The retinal immune response to injury and protein misfolding: Insights from models of traumatic brain injury, Parkinson's disease, and prion disease.

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The retinal immune response to injury and protein misfolding: Insights from models of traumatic brain injury, Parkinson’s disease, and prion disease.

by

Najiba Mammadova

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2019

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ABSTRACT

Retinal manifestations have been described in several neurodegenerative insults and disorders. The strong similarities between the retina and the brain and the accessibility of the retina has potentiated studies to investigate retinal pathology in an effort to identify biomarkers for early diagnosis, as well as for monitoring the progression of disease and efficacy of therapies as they become available. However, there is a limited understanding of retinal pathological landmarks of disease progression.

In the studies conducted in this dissertation, we investigated retinal changes associated with ocular blast injury, and protein-misfolding - in a mouse model of Parkinsonism, and in bovine spongiform encephalopathy (BSE), a transmissible spongiform encephalopathy (TSE) that affects cattle. Our work demonstrates that a neurodegenerative insult, specifically exposure to blast wave pressure, results in lasting retinal changes – activation of Müller glia, astrocytes and microglia, accumulation of phospho-tau species, and photoreceptor cell loss – that was not detected in the brain. Additionally, we report retinal changes associated with the burden of misfolded protein accumulation. Studies conducted using a transgenic mouse model of PD (TgM83, expressing human α-synuclein containing the familial PD-associated A53T mutation) demonstrate that the spread of α-synuclein and subsequent deposition in the retina may contribute to accumulation of phosphorylated tau proteins, neuroinflammation, metabolic dysregulation, and photoreceptor cell death. Additionally, we reported that Raman spectroscopy, an imaging technique that measures scattered light, can be used to accurately distinguish diseased retinal tissue from healthy retinal tissues based on their biochemical profile. Finally, we report that due to the similarities between TSEs and other protein
misfolding diseases, TSEs can be used to understand other proteinopathies. In this study, we used the differences in incubation period (the time from inoculation to the appearance of unequivocal clinical signs of disease) between classical and atypical BSEs as a model to identify the molecular factors associated with disease progression in the retina. We demonstrate that atypical BSEs, characterized by shorter incubation periods, have greater accumulation of misfolded prion protein (PrPSc), retinal glial-cell activation, neuroinflammation, and decreased autophagy. This work described a relationship between disease incubation period, neuroinflammation, and the autophagic stress response, that was previously unknown. Overall, this work provides insight into retinal changes associated with injury and protein-misfolding, and may contribute to the identification of early retinal biomarkers of disease progression and development of corresponding therapies.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation includes three manuscripts relevant to this doctoral work, which have either been published in or submitted for publication in peer-reviewed journals. These manuscripts constitute the bulk of the dissertation and are preceded by a general introduction describing the significance of the research topic and the research problem and a review of relevant literature followed by a discussion of the general findings and conclusions.

Introduction

Neurodegeneration is an umbrella term that encompasses a wide range of debilitating disorders that result in progressive degeneration of neurons due to various causes. Neurodegenerative disorders including Parkinson’s disease (PD), Alzheimer’s disease (AD), traumatic brain injury (TBI), chronic traumatic encephalopathy (CTE), transmissible spongiform encephalopathy (TSEs), and many others are progressive, share similar mechanisms of pathogenesis, and are exceedingly difficult to study because a definitive diagnosis cannot be made until postmortem histopathological evaluation [1, 2]. Due to the augmenting burden of neurodegenerative disorders on society, researchers are motivated to identify well-validated biomarkers of disease progression, and evaluate sensitive methods to screen individuals at risk prior to irreversible damage. Visual manifestations have been widely reported as a “prodromal” symptom in several neurodegenerative processes, described to occur years or even decades before any other symptoms [3-13]. Insight into the neuropathological progression that occurs in the eye is an emerging interest in the scientific community due to the potential for diagnosis earlier in the disease process than is currently possible. Retinal changes has been widely reported in patients and animal models of neurodegenerative disease.
however there is a scarcity of information on the extent of retinal involvement in the generation of visual symptoms, and we have a limited understanding of the distinguishing events that cause retinal degeneration. The purpose of these experiments was twofold: to investigate and unveil the retinal response resulting from neurodegenerative diseases or insults (i.e., non-penetrating ocular injury, PD and TSEs) in an effort to advance our understanding of the underlying retinal changes that may contribute to visual disturbances; and to identify retinal indicators of disease pathogenesis as potential biomarkers for early diagnosis and disease progression, and for monitoring the effect of therapeutic interventions as they become available.

