimulation of the P2X7 receptor on isolated microglial cells also induced changes in gene expression with parallels to those observed *in vivo* after P2X7 receptor stimulation. Specifically, the endogenous agonist ATP (Fig. 2.4b), and P2X7 receptor agonist BzATP (Fig. 2.4c) both increased expression of *Nos2* and *Arg1*.

To determine if the effects of P2X7 receptor stimulation on microglial process retraction extended to migration, chemoattraction of isolated retinal microglial cells to ATP was evaluated using a 2-part Boyden Chamber. Imaging of the filter with bound microglia confirmed elevated migration of retinal microglia towards an ATP concentration gradient (Fig. 2.4d). Measurements indicated that the number of Hoechst-stained microglial cells closely reflected total Hoechst fluorescence (Fig. 2.4e), with migration levels optimal 3 hours after the addition of cells to the chamber. Microglial migration towards 1 mM ATP was inhibited by inclusion of P2Y12 receptor

inhibitor AR-C 69931 with the ATP, but not inclusion of P2X7 receptor inhibitor A839977 with the ATP (Fig. 2.4f), suggesting the effects of P2X7 receptor stimulation are focused on retraction of ramifications and not the actual migration of retinal microglia.

Role of ATP and the P2X7 receptor in pressure-dependent microglial activation

Given that increased IOP is associated with both ATP release and microglial activation, experiments investigated the role of the P2X7 receptor in the morphological and molecular changes induced by IOP elevation. The effect of moderate transient IOP elevation on microglia cells was examined by increasing IOP to 57 mmHg for 4 hours, with retinal whole mounts fixed 24 hours later and stained for Iba1; previous studies indicated robust inflammatory responses at this point [6]. Iba1 staining revealed a noticeable change in microglial morphology in eyes exposed to elevated IOP, with larger cell bodies and shorter processes in retinal tissue exposed to elevated IOP as compared to control (Fig. 2.5a). As mechanosensitive release of ATP is an early and sustained event found after pressure elevation in bovine, mouse, rat and primate eyes [6,250,340], ATP concentration was determined as soon as pressure returned to baseline in the transient model. ATP levels sampled in the vitreal humor near the inner limiting membrane were significantly elevated in eyes subjected to increased IOP as compared to normotensive controls (Fig. 2.5b). Given the presence of elevated ATP, the response to IOP elevation was examined in retinae from P2X7$^{-/-}$ mice. Microglia cells from P2X7$^{-/-}$ mice showed a smaller change in morphology after transient IOP elevation (Fig. 2.5c). Histological quantification confirmed a clear increase in the morphological signs of activation in microglial cells from retinae of C57Bl/6J retinae following IOP elevation, and a reduced sign of activation in retina from P2X7$^{-/-}$ mice (Fig. 2.5d).

The pattern of gene change following transient IOP elevation was similar to that found after BzATP injection, with elevation of classical activation markers *Nos2* and *Tnfa*, and genes associated with the alternative activation state, *Arg1* and *Chil3* (Fig. 2.5e). The expression of genes *Tnfa* and *Arg1* increased in retina of P2X7$^{-/-}$ mice after exposure to elevated IOP, but the rise was smaller and there was no significant change in *Nos2* or *Chil3* expression (Fig. 2.5f). Overall, the change in expression of genes following IOP elevation was reduced in retina from P2X7$^{-/-}$ mice as

compared to C57Bl/6J mice (Fig. 2.5g). Altogether, both morphological and molecular changes following transient IOP elevation are consistent with a contribution from the P2X7 receptor.

As glaucoma is primarily a chronic disorder, responses were examined in retinae from C57Bl/6J mice subjected to sustained elevation of IOP via magnetic bead blockage of aqueous humor outflow (Fig. 2.6a,b). Similar morphological differences were observed in Iba1 stained cells in the sections from central retinal regions of mice with bead injection, including retraction of processes, cell soma swelling, and increased expression of Iba1 when compared to saline-treated retinae (Fig. 2.6c). Quantification of Iba1 intensity in the soma area showed significant elevation with sustained IOP elevation (Fig. 2.6d).

Retinal ganglion cell loss, microglial activation and the P2X7 receptor

The relationship between microglial activation, the P2X7 receptor and retinal ganglion cell loss was examined to better understand the consequences of microglial activation to ganglion cell health. Retinal whole mounts used above were co-stained for the ganglion cell transcription factor Brn3a and the number of cells present in each of the 24 regions (see Fig. S2.1i) were counted. In C57Bl/6J mice, IOP elevation led to a modest reduction in Brn3a-positive cells as compared to normotensive eyes (Fig. 2.7a), although little change was seen in $P2X7^{-/-}$ mice (Fig. 2.7b). Quantification confirmed a significant decline in the number of Brn3a-positive cells with IOP elevation in C57Bl/6J mice, but not $P2X7^{-/-}$ mice (Fig. 2.7c). Close overlap between microglial and retinal ganglion cells occurs throughout the retina (Fig. 2.7d), although quantification indicated the ratio of microglia to RGCs differed in peripheral regions as compared to central and middle areas (Fig. S2.5a,b), supporting the focus on these regions. Analysis of activation from individual images across retinal regions supports these findings for microglial and retinal ganglion cell quantification (Fig. S2.5b, c).

The microglial activation score was compared to ganglion cell number in all images in the central and middle areas for C57Bl/6J mice (Fig. 2.7e), and $P2X7^{-/-}$ mice (Fig. 2.7f). Images with fewer ganglion cells tended to show greater microglial activation; this relationship was greater for C75Bl/6J mice (slope -27.0) than for $P2X7^{-/-}$ mice (slope -10.7). The correlation between microglial

activation score and ganglion cell number was closer for C75Bl/6J mice with a Pearson's r of 0.442, vs 0.227 for P2X7$^{-/-}$ mice, although considerable variability across the eyes and between mice of both strains limited the strength of the overall correlation.

2.5 Discussion

The data presented in this manuscript illustrate several complex consequences of P2X7 receptor activation in microglial cells. Detailed morphological analysis indicated that administration of P2X7 receptor agonist BzATP to murine retina *in vivo* reduced branch length, and increased soma size and Iba1 expression in microglial cells, emblematic of microglia activation. Furthermore, retinal exposure to BzATP led to gene expression upregulation of *Nos2, Tnfa, Arg1,* and *Chil3*, associated with microglial activation into a mixed classical (M1)/alternative (M2) state. *Ex vivo* retinal explants demonstrated comparable morphological changes following exposure to BzATP. Isolated retinal microglial cells displayed parallel molecular changes after exposure to ATP or BzATP *in vitro*, with upregulation of classical activation marker *Nos2* and alternative activation marker *Arg1*. Transient elevation of IOP led to similar morphological changes, with larger cell bodies and shorter processes, and increased expression of *Nos2, Tnfa, Arg1,* and *Chil3.* However, both morphological and molecular changes were reduced in retinae from P2X7$^{-/-}$ mice. The similarities in the morphological and molecular changes induced by BzATP and elevated IOP, combined with the reduced responses in P2X7$^{-/-}$ mice to IOP elevation, implicate the P2X7 receptor in some of the early inflammatory responses to increased pressure in the retina.

Several observations strengthen the findings of this study. 1) The convergence of morphological evidence from *in vitro* and *in vivo* experiments supports the validity of conclusions, with rapid retraction of microglial extensions within moments of BzATP application to isolated cells complementing the detailed Sholl analysis *in vivo*; 2) Convergent molecular data from *in vitro* and *in vivo* experiments imply P2X7 receptor stimulation upregulates markers traditionally associated with both M1 and M2 activation states; 3) Parallel morphological effects of BzATP on retinal explants imply resident retinal microglial cells were sufficient to produce this response without

recruitment of external cells; 4) While BzATP can act at other purinergic receptors, use of P2X7$^{-/-}$ mice, combined with antagonist A839977 (IC_{50} 150 nm, [164]) and immunohistochemical identification strongly implicates the P2X7 receptor; 5) Boyden chamber studies suggest the P2X7 receptor effect is focused on retraction of microglial ramifications and not on chemoattractant migration; 6) Use of models for both transient and sustained elevation of IOP, combined with live-cell imaging, strengthen understanding of the time course of the response to P2X7 receptor stimulation. Overall, the data considerably extend our understanding of the P2X7 receptor, microglial activation and increased pressure.

P2X7 receptor and microglial activation states

The data above support the theory that the molecular response to transient P2X7 stimulation represents a mixed M1/M2 microglial cell state. A 4-hour stimulation of the P2X7 receptor in cultured microglia with 1 mM ATP or 200 μM BzATP upregulated classical activation gene marker *Nos2* and alternative activation gene marker *Arg1* (Fig. 2.4b,c), paralleling upregulation of classical activation markers *Nos2* and *Tnfa* and alternative activation markers *Arg1* and *Chil3* with *in vivo* administration of BzATP, or elevation of IOP (Fig. 2.1l; Fig.2.5e). P2X7 receptor stimulation had been shown to lead to upregulation of classical activation markers in in a variety of inflammatory cell types [3,52,378], with a study on the SOD-G93A model of Amyotrophic Lateral Sclerosis suggesting upregulation of alternative activation markers [103]. Given the emerging role of the P2X7 receptor in modulating phagocytosis, autophagy and lysosomal clearance by microglial cells [60], this mixed activation may have important implications for the health of aging tissues. Previous studies utilizing acute elevation of IOP in rats have focused on neurotoxic cytokine release from microglia [392]. However, directing microglia away from neuroinflammatory states is a putative therapeutic for conditions like brain trauma that are accompanied with transient ATP elevation [4,169,208]. A full understanding of putative anti-inflammatory therapies for glaucoma and their effects on microglia, such as P2X7 receptor inhibition [110,346], carbon monoxide [46,432], antibiotics [22,40], or downstream effectors [122] will help elucidate the role classical and alternative activation pathways play in retinal degeneration.

Microglial activation as an early event in retinal degeneration.

The findings above add relevant details to a growing body of evidence implicating microglial activation in various retinal degenerations including glaucoma. Activated microglia and associated inflammatory markers TNF-α and NOS-2 have been detected at the optic nerve head in human glaucomatous eyes [302,441]. Morphological changes to microglial cells were detected in 3 month old DBA/2J mice, prior to detection of neural damage [42], and documented 3 days after induction of ocular hypertension in rats [94,298]. Purinergic involvement in the microglial response was suggested by the ability of purinergic receptor blocker OxATP to inhibit the rise in CD68 expression in rat retina following transient elevation of IOP to 90 mm Hg [392]. Qualitative analysis also suggestsed antagonist Brilliant Blue G reduced staining of Iba1 following injection of hypertonic saline into rat episcleral veins [94]. The current study indicates the microglial response occurs early, with activation found 24 hours after BzATP administration (Fig. 2.1), 24 hrs after a 4 hour elevation of IOP (Fig. 2.4), and with retraction occurring within minutes of receptor stimulation *in vitro* (Fig S2.4). Within this context, the inability of P2X7 antagonist A839977 to significantly prevent microglial migration (Fig. 2.4f) distinguishes process retraction from cell migration; whether the P2X7 receptor interacts with THIK-1 channel recently implicated in microglial surveillance remains to be tested using sufficiently high agonist levels [260].

The relationship between microglial activation and retinal ganglion cell loss is complex. Detailed analysis of microglial activation and surviving ganglion cell number indicates an inverse correlation following IOP elevation which was reduced in P2X7$^{-/-}$ mice (Fig. 2.7). While it is tempting to assume this implicates microglial activation in ganglion cell death via the P2X7 receptor, several observations urge caution. For example, the variation amongst the 24 images analyzed per retina and between mice was considerable, weakening the correlation. In addition, stimulation of the P2X7 receptor can directly kill retinal ganglion cells [168], while ganglion cell death can itself lead to microglia activation [397]. Activation of A1 astrocytes has been implicated as a key step in pressure-related ganglion cell death [231,383], and the geometric relationship between astrocytes, microglial cells, ganglion cell soma and ganglion cell axons is complex, especially as the axons are thought

to be a primary target in glaucomatous pathology [387,403]. P2X7 receptor inhibition has been implicated as a therapeutic strategy for several retinal diseases including Age-related Macular Degeneration [110,309], Diabetic Retinopathy [71,116,325,326], and glaucoma [247,281,323,346,392]. While topical administration of a P2X7 receptor antagonist preserved general RGC function in the DBA2/J model [346], recent work suggests the P2X7 receptor also has beneficial effects on retinal ganglion cells after moderate IOP elevation [421], and can trigger the release of neuroprotective cytokine IL-3 [233]. These complex responses may also reflect the mixed microglial activation state response identified in the present study. The precise relationship between microglial activation, ganglion cell death and the P2X7 receptor is likely context dependent and awaits future clarification.

In summary, our results support a model whereby P2X7 receptor stimulation alone is sufficient to cause microglial activation, and that this activation occurs rapidly after receptor stimulation or following ATP release with elevation of intraocular pressure. Furthermore, although the P2X7 receptor is traditionally associated with its proinflammatory role[3], P2X7 receptor stimulation here led to a mixed activation state in microglial cells, suggesting the response is complex. Microglia activation was increased with elevated IOP, reduced in $P2X7^{-/-}$ mice and was loosely associated with loss of retinal ganglion cells following increased IOP. The impact of the mixed M1/M2 activation state on the response to P2X7 receptor stimulation, and the corresponding influence on both inflammation and phagocytosis/clearance needs to be determined [60]. As P2X7 receptor modulation is being targeted for retinal disorders [110], the quantitative approaches used in this study can add to a deeper understanding of P2X7 receptor signaling and any putative beneficial effects.

2.6 Figures

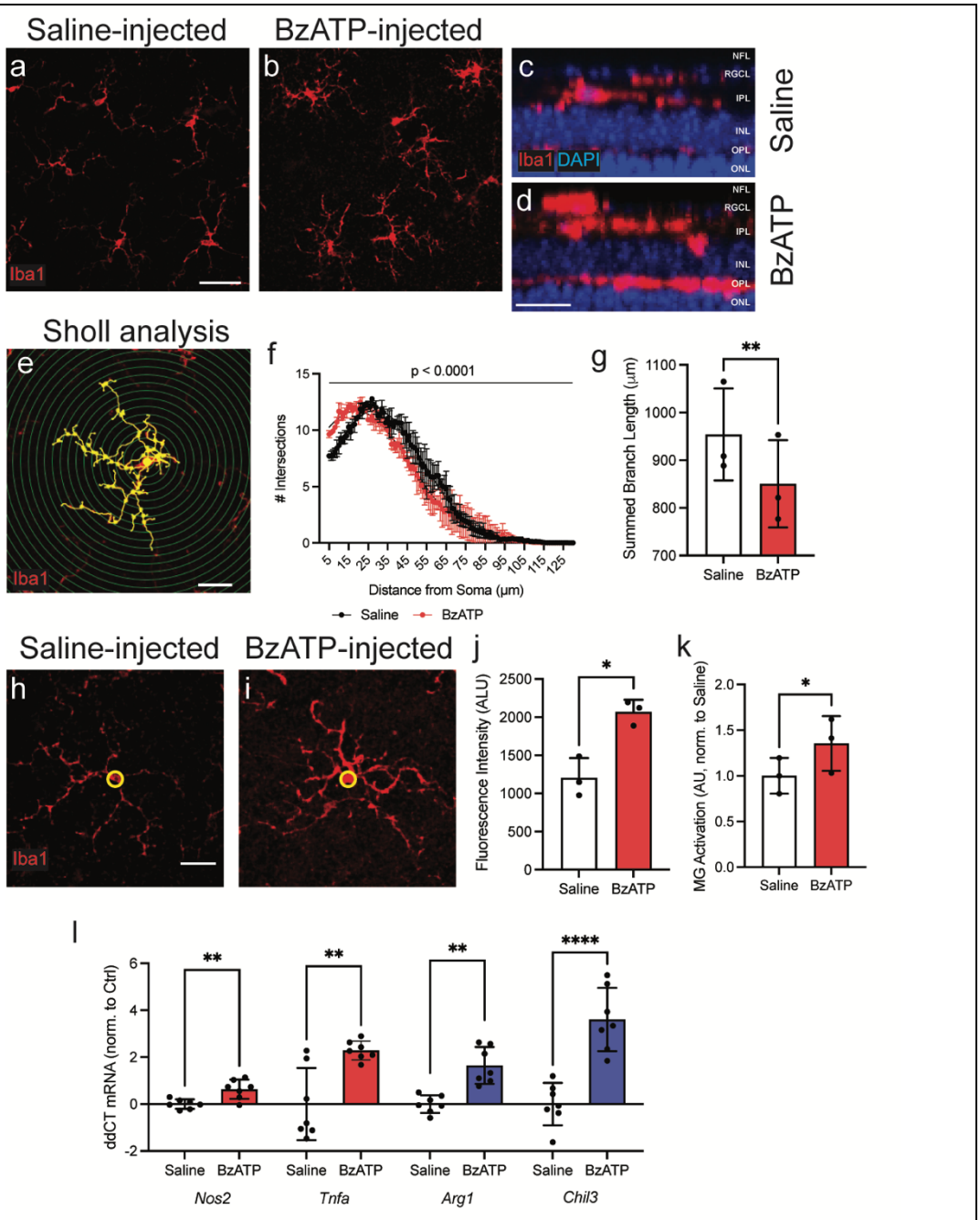


Figure 2.1. Retinal P2X7 receptor stimulation leads to activated microglia morphology and gene expression. Representative images from retinae fixed 24 hrs after intravitreal injection with saline (**a**) or 250 µM BzATP (**b**) indicated BzATP exposure led to greater Iba1 expression and altered morphology. **c, d** Z-projections of retina whole mounts demonstrate increased Iba1 staining in the IPL and RGC layers of the retina with BzATP exposure. **e** Representation of tracing and conversion of Iba1 staining to a binary image for Sholl analysis. **f.** Sholl analysis indicated reduced branching complexity of microglia exposed to BzATP (2-way RM ANOVA with

Sidak's MC Test; n=3 mice; Significance represents Interaction value of ANOVA). **g** Summed branch length is reduced in microglia exposed to BzATP as compared to Saline (paired Student's t-test; n=3 mice). Cell soma size and Iba1 intensity are determined in circled area (yellow ring, 5 µm diameter) of microglia cell body from retinae exposed to (**h**) saline vs. (**i**) BzATP. **j** Quantification of Iba1 intensity in selected area (paired Student's t-test; n=3 mice). **k** Observer scoring of images taken from saline- or BzATP-exposed retinae showing Iba1-positive microglia are activated with BzATP exposure (paired Student's t-test; n=3 mice; normalized to average saline value). **l** Expression of both classical M1- activation genes *Nos2, Tnfa*, and alternative M2 activation genes *Arg1, Chil3* are elevated in retinae exposed to BzATP (paired Student's t-test; n=7 mice)**.** Statistical significance shown at *p<0.05, **p<0.01, ****p<0.0001. Scale bars represent 40 µm (**a**), 15 µm (**d**), 25 µm (**e, h**).

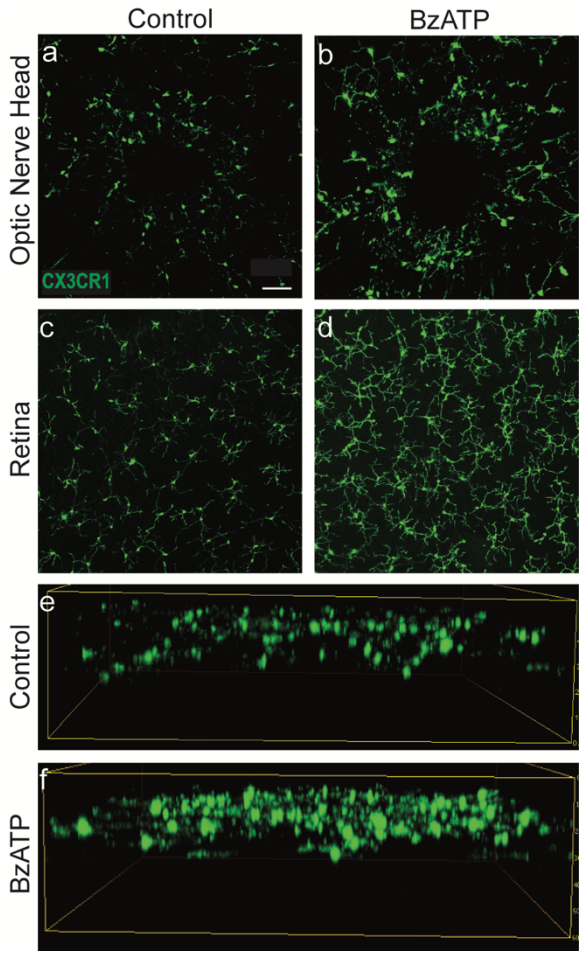


Figure 2.2. *Ex vivo* retinal whole mount P2X7 receptor stimulation activates microglia. a, b Retinal whole mounts isolated from Cx3CR1$^{+/GFP}$ mice revealed increased fluorescence at the optic nerve head compared to control media (**a**) after exposure to 200 μM BzATP for 2 hours in a petri dish (**b**). This elevation in fluorescence was also seen in the Middle Nasal areas when comparing control media (**c**) to BzATP exposure (**d**). e, f Z-projection of Middle-Nasal retinae exposed to control media or BzATP. Bar = 50 μm.