**Literature Review**

**Retinal histology and functional organization**

The neural retina is the innermost light-sensitive layer of tissue in the eye. Due to its neuroectodermal origin, it is part of the central nervous system (CNS), and it can be visualized non-invasively. The vertebrate retina consists of three layers of neurons, interconnected by two layers of synapses, and supported by an outer layer of pigmented epithelial cells (Fig 1). The outermost layer of the retina is the retinal pigment epithelium (RPE), which is a monolayer of pigmented, cuboid cells. This is followed by the outer nuclear layer (ONL) or the photoreceptor cell layer that consists of cell bodies of photoreceptor cells (i.e., rods and cones). Cones mediate high-resolution color vision during daylight (photopic vision) and are abundant in the fovea, while rods mediate lower-resolution vision in dim lighting (scotopic vision) and are the predominant photoreceptor type in the vertebrate retina. The inner nuclear layer (INL) immediately follows the ONL, and contains cell bodies of horizontal, bipolar and amacrine cells. The INL is followed by the ganglion cell layer (GCL), that is
Figure 1: Schematic representation of retinal anatomy.

comprised of the cell bodies of ganglion cells and displaced amacrine cells. These five major cell types are interconnected by synapses present in the outer and inner plexiform layers. Specifically, the outer plexiform layer (OPL) contains axon terminals of photoreceptor cells (rod spherules or cone pedicles), and dendrites of bipolar cells and horizontal cells. The inner plexiform layer (IPL) comprises the synapse between the axons of bipolar cells and dendrites of amacrine and ganglion cells. The innermost layer of the retina (OFL) consists of unmyelinated ganglion cell axons, that give rise to the optic nerve as they exit the eye at the optic disk. Other supporting structures within the retina include glial cells (Müller glia, astrocytes, and microglia), endothelial cells and pericytes that constitute the retinal vessels
Cell bodies of Müller glia are located in the inner nuclear layer with their processes spanning across the entire retina forming the outer limiting membrane (i.e., the junction between photoreceptor cell bodies and outer segments) and the inner limiting membrane (i.e., the innermost boundary of the retina). Retinal astrocytes are confined to the optic fiber layer (OFL), correlating to the distribution of retinal blood vessels. Microglia are distributed throughout the retina. They are found most often in the outer plexiform layer (OPL) and inner plexiform layer (IPL). Mammalian retinal cell types have been extensively characterized and can be identified using a number of immunohistochemical markers (Table 1).

**Table 1: Markers for the immunohistochemical detection of specific retinal cell types.**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Detects</th>
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<tr>
<td>Cytokeratin [17, 18], RPE65 [17, 18]</td>
<td>Retinal pigment epithelium (RPE)</td>
</tr>
<tr>
<td>Rom-1 [18]</td>
<td>Outer segments of photoreceptor cells</td>
</tr>
<tr>
<td>Recoverin [18]</td>
<td>Photoreceptor cells</td>
</tr>
<tr>
<td>Rho4D [17, 18]</td>
<td>Rods</td>
</tr>
<tr>
<td>Transducin-γ [17, 18]</td>
<td>Cones</td>
</tr>
<tr>
<td>Calbindin [18]</td>
<td>Horizontal cells</td>
</tr>
<tr>
<td>PKC-α [19, 20]</td>
<td>Bipolar cells</td>
</tr>
<tr>
<td>Parvalbumin [17, 18], Syntaxin 1 [21]</td>
<td>Amaacrine cells</td>
</tr>
<tr>
<td>Choline acetyltransferase (ChAT) [20, 22, 23]</td>
<td>Cholinergic amacrine cells</td>
</tr>
<tr>
<td>NeuN [17], Brn3 [24, 25], MAP-1/2 [26, 27]</td>
<td>Ganglion cells</td>
</tr>
<tr>
<td>GFAP [18], Vimentin [17], Glutamine synthetase (GS) [20, 28]</td>
<td>Müller glia and astrocytes</td>
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<tr>
<td>Ionized calcium binding adaptor molecule 1 (Iba1), CD11b, TMEM119</td>
<td>Microglia</td>
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The general system of information processing in the retina is based on a direct/vertical pathway of neural signals that are transmitted by the light-sensitive photoreceptor cells to
bipolar cells and then to ganglion cells, the final output neurons of the retina. Ganglion cells then fire action potentials. These electrical signals are transmitted down the optic nerve to visual centers in the brain. This vertical pathway is influenced by a horizontal pathway, specifically modulatory signals from horizontal cells, that synapse in the outer plexiform layer and affect photoreceptor/bipolar cell interactions and amacrine cells that synapse in the inner plexiform layer and affect bipolar/ganglion cell interactions [4]. The primary neurotransmitter of the vertical signaling system is glutamate that acts via excitatory ionotropic and inhibitory metabotropic glutamate receptors, while the horizontal pathway is primarily mediated by inhibitory neurotransmitters, GABA and glycine [4]. Briefly, in the dark, photoreceptors exist in a depolarized state, in which they continuously releasing glutamate [4]. Upon exposure to light, photoreceptors hyperpolarize, which is characterized by a graded change in membrane potential and a corresponding decrease in the rate of glutamate release onto postsynaptic bipolar cells [29]. This results in either disinhibition of ON-bipolar cells or inhibition of OFF-bipolar cells. Bipolar cells can be subdivided into an ON-type (depolarizing to central illumination/hyperpolarizing to surround illumination) or OFF-type (hyperpolarizing central illumination/depolarizing to surround illumination). Cone photoreceptors communicate directly with ON/OFF bipolar cells that relay signals to ON/OFF ganglion cells, while rod photoreceptors only communicate with metabotropic ON bipolar cells that relay signals to ganglion cells via AII and GABAergic A17 amacrine cells [29]. The receptive field of ganglion cells are modulated by horizontal and amacrine cells primarily to allow for fine visual discrimination. The size and photosensitivity of a receptive field depends on the extent of synapses made in the outer plexiform layer and inner plexiform layer. For example, receptive fields in the peripheral retina are larger due to increased convergence of rods onto bipolar cells,
while receptive fields in the macula (central retina) are smaller. Visual information in the retina is extensively modified before it reaches visual centers in the brain [4, 29].

**Retinal neuroinflammation and autophagic protein clearance**

*Key players in the retinal immune response: Müller glia, astrocytes, and microglia.*

The activation of retinal macroglia, namely Müller glia and astrocytes, is widely reported in response to stressors including blast injury and protein-misfolding diseases, and chronic activation has been shown to exacerbate retinal degeneration [30-35]. Evidence suggests that in the injured retina, morphological, molecular, and functional responses of Müller glia may be highly influenced by activated microglia with both beneficial and detrimental effects (reviewed in [36]). Microglial response to injury is a highly regulated mechanism consisting of phenotypic and morphological changes, as well as production of inflammatory cytokines, chemokines, and cell adhesion molecules. A prolonged or chronic response will propagate neuroinflammation, increased oxidative and nitrosative injury and consequent retinal tissue damage [37-42]. The dual adaptive and maladaptive nature of Müller glial and microglial responses to retinal injury have been vastly studied, and investigations of the mechanisms that shape the overall immune response in the retina due to injury are ongoing.

*Retinal macroglia: Müller glia and astrocytes*

Müller glia and astrocytes are the two types of macroglia found in the retina. Müller glia are the principal radial glial cells of the retina that represent 90% of the retinal glia, and were first described in 1851 as “radial fibers” by German anatomist, Heinrich Müller. Developmentally, Müller glia and retinal neurons are derived from a single progenitor cell in distinct phases [43]. Retinal astrocytes are star-shaped glial cells first described by Ramón y
Cajal in the 19th century [44]. Müller glia and astrocytes provide structural stability and metabolic and homeostatic support for retinal neurons by performing a variety of functions. Müller glia and astrocytes: (i) regulate the composition of extracellular space fluid, including extracellular ions (e.g., potassium ions) and neurotransmitters (e.g., GABA and glutamate); (ii) clear debris and neural waste products such as carbon dioxide and ammonia; (iii) regulate synaptic activity in the inner retina; (iv) regulate the blood-retinal barrier (BRB) and protect the retina in case of mechanical trauma; and (iv) produce neurotrophic factors, such as pigment epithelium-derived factor (PEDF), glia cell line-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF), which are essential for maintenance of BRB integrity as well as neuronal survival [34, 45-48].