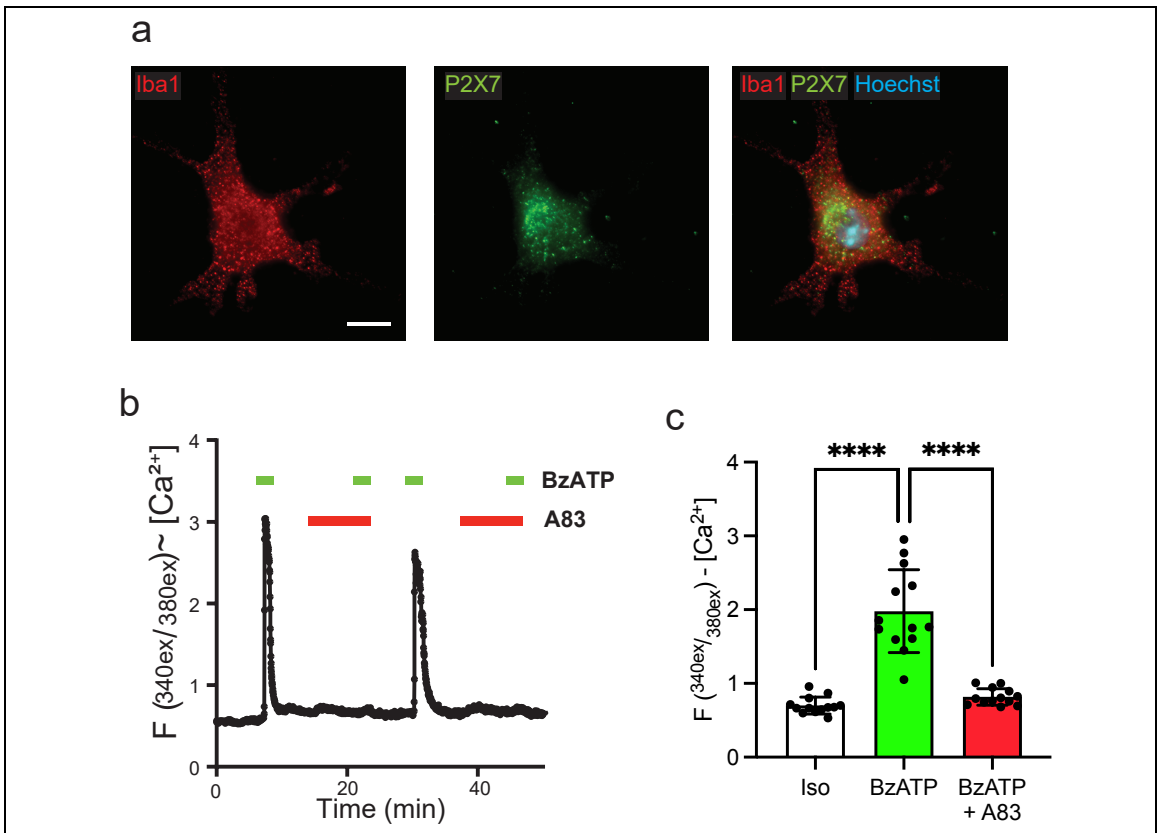


Figure 2.3. Isolated microglial cells express P2X7 receptor on protein and functional levels. a Immunostaining of P2X7 receptor (green) in primary retinal microglial cells stained positive for Iba1 (red). Bar = 10µm. **b** Representative trace from a retinal microglia cell loaded with Fura-2 showing elevation in cytoplasmic Ca^{2+} in response to BzATP (100 µM, Mg^{2+} free solution). Data displayed as the ratio of light excited at 340/380 nm, emitted > 520 nm (referred to as "F(340/380)"). The response to BzATP was transiently reduced by 1µM P2X7 antagonist A839977 (A83). **c** Quantification of the response in multiple cells (RM 1-way ANOVA with Sidak's MC Test; n=12 cells from 3 culture preparations).

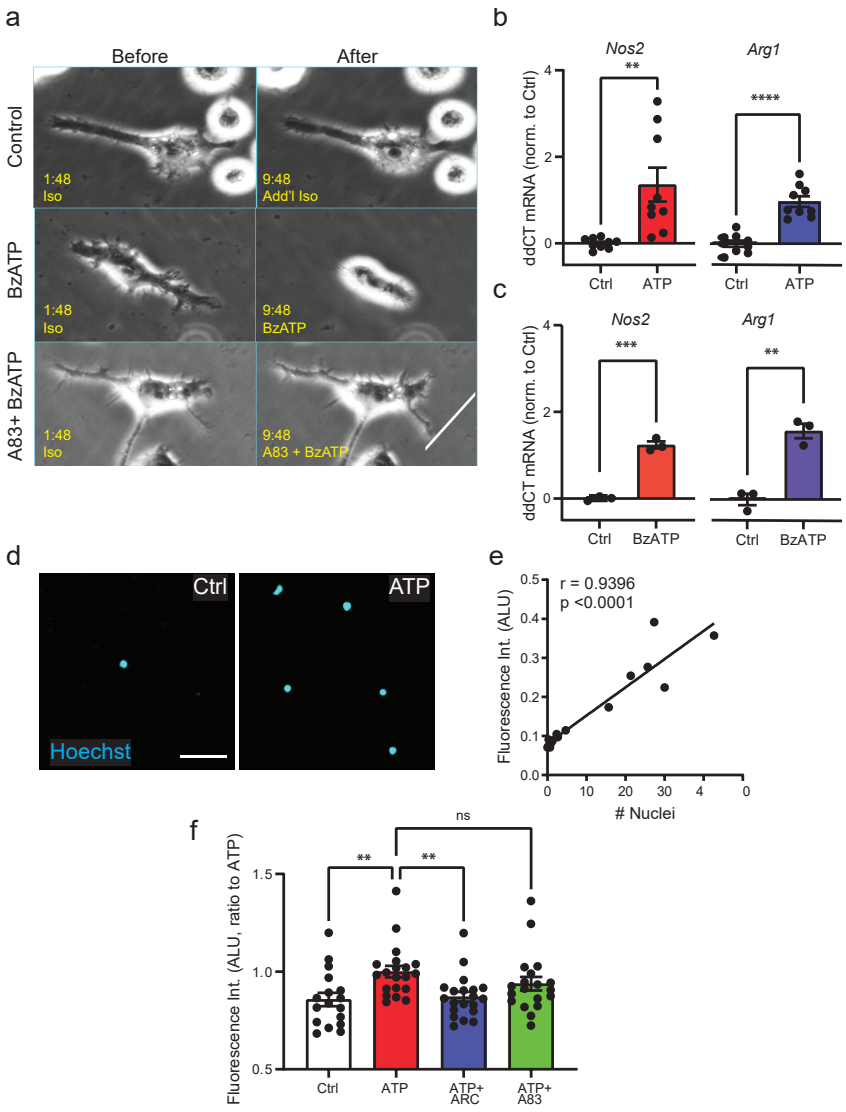


Figure 2.4. Isolated retinal microglia respond to P2X7 receptor stimulation with rapid retraction, gene activation but not migration. a Images of isolated retinal microglial cells taken before (left) and ~ 8 min after (right) application of isotonic solution (Control), 250 μM BzATP (BzATP), or 250 μM BzATP (BzATP) ± 10 μM A839977 (A83) suggests P2X7 receptor leads to process retraction *in vitro*. Similar responses were found in >7 experiments. Bar=10μm, real time in min on image; solution added at minute 3:00; see Fig. S4 for video. Elevated expression of *Nos2* and *Arg1* was detected in cultured retinal microglial cells following 4 hr exposure to 1 mM ATP (**b,** n=9 wells from 3 culture preparations) or 200 μM BzATP (**c,** n=3 wells from 1 culture preparation; unpaired Student's t-test). **d** Representative images of isolated retinal microglial cells with Hoechst-stained nuclei after passing through a Boyden chamber, indicated that microglia migrate towards a 1 mM ATP gradient. Bar=50 μm. **e** Correlation between number of Hoechst-stained nuclei in brain microglia per well and fluorescence at 340ex/527em (Pearson's correlation r = 0.9396 with p = 0.0001; n=17 wells from 1 culture preparation). **f** Migration of retinal microglia towards 1 mM ATP was inhibited by exposure to 10 μM P2Y12 inhibitor AR-C 69931 (ARC) in the presence of ATP but not 1 μM A839977 (A83) in the presence of ATP. (1-way ANOVA with Sidak's MC Test; n=17 Ctrl, 20 ATP, 20 ARC, 19 A83 wells from 4 culture

preparations). Statistical significance shown as **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.

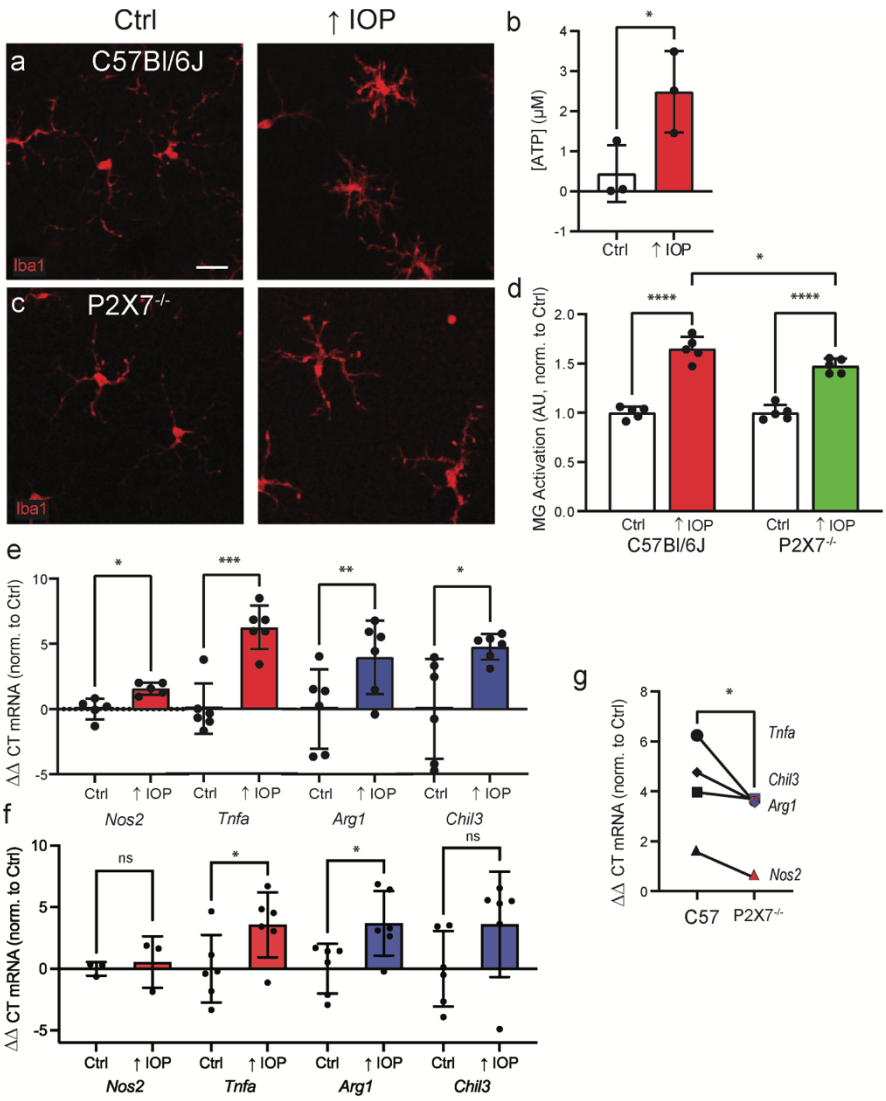


Figure 2.5. Elevation of IOP releases ATP and activates microglia through P2X7 receptor involvement. a Representative image of staining for Iba1 in a retinal whole mount from an unpressurized C57Bl/6J mouse eye (Ctrl, left) and from an analogous region 20 hrs after elevation of IOP to 57 mmHg for 4 hrs (right). Retinal microglia subject to IOP elevation showed increased soma size, increased staining for Iba1, and shorter, thicker projections. Bar=20μm. **b** Increase in ATP concentration of posterior vitreous humor after elevation of IOP (paired Student's t-test; retinal pairs from 3 mice). **c** Iba1 staining from a P2X7$^{-/-}$ mouse indicates reduced morphological activation after elevation of IOP (right) when compared to unpressurized retina (left). **d** Quantification of morphological activation of microglia across central and middle regions suggests IOP elevation triggered greater morphological activation in C57Bl/6J than P2X7$^{-/-}$ retinae (2-way ANOVA with Tukey's MC test; dots are n=10 retinae from 5 mice per C57Bl/6J, P2X7$^{-/-}$ strain; data normalized to unpressurized score for each strain). qPCR showing increased expression of *Nos2, Tnfa, Arg1,* and *Chil3* in the retina after elevation of IOP in C57Bl/6J (**e**) mice. Dots represent change in expression from a single mouse, with expression

normalized to the average ΔΔCT value of unpressurized contralateral eyes (paired Student's t-tests; *Nos2:* n= 5 mice, *Tnfa, Arg1, Chil3:* n= 6 mice). **f** A small but significant increase was observed in retinal gene expression of *Arg1*, and *Tnfa,* but not *Nos2* or *Chil3* after IOP elevation in P2X7$^{-/-}$ mice (Paired Student's t-tests; *Nos2* n=6 retinae from 3 mice, *Tnfa, Arg1, Chil3,* n=12 retinae from 6 mice). **g** Relative change in retinal expression of key genes after elevation of IOP in C57Bl/6J mice compared to P2X7$^{-/-}$ mice. Values represent mean ΔΔCT levels for each gene compared to unpressurized control retinae (unpaired Student's t-test; n=5 genes). Statistical significance shown as *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001, ns=not significant.

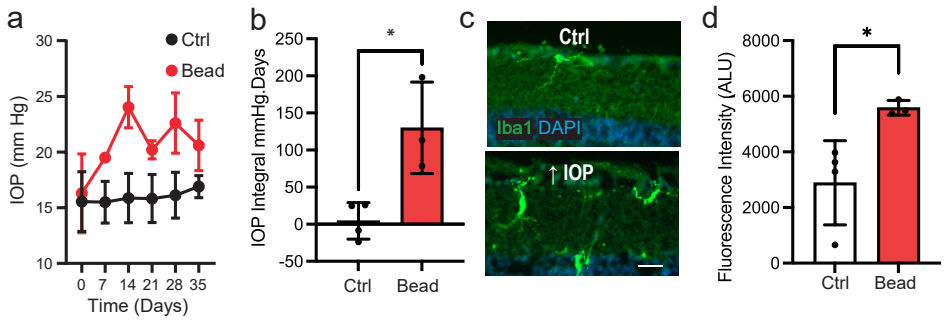


Figure 2.6. Microglial activation in bead model of sustained ocular hypertension. a Weekly IOP measurements from mice injected with magnetic beads (red) or saline control (black; 1-way RM ANOVA with Sidak's MC test). b The IOP integral (right) expressed as summed mmHg days exposure over baseline IOP, for bead and saline-injected eyes (unpaired Student's t-test). c Representative staining for Iba1 in cryosections 7 weeks after injection with saline (top) or beads (bottom), suggesting sustained elevation of IOP also induces changes in the microglial phenotype emblematic of activation. Bar=50 µm. d Quantification of a 5 µm area surrounding the soma indicates significant elevation of Iba1 intensity per cell in bead-injected mice (unpaired Student's t-test). All data from Ctrl: n=4 retinae from 3 mice, Bead: n=3 retinae from 3 mice. Statistical significance shown as *p<0.05.

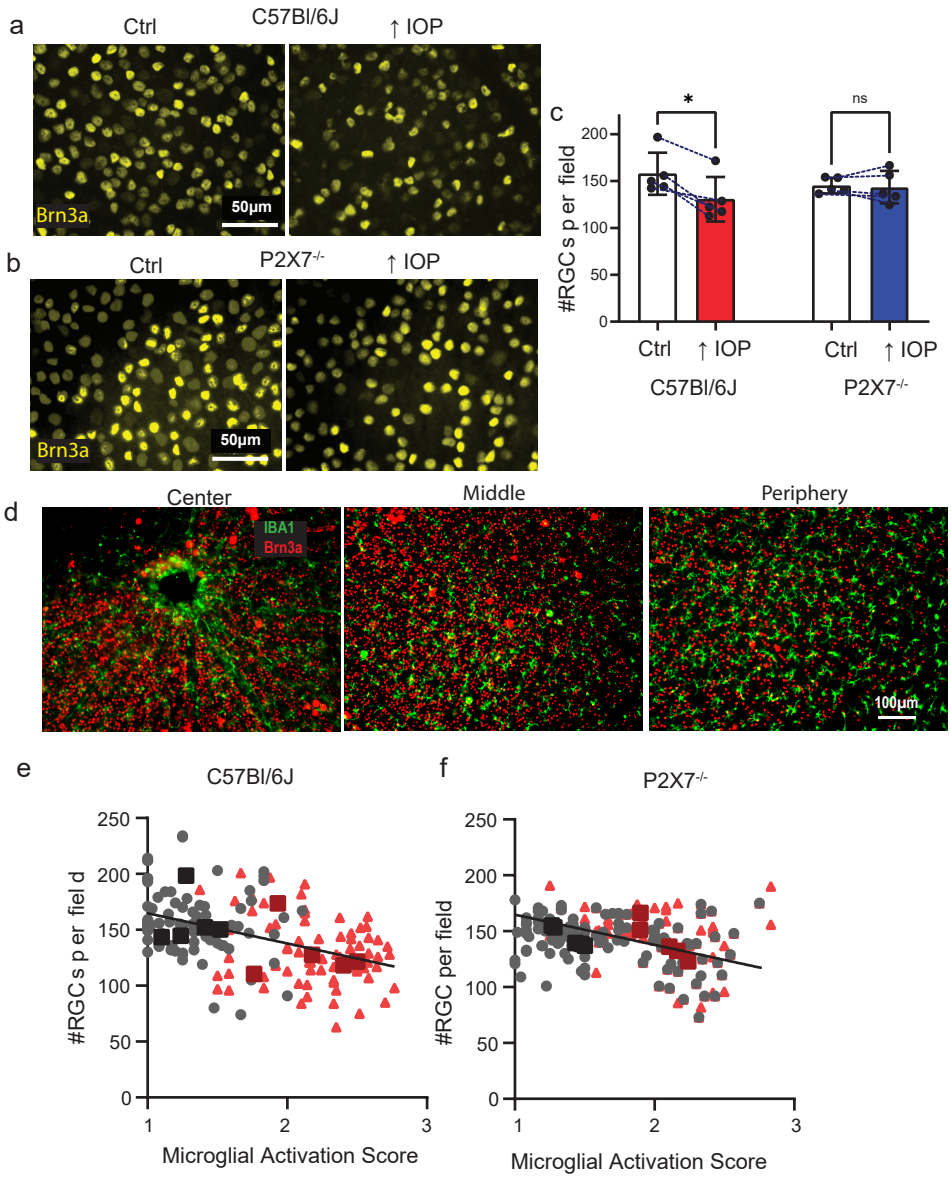
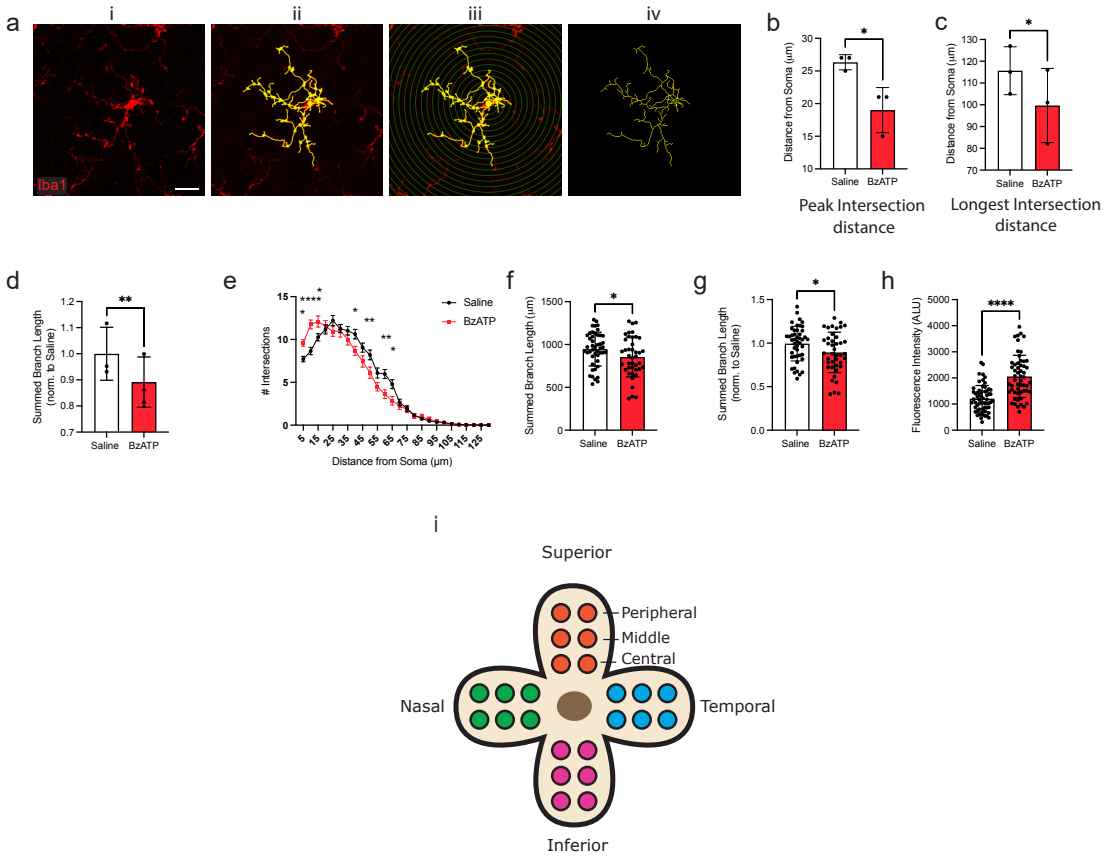
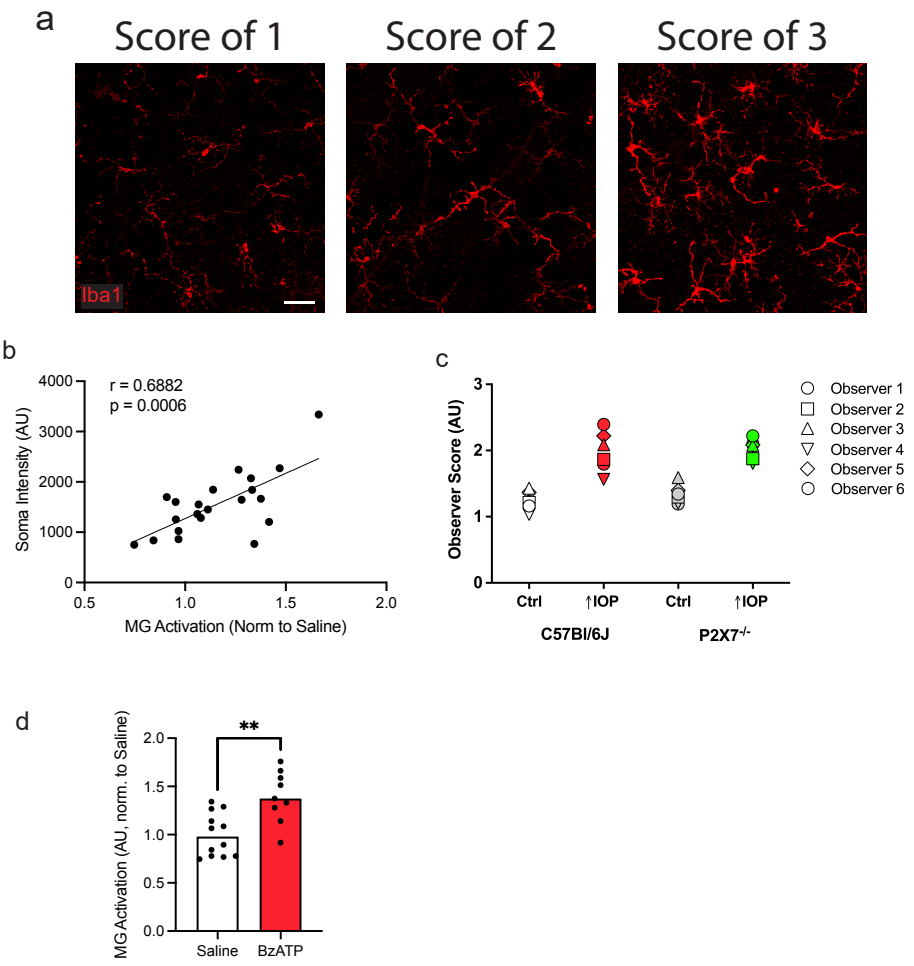