Under nearly every challenge to the retina, the activation of Müller glia and astrocytes is indicated by hypertrophy, and an increase in distribution of intermediate filament proteins including glial fibrillary acidic protein (GFAP) and vimentin (often evident throughout their basal and apical processes) and other molecules (e.g., glutamine synthetase, and MMPs) [49, 50]. An increase in GFAP in Müller glia and astrocytes is a predominant feature and a sensitive indicator of ocular diseases, traumatic brain injury, and neurodegenerative diseases such as PD, AD, and TSEs, and a widely used biomarker protein [51]. Activation of Müller glia and astrocytes have been shown to be beneficial to post-injury recovery (i.e., prevention of glutamate neurotoxicity and release of trophic factors and interleukins that serve to protect neurons from cell death [52-55]). However, excessive activation and the associated inflammatory responses will negatively impact structural and functional recovery due to a loss of function of Müller glia [33, 35, 47, 49, 52, 56]. For example, the release of vascular endothelial growth factor (VEGF) by activated Müller glia in response to injury has been
shown to be neuroprotective at low concentrations by inducing angiogenesis, synaptogenesis, and detoxification of reactive oxygen species [57, 58]. Conversely, prolonged expression of VEGF by both Müller glia and astrocytes can lead to the breakdown of retinal vasculature, including the blood retina barrier, and induce lymphocyte infiltration and cell death [59, 60]. There seems to be a continuum of states of Müller glial activation however, the mechanisms that regulate the transition between protective and detrimental gliosis are ill-defined. In the acute stages of Müller glial activation, if the trigger is removed, no changes in functionality or tissue alterations are detected [56, 61]. Prolonged or chronic activation of Müller glia may result in cellular hypertrophy and loss of function that contributes to reduced neuronal viability [61].

Activation of astrocytes results in common features including hypertrophy accompanied by the up-regulation of intermediate filament proteins, GFAP and vimentin, and release of growth factors [61]. Although microglia are the primary mediators of the retinal inflammatory response, reports demonstrate that activated astrocytes secrete inflammatory cytokines (e.g., IL-1β, IL-6, and IL-8) [62] and chemokines involved in recruitment of microglia and T-cells (e.g., CCL2, CCL5, CXCL12) [63, 64]. Activation of retinal Müller glia and astrocytes is a ubiquitous response to a diverse array of retinal injuries and stressors, however the function of retinal gliosis is ambiguous, and the mechanisms involved have not been fully characterized.

**Retinal microglia and neuroinflammation**

First described by Pío del Río-Hortega in 1932, retinal microglia have been well documented, and generally thought to be of hemopoietic origin with monocytes being their precursor cells [65, 66]. In this regard, microglia can be compared to resident tissue macrophages of other organs. However, their origin is still a matter of debate (reviewed in [67]). In the central nervous system, microglia are considered to be the primary orchestrators
of the inflammatory response [68-70]. Despite differences in the triggering event, retinal microglial activation is a predominant characteristic of ocular diseases [67, 71] and other neurodegenerative conditions, such as traumatic brain injury [72], PD [73], AD, prion diseases [74], and multiple sclerosis [72-76]. One of the more outstanding characteristics of microglia is their morphology, most commonly ramified/quiescent or rod-like/amoeboid [72, 73, 77].

Under normal physiological conditions microglia exist in the resting or ramified state, characterized by small cell bodies, high motility [68, 78], and elaborated thin processes that they can retract or extend in order to surveil or “police” their environment [77]. In response to tissue injury or stress, microglia acquire an activated state, accompanied by an ameboid morphology with larger cell bodies and retracted/thick processes. Other morphological states of microglia have also been observed including a “reactive” phenotype characterized by small, spherical cell bodies that can be rod-shaped [79-82]. Recently, a major push of the microglia literature has been to define distinct activation states (e.g., M1, M2a, M2b, M2c) characterized by the expression of either pro- or anti-inflammatory cytokines, in which microglia are performing a limited set of functions (reviewed in [83]). M1 or classical activation is characterized by the increased expression of pro-inflammatory cytokines (e.g., IL-1β, TNF-α, IL-6, and IL-23) and cell surface markers (CD40, CD86, CD16/32), and is generally considered as a harmful state often associated with tissue damage [83, 84]. M2 or alternative activation is characterized by upregulation of anti-inflammatory molecules and receptors, such as IL-4 [83] and mannose receptor (CD206) [85, 86]. This activation state is implicated in a range of regulatory and homeostatic functions, including resolution of inflammation (M2a) and phagocytosis to promote wound healing (M2c) and tissue repair [87-89]. A recent study quantitatively analyzed the spatio-temporal distribution of specific microglial morphological
states in the brain, in an effort to relate morphology and function [90]. However, contradicting studies demonstrate that although microglial activation is accompanied by specific changes in morphology (surface markers and/or receptors [91, 92]), microglia perform a diverse set of functions at any given time.