Figure 2.7. Ganglion cell death and microglial activation. a Representative images show that staining for RGC marker Brn3a is decreased in retinae 1 day after a 4 hr IOP elevation (right) compared to unpressurized C57Bl/6J mice (Ctrl; left). **b** A decrease in Brn3a staining was not observed in retinae from P2X7$^{-/-}$ mice after IOP elevation as compared to unpressurized control eyes. **c** There was a significant decline in Brn3a-labeled RGCs in retinae exposed to elevated IOP compared to normotensive controls from C57Bl/6J eyes, but not P2X7$^{-/-}$ mice (*p=0.0017, paired t-test with line connecting normotensive and elevated IOP from same mouse, n=5 mice, mean of ~ 24 images per eye). **d** Retinal whole mount from a C57Bl/6J mouse showing the spatial relationship between RGCs stained with Brn3a (red) and microglia stained with Iba1 (green); images show staining across the central region with the optic nerve head (left), the middle region (center), and peripheral areas (right), of the retina. Relationship between RGC number and microglial activation score for C57Bl/6J (**e**) and P2X7$^{-/-}$ mice (**f**) under normotensive (grey) and elevated IOP (red) conditions. Small symbols from individual images (up to 24 per eye) while larger squares represent mean values per eye (n=5 per condition). Lines are linear

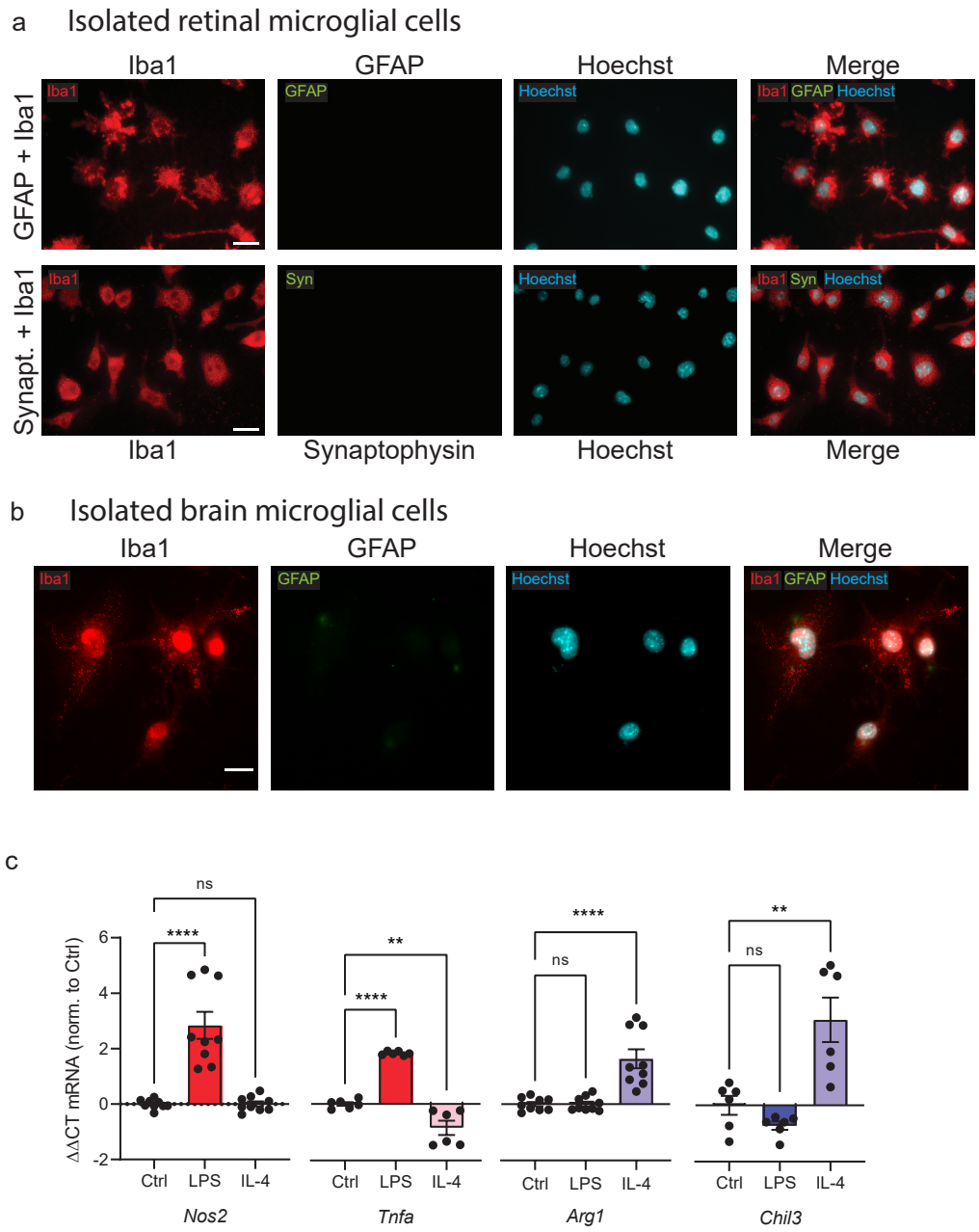
regression fit to image data (C57Bl/6J: $F=37.70$, $(1,155)$ $p<0.0001$, $R^2=0.196$; $P2X7^{-/-}$ $F=8.22$, $(1,152)$, $p=0.0047$, $R^2=0.05$).



Supplementary Figure S2.1. Details of Sholl and other image analysis. a Representations of i- Iba1 staining; ii- image tracing, iii - concentric rings placed every 5µm for analysis of crossing and length; and iv - conversion to a binary image. Bar = 25 µm. **b, c** Tracings were analyzed for distance from soma at which peak number of intersections with concentric rings (**b**) and farthest intersection with concentric ring (**c**) occurred (paired Student's t-test; n = 6 retinae of 3 mice). **d** Total summed branch length averaged per mouse was reduced by approximately 10% (paired Student's t-test; n = 6 retinae of 3 mice; Normalized to mean saline injected= 1.0000, mean 250 µM BzATP = 0.8914). **e** Sholl analysis performed with individual cells (2-way Repeated Measures ANOVA with Sidak's Multiple Comparison's Test; n = 41 Saline, 46 BzATP) cells from 6 retinae of 3 mice; significance represents Multiple Comparison's, data measured every 5 µm). **f**, **g** Absolute (**f**) and normalized (**g**, to avg saline) of Sholl data from individual images confirms reduction in branch length of microglia exposed to BzATP (unpaired Student's t-test; n = 41 Saline, 46 BzATP cells from 6 retinae of 3 mice; Normalized mean saline = 0.9941, mean BzATP = 0.8930). **h** Quantification of Iba1 intensity in 5µm ring around soma of individual cells from 6 retinae from 3 mice (unpaired Student's t-test; n = 60 saline, 55 BzATP cells from 6 retinae of 3 mice). i. Schematic diagram indicating approximate placement of images analyzed for microglia and RGCs. *p<0.05, **p<0.01, ****p<0.0001.

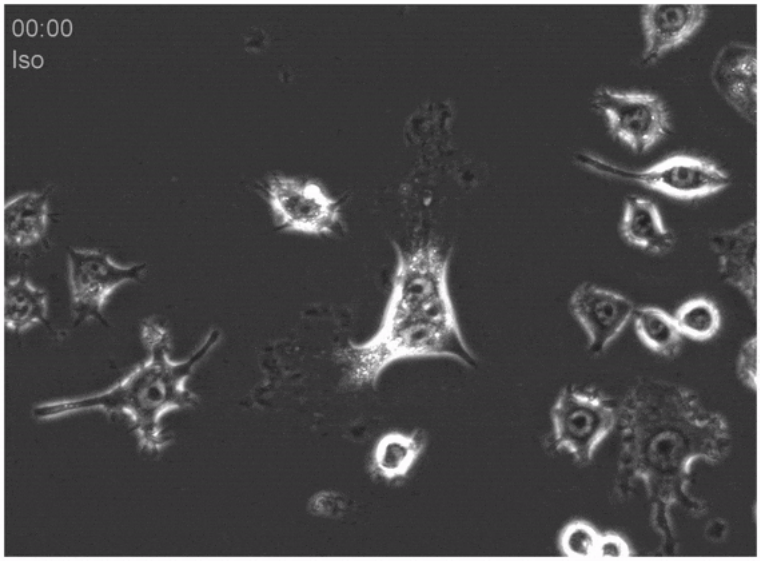


Supplementary Figure S2.2. Validation of observer scoring. a Representative images derived from Iba1-immunostained retinal whole mounts indicating a score of 1, 2, or 3. Bar=50µm. **b** A significant correlation between observer scoring and microglial Iba1-soma intensity was found. Observer scoring was normalized to the mean of 3 saline injected mice, while soma intensity was quantified in a 5µm diameter ring as described in Figure 1. Each dot represents the mean value among masked, trained observers (Pearson's correlation r = 0.6882 with p = 0.0006; n = 21 images, 6 retinae from 3 mice). **c** Scoring of microglial activation shows homogeneity between trained observers. **d** Observer scoring of images taken from saline- or BzATP-exposed retinae analyzed by image (unpaired Student's t-test; n = 12 saline, 9 BzATP images, from 6 retinae of 3 mice). **p<0.01. Bar = 50 µm.



Supplementary Figure S2.3. Confirmation of cellular identity of isolated microglial cells. **a** Immunocytochemistry indicating absence of glial marker GFAP (top) or neuronal marker synaptophysin (Synapt, bottom) in primary cultures of retinal microglial cells. Bar=20µm. **b** Isolated brain microglial cells stained for Iba1 but not GFAP. Bar=20µm. **c** qPCR results from cultured retinal microglial cells exposed for 4 hrs to DMSO (Ctrl), 10 ng/ml LPS (LPS), or 10 ng/ml IL-4 (IL4); changes in relative expression of mRNA for *Nos2, Tnfa, Arg1, and Chil3* were consistent with microglial cells polarization. (n=6-9 samples from 2-3 biological replicates).

a



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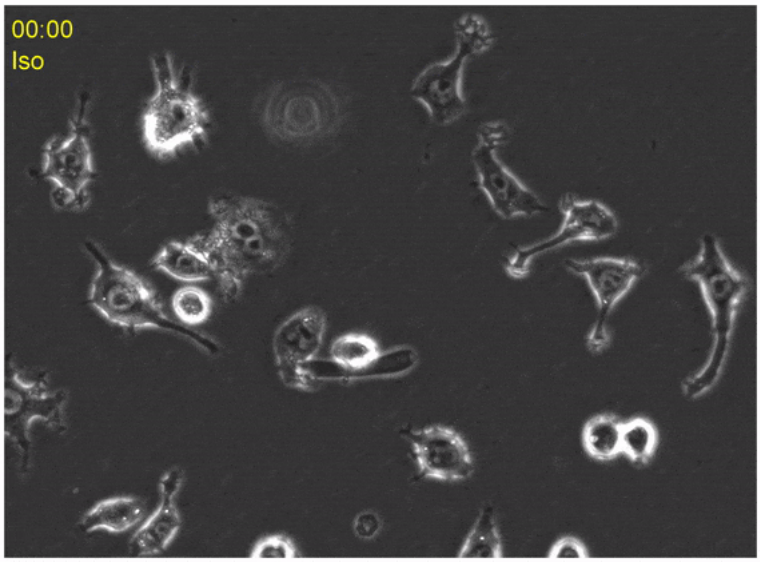
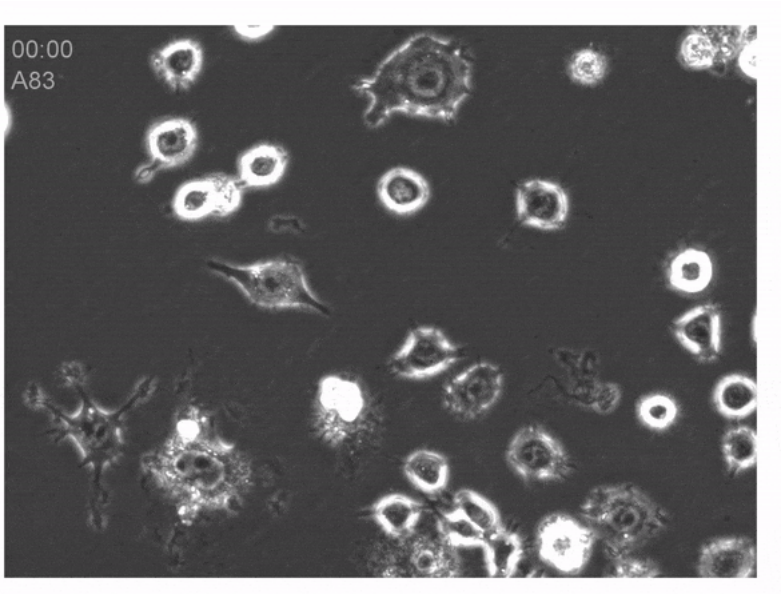
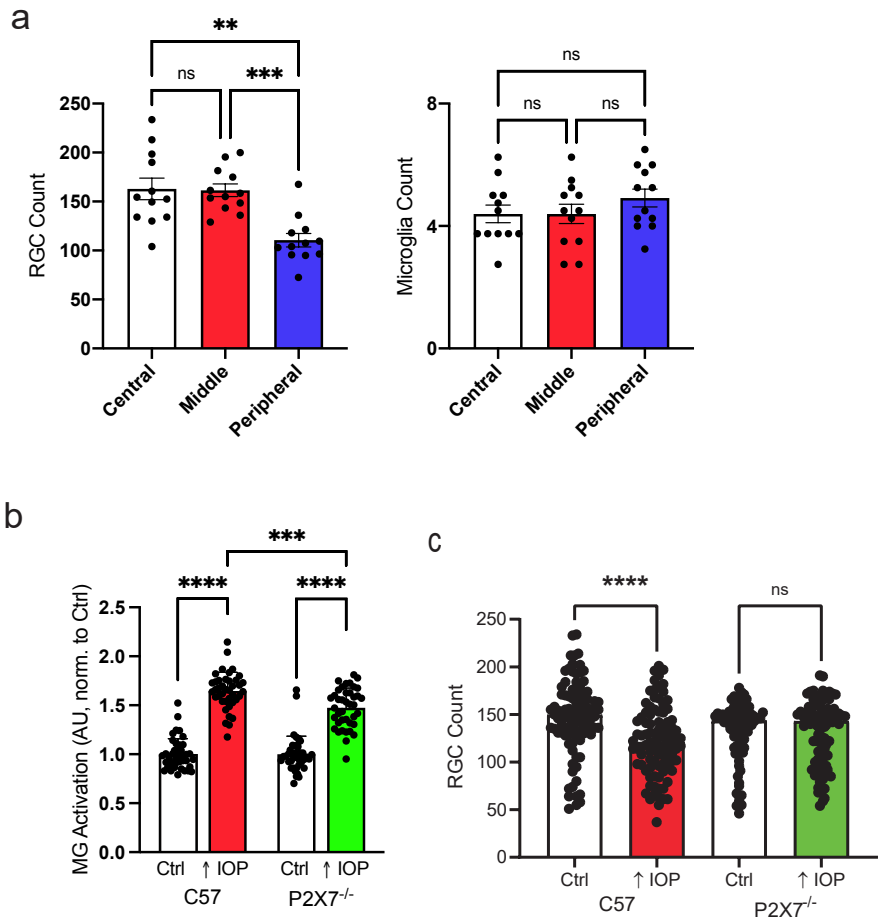


Supplementary Figure S2.4. Videos demonstrating microglial retraction with exposure to BzATP. Videos demonstrating microglial retraction with exposure to BzATP. 15 minute videos were recorded at 15 frames per second, with all solutions added at t = 3 min. **a** Addition of fresh control solution led to few morphological changes. **b** Addition of 250 μM BzATP resulted in rapid retraction of microglial extensions and rounding of cells.

c



Supplementary Figure S2.4 (continued). c In the presence of 10 μM A839977 (and following 30 min preexposure to 10 μM A839977) ,250 μM BzATP induced little retraction. All solutions Mg^{2+}-free to prevent block of the P2X7 receptor.



Supplementary Figure S2.5. Microglia and cell counts in the retina. a RGCs (left) and microglia (right) cell numbers were counted per image and averaged within superior inferior, nasal, or temporal regions. There was a significant difference in distribution of RGCs in peripheral retinal areas (RM 1-way ANOVA with Sidak's MC test; n=12 regions from 3 mice). **b** Quantification of morphological activation from individual images of microglia across central and middle regions suggests greater activation in C57Bl/6J retinae than in P2X7$^{-/-}$ retinae (2-way ANOVA with Tukey's MC test; n=52 regions from 10 retinae derived from 5 mice per C57Bl/6J, P2X7$^{-/-}$ strain; data normalized to unstimulated score for each strain). **c** RGC numbers counted in central and middle fields under normotensive (Ctrl) and increased IOP in mice from both genotypes. Statistical significance shown as ** p<0.01, ***p<0.001, ****p<0.0001, ns=not significant.

CHAPTER 3: STIMULATION OF THE P2X7 RECEPTOR CONTRIBUTES TO GENERATION OF NEUROTOXIC REACTIVE ASTROCYTES

3.1 Abstract

Neuronal death is associated with polarization of astrocytes into the A1 neuroinflammatory state. While this polarization can be initiated by release of C1q, TNF-α and IL-1α from microglial cells, the upstream effectors stimulating their release are unclear. This preliminary study investigates the role of P2X7 receptor (P2X7R) stimulation in the increase in these inflammatory signals and the effects on transcription of astrocyte inflammatory genes. 250 μM of P2X7R agonist BzATP. qPCR was performed on retinae 24 hrs after intravireal injection of 250 μM P2X7 agonist BzATP. Additionally elevation of intraocular pressure (IOP) was applied to C57Bl/6J or P2X7$^{-/-}$ retinae for 4 hrs, with subsequent isolation of retinae 22 hrs later for qPCR analysis. Intravitreal injection of P2X7 receptor agonist BzATP increased expression of *Tnfa*, *Il1a*, and *C1qa*. Levels of pan-reactive, A1-specific, and 2 A2-specifc genes were elevated, with activation was most consistent with the A1 state. Preliminary data evaluating the effects of IOP on retinal gene expression in C57Bl/6J mice indicated elevated expression of *Tnfa* and *Il1a*, A1-specific gene *Serping1*, and upregulation of A2-specifc genes *CD14*, *Ptx3,* and *Clcf1*. Trending upregulation ($p<0.1$) was observed of pan reactive astrocyte genes *Gfap* and *Vim*, and A1-specific genes *C3* and *H2T23*. Elevation of IOP in P2X7$^{-/-}$ retinae resulted in no upregulation of microglia or astrocyte inflammatory genes. Taken all together, this implicates P2X7R stimulation in promotion of microglia inflammation, and astrocyte polarization into an A1 neurotoxic state. Surprisingly, preliminary data suggests that P2X7R involvement in the elevation of IOP is less clear, with upregulation of A2 neuroprotective genes, and trending upregulation of A1 genes observed in C57Bl/6J retinae but not P2X7$^{-/-}$ retinae. Caution should be merited before any encompassing conclusion can be made, as further experimentation to support this data is necessary.

3.2 Introduction

Mechanical strain is associated with the release of ATP in many tissues[20,81,426], where it mediates an inflammatory response through ionotropic P2X or metabotropic P2Y receptors[51,234]. The P2X7 receptor (P2X7R) is a major inflammatory receptor of the Central Nervous System (CNS)[3]. Stimulation of P2X7R with high concentrations of ATP lead to activation of the NLRP3 inflammasome and release of inflammatory cytokines[3,129,359]. Signaling mechanisms elucidated in models of mechanical strain provide valuable insight into pathology of glaucoma, where symptomatic elevated intraocular pressure contributes to optic nerve damage and blindness through unknown mechanisms[415,428].

Recent work has pointed to the loss of neural populations in models of mechanical strain as mediated by neurotoxic astrocytes[145,383]. Reactive astrocytes have been detected in several models of neurodegeneration[145,231,383,442]. Astrocytes respond to inflammatory effectors by adopting A1 pro-inflammatory or A2-neuroprotective forms, as defined by transcriptional alterations[444]. Neurotoxic astrocytes were induced by exposure to cytokines IL-1α, C1q, and TNF-α *in vitro* leading to loss of neurons[231]. Deletion of *Il1a*, *Tnfa*, and *C1qa* protected neurons and reduced neurotoxic astrocyte gene upregulation in mouse models of chronic elevation of IOP[145,383]. However, upstream mediators that lead to release of these cytokines are unclear.

Microglia are implicated as primary effectors of release[230,231]. Microglia are the primary innate immune effector cells of the retina, where they play roles in retinal homeostasis, cytokine release, and phagocytosis of apoptotic cells[374]. Microglia activation into an inflammatory state is contributes to neuron loss in the DBA/2J model of elevated IOP[42] and in human glaucoma[441]. Microglia are the major source of IL-1α[412], C1q[47,111,320], and TNF-α[330]. Moreover, microglia are the major cell type to express P2X7R[189]. P2X7 receptor stimulation results in release of IL-1α[129] and TNF-α (REF). Taken together, these implicate microglial P2X7R as key mediators of neurotoxic astrogliosis developed from elevated IOP.