Retinal microglia perform many functions in the retina in response/contribution to various pathological states that can be both beneficial and detrimental. Microglia express numerous cell-surface markers and receptors that relate to their function, and can be used for their identification (e.g., Iba1, CD11b, CXCR3) [93]. While only a few markers can be used to differentiate between quiescent/resting and activated microglia (e.g., B7-1, MHCII, OX-41) the extent of some markers can be used to differentiate between resting and reactive microglia (i.e., F4/80, CD45, and CD68 are expressed higher in activated microglia) [67, 71, 93]. Reports show up-regulation of Iba1, CD11b, and/or CD68 in retinal microglia in response to optic nerve crush [94], ocular blast-injury [72], α-synuclein [73], and misfolded prion proteins (PrPSc) [74]. Under normal physiological conditions, retinal microglia participate in synaptic pruning [95-97], and phagocytosis of apoptotic neurons [98-102], antigen presentation to provoke T-cell effector function [103-106], and modulation of inflammation [107, 108]. Additionally, many receptors on microglia play a key role in the initiation or modulation of their pathological response, acting as molecular triggers of microglial activation. Retinal microglia express TLR1-9, members of the Toll-like receptor family, that recognize and are activated by pattern recognition receptors (PRRs), which are components central to host retinal innate immunity. Specific PRRs, in this case TLRs, identify a specific array of pathogen-associated molecular patterns (PAMPs) (often linked with cell stress and microbial pathogens), and/or damage-associated molecular patterns (DAMPs) [109]. DAMPs are endogenous
molecules released by stressed or damaged cells, and include heat-shock proteins (HSPs), high-mobility group box 1 (HMGB1), IL-1α, ATP, DNA, mitochondrial reactive oxygen species (mROS), among many others. [109, 110]. Reports describe microglial TLR-2 as the primary receptor for amyloid-β peptide in AD [111, 112], and α-synuclein in PD, dementia with Lewy bodies (DLB), and multiple system atrophy (MSA) [113], however this has not been described in retinal microglia. TLR-mediated activation of microglia leads to an inflammatory cascade characterized by the release of a multitude of cytokines (e.g., TNF-α, IL-1β, IL-6), chemokines, nitric oxide synthase, and other pro-inflammatory molecules. Madeira et al. describe that retinal microglia-mediated secretion of TNF-α, IL-6, and IL-1β is markedly implicated in many retinal neurodegenerative diseases (e.g., glaucoma, age-related macular degeneration (AMD), diabetic retinopathy) [114]. Specifically, studies report that downstream signaling of TNF-α and IL-1β via receptors TNFR1/2 and IL-1R1/2 respectively, induces apoptosis of retinal ganglion cells in glaucomatous neurodegeneration [115, 116]. Moreover, TNF-α and IL-1β can enhance the expression of cell-adhesion molecules (e.g., intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)) that compromise the integrity of the BRB and further facilitate the neuroinflammatory cascade by recruiting leukocytes into the retina [67, 117]. Although the influence of microglia and reactive gliosis on the pathogenesis of protein-misfolding diseases (e.g., PD, AD, and prion diseases) has been well characterized (reviewed in [118], [119], and [120] respectively), limited reports describe retinal microglia-mediated neuroinflammation in response to misfolded proteins [73]. Liu and colleagues reported that injection of amyloid-β into the eyes of rats leads to retinal microglial activation, and upregulation of pro-inflammatory cytokine genes (IL-6, TNF-α, IL-1β, and IL-18) in the RPE and inner retina, possibly suggesting involvement of the NLRP3 inflammasome [121].
Greenlee et al., have demonstrated retinal microglial activation in response to PrP^{Sc} in transmissible spongiform encephalopathies (TSEs), specifically a mouse model of scrapie (TSE that affects sheep and goats) [74] and bovine spongiform encephalopathy (BSE) (TSE that affects cattle) [122]. Additionally, we have recently demonstrated retinal microglial activation characterized by increased Iba1, and CD68 in a mouse model of PD [73], and microglia-mediated NLRP3 inflammasome activation in retinas of BSE-inoculated cattle (manuscript in preparation to be submitted to Acta Neuropath).

Present primarily in innate immune cells (macrophages, monocytes, dendritic cells, and microglia), inflammasomes are cytosolic multiprotein complexes that detect stressors and regulate the activation and secretion of pro-inflammatory cytokines (IL-1β and IL-18) [123]. Among several existing inflammasome complexes, NLRP3 is the best characterized, so termed because the NLRP3 protein in the multiprotein complex belongs to the family of nucleotide-binding and oligomerization domain-like receptors (NLRs), and is also known as “pyrin domain-containing protein 3” [123]. In addition to the NLRP3 protein, the NLRP3 inflammasome contains adapter protein apoptosis-associated speck-like protein (ASC) and pro-caspase 1 (precursor molecule of caspase-1) [123]. Activation of the NLRP3 inflammasome is thought to occur in two steps: 1) TLRs recognize a variety of stressors, including PAMPs or DAMPs which serves as a priming or initiation signal that leads to the activation of nuclear factor κB (NF-κB) and subsequent transcription of key inflammasome components (inactive NLRP3, proIL-1β, and proIL-18); and 2) the assembly of inactive inflammasome components (NLRP3, ASC, and procaspa-1) and subsequent conversion of procaspa-1 to active caspa-1, triggers the production and secretion of active IL-1β and IL-18 [123]. Inappropriate or prolonged activation of the NLRP3 inflammasome has been
implicated in the pathogenesis of traumatic brain injury [124] and protein-misfolding diseases including PD, AD, and prion diseases [125-129]. In a mouse model of diabetic retinopathy, a recent report describes increased mRNA levels of several NLRP3 inflammasome components (NLRP3, ASC, and caspase-1), as well increased activation of GFAP+ and Iba1+ macro- and microglia respectively, suggesting that NLRP3-mediated inflammation may contribute to retinal pathology (e.g., neovascularization, and reduced retinal thickness) detected in the advanced stages of diabetic retinopathy [130]. Activation of the NLRP3 inflammasome has also been described in the pathogenesis of age-related macular degeneration (AMD) [131], optic nerve injury [132, 133], and recently glaucoma [134]. However, reports describing microglia-mediated NLRP3 inflammasome activation in the retina, in response to misfolded proteins are lacking.

*Interplay of retinal neuroinflammation and autophagy*

Autophagy is a highly conserved intracellular system for the degradation of cytosolic components, including damaged organelles and proteins [135]. There are three mechanistically distinct forms of autophagy that coexist in mammalian cells – microautophagy, characterized by direct lysosomal engulfment of cytoplasmic cargo; chaperone-mediated autophagy (CMA), in which chaperones (i.e., heat shock proteins) transport specific proteins marked for degradation directly from the cytosol to the lysosomes; and macroautophagy, in which bulk cytoplasmic contents (e.g., damaged organelles, and large protein aggregates) are degraded within an autolysosome (i.e., an autophagosome, or a double-membrane sequestering vesicle, fused with a lysosome). Other forms of more specialized autophagy that have been described include mitophagy (mitochondrial degradation) [136], xenophagy (selective degradation of intracellular viruses and bacteria) [95, 137, 138], and pexophagy (degradation of peroxisomes)
Macroautophagy, herein referred to as autophagy, can be broken down into three general stages, including induction, vesicle nucleation/elongation (autophagosome formation), and fusion and degradation, with several key protein complexes involved in each stage [140, 141]. Under normal conditions, mammalian cells have an intrinsically active basal level of autophagy, essential for proper maintenance of cellular homeostasis. However, increasing studies demonstrate the pathophysiological roles of autophagy, such as clearance of intracellular protein aggregates in a variety of neurodegenerative diseases, most commonly PD, AD, HD, and TSEs (described in [142-144]). A plethora of reports show that enhanced autophagy in animal models of these diseases results in clearance of the aggregated proteins and ameliorates clinical signs of neurodegeneration, demonstrating a neuroprotective role of autophagy. Conversely, in the retina, overactivation of autophagy promotes apoptosis, best characterized in AMD, DR, and glaucoma [141, 144-153]. In this regard, autophagy has been described as a “double-edged sword” with both beneficial and detrimental roles [154]. The interplay or more specifically, the inverse regulation between neuroinflammation and autophagy has gained increasing attention [155, 156], with one of the first reports describing that when stimulated with lipopolysaccharide (LPS), microglia of autophagy-related protein 16-1 (Atg16L1) knockout mice, respond with increased expression of caspase-1 and IL-1β [147]. Limited reports describe concurrent changes in retinal autophagy and neuroinflammation in response to misfolded proteins. Recently, we reported retinal pathologies in response to α-synuclein in a mouse model of PD, specifically increased microglial activation, which paralleled a decrease in autphagic proteins, ULK1 (involved in the induction phase) and LC3II (involved in autophagosome formation) [73]. Additionally, we have demonstrated an inverse relationship between neuroinflammation and autophagy in
Retinas from animals inoculated with an agent of BSE (*manuscript in preparation to be submitted to Acta Neuropath.*).