Using *in vivo* administration P2X7R agonist BzATP and elevation of IOP in models where the P2X7 receptor is deleted ($P2X7^{-/-}$), we confirm the central role that the receptor has in triggering

astrogliosis. We demonstrate that retinal injections of BzATP upregulate gene expression of *Tnfa*, *Il1a*, and *C1qa*, as well as genes associated with A1-specific astrocytes. Additionally, elevation of IOP in C57Bl/6J mice upregulated retinal expression of *Tnfa* and *Il1a*, with significant or trending upregulation of both A1- and A2-specific astrocyte transcripts. Gene upregulation of *Tnfa*, *Il1a*, or astrocyte inflammation was not observed in P2X7$^{-/-}$ that was subjected to elevated IOP. Thus, we demonstrate that P2X7R stimulation mediates factors associated with astrocyte inflammation that accompany IOP-induced mechanical strain.

3.3 Methods

Animals care and use

All procedures were performed in strict accordance with the National Research Council's "Guide for the Care and Use of Laboratory Animals". All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) in protocol #803584. All animals were housed on a 12:12 light:dark cycle in temperature-controlled rooms with food and water ad libitum. Mice (C57Bl/6J wild type and P2XR7-/- B6.129P2-P2rx7tm1Gab/J Pfizer were obtained from Jackson Laboratories (Bar Harbor, ME).

Intravitreal Injections

Intravitreal injections were performed as previously described[168]. In brief, C57Bl/6J mice were anesthetized with 1.5% isofluorane, and subsequently injected under a dissecting microscope using a micropipette attached to a microsyringe (Drummond Scientific Co., Broomall, PA, USA). 1.5 µl was injected the vitreous cavity approximately 0.5 mm anterior to the limbus, into superior nasal region of the sclera. Injections consisted of Sterile Balanced Saline solution as a control with or without 250 µM Benzoylbenzoyl-ATP (BzATP, #B6396, Sigma Aldrich). Retinae were isolated 24 hrs later. The concentration and time course of isolation after administration was chosen based upon previous trials[6,247].

Table 3.1: Primers used for qPCR

Gene name	Genbank accession	Primer (F: 5'-3'; R: 3'-5')	Size (bp)
Tnfa	NM_013693.3	F: AAATGGCCTCCCTCTCATCAG R: GTCACTCGAATTTTGAGAAGATGATC	73
Il1a	NM_010554.4	F: CAACGTCAAGCAACGGGAAG R: AAGGTGCTGATCTGGGTTGG	126
C1qa	NM_007572.2	F: GAAGGGCGTGAAAGGCAATC R: CAAGCGTCATTGGGTTCTGC	86
Gfap	NM_001131020.1	F: CCTGCCAGCTCTCCCT R: AAAGGTGTGGCTGAAATGCG	216
Steap4	NM_054098.3	F: CCCGAATCGTGTCTTTCCTA R: GGCCTGAGTAATGGTTGCAT	262
Vim	NM_011701.4	F: GATGGCCCTGGACATTGAGA R: TTGAGTGGGTGTCAACCAGAG	146
Lcn2	NM_008491.1	F: CCAGTTCGCCATGGTATTTT R: CACACTCACCACCCATTCAG	206
SerpinA3N	NM_009252.2	F: GCTGGCTGGTTTCAGCTCT R: ATCCATTCCCAACGTGCCAT	127
Aspg	NM_001081169.1	F: GCTGCTGGCCATTTACACTG R: GTGGGCCTGTGCATACTCTT	133
C3	NM_009778.3	F: TTCCTTCACTATGGGACCAGC R: CTCCAGCCGTAGGACATTGG	127
Serping1	NM_009776.3	F: ACAGCCCCCTCTGAATTCTT R: GGATGCTCTCCAAGTTGCTC	299
H2D1	NM_010380.3	F: TCCGAGATTGTAAAGCGTGAAGA R: ACAGGGCAGTGCAGGGATAG	204
H2T23	NM_010398.3	F: GGACCGCGAATGACATAGC R: GCACCTCAGGGTGACTTCAT	212
Amigo2	NM_178114.4	F: GAGGCGACCATAATGTCGTT R: GCATCCAACAGTCCGATTCT	263
Fkbp5	NM_010220.4	F: TATGCTTATGGCTCGGCTGG R: CAGCCTTCCAGGTGGACTTT	194
Fbln5	NM_001361987.1	F: CTTCAGATGCAAGCAACAA R: AGGCAGTGTCAGAGGCCTTA	281
CD14	NM_009841.4	F: GGACTGATCTCAGCCCTCTG R: GCTTCAGCCCAGTGAAAGAC	232
Ptx3	NM_008987.3	F: AACAAGCTCTGTTGCCCATT R: TCCCAAATGGAACATTGGAT	147
Clcf1	NM_019952.5	F: CTTCAATCCTCCTCGACTGG R: TACGTCGGAGTTCAGCTGTG	176
Emp1	NM_010128.4	F: GAGACACTGGCCAGAAAAGC R: TAAAAGGCAAGGGAATGCAC	183
Slc10a6	NM_029415.2	F: GCTTCGGTGGTATGATGCTT R: CCACAGGCTTTTCTGGTGAT	217
GAPDH	NM_017008	F: TCACCACCATGGAGAAGGC R: GCTAAGCAGTTGGTGGTGCA	169

Quantitative PCR

Evaluation of RNA expression utilizing qPCR was based on published methods described previously[247]. In brief, after homogenizing in TRIzol reagent (#15596018, Invitrogen), RNA derived

from mice retinae was purified with an RNeasy mini kit (#79254, Qiagen, Inc.) and converted to cDNA was performed using the High Capacity cDNA Reverse Transcription Kit (#4368814, Applied Biosystems). Gene expression was assayed using PowerUp Sybr Green (#A25742, Applied Biosystems) on the Quant Studio 3 Real-Time PCR system (Applied Biosystems Corp.). Analysis was performed employing the delta-delta CT approach without conversion to RQ values as described[247]. Primers are listed in Table 3.1.

Data analysis

Data are displayed as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism software version 9.0.0 (Graphpad Software, Inc. San Diego Ca, USA). Significant differences between two related groups were assessed by paired Student's t-tests. Results returning $p<0.05$ were considered significant.

3.4 Results

BzATP injection increases expression of microglial genes associated with astrocyte inflammation.

We first determined if P2X7R stimulation was sufficient to increase expression of genes that are associated with inducing astrocytes into an inflammatory state. BzATP (250 μM) or Saline was injected intravitreally into the eyes of C57Bl/6J mice, and retinae were removed after 24 hours. Intravitreal injections of P2X7R agonist BzATP increased retinal expression of *Tnfa*, *Il1a*, and *C1qa* (Figure 3.1A).

As cytokines TNF-α, IL-1α, and C1q are implicated promoting astrocyte inflammation into a neurotoxic reactive state[230,231], we sought to determine the changes in transcripts associated with reactive astrocytes. Retinal exposure to BzATP upregulated Pan-reactive genes (*Gfap, Steap4, Vim, Lcn2, SerpinA3N,* and *Aspg*) (Figure 3.2A), A1-specific genes (*C3, Serping1, H2T23, H2D1,* and *Amigo2*, but not *Fkbp5* or *Fbln5*) (Figure 3.2B), and A2-specific genes (*CD14* and *Ptx3*, but not *Clcf1, Emp1, Slc10a6*) (Figure 3.2C).

Role of P2X7R in microglial and astrocyte inflammation after transient elevation of IOP.

As chronic elevation of IOP has generated neurotoxic reactive astrocytes in the retina[145,383], we sought to determine the role of P2X7R with transient elevation of IOP. In preliminary work, the IOP of eyes from C57Bl/6J or P2X7$^{-/-}$ was elevated for 4 hrs and examined 24 hrs later; previous studies have pointed to robust gene expression responses at this timepoint[6]. IOP elevation resulted in increased expression of *Tnfa* and *Il1a* in the retinae from C57Bl/6J mice (Figure 3.3A). Interestingly, gene expression increases were not significant in retinae from P2X7$^{-/-}$ mice (Figure 3.3B). However, direct comparisons of normalized mean gene expression of *Tnfa, Il1a,* and *C1qa* was insignificant, possibly indicated a need for a more robust sample set.

Gene expression of astrocyte inflammatory genes after transient elevation of IOP was subsequently assayed. In C57Bl/6J mice, Nonsignificant or trending significant upregulation of Pan-reactive transcripts was observed (Figure 3.4A). A1-specific gene *Serping1* was significantly elevated, with trending significant upregulation of *C3* and *H2T23*. Intriguingly, A2-specific transcripts *CD14*, *Ptx3*, and *Clcf1* were all significantly regulated. No inflammatory genes were significantly upregulated in retinae from P2X7$^{-/-}$ mice (Figure 3.4B). Comparing the mean normalized gene expression following transient elevation of IOP confirmed a reduced gene expression of astrocyte inflammatory genes in P2X7$^{-/-}$ mice as compared to C57Bl/6J mice (Figure 3.4C) indicating a role for P2X7R in inflammatory responses from elevated IOP.

3.5 Discussion

The factors that link the loss of neural populations to the mechanical strain that is observed with high IOP still remain uncertain. In patients, reduction of IOP has not proven consistent in preserving retinal ganglion cell populations[428], or maintaining visual function[379]. In patient samples, and in many animal models of IOP have demonstrated that inflammation is a considerable component of disease progression[415]. Recent work has pointed to a role of inflammatory neurotoxic astrocytes in loss of neural populations[230,231]. New therapies targeting intermediary processes may prove fruitful in reducing inflammation. Herein, we examined the role that P2X7 receptor plays in elevation of microglia cytokines and inducing neurotoxic astrocytes. Direct injection of P2X7-

selective agonist BzATP led to elevation gene expression of microglia inflammatory markers *Tnfa*, *Il1a*, and *C1qa*. Additionally, stimulation of retinal P2X7 receptor led to increased gene expression of several pan-reactive and A1-specific astrocyte genes. Transient elevation of IOP for 4 hours in retinae of C57Bl/6J mice resulted in upregulation of *Tnfa* and *Il1a*, and upregulation of both A1- and A2-specific genes. However, elevation of inflammatory markers was not observed after elevated IOP in retinae from P2X7R$^{-/-}$ mice, implicating P2X7R as upstream of astrocyte inflammation.

The impact of P2X7R stimulation on astrocyte inflammation state has not been explored. Retinal exposure to BzATP for 24 hrs demonstrated that P2X7R stimulation results in polarization to A1 neurotoxicity. P2X7R stimulation upregulated gene expression of complement cascade component genes *C3* and *Serping1*, as well as Major Histocompatibility Complex 1 (MHC I) genes *H2T23* and *H2D1*. *CD14* is considered indicative of A2 anti-inflammatory phenotype. However, the protein role in inflammation is complex, as it as membrane-bound CD14 acts as a co receptor for TL4 in LPS-mediated signaling[446]. Interestingly P2X7R stimulation prior to LPS-TLR4 stimulation has reduced inflammatory response in peripheral monocytes by increasing exocytosis of CD14 and depleting its membrane-bound counterpart[5]. Similarly, *Serping1* protein product functions as a complement component C1 inhibitor[70]. In that manner, P2X7R stimulation resulted in a neuroinflammatory state, without clear polarization. It should be noted, however, that definitions of astrocyte polarization are contextual, and likely represent a more complex phenomenon[26,101].

Transient elevation of IOP additionally resulted in expression of astrocyte inflammatory genes. This supports work using the microbead model of chronic elevation of IOP leading to upregulation of neurotoxic reactive astrocytes in ASCA2+ cell populations which implicates A1-astrocyte reactivity in the retina[145,383]. In both studies, astrocyte inflammation and loss of RGCs from elevated IOP in mice where *Il1a*, *Tnfa*, and *C1qa* were deleted[145], indicating that gene upregulation and cytokine release are necessary for astrocyte inflammation and RGC loss from elevated IOP, within the model at a minimum.

The P2X7 receptor is widely expressed, but at higher density on microglia within the CNS[189,395]. However, this study did not determine the cell specific expression of the genes that were evlauated. Many of the genes attributed to neurotoxic reactive astrocytes are implicated in the complement pathway and antigen presentation, microglia may contribute to the pool of upregulated genes. For example, previous work indicates that Müller glia are the primary cell population expressing *C3*[320], but C3 protein expression has been detected in microglia/macrophage populations in murine models and human patient samples of Amyotrophic Retinal Degeneration (AMD)[299] and Retinitis Pigmentosa (RP)[373]. As most previous work, including specifically in models of elevated IOP, focus only on one cell population, it can be said that astrocytes and Müller glia are contributors to gene upregulation within the retina, and are likely polarized into a neuroinflammatory state.

Similarly, the exact contribution of microglia remains uncertain. Microglia are postulated to be the upstream regulators of neurotoxic reactive astrocytes[230]. Though they are likely to be the cell population that is the primary source of TNF-α[330] and IL-1α[15], cell-specific identification of these factors *in vivo* has not been evaluated. *In vitro*, polarization of microglia with LPS led to the release of TNF-α, IL-1α, and C1q into cell supernatants, which then polarized *in vitro* astrocytes into an A1 neurotoxic phenotype[231]. This suggests that microglia are sufficient to induce neurotoxic astrocytes. Yet ablation of microglia with PLX5622 and subsequent application of the optic nerve crush (ONC) model of elevated IOP did not rescue the RGC loss that was observed when microglia ablation was not performed[231]. This may be due to infiltration from peripheral macrophages that are present in CD11b+CD11c+ populations that are early contributors to inflammation in mouse models of chronic elevated IOP[383,414]. However, the early timepoint (24 hrs) minimizes the chance of peripheral monocytes to migrate into the retina. Furthermore, work has demonstrated that BzATP administration to *ex vivo* retinae was sufficient to induce phenotypic activation of resident microglia, indicating that P2X7R stimulation of resident microglia is sufficient to induce an inflammatory response. Taken all together, the data generated in this study supports a model whereby microglial P2X7R stimulation on microglia promotes the release of cytokines that induce neurotoxic reactive

astrocytes (Figure 3.5). Regardless, the question of cell specific origin of *Tnfa*, *Il1a*, and *C1qa* remains an important one that can be addressed with future research.

In conclusion, our results extend the role of P2X7R as a means to induce neurotoxic reactive astrocytes, Furthermore, preliminary data indicates that transient elevation of IOP may promote reactive astrocyte inflammation through stimulation of P2X7R.

3.6 Figures

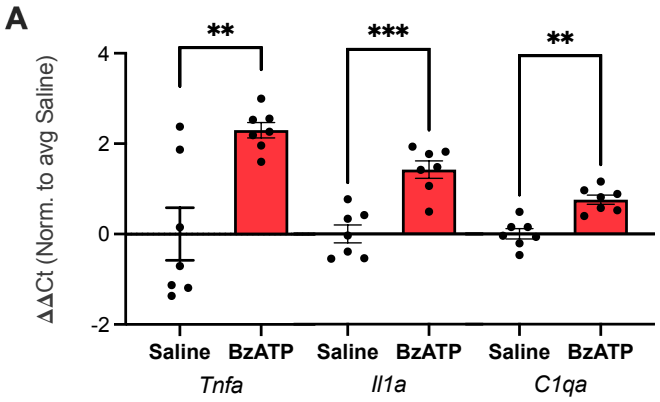


Figure 3.1. BzATP-injected retinae express microglia activation genes. A Retinae that were injected with saline (Saline) or 250 μM BzATP (BzATP) increased gene expression of *Tnfa*, *C1qa*, and *Il1a* after 24hrs, (n = 7 Saline and 7 BzATP-injected retinae; Paired Student's t-test; samples normalized to average of Saline-injected eyes). ** p<0.01, *** p<0.001.

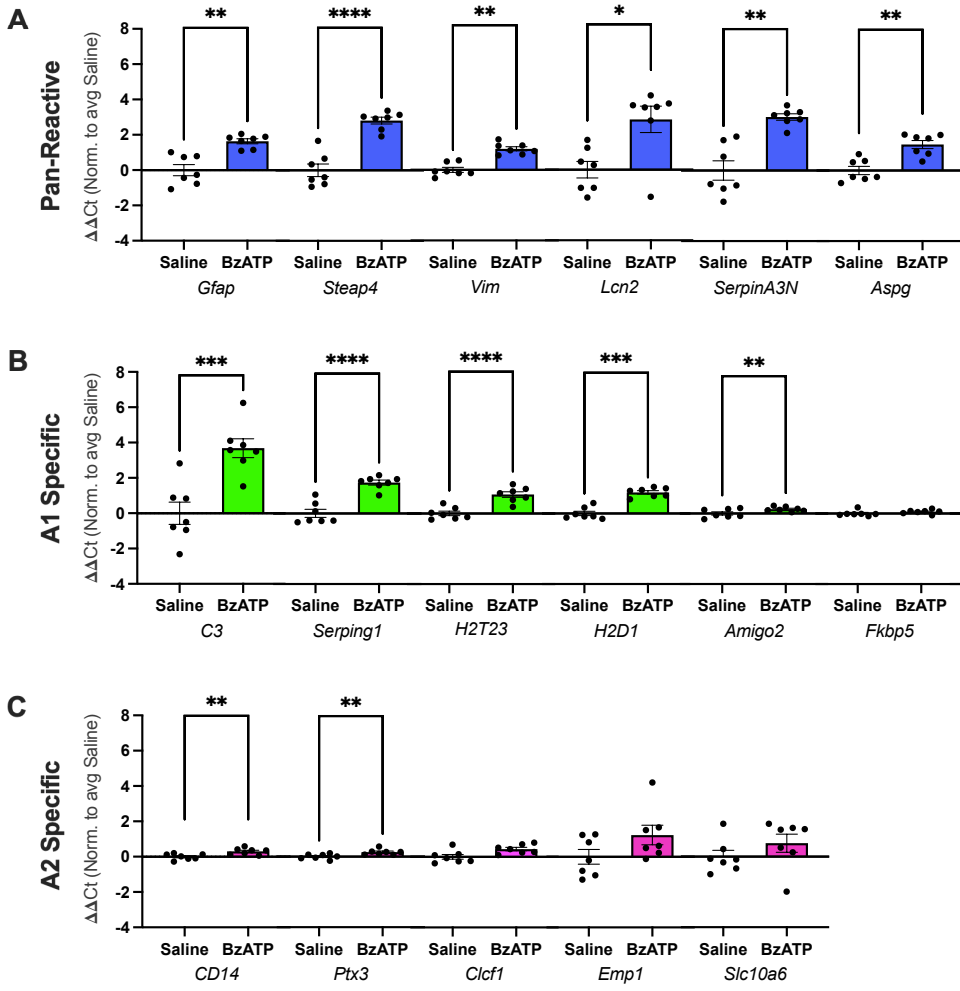


Figure 3.2. P2X7R stimulation leads to elevated gene expression of neurotoxic astrocyte genes. A Transcripts of pan-reactive astrocyte inflammation are upregulated in retinae exposed to BzATP **B** Astrocyte A1-specific genes *C3, Serping1, H2D1, H2T23,* and *Amigo2* are significantly upregulated. **C** Astrocyte A2-specific genes *Cd14* and *Ptx3* are significantly upregulated. All data represented as paired Student's t-test, normalized to average of Saline eyes; n = 7 Saline and 7 BzATP-injected retinae. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

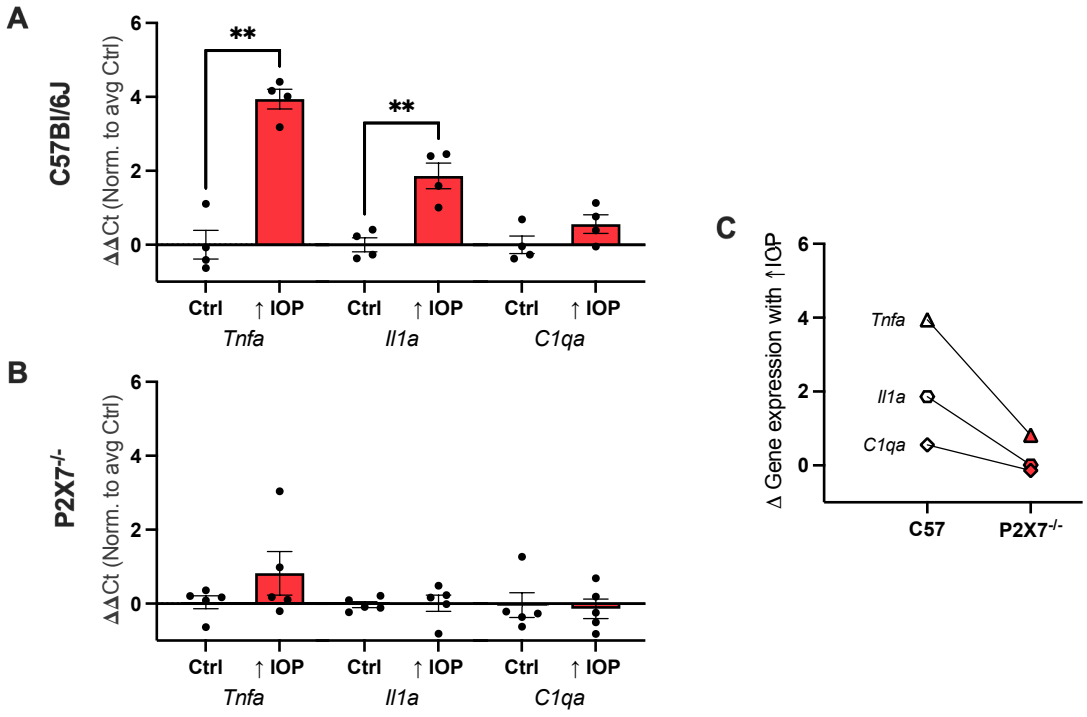


Figure 3.3. Elevated IOP leads to upregulation of genes associated with induction of neurotoxic astrocytes. A Elevation of IOP for 4 hrs followed with tissue harvest 24 hrs later resulted in elevated expression of *Tnfa* and *Il1a* in retinae from C57Bl/6J mice. **B** Similar elevation of IOP did not lead to significant gene expression changes in retinae from P2X7$^{-/-}$ mice. Data in **A**, **B** represented normalized to average of Ctrl eyes (Paired Student's t-test; n = 4 Ctrl, 4 IOP eyes from P2RX7$^{-/-}$ mice (**A**); n = 5 Ctrl, 5 IOP eyes from C57Bl/7J mice (**B**). **C** Relative change in expression of *Tnfa*, *Il1a*, and *C1qa* in retinae exposed to elevated in IOP in C57Bl/6J mice compared to P2RX7$^{-/-}$ mice. Data is represented as mean ΔΔCT levels for each gene in IOP-elevated C57Bl/6J retinae compared to IOP-elevated P2X7$^{-/-}$ retinae (n=3 genes). ** p<0.01

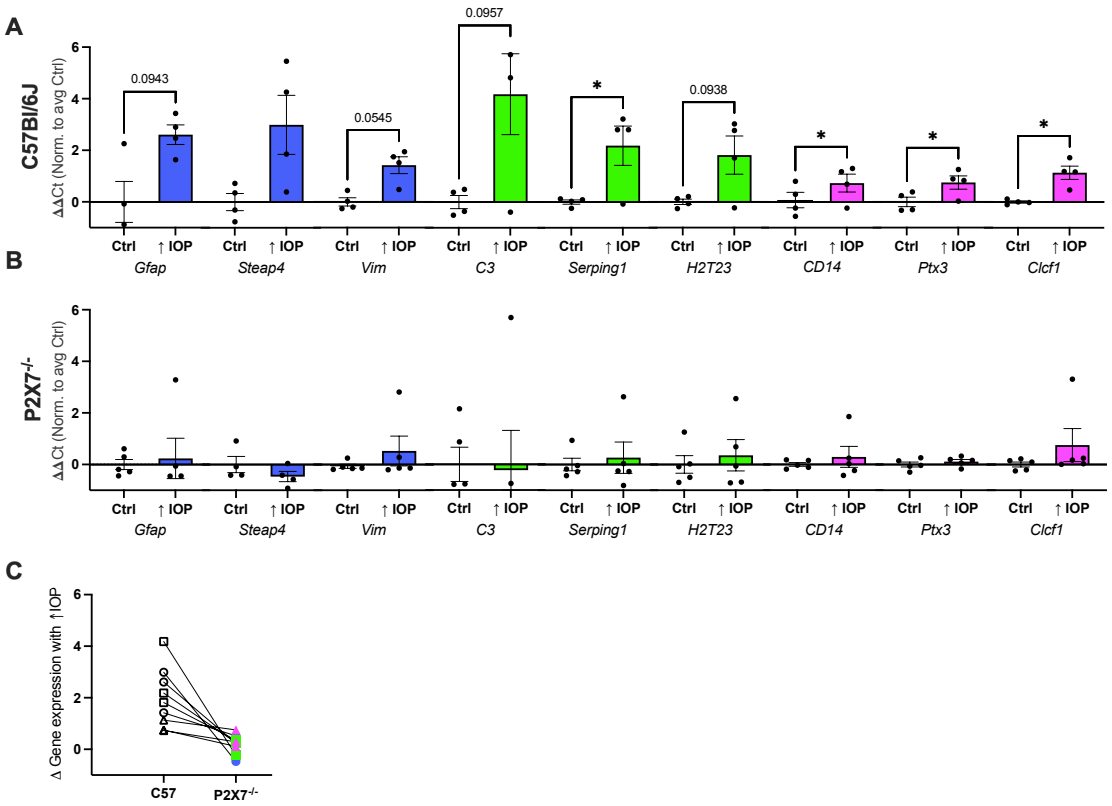


Figure 3.4. Elevation of IOP leads to expression of astrocyte reactivity genes. A Elevation of IOP for 4 hrs followed and tissue harvest 24 hrs later resulted in significant or trending expression neurotoxic reactive astrocytes in retinae from C57Bl/6J mice. **B** Elevation of IOP did not lead to significant gene expression changes in retinae from P2X7$^{-/-}$ mice. Data in **A**, **B** represented normalized to average of Ctrl eyes (Paired Student's t-test; n = 4 Ctrl, 4 IOP eyes from C57Bl/7J mice (**A**); n = 5 Ctrl, 5 IOP (*Steap4* n = 4 Ctrl, 4 IOP) eyes from P2RX7$^{-/-}$ mice (**B**). **C** Relative change in expression of reactive astrocytes in retinae exposed to elevated in IOP in C57Bl/6J mice compared to P2RX7$^{-/-}$ mice. Data is represented as mean ΔΔCT levels for each gene in IOP-elevated C57Bl/6J retinae compared to IOP-elevated P2X7$^{-/-}$ retinae (n=9 genes). *p<0.05,** p<0.01.

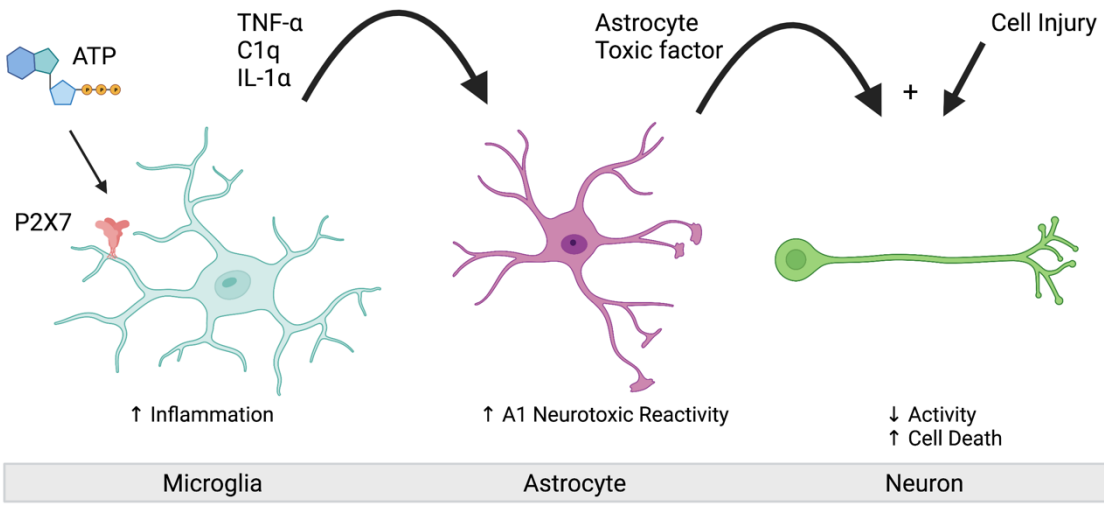


Figure 3.5. Proposed model of P2X7-medated Astrocyte Neurotoxicity. P2X7 stimulation leads to upregulation and release of factors TNF-α, IL-1α, and C1q. Released factors induce factors in astrocytes emblematic of A1 Neurotoxicity. Astrocytes release an unknown neurotoxic factor that acts in concert with independent cell injury to lead to neural death.

CHAPTER 4: THE P2X7 RECEPTOR IN MICROGLIAL CELLS MODULATES THE ENDOLYSOSOMAL AXIS, AUTOPHAGY AND PHAGOCYTOSIS *

4.1 Abstract

Microglial cells regulate neural homeostasis by coordinating both immune responses and clearance of debris, and the P2X7receptor for extracellular ATP plays a central role in both functions. The P2X7receptor is primarily known in microglial cells for its immune signaling and NLRP3 inflammasome activation. However, the receptor also affects the clearance of extracellular and intracellular debris through modifications of lysosomal function, phagocytosis, and autophagy. In the absence of an agonist, the P2X7receptor acts as a scavenger receptor to phagocytose material. Transient receptor stimulation induces autophagy and increases LC3-II levels, likely through calcium-dependent phosphorylation of AMPK, and activates microglia to an M1 or mixed M1/M2 state. We show an increased expression of *Nos2* and *Tnfa* and a decreased expression of *Chil3* (*Ym1*) from primary cultures of brain microglia exposed to high levels of ATP. Sustained stimulation can reduce lysosomal function in microglia by increasing lysosomal pH and slowing autophagosome-lysosome fusion. P2X7 receptor stimulation can also cause lysosomal leakage, and the subsequent rise in cytoplasmic cathepsin B activates the NLRP3 inflammasome leading to caspase-1 cleavage and IL-1β maturation and release. Support for P2X7 receptor activation of the inflammasome following lysosomal leakage comes from data on primary microglia showing IL-1β release following receptor stimulation is inhibited by cathepsin B blocker CA-074. This pathway bridges endolysosomal and inflammatory roles and may provide a key mechanism for the increased inflammation found in age-dependent neurodegenerations characterized by excessive lysosomal accumulations. Regardless of whether the inflammasome is activated via this lysosomal leakage or the better-known K^+-efflux pathway, the inflammatory impact of P2X7 receptor stimulation

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* Some text and figures taken or modified from Campagno, K. E. & Mitchell, C. H. The P2X7 Receptor in Microglial Cells Modulates the Endolysosomal Axis, Autophagy, and Phagocytosis. (2021) *Front Cell Neurosci* **15**, 645244.

is balanced between the autophagic reduction of inflammasome components and their increase following P2X7-mediated priming. In summary, the P2X7receptor modulates clearance of extracellular debris by microglial cells and mediates lysosomal damage that can activate the NLRP3 inflammasome. A better understanding of how the P2X7receptor alters phagocytosis, lysosomal health, inflammation, and autophagy can lead to therapies that balance the inflammatory and clearance roles of microglial cells.

4.2 Introduction

Microglia act as the innate immune effector cells of the central nervous system (CNS) [123,209] and their contribution to inflammatory signaling pathways between neurons, astrocytes and other components of neural tissue largely sets the magnitude of the immune responses. Although microglia constitute a relatively small number of cells in the CNS [283], their position as the primary defensive cells in neural systems places them at the crossroads of health and disease. The inflammatory responses mediated by microglial cells are regulated by multiple stimuli that help ensure an appropriate response. Extracellular ATP acting at the $P2X_7$ receptor is widely recognized as a pivotal player in microglial signaling for triggering the release of neurotrophic factors, cytokines such as IL-6, and the activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome [199].

In addition to their role in inflammatory signaling, microglial cells make major contributions to the clearance of material from extracellular space. Their ability to phagocytose and degrade apoptotic cells, bacteria and engulfed synaptic material contributes to neural homeostasis [30,300]. Furthermore, the dysfunctional clearance of proteins and lipids by microglia is increasingly implicated in neurodegenerative disorders. For example, undegraded myelin and lipid droplets have been observed within microglia from aging models [266,350]. The incomplete removal of amyloid beta (Aβ) deposits contributes to waste accumulation and “microglial exhaustion” in pathologies such as Alzheimer’s disease [388,389]. These observations suggest that defective clearance of waste material by microglia may contribute to the mechanisms underlying some age-related neurodegenerations.

The multiple steps required for the phagocytosis and degradation of extracellular waste material by microglial cells are tightly coordinated. Several lines of evidence suggest the $P2X_7$ receptor is involved in regulating microglial clearance, including effects on phagocytosis, autophagy, and lysosomal function. This review examines the evidence for the role of the $P2X_7$ receptor in regulating these steps and highlights the role of the $P2X_7$ receptor in modulating both the clearance and inflammatory components of microglial function.

4.3 Extracellular ATP and $P2X_7$ receptors

Purinergic signaling pathways evolved early, and the ubiquitous presence of high cytoplasmic adenosine triphosphate (ATP) levels, combined with multiple pathways for ATP release, ensured widespread use of ATP as an extracellular transmitter. ATP can be released physiologically through ion channels or from vesicles loaded with ATP [21,95]. Increased extracellular ATP is often associated with pathological stress; this rise in extracellular ATP concentration can result from an upregulation of channel and vesicular release pathways, in addition to the release of cytoplasmic ATP upon cell rupture. Signaling is largely mediated by stimulation of ionotropic P2X or metabotropic P2Y receptors by ATP, the dephosphorylated nucleotide adenosine diphosphate (ADP) or pyrimidine analogues [59]. Adenosine produced by the subsequent dephosphorylation can act at a distinct set of metabotropic receptors, adding to the complexity. This also emphasized the role of extracellular dephosphorylating enzymes in coordinating purinergic signals.

While most cell types possess some combination of purinergic receptors, purinergic signaling plays a particularly important role in microglial cells. The $P2Y_{12}$ receptor for ADP is widely recognized as a marker for microglial cells; $P2Y_{12}$ receptor expression is greatest on the ramified processes, and stimulation makes a major contribution to chemoattractant responses of microglial cells [277,286]. Stimulation of the adenosine A_{2A} receptor on microglial cells contributes to the characteristic amoeboid morphology during microglial activation accompanying brain inflammation[315].

The ionotropic $P2X_7$ receptor is especially relevant to immune responses in microglial cells due to its requirement for high concentrations of ATP, its lack of inactivation, the distribution of particular splice variants amongst immune cells, the multiple interactions of its extended cytoplasmic tail, and its association with the opening of a large transmembrane pore [90]. Receptor opening is stimulated by levels of ATP approaching the millimolar range, several orders of magnitude higher than required for other P2X or P2Y receptors [73]. While this was originally interpreted to mean the receptor was only activated by large levels of ATP released following cell rupture, the presence of the $P2X_7$ receptor on several post-mitotic cells, combined with the identification of pannexin channels as ATP conduits localized adjacent to $P2X_7$ receptors, provided for a role for the receptor in more widespread signaling scenarios [279]. Like other members of the P2X family, binding of ATP leads to the opening of a cation-selective channel that allows Ca^+ and Na^+ influx, and K^+ efflux. However, in some circumstances stimulation also leads to the opening of a pore permeable to larger macromolecules up to 900 Da; whether this pore represents a more dilated state of the $P2X_7$ channel or the recruitment of additional proteins such as pannexin channels remains unresolved, despite intense investigation [91]. The lack of $P2X_7$ channel inactivation in the continued presence of agonist has been attributed to the palmitoylated C-cys anchor retaining the gate in the open position [273]. This lack of inactivation makes regulation of agonist levels critical for both initiating the signal and also shutting it down in a timely manner; there are parallels with the NMDA channel for glutamate in both the central role in signaling and the damage following overstimulation of both channels. The human $P2X_7$ receptor possesses multiple splice variants, and the variant including the large 240 AA carboxy tail is usually associated with immune cells; this carboxy tail contains many of the binding sites associated with complex inflammatory interactions [75,189,210].

While the $P2X_7$ receptor was originally referred to as “death receptor”, more recent work indicates the contribution of the receptor is much more nuanced, with participation in a variety of signaling events; stimulation can even lead to proliferation in microglial cells [28]. These varied outcomes from the $P2X_7$ receptor may be explained by differences in splice variant expression,

agonist availability and other factors still under investigation [2,89]. While excessive stimulation can lead to microglial cell death, the effects of the $P2X_7$ receptor on the endolysosomal axis discussed here usually do not lead to cellular demise, but have sustained pathological consequences for microglial clearance instead.

4.4 Microglia, inflammation, and clearance

Microglia are morphologically plastic cells, existing in a variety of states in response to environmental stimuli. In the "resting" state, often referred to as M0, microglia engage in a constant sampling of their surroundings, with long ramified extensions surveying the neuronal milieu [305]. Microglia are constantly molded by environmental cues and respond to localized differences, so that even in the resting state the population shows great heterogeneity [222]. Once microglia are challenged with immunomodulatory substances, they enter an "activated" state in an attempt to maintain homeostasis. Although there is a range of "activated" states, they have traditionally been characterized into pro-inflammatory ("classical activation", M1) and pro-phagocytic ("alternative activation", M2) states [77]. The M2 state itself can be subdivided into the M2a state associated with repair/regeneration functions, the M2b state associated with immunomodulatory functions, and the M2c state associated with an acquired deactivation phenotype [67,264,267]. Progression into these activation states follows clear triggers; application of lipopolysaccharide (LPS) or Interferon gamma (IFN-γ) leads to the M1-like states, application of Interleukin (IL) IL-4 or IL-13 triggers the M2a state, ligation of immunoglobulin Fc-gamma-receptors that results in IL-12 expression, increased IL-10 secretion, and HLA-DR expression leads to the M2b state, while IL-10 is associated with the M2c state [4]. The states themselves are defined by proteomic or genetic markers, with IL-1β (*Il1b)* or NOS2/iNOS (*Nos2*) for M1, Arg1 (*Arg1*) or Chil3/YM1 (*Chil3*) for M2a, IL-10 (*Il10*) or CCL1 (*Ccl1*) for M2b and CD163 (*Cd163*), MMP8 (*Mmp8*), and VCAN (*Vcan*) for M2c [33,65,67,255,264,267,390,424]. However, recent investigations indicate this categorization is far too simplistic, and the different microglial states are better represented as a spectrum of combinations [243,288]. That said, the basic divisions between microglial states associated with increased inflammatory signals and those

linked to increased phagocytosis are important to consider when interpreting the varied effects of the $P2X_7$ receptor, and would benefit from a more thorough examination in the future.

The functional responses associated with the various microglial states usually help maintain the optimal activity of the neural tissue, a connection that can by overlooked when research is focused on pathology. However, the central role of microglia in coordinating the immune responses can lead to aberrant behavior upon excessive stimulation or impaired restraint. Microglia dysfunction has been implicated in several disease states, including schizophrenia [406], autism [228], ischemic stroke [193], traumatic brain injury [244], Alzheimer's disease [389], Parkinson's disease [108] and frontotemporal dementia [159]. Understanding how to maintain the beneficial activities of microglia while limiting their destructive actions remains a key challenge, and the $P2X_7$ receptor is likely to contribute to the balance with its central role in coordinating both inflammation and clearance.

The effects of the $P2X_7$ receptor on microglial clearance are less well studied than those on inflammation, and throughout this review, results obtained from primary and cultured microglial cells are supplemented by work on macrophages and monocytes. While microglial cells share many features with their peripheral cousins the macrophages, including recognition through the widely used markers Iba1 or Cx3CR1, microglia differ from peripheral macrophages[224,337]. For example, microglia differ from macrophages in protein expression [25,54], turnover [11,220], inflammatory response [48,447], and physiological regulation [261-263]. Microglia originate from early myeloid progenitor cells that migrate from the yolk sac, with minimal CNS contribution from peripheral macrophages [24,127,132]. Of particular relevance here is the increased expression of degradative lysosomal enzymes such as dipeptidyl peptidase, tripeptidyl peptidase and Cathepsin D (CatD) in microglial cells as compared to macrophages [263]. Also important in this regard are differences between microglial cells from inflamed CNS and those recruited from blood-derived monocytes; the substantial differences recently identified between these cell types implies their response to the $P2X_7$ receptor could well differ [39,217,439]. While the relative paucity of data from microglial cells on how the $P2X_7$ receptor alters clearance means that some of the information presented in this review

has been obtained from macrophages, the reader should be aware that application of results from macrophages to microglial cells has limitations.

4.5 $P2X_7$ receptor and microglial states

The $P2X_7$ receptor is widely recognized for its inflammatory actions. Stimulation of the $P2X_7$ receptor can result in the secretion of proinflammatory cytokines IL-1β and IL-6 in many cell types [3,52,367]. Additionally, stimulation of the $P2X_7$ receptor leads to the nuclear translocation of transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) which has been demonstrated to elevate gene expression of *Tnfa, Cox2, and Il1b* in microglia [106]. While these markers are widely associated with the proinflammatory M1 state of microglia, the pattern of genes activated by stimulation of the $P2X_7$ receptor in microglial cells is likely to be complex. M2 state markers Arg1 and CD163 occurred following 15 minute stimulation with Benzoylbenzoyl-ATP (BzATP), a selective agonist of $P2X_7$ [103], which may indicate a mixed activation state similar to what has been observed in microglia following traumatic brain injury [288]. Furthermore, isolated rat primary microglia challenged with LPS revealed upregulated expression of gene *Arg1* and its protein product [243]. A direct comparison of LPS and IL-4 challenge to primary mouse microglia indicate that IL-4 induces an exponentially higher expression of *Arg1* compared to LPS [67], however, suggesting the response is complex. Microglial activation state and autophagy may be interrelated, with autophagy induction polarizing microglia towards the M1 state, while induction of M2 state can itself increase autophagy [103].

4.6 $P2X_7$ receptors and phagocytosis by microglial cells

Phagocytosis of extracellular material is a central function of microglial cells. Materials bound to phagocytic receptors are taken in through the phagocytic cup and delivered to the endocytic system for pathogen destruction, degradation, or sorting [377]. Induction of the M2a state leads to an increase in cell-specific receptors associated with phagocytosis such as CD163, TREM2, and Dectin-1 [85,204,212,303,430,450]. However, expression of phagocytosis receptors does not necessarily correlate with increased phagocytosis, and the relationship between induction into the

M2a state and the rate of phagocytosis is a matter of debate [265]. Induction of both M1 and M2 microglial phenotypes was found to reduce deposits of amyloid beta *in vivo* [404], while addition of IL-4 suppressed macrophage phagocytosis of beads *in vitro* [287]. Stimulation of receptors such as TREM2 and MerTK modulate microglial phagocytosis [178,307], but the relationship between the $P2X_7$ receptor and microglial phagocytosis by microglia are more complex. For example, $P2X_7$ receptor expression was not upregulated with the challenge of lipopolysaccharides [338], but was upregulated with exposure to amyloid beta (1-42) [275], suggesting $P2X_7$ expression is a specific response.

One of the most intriguing connections between the $P2X_7$ receptor and phagocytosis involves its identity as a scavenger receptor, whereby the receptor increases phagocytosis in the absence of external agonist ATP [139-141] (Figure 4.1A). This role of the $P2X_7$ receptor differs considerably from other functions in that activity as a scavenger receptor is reduced when extracellular ATP rises. For example, phagocytic uptake of non-opsonized beads by cells of the human monocyte THP-1 cell line was reduced by blocking the receptor with antibodies against the extracellular domain of the $P2X_7$ receptor [139]. A similar reduction in phagocytosis of *E. coli* bacteria by human derived monocytes was also induced by a $P2X_7$ receptor antibody [316]. Pharmacological support for the $P2X_7$ receptor contribution was shown in human microglial cells when stimulation of the $P2X_7$ receptor with agonist BzATP reduced phagocytosis of fluorescent-tagged *E. coli* bioparticles, while $P2X_7$ antagonist A438079 reversed the effect of BzATP and enhanced phagocytosis of these bioparticles [179]. Further support for a role for the unstimulated $P2X_7$ receptor in phagocytosis was shown on a molecular level in experiments where siRNA knockdown of the $P2X_7$ receptor reduced phagocytosis in the absence of agonist in isolated human monocytes, while overexpression of the $P2X_7$ receptor in HEK293 increased phagocytosis [139,140].

Details about the mechanisms of $P2X_7$ as a scavenger receptor show a complex pattern. Extracellular residues 306-320 of the $P2X_7$ receptor were shown to bind to beads, bacteria and apoptotic cells [140]. Intracellular regions of the $P2X_7$ receptor are bound to non-muscle myosin heavy chain IIA (NMMHC IIA) and thus the cytoskeleton [138,139], consistent with a pathway for phagocytosis of material into the cell. The addition of ATP led to dissociation of NMMHC IIA from the $P2X_7$

receptor and reduced phagocytosis with roughly the same kinetics as channel gating, suggesting a common link. Overexpression of NMMHC IIA reduced the pore-opening activity that occurred with $P2X_7$ receptor agonist presentation, reinforcing the notion that the receptor may switch between its phagocytic and conductive identities [138]. However, $P2X_7$ receptor selective antagonists blocked pore formation but not phagocytic activity in human monocytes [316], indicating that phagocytosis is driven by the closed state of the receptor, and antagonist binding sites are distinct from the phagocytic targets [141]. This suggests these $P2X_7$ antagonists can block channel activity without affecting phagocytosis, a useful selection trait if the goal is to reduce the balance between inflammatory activity and phagocytosis [110]. Transient stimulation of the $P2X_7$ receptor in human-derived microglia decoupled the receptor from the cytoskeleton and eliminated its ability to phagocytose bioparticles while also activating caspase-1 [138,139]. Overall, the ability of the $P2X_7$ receptor to enhance phagocytosis in the absence of extracellular ATP presents a critical step in understanding how the receptor manipulates microglial clearance of extracellular debris. Moreover, $P2X_7$ receptor phagocytic activity is likely restricted to microglia, as phagocytosis by human monocyte-derived macrophages was inhibited with a glycoprotein-rich fraction of serum[137] (Gu et al. 2012). Readers are directed to the excellent review by Gu and Wiley for more details about the $P2X_7$ receptor as scavenger receptor [141].

While the ability of the $P2X_7$ receptor to act as a scavenger receptor in the absence of an agonist is strongly supported by the evidence above, it should be noted that not all experimental findings agree with this concept. For example, recent work in murine bone marrow-derived macrophages (BMDMs) suggests that stimulation of the $P2X_7$ receptor enhances uptake of pHRodo-tagged bacterial bioparticles, as uptake was reduced following exposure to $P2X_7$ receptor inhibitor A740003 and by reduction of extracellular ATP with the hydrolyzing enzyme apyrase [453]; phagocytosis was not inhibited with antagonists AZ10606120 and A438079, however [8]. Whether these differences indicate a characteristic of BMDMs or just experimental deviation remains to be determined.

4.7 $P2X_7$ and regulation of lysosome pH and autophagosome maturation

Lysosomes are membrane bound organelles with an acidic lumen that are responsible for the degradation of extracellular materials delivered through the endocytic pathway, or of intracellular materials delivered via autophagy. Degradation is accomplished by several intraluminal hydrolases, whose enzymatic activity is optimally at low pH levels [386]. In addition to being necessary for optimal degradative activity, a low lysosomal pH is important for maintaining the electrical potential across the lysosomal membrane, the transport of signaling molecules stored in lysosomes such as Ca^{2+} and ATP, and for the fusion of the lysosomal membrane with incoming autophagosomes and endosomes [322,435]. Elevation of lysosomal pH reduces degradation of phagocytosed material in numerous cell types [357] and leads to autophagic backup and reduced protein degradation [206], making the restoration of lysosomal pH a key target for amelioration of diseases of accumulation [144].

The ability of $P2X_7$ receptor stimulation to elevate lysosomal pH in numerous cell types including microglia, has important implications for clearance (Figures 4.1B,C). The $P2X_7$ receptor deacidified lysosomes in microglial cells; stimulation of the MG6 microglia cell line with ATP [402] or the BV2 microglial cell line with $P2X_7$ receptor agonist BzATP [365] elevated lysosomal pH. The ability of $P2X_7$ receptor antagonists to prevent this de-acidification supported a role for the $P2X_7$ receptor in this rise in lysosomal pH. It should be noted, however, that $P2X_7$ receptor stimulation of macrophages infected with bacteria acidified lysophagosomes [104].

The relatively high lysosomal pH of microglial cells under baseline conditions differs from peripheral macrophages and other cells, with a reported value of six as compared to 4.5-5 in most cell types [263]. However, the lysosomal pH in microglia is dynamic, and several pathways are involved in lowering the luminal pH in preparation for degradative activity. For example, stimulation with microglial activating compound Macrophage Colony Stimulating Factor (MCSF) or LPS lowered lysosomal pH and enhanced degradation of protofibrillar amyloid beta (fAβ) [263]. MCSF putatively polarizes microglia to the M2 state while LPS polarizes them towards the M1 state [65,169]. This study also demonstrated that forskolin lowered lysosomal pH and increased fAβ degradation,

and the enhanced degradation induced by MCSF was blocked by inhibition of protein kinase A or chloride transport, consistent with cyclic adenosine monophosphate (cAMP) and chloride contributing to lysosomal acidification. The movement of chloride transporter CLC-7 into the lysosomal membrane to enact Cl^- counterion transport was subsequently identified as a key mechanistic step in luminal acidification [261]. Future experiments are needed to determine whether baseline lysosomal pH differs in microglia of the pro-phagocytic M2 state, and whether the rise in lysosomal pH associated with $P2X_7$ receptor stimulation is altered in this subset of cells.

A rise in lysosomal pH will usually reduce rates of degradation or autophagy. After extracellular debris is engulfed into the cell via phagocytosis, it moves through a membrane enclosure called an autophagosome, which eventually binds to and fuses with the lysosome for hydrolase-mediated degradation. There are suggestions that stimulation of the $P2X_7$ receptor may reduce autophagosome-lysosome fusion to further reduce bacterial clearance. ATP reduced the fluorescence signal in MG6 microglial cells that had phagocytosed pHrodo-BioParticles [400]; as pHrodo-BioParticles fluoresce brighter in the lower pH levels of lysosomes, this implied a reduced delivery to the lysosomal lumen, although this is also consistent with a de-acidification of that compartment. Furthermore, exposure to 2.5 mM ATP for 4 hours resulted in detection of transferrin receptor protein, the intermediate form of endolysosomal protease CatD and autophagosomal lipid marker microtubule-associated protein 1A/1B-light chain 3-II (LC3-II) into the supernatant, consistent with incomplete autophagosome-lysosome maturation and elevated secretion into the extracellular space [402] via a non-classical secretory pathway [98]. Lysosome destabilization can also lead to release of lysosomal cathepsins into the cytoplasm [295]. Intracellular cathepsin B (CatB) is known to promote cleavage of cytokine IL-1β via caspase 1 after stimulation of the $P2X_7$ receptor (Figure 2). Furthermore, CatB activity can polarize microglia closer to M1, with microglia isolated from hippocampi after hypoxia/ischemia expressing significantly higher levels of *Nos2, Tnfa, and IL1b* not observed in those from $CatB^{-/-}$ mice [304].

The above contrasts with *in vitro* studies of human and mouse macrophages also suggest that the $P2X_7$ receptor can enhance phagosome-lysosome fusion and increases degradation of

bacteria in a calcium and phospholipase D (PLD)-dependent manner [80,82,215,384]. Mouse macrophages that internalized *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) followed by exposure to 3mM ATP for 4 hrs showed increased colocalization between BCG and lysosome marking-dye Lysotracker Red [104]. $P2X_7$ receptor stimulation reduced the viability of *Mycobacterium tuberculosis* in primary human macrophages and THP-1 derived macrophages, dependent upon Ca^{2+} influx and activation of PLD [214,215]. Inhibition of the PLD was independent of Ca^{2+} influx, and correlated with the reduction of phagosome-lysosome fusion. However, internal calcium stores were involved with the loss of internalized *M. bovis* that were associated with high levels of extracellular ATP [384]. These differences between effects of the $P2X_7$ receptor on phagocytosis by macrophages versus microglia again stress the need for further examination on microglia alone.

4.8 Manipulation of autophagy by the $P2X_7$ receptor

Autophagy is the process by which intracellular material is identified, packaged, and trafficked for lysosomal degradation, and several lines of evidence suggest the $P2X_7$ receptor can modulate the autophagy process (Figure 4.1). Autophagy is normally prevented by the mammalian target of rapamycin 1 (mTOR1); inhibition of mTOR by AMP-activated protein kinase (AMPK) can thus initiate autophagy [162,301]. Recruitment of LC3 to membranes leads to the formation of nascent autophagosomes, and increased clustering of the membrane-bound phosphatidlyethanoliamine-conjugated LC3-II is a common marker for autophagy initiation [206]. Autophagy, by definition, focuses on processing material produced within microglial cells, but autophagic and phagocytic pathways run in parallel. While autophagy [327], phagocytosis [371,377] and their intersection with inflammation [391] within microglia have been individually reviewed, the specific effects of $P2X_7$ receptor stimulation on the autophagic pathway in microglia merit special attention.

Transient stimulation of the $P2X_7$ receptor with ATP or BzATP elevates LC3-II levels in isolated primary mouse microglia, with LC3-II returning to basal levels 4 hrs after receptor stimulation [402]. A similar rise in LC3 was found in the MG6 [402] and BV2 [365] microglial cell lines. Receptor involvement is supported by a reduced LC3-II response to stimulation in microglial cells

from $P2X_7^{-/-}$ mice and in the presence of $P2X_7$ antagonists. The rise in LC3-II was greater in microglial cells from the SOD1-G93A mouse model of Amyotrophic Lateral Sclerosis (ALS) than the wild type, and peaked 15 min after agonist presentation [103].

Elevation of LC3-II can represent autophagic induction or autophagic backup [206]; the ability of the $P2X_7$ receptor to elevate lysosomal pH, combined with the autophagic reduction that accompanies lysosomal de-acidification [197], demands particular care when investigating how the $P2X_7$ receptor increases LC3-II levels. Levels of LC3-II were increased further when proton pump inhibitor Bafilomycin A1 was given concurrently with BzATP [103]; while this is a classical test to distinguish between autophagy induction and inhibition, the relatively small increase, combined with the absence of absolute pH measurements, does not rule out an effect of lysosomal de-acidification on rising LC3-II levels. The BzATP-mediated rise in LC3-II was dependent upon extracellular calcium in microglia and THP monocytes [32,402], but extracellular calcium was also necessary for lysosomal deacidification [142]. However, additional observations support the ability of the $P2X_7$ receptor to raise LC3-II by inducing autophagy, independent of its effects on lysosomal pH. In microglia derived from the superoxide dismutase 1 (SOD1)-G93A mouse model of Amyotrophic Lateral Sclerosis (ALS), sequestosome-1 SQSTM1/p62 levels were reduced by 15 min BzATP but increased by a sustained stimulation with BzATP for 6 hours [103]. As SQSTM1/p62 normally binds to cargo targeted for autophagic degradation by the lysosome, and is itself degraded, p62 accumulation is a standard indicator of autophagic backup due to a reduced lysosomal function [205]. However, SQSTM1/p62 is a multifunctional protein implicated in several cellular interactions such as adipogenesis, nutrient sensing, oxidative stress reactions though nuclear factor erythroid 2-related factor (Nrf2), and regulation of NF-κB. Additionally, both Nrf2 and NF-κB have transcriptionally upregulated expression of SQSTM1/p62 [331,353]. As such, the complex time course linking the $P2X_7$ receptor to p62 may reflect involvement of pathways in addition to degradation.

The activation of the AMPK pathway following stimulation of $P2X_7$ receptors also provides support for autophagic induction by the $P2X_7$ receptor, given the role of AMPK in regulating

autophagy induction [201]. For example, BV2 cells stimulated with BzATP showed an elevation of p-AMPK, consistent with autophagy induction [365]. LC3-II protein levels were reduced with siRNA against AMPK, AMPK inhibition with Compound C, or addition of mitochondrial antioxidant MitoTEMPO. The activation of AMPK by calcium-sensitive calcium/calmodulin-dependent protein kinase kinase beta (CaMKKβ) [36] may link calcium influx through the $P2X_7$ channel and AMPK activation. The demonstration of rapid phosphorylation of mTOR after BzATP application also supports autophagy induction downstream from AMPK-activation [103]. Whether $P2X_7$ receptor signaling leads to canonical inhibition of mTOR, activation of AMPK, or additional pathways requires further clarification. Future investigations must control for the effect of $P2X_7$ receptors on lysosomal pH, and the considerable feedback systems mediated by Transcription factor EB (TFEB) that are activated by this rise in pH [451]. However, the identification of intersections between the $P2X_7$ receptor and autophagy pathways is an active field of investigation in microglial research and multiple targets should soon emerge.

4.9 Effect of priming and autophagy on $P2X_7$ receptor mediated inflammatory responses

While this review is focused on how the $P2X_7$ receptor modifies microglial clearance, autophagic clearance can also modulate the impact of the $P2X_7$ receptor on inflammation. The $P2X_7$ receptor is well known for its association with increased inflammatory signals and cytokine release (Figure 4.2); its interaction with the NLRP3 inflammasome is perhaps the most widely recognized [90], and the $P2X_7$ receptor was the most potent activator of the NLRP3 Inflammasome in N13 microglial cells [114]. The NLRP3 inflammasome is a multimeric compound consisting of NOD-, LRR- and pyrin domain-containing protein 3, the apoptosis-associated speck-like protein containing caspase recruitment domain (ASC), and procaspase-1 [396]. The resulting release of master cytokine IL-1β can influence many immune signals, and the inflammasome is tightly regulated to reflect its central role. In the first “priming” step, the availability of inflammasome components is controlled. In the second step, these components assemble together to form the inflammasome itself, resulting in sequential cleavage of caspase 1 and then cytokines IL-1β and

IL-18 into their mature forms. These mature cytokines exit the cell through the cleaved gasdermin D pore, enabling cytokine release into extracellular space where they initiate a variety of inflammatory responses. The efflux of K^+ from the cell is a key signal to assemble component proteins into the inflammasome, and this efflux is frequently associated with opening of the $P2X_7$ receptor [196,294,396].

While the $P2X_7$ receptor is closely associated with the secondary activation stage of the NLRP3 inflammasome, reports also link it to the first priming phase. Priming is often initiated experimentally with the Toll-Like Receptor 4 agonist LPS, which leads to translocation of transcription factor NF-κB to the nucleus, resulting in the transcription of inflammasome components [191]. However, a number of studies suggest that the $P2X_7$ receptor can also fulfill the priming step, initiating translocation and IL-1β transcription itself. N9 mouse microglia cells exposed to ATP or BzATP demonstrated NF-κB activation, though the response was delayed when compared to LPS-mediated activation [106]. This study also demonstrated that the NF-κB/DNA complex induced by $P2X_7$ receptor signaling differed from that induced by LPS, in that ATP induced a p65 homodimer, rather than the p65/p50 heterodimers found with LPS. Work in HEK293T cells and the RAW264.7 macrophage cell line indicate that the $P2X_7$ receptor also interacts with myeloid differentiation primary-response protein 88 (MyD88), a common adaptor protein for TLRs [242]. $P2X_7$ receptor-induced NF-κB translocation has been demonstrated in osteoclasts [211] and osteoblasts [124], suggesting that $P2X_7$ receptor activation of NF-κB may be a common mechanism for priming inflammasome components. In astrocytes, data showing inflammasome priming of isolated microglial cells by the $P2X_7$ receptor was supplemented by a role *in vivo*, in which increased expression of IL-1β followed injection of BzATP, and the pressure-dependent rise in IL-1β was absent in $P2X_7^{-/-}$ mice [6]. This ability of the $P2X_7$ receptor activation to both prime and activate the NLRP3 inflammasome places it in a very powerful position to regulate innate immune responses.

Numerous studies highlight interactions between autophagy and the NLRP3 inflammasome [29]; while the specific role of the $P2X_7$ receptor is usually not involved, autophagic degradation of inflammasome components is predicted to regulate the ability of the $P2X_7$ receptor

to activate the NLRP3 inflammasome. Increased autophagy is associated with decreased NLRP3 Inflammasome function *in vivo* in rodent microglial cells [63], and in primary microglia cells [63,68,150]. Autophagic degradation of inflammasome components was also suggested by studies in the BV2 cell line [150,202,366,438], in THP-1 human macrophages [238], and in BMDCs or immortalized bone marrow-derived macrophages [152,394]. Induction of autophagy by a small molecule reduced NLRP3 expression in microglial cells and inflammasome activation [150]. Suppressed caspase-1 activation may lead to enhanced autophagy in microglial cells following inflammasome silencing [216]. Primary microglia derived from mice deficient in autophagy gene Beclin-1 ($BECN^{-/-}$) had less colocalization between NLRP3 and LC3 than their wild-type counterparts, and increased IL-1β levels, suggesting that autophagic degradation can reduce the NLRP3 inflammasome response [167]. Colocalization between ASC or NLRP3 and LAMP-1 after induction of autophagy was demonstrated in THP-1 cells [369]. Similarly, IL-1β colocalized with LC3-GFP puncta in immortalized BMDMs [152]. Deficits in autophagy and clearance are predicted to promote $P2X_7$-mediated inflammation, as accumulation of lipid droplets within microglia resulted in reduced phagocytosis and increased secretion of proinflammatory cytokines IL-1β and Tnfα after LPS challenge [266]. In BMDMs, autophagy induction increased IL-1β secretion dependent upon ATG5 [99]. However, degradation of inflammasome components through the autophagy pathway is generally expected to reduce the ability of the $P2X_7$ receptor to initiate assembly and activation of the inflammasome in microglial cells. Overall, the balance between the degradation of inflammasome proteins and their upregulation via priming initiated by $P2X_7$ receptor stimulation will influence the consequences of NLRP3 inflammasome activation by the $P2X_7$ receptor on microglial cells (Figure 4.2).

4.10 The $P2X_7$ receptor activates the NLRP3 inflammasome via lysosomal leakage and cathepsin B

A particularly important connection linking the $P2X_7$ receptor, lysosomes and inflammation involves the ability of the $P2X_7$ receptor to cause lysosomal destabilization and subsequent activation of the NLRP3 inflammasome through actions of cathepsin B. In BMDMs, destabilized

lysosomes led cathepsin B to activate caspase-1 and cleave IL-1β for release [66]. In the THP-1 monocytic cell line, *S. pneumoniae* triggered IL-1β release in an ATP- and cathepsin B-dependent manner [163]. In BV-2 cells, increased leakage of cathepsin B into the cytoplasm following BzATP application was shown using the Magic Red assay, while inhibition of cathepsin B with CA-074 prevented the BzATP-induced cell death [365]. In our studies on primary mouse brain microglial cells, the ATP-dependent secretion of IL-1β was reduced by cathepsin-B inhibitor CA-074 (Figure 4.3). While these findings suggest a critical mechanism by which the $P2X_7$ receptor can activate the NLRP3 inflammasome by disrupting lysosomal integrity, additional work is needed to understand how receptor activation leads to lysosomal leakage and whether this is related to its ability to increase lysosomal pH. Experiments to determine whether compromised lysosomes filled with partially degraded waste material are more susceptible to this lysosomal leakage are warranted.

4.11 Discussion

This review article has presented emerging evidence for the role of the $P2X_7$ receptor in regulating clearance by microglial cells. While the contributions of the $P2X_7$ receptor to immune signaling are well known [3,314], its ability to modulate microglial clearance has been less well studied. The ability of microglial cells to clear extracellular and intracellular material occurs at several key steps including the unstimulated receptor acting as a scavenger receptor to phagocytose extracellular material into the microglial cells, the transient receptor stimulation enhancing autophagy through a p-AMPK pathway and the sustained receptor stimulation elevating lysosomal pH and reducing degradation. Given its role at the crossroads between inflammation and clearance, the ability of the $P2X_7$ receptor to activate the NLRP3 inflammasome following lysosomal leakage is particularly relevant. Also, regulating the availability of inflammasome components by balancing their decrease through autophagy with their increase through priming has particular relevance as the $P2X_7$ receptor can itself increase priming. We propose the effects of the $P2X_7$ receptor on clearance pathways in microglia are as significant as its effects on inflammatory signaling. Given that impaired clearance by microglial cells is increasingly implicated in the pathogenesis of

neurodegenerative diseases [12], regulation of this clearance by the $P2X_7$ receptor warrants further investigation, and identifies potential points of intervention.

Given the receptor involvement in both clearance and inflammation, several points of particular relevance emerge. First, the ability of the $P2X_7$ receptor to act as a scavenger receptor offers several new directions for therapeutics [110,141]. Because this role requires the absence of an agonist, reducing extracellular ATP levels may provide the double benefit of increasing phagocytosis while reducing inflammation. Overexpression of intracellular binding protein NMMHC IIA reduced $P2X_7$ receptor pore opening, suggesting that shifting the receptor towards this phagocytic role may also reduce its cytotoxic actions.

Second, the relatively high baseline level of pH in the lumen of microglial lysosomes implies that regulation of this pH is a powerful mechanism through which external and internal signals can increase clearance. Given that stimulation of the $P2X_7$ receptor raises lysosomal pH, pH may be lowered by receptor antagonists, or by reducing levels of agonist ATP by increasing enzymatic degradation of extracellular ATP and blocking ATP release through sites like pannexins. Elevation of cAMP and chloride channel function lowered lysosomal pH in microglial cells [261,263], identifying a potential pathway to enhance clearance. Our work indicates the elevation of cAMP and PKA can acidify compromised lysosomes in epithelial cells [237], and that stimulation of plasma membrane receptors linked to stimulatory G proteins, such as the dopamine D5 receptor, can lower lysosomal pH and increase the clearance of phagocytosed waste by impaired lysosomes [143]. Increased clearance can also be induced by stimulating the cystic-fibrosis transmembrane conductance regulator (CFTR) chloride channel localized to the lysosomal membrane [236], or by acid nanoparticles transported to the lysosomal lumen [13,246]. Antagonists targeted to the $P2Y_{12}$ receptor were recently shown to lower lysosomal pH and improve clearance; of note, oral delivery of the FDA–approved antagonist ticagrelor (Brilinta) lowered lysosomal pH and was protective in a mouse model of neurodegeneration [248,249]. Whether this drug can cross the blood-brain barrier to reach microglial cells, and whether it will interfere with other microglial functions regulated by the $P2Y_{12}$

receptor remains to be determined. However these studies provide a proof of concept for pharmacologic restoration of an acidic lysosomal lumen to enhance clearance.

Third, the ability of the $P2X_7$ receptor to activate the NLRP3 inflammasome following lysosomal leakage of cathepsin B identifies another pathway through which limiting $P2X_7$ receptor stimulation may be of benefit. Given that lysosomal accumulations and inflammatory signaling are both emerging as key steps in aging-dependent neurodegenerations, it would be interesting to see whether leakage of cathepsin B occurs more frequently from compromised lysosomes, and whether this becomes a dominant pathway to NLRP3 inflammasome activation in aging microglial cell.

Finally, manipulating the balance between increasing levels of inflammasome components through priming and decreasing them following their autophagic removal will have a considerable impact on the immune signals emerging from the microglial cell. In this regard, the ability of the $P2X_7$ receptor to trigger both the stage one “priming” of the inflammasome as well as the stage two “activation” phase suggests the $P2X_7$ receptor, and its agonist extracellular ATP, have important consequences for the neuroinflammatory environment. The autophagic degradation of inflammasome components represents an “anti-priming” and emphasizes how the ratio of increasing and decreasing relevant inflammasome proteins can control the gain on the inflammasome responses triggered by the $P2X_7$ receptor, including those activated by lysosomal leakage.

While some therapeutic options of $P2X_7$ receptor inhibition have been explored [52] a deeper understanding of the intersection between the $P2X_7$ receptor, clearance and inflammation will assist treatments for neurodegenerative diseases in which both problems present. In age-related macular degeneration, where increased accumulations and loss of microglia clearance occur, shifting the $P2X_7$ receptor away from its inflammatory contributions and towards its scavenging role may prove advantageous [110,418]. In Alzheimer’s disease, aged microglia incompletely digest amyloid beta plaques, increasing local inflammation and pathogenesis [157,389]. The detrimental effects of Rapamycin to neurons in preclinical models of Alzheimer’s disease after plaque deposition (when clinical diagnosis would likely occur) illustrate the complexity of manipulating a system as tightly

regulated as autophagy [62,190], and stress the need for better understanding and a more nuanced approach. Finding therapeutic modalities that ameliorate autophagic reduction, or ones that can be targeted away from neurons, is crucial. In conditions of trauma such as spinal cord injury that are accompanied by elevated ATP and autophagic dysregulation [120,225], the modulation of microglia activation state has been proposed to enhance repair [4,169,208]. However, treatment timing is critical, as transient $P2X_7$ receptor stimulation may enhance autophagy and promote M2a microglia, while sustained stimulation can inhibit autophagy and promote proinflammatory M1 microglia [103,183]. The $P2X_7$ receptor plays a complex role in microglial cells in balancing clearance with inflammatory signals; deeper mechanistic investigations may reveal more specific therapeutic approaches.

These above observations come with multiple caveats, and there remain some inconsistencies. For example, microglial responses differ from those in macrophages on several levels, such as the effect on phagosome-lysosome fusion. As many of the findings come from *in vitro* experiments, even results obtained from “microglial” cells can vary with the use of cell lines or primary culture. *In vitro* microglia have been demonstrated to lose transcriptional identity in as little as 8 days after isolation [24]. With a cell type so sensitive to extracellular environments, even small changes in the protocol can alter responsiveness. In addition, microglial cells *in vitro* are removed from the challenges of the neurodegenerative environment, and their need to phagocytose waste material, along with the presence of accumulating waste material, will be altered significantly. Closer attention to the effect of the microglial state on responses to the $P2X_7$ receptor is also needed.

In summary, the $P2X_7$ receptor can modulate both inflammatory and clearance pathways in microglial cells. Reducing extracellular ATP levels to shift the receptor from an inflammatory trigger to a scavenger receptor will likely be of benefit in neuroinflammatory diseases associated with increased waste material. Limiting conditions in which receptor stimulation leads to lysosomal leakage will also help reduce NLRP3 inflammasome activation. The $P2X_7$ receptor makes a complex contribution to microglial function, and greater attention in future experiments to attributes

such as microglial polarization and lysosomal waste accumulation will help clarify these interactions.

4.12 Figures

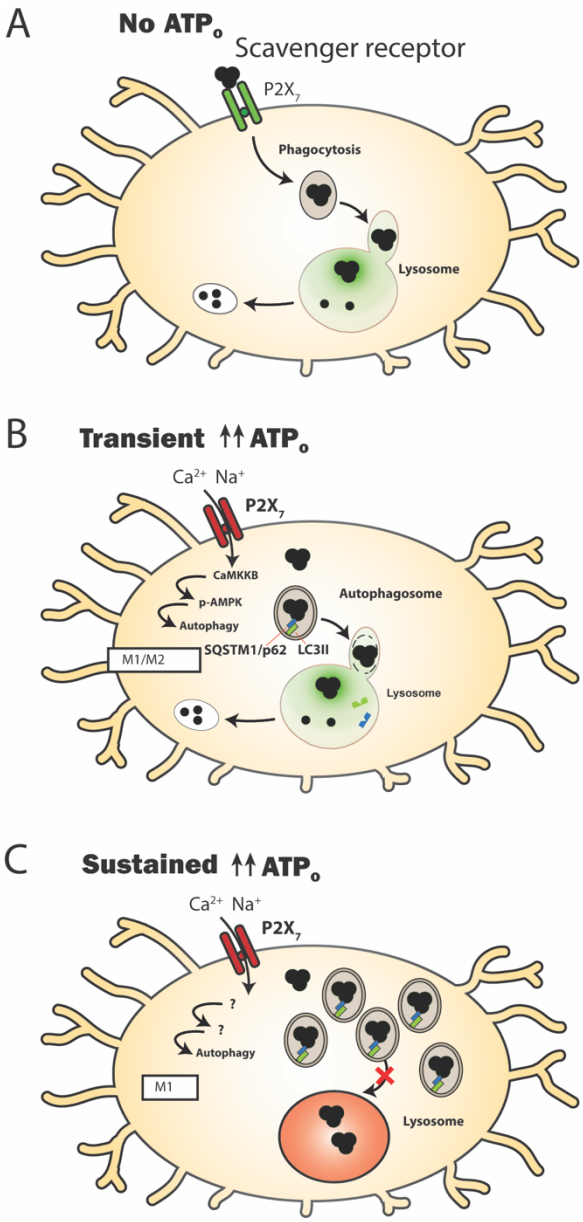

Figure 4.1. P2X7 receptor modulation of clearance pathways in microglial cells. A Under control conditions when extracellular ATP levels (ATP_0) are too low to activate the P2X7 receptor, it acts as a scavenger receptor to aid in the phagocytosis of extracellular waste. The luminal pH of lysosomes in microglial cells is sufficiently acidic (green) to enable efficient degradative enzyme activity. **B** Following transient stimulation with high concentrations of ATP_0, the P2X7 receptor no longer acts as a scavenger receptor. Autophagy is stimulated, shown by a transient rise in levels of autophagosomal lipid marker microtubule-associated protein 1A/1B-light chain 3-II (LC3-II). Material is degraded and microglia express genes and proteins associated with a mixed M1/M2 activation state. **C** A more prolonged stimulation of the P2X7receptor leads to an elevated lysosome pH (red), with autophagosomal contents being delivered extracellularly instead of to the lysosome. Markers of accumulation, including LC3-II

(green) and sequestosome-1 (SQSTM1/p62; blue) are elevated. Microglia express genes and proteins associated with an M2 activation state.

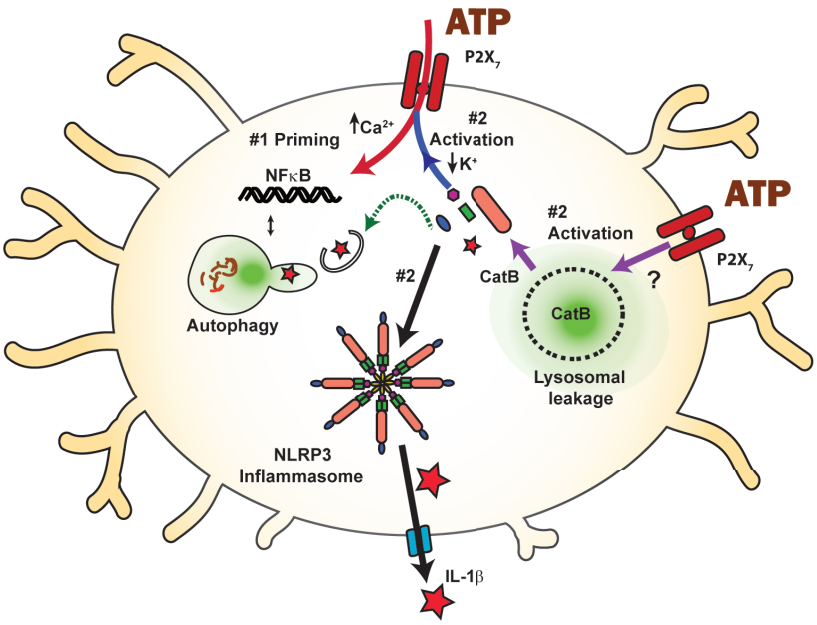

Figure 4.2. Inflammasome activation by the P2X$_7$ receptor and the balance between priming and autophagic degradation of inflammasome components. Stimulation of the P2X7 receptor triggers the assembly and activation (step #2) of the NLRP3 inflammasome following efflux of K^+ through the open channel (blue arrow), eventually leading to cleavage of pro IL-1β (red star) into the mature form and release through the gasdermin D pore (blue). The P2X$_7$ receptor can also activate the NLRP3 inflammasome and subsequent IL-1β release by raising cytoplasmic cathepsin B (CatB), following its initiation of lysosomal leakage through an unknown mechanism (purple arrows). P2X$_7$ receptor stimulation can prime inflammasome components via NF-κB (step #1, red arrow) and increase available inflammasome components. Component levels can also be decreased following autophagic degradation (green arrow), limiting the inflammatory potential of P2X$_7$ receptor stimulation. The balance between priming and autophagic degradation will influence the inflammatory impact of P2X$_7$ receptor stimulation.

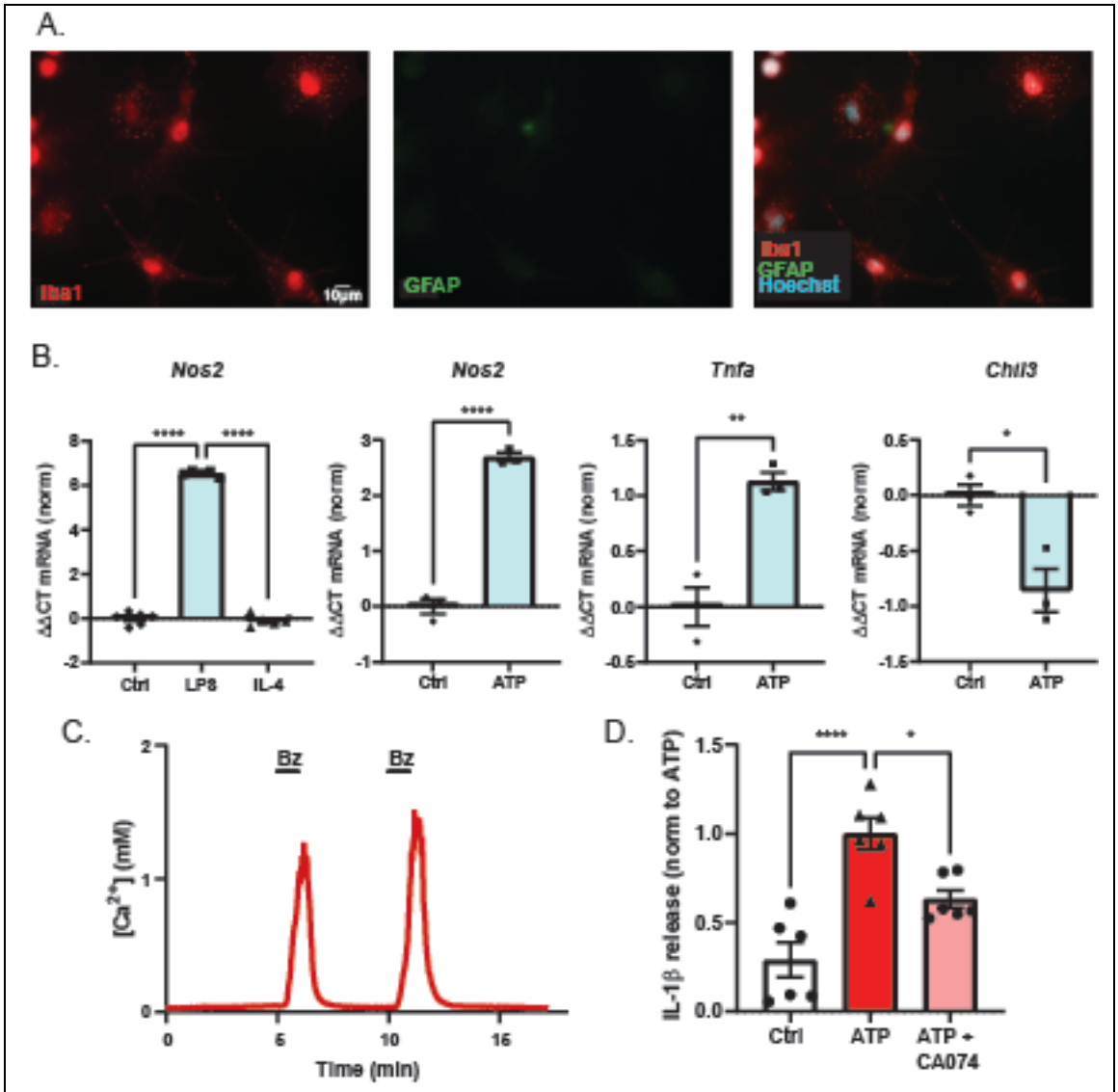

Figure 4.3. ATP-dependent IL-1β release from primary microglial cells involves cathepsin B. **A** Purity of primary mouse brain microglia cultures shown with high staining for marker Iba1 (red) and low staining for astrocytic marker GFAP (green). **B** Primary microglial cultures increased expression of *Nos2* with 4 hours exposure of LPS but not IL-4, as expected for microglial cells polarized into the M1-state (****p<0.001). Exposure to 1 mM ATP for 4 hours increased expression of *Nos2* (****p<0.001) and *Tnfa* (**p=0.0042) and decreased expression of *Chil3* (*p=0.014) as compared to control (Ctrl) using quantitative PCR. **C** Evidence of functional $P2X_7$ receptors in primary murine microglial cells indicated by the robust, reversible and repeatable elevation of cytoplasmic calcium when briefly exposed to 100μM BzATP (line); cells were loaded with Ca^{2+} indicator fura-2. D. Release of cytokine IL-1β triggered by exposure to 1 mM ATP for 1 hour was reduced by cathepsin B inhibitor CA-074 (10μM). Microglial cells were primed with 500 ng/ml LPS for 4 hours and IL-1β was measured with an ELISA. Approaches as described previously[233,247,367]. All work was approved by the University of Pennsylvania IACUC.

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

5.1 General conclusions

In this dissertation, we expand upon the role that P2X7 receptor has on microglia and astrocytes within the retina. Results from Chapter 2 implicate P2X7 receptor signaling in microglial morphological alterations, and activation into a mixed classical/alternative state with data derived from *in vivo* retinal injections and *in vitro* manipulations of cultured microglia. Elevation of intraocular pressure (IOP) resulted in similar morphological and molecular changes, and results that were dampened in retinae from P2X7$^{-/-}$ mice. Loss of retinal ganglion cells (RGCs) was observed after transient elevation of IOP in C57Bl/6J mice, and those losses were reduced in retina of P2X7$^{-/-}$ mice. Chapter 3 results revealed that direct stimulation of P2X7R in the retina increased the expression of genes *Tnfa*, *Il1a*, and *C1qa,* and induced gene expression indicative of a state of neurotoxic astrocytes. Preliminary data whereby transient IOP elevation applied to C57Bl/6J retinae elevated gene expression of *Tnfa* and *Il1a,* and trending expression of neurotoxic reactive astrocyte genes. Inflammatory microglia and astrocyte gene expression was reduced in retinae from P2X7$^{-/-}$ subjected to elevated IOP, as compared to C57Bl/6J retinae. A literature review that comprises Chapter 4 demonstrates that microglial P2X7R signaling reduces P2X7R scavenger receptor activities, and transiently induces autophagy, while prolonged stimulation points to evidence of autophagy inhibition. Furthermore, original data suggest that stimulation of P2X7R in isolated brain microglia compromises the lysosome and leads to release of lysosomal cathepsin B (CatB), which promotes the expression of cytokine IL-1β. As microglial cell state influences phagocytic receptor expression, modulation of cell state into a less inflammatory phenotype may promote phagocytosis. In this, we will discuss the implications of this work in detail, point to limitations in this research and outline future experiments that will elucidate the role that microglial P2X7R plays in retinal health and disease.

5.2 Activation of microglia is an early event after elevation of IOP

Data presented in Chapter 2 support the hypothesis that P2X7R stimulation leads to inflammatory activation of microglia and contribute to loss of retinal ganglion cells. We observed

that injection of P2X7R agonist BzATP into the retina for 24 hrs resulted in the elevation of expression of microglia marker Iba1, retraction of processes as determined through Sholl analysis, as well as elevated expression of pro-inflammatory genes *Nos2* and *Tnfa* and anti-inflammatory genes *Arg1* and *Chil3* all in concert with microglia activation (Figure 2.1). BzATP-induced elevation of Iba1 expression correlated with scoring of activation by trained observers (Figure 2.1), lending strength to the multiple methodologies we employed. Elevation of IOP for 4 hrs followed by tissue harvest 24 hrs later additionally led to activation of microglia, increased pro- and anti-inflammatory gene expression (Figure 2.5), and loss of RGCs in retinae of C57Bl/6J mice, but not P2X7$^{-/-}$ mice (Figure 2.7). Furthermore, cultured microglia exposed to BzATP for a brief period (<3 min) retracted processes indicating that P2X7R stimulation alone is sufficient to lead to activation of microglia (Figure 2.4, S2.4). Taken all together, microglia activation is an early event and may contribute to the loss of RGCs that arises from elevation of IOP.

The work in Chapter 2 supports prior work pointing to microglia activation preceding RGC loss in other mouse models of glaucoma that utilize the elevation of IOP. In the DBA/2J genetic model, microglia activation was detected at 1 month, prior to the onset of RGC degeneration. In models of rat Chronic Ocular Hypertension (COH), where pressure is elevated after cauterization of episcleral veins (ECV), elevated Iba1 or CD68 expression was detected after several days[94,298], prior to loss of RGCs. Elevation of IOP to 90 mm Hg in rat retinae increased expression of microglial activation marker CD68 as well, the effects of which were inhibited by purinergic receptor blocker OxATP, implicating a role of ATP[392]. The observations that microglia activate with transient elevation of IOP expands upon previous literature to further implicate microglial activation as an early event contributing to progression of progression of glaucoma.

Microglial morphological activation and gene expression were reduced in retinae from P2X7$^{-/-}$ mice when compared to retinae from C57Bl/6J mice, 24 hrs after elevation of IOP (Figure 2.5). Additionally ATP levels were elevated shortly after elevation of IOP, and prior to evaluation of morphology (Figure 2.5). While it is possible that the ATP that could contribute to activation of microglia was derived from dying RGCs[88], the time course points to extracellular ATP as originating

elsewhere. ATP is released from pannexin hemichannels located on neurons and astrocytes that respond to mechanical strain[172,433]. Future experiments utilizing this model will examine upstream and concurrent effectors to elucidate the role of P2X7R signaling in microglial activation from elevated IOP. Co-administration of pannexin inhibitors, such as probenecid[429] with IOP elevation will clarify the contribution of pannexin-mediated ATP release to microglial activation. If inhibition of pannexin channels reduces microglial activation, these results will also affirm that microglial activation precedes loss of RGCs. In addition to upstream mediators, evaluation of concurrent factors may clarify the influences on P2X7R-mediated microglial activation *in vivo*. Loss of immunoquiescent cytokines, such as Cx3CR1 ligand Cx3CL1 (fraktalkine), from elevated IOP may contribute to inflammation *in vivo*. Indeed, during progression of pathology in mouse models of Alzheimer's disease (AD), early loss of Cx3CL1 expression contributed to microglial activation[105,133]. Conversely, exogenous administration of Cx3CL1 reduced activation and phagocytosis by activated microglia in a mouse model of Retinitis Pigmentosa[443]. Examination of upstream effectors and concurrent factors that promote immunoquiescence may provide a fruitful direction concurrent to understanding contributions P2X7-mediated inflammation, and illuminate the mechanistic order of events that lead to loss of RGCs.

Detailed analysis of the work in chapter 2 indicates an inverse relationship where increased microglial activation after IOP elevation correlates to loss of RGC populations. However, attribution of loss of RGCs to microglia activation is complicated by several factors. Direct stimulation of RGC P2X7R has led resulted in death *in vitro*. Furthermore, the role of P2X7R may be influenced in a cell-specific or contextual manner. P2X7R stimulation on RGCs led to release of neuroprotective cytokine IL-3[233], while deletion of the receptor reduced function of a subset of RGCs after elevation of IOP[421]. Determining the contribution of microglial activation to loss of RGC populations will require an understanding of the cell-specific roles of P2X7R stimulation and the downstream effectors of microglial activation and their effects on RGCs.

There is precedent for early activation contributing to loss of retinal cell populations. Stimulation of microglial immunoquiescent receptor Cx3CR1 reduced microglial activation[422,443].

Deletion of the receptor resulted in a greater loss of RGCs after transient elevation of IOP[422], and increased cytokine secretion and phagocytosis of photoreceptor material in the Rd1 mouse model of Retinitis Pigmentosa[443]. Inhibition of activation with antibiotic minocycline after elevation of IOP[422] or in the DBA/2J chronic IOP model[40] reduced RGC loss as well. Activation of microglia was observed at an earlier timepoint in our work presented in Chapter 2, suggesting that microglial contributions to RGC loss may be substantial. Furthermore, the morphological and molecular changes observed with BzATP administration, as well as the inhibition of activation and RGC loss observed in P2X7$^{-/-}$ mice implicates P2X7R stimulation as having a substantial role in microglial activation.

The P2X7 receptor is expressed at higher density on microglia, as compared to other cell types within the CNS[75,189]. Within the CNS, pathological expression of TNF-α[330] and and expression of IL-1β[102] are localized microglia. Preservation of RGCs after elevated IOP was observed in chronic models of IOP within mice whereby the genes *Tnfa*, *Il1a*, and *C1qa* were deleted[145,383] under the hypothesis that neuron death is not driven by cytokines, but by neurotoxic reactive astrocytes[231]. The data shown in Chapter 3 demonstrated upregulation of genes associated with reactive astrocytes in retinae that were exposed to BzATP (Figures 3.1, 3.2) and preliminary data suggest a similar response may occur following transient elevation of IOP (Figures 3.3, 3.4). This preliminary data suggest elevation of IOP in P2X7$^{-/-}$ mice did not upregulate neurotoxic astrocyte genes; confirmation would lend support to the theory that microglial P2X7R stimulation plays a key role in the process.

The role of microglia within this model can be confirmed using microglia depletion methods, such as administration of PLX5622. In this case, the transient elevation of IOP model that was utilized to develop these data will prove useful. Chronic microglia depletion (>30 days) in mature mouse retinae led to photoreceptor synapse loss in the OPL[425] providing for confounding effects for depletion of microglia in chronic models of IOP. Cell-specific deletion of P2X7R will further elucidate the role of microglia. P2X7 receptor signaling on retinal ganglion cell populations has been demonstrated to have both neurotoxic[168] and suggestively neuroprotective[421] effects, which

may be reflective of diverse and cell-specific roles[194]. Research into the role of microglia-specific P2X7R signaling is limited by the absence of tools to selectively ablate the P2X7R on microglia alone. The only readily available mouse strains have deleted P2X7R from the entire mouse[174,185]. Mouse models utilizing Cre-inducible deletion of P2X7R will provide cell specific information about P2X7R signaling (for example, see Douguet *et al*, 2021[96]). Nevertheless, the models in use still provide valuable information about P2X7R- and microglia-mediated inflammation.

Understanding the contribution of microglia activation to the loss of neural populations will improve treatments of neurodegenerative diseases. Current therapeutics for glaucoma, a disease of elevated intraocular pressure, rely on alleviation of IOP with minimal consideration to the mechanistic effects on loss of RGCs. Determining the mechanisms of activation, the downstream effects (examined in Chapters 2 and 3) and upstream effectors of microglia activation may aid in developing new therapies to reduce retinal loss. While determining the order of events regarding microglial activation and neuron loss requires considerable resources to answer fully, and therefore remained outside the scope of this study, future experiments to answer the issue can focus on determination upstream, concurrent, and downstream factors that are related to P2X7R stimulation.

5.3 P2X7R stimulation and microglial activation states

Despite our initial hypothesis that retinal administration of BzATP would lead to gene expression of proinflammatory genes only, P2X7R stimulation resulted in upregulation of classical activation genes *Nos2* and *Tnfa* and alternative activation genes *Arg1* and *Chil3* was observed. *In vitro* microglia paralleled this gene expression, with upregulation of *Nos2* and *Arg1* after 4 hr exposure of either ATP or BzATP. Transient elevation of IOP in retinae from C57Bl/6J mice again led to elevation of gene expression of *Nos2*, *Tnfa, Arg1,* and *Chil3*, all of which were reduced in retinae from P2X7$^{-/-}$ mice.

P2X7R-induced upregulation of *Tnfa* and *Nos2* is known to occur through NF-κB[118,297]. The mechanism of upregulation of *Arg1* with P2X7R stimulation of microglia is not known. *Arg1* is upregulated by transcription factor STAT6[56,169]. The main activator of STAT6 is IL-4[169]. Stimulation

of neuronal P2X7R with BzATP results in secretion of IL-4[233]. Within microglia, it remains to be determined if IL-4 is released in a similar manner, and autostimulates IL-4R on microglia.

Our data were limited to gene expression measurements of whole retinae or on a collections of cells. Once stimulated, gene and protein expression of NOS-2/iNOS (*Nos2*)[19], TNF-α (*Tnfa*)[330], and Arg1 (*Arg1*) [419,434] colocalize to microglia within the CNS. Gene expression elevation of *Arg1* and *Nos2* may simultaneously occur within individual microglia, or may occur differently among microglia. Regardless, any signal differences are lost with bulk analysis. Future experiments on individual cell types utilizing flow cytometry, cell specific isolation, or immunohistochemical staining will provide more information on this matter.

If expression of *Nos2* and *Arg1* are upregulated simultaneously within the same cell, this will add evidence to the complexity of P2X7R signaling. While the majority of consequences of receptor stimulation in the literature are detrimental, protective actions occur too. For example proliferation or cytotoxicity after exposure to ATP likely due to time or concentration differences[1,89]. The upregulation of *Arg1* may represent an early-phase anti-inflammatory response; transient stimulation of P2X7R in microglia in the SOD-G93A mouse model of Amyotrophic Lateral Sclerosis (ALS) led to expression alternative activation marker proteins ARG-1 and CD163. Interestingly, elevation and subsequent reduction of these markers coincide with upregulation of autophagic markers, with elevation of LC3B-II and reduction of phosphorylated mTOR occurred at the same time[103]. Markers were then reversed with extended exposure to ATP for 6 hrs[103]. This transition from adoption of anti-inflammatory markers to pro-inflammatory markers is observed in models of spinal cord injury as well. 1 – 3 days after spinal cord injury, concurrent upregulation of classical and alternative activation markers was observed in macrophage and microglia populations, before transitioning to exclusively pro-inflammatory 14 days after injury[4,200]. The causes of the transition are multifactorial. Myelin phagocytosis early after injury promoting alternative activation, and TNF or iron-rich blood cells promoting classical activation[213]. Interestingly, administration of IL-4 promoted injury recovery and alternative activation of microglia[115], while elimination of microglia entirely exacerbates the injury[23] pointing to the value of anti-inflammatory properties that microglia

adopt early after injury. Of note, these anti-inflammatory effects of P2X7R have not been specifically addressed in chronic models of elevation of IOP, leaving gaps of understanding of the diversity of P2X7R actions. More detailed information regarding this transition may be addressed with a full understanding of timecourse with P2X7R stimulation or elevation of IOP.

The multifaceted nature of P2X7R inflammatory response should be taken into consideration with proposals for the use of antagonists to ameliorate diseases. Inhibition of P2X7R exacerbated early negative effects in models of ischemia, ALS, and epilepsy, while contradictory effects in models of Alzheimer's were observed[194]. Inhibition of P2X7R in glaucoma models of elevated IOP appear to reduce disease progression[94,168,323,346,392], yet beneficial effects have been noted. Transient elevation of IOP resulted in P2X7$^{-/-}$ retinae led to exacerbation of dysfunction in a subtype of retinal ganglion cell[421]. Mechanical strain or BzATP applied to neurons resulted in elevated release of neuroprotective cytokine IL-3[233]. IL-3 stimulation of microglia has recently been shown to reduce plaque deposition and cognitive impairments in the 5xFAD mouse model of Alzheimer's disease[272]. Taken together, this suggests the need for exploration of therapies to target intracellular effectors of P2X7R stimulation to determine if the beneficial effects of P2X7R inhibition are recapitulated, or of combinations of therapies to preserve P2X7R-mediated benefits.

Moreover, manipulation of P2X7R-mediated inflammation can be accomplished with influencing the balance between pro-and anti-inflammatory processes themselves. NOS2 and arginase 1 utilize the same substrate in the Krebs cycle: L-arginine. Induction of arginase 1 and depletes L-arginine and dampens NOS2 signaling[57], and manipulation of arginase 1 is a therapeutic option for acute retinal injuries[112]. Alterations or modulation of arginase 1 remain an interesting direction to explore for amelioration of P2X7R-induced inflammation.

If further experimentation finds that *Arg1* and *Nos2* upregulated in distinct populations of microglia rather than a mixed-activation state, it may elucidate information regarding the complexity of P2X7R signaling in a time- or concentration-specific manner[1,89]. Immunohistochemistry is currently being performed to determine cell-specific expression of cytokines to support work in

Chapters 2 and 3. Evaluation of distinct microglia should also utilize cell-specific isolation or RNAscope to delineate changes to each cell population.

Measuring concentration-specific effects of P2X7R stimulation *in* vivo are challenging. Accurate measures of ATP concentration are difficult, and the distribution of ATP within the retina have not been determined. Mechanical strain induced with elevation IOP is greatest at the optic nerve head[372], suggesting that ATP release throughout the retina may be inconsistent. Degradation of ATP with ectonucleases such as NTPDase (CD39) further confounds assessments of ATP[251,343]. A detailed assessment of differences in location-specific upregulation of pro- or anti-inflammatory markers utilizing retinal whole mount models may offer clues as to differential exposure to ATP while providing a snapshot of P2X7R signaling. Furthermore, although the spatial correlation between morphology activation of microglial cells and retinal ganglion cell loss was not particularly strong (Figure 2.7), an assessment of cell-specific gene expression differences may provide information regarding local activation and inflammatory mechanisms by demonstrating if pro-inflammatory markers were upregulated where RGCs were reduced, or vice versa, where anti-inflammatory markers were upregulated, and RGCs preserved.

Use of classical/M1 and alternative/M2 activation is a simplistic model of a complex phenomenon[26,336]. Current developments utilizing RNA sequencing offer more nuanced characterization of microglia[268] that are not captured within this study. Nevertheless, arginase 1 has proven to be a useful marker and regulator to reduce Nos2-mediated release of reactive oxygen species[57], indicating that identification of expression of either *Arg1* or *Nos2* still provides useful information. The pro-inflammatory state of classical activation is supported with expression of *Tnfa*, a prototypical inflammatory marker, while *Chil3* is upregulated with IL-4/STAT6 signaling[67,329] (Figure S2.3), which has been implicated in repressing inflammatory enhancers and *NRLP3* expression in macrophages[86]. Nevertheless, terminology utilizing classical- or alternative-activation remain in common use to explain complicated phenomena.

5.4 P2X7R stimulation induces reactive astrocytes

Preliminary data presented in Chapter 3 indicates that P2X7R stimulation is sufficient to induce gliosis. Retinal exposure to BzATP for 24 hrs induced gene expression elevation of cytokines *Tnfa*, *Il1a*, and *C1qa* (Figure 3.1) that are linked to induction of reactive astrocytes[231]. The same tissue samples exhibited elevated expression of transcripts associated with reactive astrocytes, with more consistent upregulation of A1 neurotoxic polarization[101,444] (Figure 3.3). Elevation of IOP in C57Bl/6J mice also elevated expression of *Tnfa* and *Il1a*, with trending upregulation of *C1qa* (Figure 3.2), while trending upregulation is observed of genes associated with astrocyte inflammation (Figure 3.4). Interestingly, the upregulation of putative A2 genes *CD14* and *Ptx3* was observed was highly significant, though the mean expression level was lower than A1 genes. While this may reflect a statistical phenomenon that will change with further experimentation, this may also reflect a general anti-inflammatory reaction that is independent of P2XR signaling. Importantly, upregulation of genes associated with reactive astrocytes was not observed after IOP in P2X7R$^{-/-}$ (Figure 3.4), implying that P2X7R signaling is necessary to induce A1-neurotoxic reactive astrocytes, while A2-neuroprotective astrocytes may be a common counteractive anti-inflammatory pathway induced with any onset of inflammation. Furthermore, as with microglial states that change with disease progression, the elevation of A2-specific transcripts may reflect evaluation at an early timepoint.

The mechanisms connecting elevation of IOP to astrocyte inflammation through P2X7R-mediated release of cytokines is supported with previous data. Several studies demonstrate that P2X7R inhibition reduces negative outcomes in models of IOP[94,323,346,392], and P2X7R stimulation induces expression of TNF-α and IL-1α through activation of NF-κB[129,210,242]. The data provided in Chapters 2 and 3 offer a pathway whereby stimulation of microglial P2X7R may cause activation of microglia, neurotoxic astrocytes, and subsequent death of neuron populations, although future experiments are needed to address the details.

The cell-specific origin of the *Tnfa*, *Il1a*, and *C1qa* needs to be determined. Substantial literature within the retina itself points to cell populations that release these cytokines that induce

neurotoxic astrocytes. C1q expression is localized to microglia within the retina[47] in accordance with the role that the complement system plays in innate immunity, host defense, and synapse engulfment[14,293]. Similar localization to microglia of protein IL-1α protein has been localized to spinal cords[15] or after infection with *Toxoplasma gondii*[16]. TNF-α is strongly expressed by microglia[330], but expression has also been found in cultured astrocytes[360]. While determining the specific role that microglia play in inducing neurotoxic astrocytes can theoretically be determined through microglia depletion deletion of microglia using PLX-3397 did not reduce loss of RGCs after ONC[231]. LPS injection into mouse retinae did not eliminate upregulation of astrogliosis transcripts either, although this was attributed to incomplete depletion of microglia[231]. A comparison of the effects of LPS between depleted retinae and non-depleted retinae was not performed in that study, but would offer useful information regarding the extent of inflammation reduction that occurs with microglia elimination. That information, paired again with microglia-specific deletion of P2X7R, will elucidate the role of P2X7R in microglia signaling; if no reduction of inflammation is observed, a parallel, rather than sequential, induction of microglia and astroglial populations would appear to be a likelier outcome. The role of reactive neurotoxic astrocytes in the loss of neural populations is currently unknown[101,230-232], but will be elucidated further with mechanistic targeting of specific cell populations.

5.5 Intersection between P2X7R inflammation, phagocytosis, and autophagy

As P2X7R stimulation interacts with many intracellular signaling pathways[210,314], we sought to review the role it plays in microglia processes of autophagy and phagocytosis[60]. Absent stimulation with ATP, P2X7R can act as a phagocytic scavenger for the CNS. Upon transient stimulation with ATP, markers of induction of autophagy, such as LC3-II, are upregulated. However, prolonged stimulation of P2X7R elevates the pH of the lysosome which likely contributes leak of Cathepsin B out of the lysosome, leading to IL-1β release (Figure 4.3). Emerging data suggest that autophagic or phagocytic processes and inflammatory processes may regulate each other in microglia. Induction of autophagy degrades NLRP3 inflammasome components[63,68,150], while

polarization into an anti-inflammatory state increases recycling of phagocytic receptors[85,204,212,303,430,450]. As microglia are the primary phagocytic cells in the CNS, P2X7R may represent a central regulator within the CNS. However, the intricacies still need to be explored.

The discovery that P2X7R acts as a scavenger receptor within the CNS[141], and that scavenger activity is unaffected by channel inhibition[140] offers new direction into use of P2X7R therapeutics. P2X7R-mediated phagocytosis is believed to play a role in synaptic pruning during neurogenesis[140,141], yet its role in the adult brain is unclear.

The process of phagocytosis mediated by several mechanisms[109], and the overall phagocytic response to P2X7R stimulation is likely substrate and receptor dependent. While stimulation of Stimulation of P2X7R reduced phagocytosis of apoptotic neurons[140] it also upregulates expression of phagocytic-mediated components, such as complement proteins (Chapter 4). This is further complicated in disease models, where gross evaluation of outcomes that incorporate P2X7R expression levels or location are confounded by the effects of P2X7R-mediated inflammation[141] which contribute to cell death, or by possible compensatory phagocytic mechanisms. Nevertheless, a comparison between the *in vivo* phagocytic effects of P2X7R antagonism and deletion of the receptor may prove useful.

The intracellular signaling mechanisms, whereby microglia polarization influences phagocytic properties, have not been clearly defined. Polarization away from the alternative activation/M2 phenotype reduces phagocytosis due to reduction of STAT6 signaling[56] or reduction of TREM2 expression[240]. Although P2X7R signaling upregulated *Arg1*, the effects of stimulation on STAT6 or TREM2, which itself signals via STAT6[240], have not been studied. Interestingly, *in vitro* phagocytosis of myelin reduces expression of inflammatory markers by microglia, and elevates arginase 1 expression [213]. Nevertheless, modulation of polarization via IL-4/STAT6 may prove valuable to understand effects on phagocytosis, which is putatively reduced with P2X7R stimulation. Understanding how modulates the M2-like genes modulates phagocytosis will have implications for both the inflammatory and the phagocytic functions of P2X7R.

P2X7R stimulation with ATP after LPS priming *in vitro* led to IL-1β release, and was reduced by inhibition of Cathepsin B with CA-074 (Figure 4.3). In Bone-Marrow-Derived Macrophages, CatB interaction with NLRP3 is necessary for Caspase 1 activation and pro-IL-1β cleavage to its mature form, and may play a role in inflammatory processes in neurodegenerative diseases[165]. Extended stimulation of P2X7R additionally led to elevation of pH of the lysosome[402]. Lysosomal pH elevation is associated with destabilization, which may contribute to release of lysosomal CatB and subsequent inflammation[295]. Therapies to reduce the pH of the lysosome, such as elevation of cAMP [74,261,263], or the use of acidic nanoparticles[13,278], may reduce CatB-mediated inflammation. Understanding the role of pH microglia lysosomes is hampered by a lack of tools to measure pH *in vivo* coupled with the limitations of *in vitro* microglia research[24,25]. Nevertheless, lysosomal modulation remains an interesting avenue of research.

5.6 Current clinical applications of P2X7R antagonists

The ultimate goal of this research is to improve on therapies to ameliorate inflammation in neurodegenerative diseases. The enclosed dissertation focuses on a model of glaucoma whereby elevated intraocular pressure creates strain and ATP release through pannexin hemichannels, rather than ATP released through cell apoptosis. Clinical inhibition of P2X7R may prove a viable therapeutic option in glaucoma or similar chronic neurodegenerative diseases. Therapeutic inhibition of P2X7R has been proposed for general inflammatory conditions[52], cancer[52,97], retinal-specific diseases[110], neuropsychiatric conditions[27], as well as assorted neurodegenerative diseases[173]. Currently, there are fifteen ongoing and completed clinical trials (and one terminated) evaluating P2X7R and therapies for Rheumatoid arthritis, cancer, compound receptor occupancy, or single-nucleotide polymorphisms (Clinicaltrials.gov, Table 5.1). Interestingly, application towards neurodegenerative diseases has not been a focus of clinical evaluation of P2X7 receptor inhibitors, though one inhibitor is capable of crossing the blood-brain barrier [411]. Published literature of Phase 1 or 2 results indicates that P2X7R inhibitors have a favorable safety profile[7,126,385,411]. Yet clinical

administration of P2X7R therapies has yielded disappointing results[90,218]. One hypothesis of this is that this is because the new class of P2X7R inhibitors are noncompetitive allosteric modulators,

Table 5.1 Clinical trials utilizing P2X7R inhibition or assessment.

NCT	Study Title	Phase	Intervention	Status
NCT00293189	Gene-Polymorphies in the P2X7 Gene in Patients With Osteoporotic Fractures	n/a (obs.)	P2X7R assessment	Unknown
NCT00471120	Feasibility Study: Accuracy of Biomarker in Detection of Endometrial Cancer	n/a	P2X7 assay	Terminated
NCT00628095	Study of CE-224,535 A Twice Daily Pill To Control Rheumatoid Arthritis In Patients Who Have Not Totally Improved With Methotrexate	2, 3	CE-224,535	Completed
NCT00697983	Cohort Study on Associations Between Purinergic Receptor SNPs and Osteoporosis Risk	n/a (obs.)	P2X7R assessment	Completed
NCT00849134	First Time in Human Study Evaluating the Safety, Tolerability, Pharmacokinetics, Pharmacodynamics and the Effect of Food of Single Assending Doses of GSK1482160.	1	GSK1482160	Completed
NCT01440413	Study of the Response to a Neoadjuvant Chemotherapy Based on the Antitumor Immune Response in Localized Breast Cancer	n/a (obs.)	P2X7R assessment	Active
NCT01898767	Eicosanoid Lipids by Airway Cells During Infection With Human Rhinoviruses	n/a (obs.)	P2X7R assessment	Completed
NCT02082821	A P2X7R Single Nucleotide Mutation Promotes Chronic Allograft Vasculopathy	n/a (obs.)	P2X7R assessment	Completed
NCT02293811	Decoding of the Expression of Tumor Suppressor P2RX7 in Inflammatory and Malignant Colonic Mucosa	n/a (obs.)	P2X7R assessment	Unknown
NCT02587819	Investigation of the Safety and Tolerability of BSCT (Anti-nf-P2X7) 10% Ointment	1	BSCT 10% ointment	Completed
NCT03088644	A Study to Investigate P2X7 Receptor Occupancy by JNJ-54175446 With the Newly Developed P2X7 Receptor Positron Emission Tomography (PET) Tracer 18F-JNJ-64413739	1	JNJ-54175446	Completed
NCT03437590	A Positron Emission Tomography (PET) Study to Investigate P2X7 Receptor Occupancy by JNJ-55308942 Using [18F]-JNJ-64413739	1	JNJ-55308942	Completed
NCT03918616	P2X7 Receptor, Inflammation and Neurodegenerative Diseases	n/a (obs.)	P2X7R assessment	Completed
NCT04116606	Antidepressant Trial With P2X7 Antagonist JNJ-54175446	2	JNJ-54175446	Recruiting
NCT04122937	Defining Inflammation Related to Peritoneal Carcinomatosis in Women With Ovarian or Colon Cancer	n/a (obs.)	P2X7R assessment	Completed
NCT04126772	Multimodal Imaging of MS Reveals the Smoldering Inflammation	n/a (obs.)	P2X7R assessment	Recruiting

Obs. – Observational. From *clinicaltrials.gov*

rather than competitive inhibitors[195] to augment target specificity or reduce therapeutic concentration[8,9,31]. However, therapeutic inhibition of P2X7R with allosteric modulators is hindered with conformational changes in the presence of ATP[195]. The high concentration of ATP in diseases of high cell turnover, such as cancer[89] may contribute to the lack of clinical efficacy. As the mechanism of ATP release that ultimately stimulates P2X7R may be considerably less, or reduced through coadministration of pannexin inhibitors, clinical application of P2X7R antagonists may be a viable option for reducing neuroinflammatory contributions to glaucoma.

5.8 Summary

The varied roles P2X7R inhabits establish the receptor as a key mediator for inflammatory, phagocytic, and autophagic pathways. Chapter 2 demonstrates the role of P2X7R stimulation in activation of microglia, leading to morphological alterations and expression of both pro- and anti-inflammatory genes. Chapters 2 and 3 indicate a mechanism by which elevated IOP stimulates microglia P2X7R, induces reactive astrocytes, and leads to death of RGCs. Future experiments are limited by the lack of models where the P2X7 receptor is deleted in a cell-specific manner. Nevertheless, cell-specific isolation and more granular study of microglia activation in a location-based manner will elucidate the role of microglia activation and astrogliosis in loss of RGC populations. Chapter 4 reviews the diverse roles that P2X7R plays in phagocytosis modulating autophagy in microglia, while outlining the need to explore the roles that autophagy, lysosomal modulation, or cell-state modulation may play on P2X7R inflammatory processes. Modulation of P2X7R may offer a viable option for abatement of inflammation in glaucoma, or for favoring phagocytic processes of the receptor.

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