**Retinal response to blast injury and protein-misfolding disorders**

*The retina in blast-induced ocular injury*

Traumatic brain injury (TBI) is a complex injury with a broad spectrum of clinical manifestations, that result from a blow to the head due to a variety of causes including motor accidents, sports-related injuries, assaults, and military conflict. TBI can be broadly classified as penetrating or non-penetrating, and more specifically categorized according to injury severity and physical mechanism (i.e., the causative force associated with the injury) [157]. Blast-induced injuries, currently designated as the ‘signature injury’ of military conflict, are traumatic brain injuries that result from blast wave pressure, a complex pressure wave or an instantaneous and severe rise in atmospheric pressure generated by an explosion. The mechanism of blast-induced injury is categorized according to four groups: *primary blast injury*, resulting from the direct effect of the blast shockwave which causes a shearing stress force on bodily organs and tissues; *secondary or penetrating blast injury*, resulting from the impact of fragments, flying debris, etc.; *tertiary blast injury* resulting from environmental contamination (burns, inhalation injury, exposure to toxic substances); and/or *quaternary blast injury*, resulting from the body being physically propelled into objects (crash injuries, blunt trauma, traumatic amputations, etc.) [157]. The nature of 21st century military conflict has caused a dramatic emergence of primary blast injuries, and has taught physicians many lessons about TBI. Most commonly reported manifestations of primary blast injuries include damage to gas-supported organs - the lungs, gastrointestinal tract, and tympanic membranes - as well as denser tissues, most notably the brain and eyes [158].
Between the years 2000 and 2011, over 54,000 military personnel experienced visual dysfunction and/or loss due to blast insult, with an estimated cost of $24.3 billion in rehabilitation and lost wages over the lifetime of combat veterans [159]. While TBI-related visual symptoms differ from patient to patient due to the cause of the injury (i.e., the direction or intensity of the blast force, part of the visual system affected, patient-specific conditions such as genetic predispositions or previous head injury, etc.), common visual manifestations of blast-injury include: photophobia, loss of visual acuity and color discrimination, contrast sensitivity, visual field defects, and more. Currently, diagnosis and therapeutic interventions for penetrating eye injuries from fragmentation are readily available, however non-penetrating ocular injuries due to blast wave pressure are often not apparent. Too often however, veterans without obvious outward signs of trauma (i.e., “normal” routine ophthalmic exams) are left undiagnosed and afflicted with lifelong visual deficits and permanent loss of vision. [160]. Currently, underlying retinal pathologies are poorly understood.

Pathologic processes triggered by the shearing impact of blast wave pressure due to TBI are often separated into an initial primary damage phase characterized by diffuse axonal injury and brain edema invariably resulting in necrotic cell death, and an extended secondary phase encompassed by a neuroinflammatory cascade [161]. Within the CNS, neuroinflammation is initiated at the time of insult in an effort to restore cellular and molecular homeostasis, however this response is often dysregulated leading to progressive neurodegeneration and apoptotic cell death. This response is recognized as a critical factor in exacerbating neuronal injury. Specifically, cellular damage causes cellular membrane disruption, as well as release of various endogenous factors including RNA, DNA, and heat shock proteins, all acting as damage associated molecular patterns (DAMPs) [161, 162].
DAMPs will bind to toll-like receptors (TLRs) activating NF-κB and mitogen-activated protein kinase (MAPK) pathways triggering rapid release of pro-inflammatory mediators from resident activated macro- and microglial cells [161, 162]. Sustained molecular signals from damaged host cells promotes chronic microglial activation, neuroinflammation, oxidative stress and neurodegeneration [161, 162]. When non-penetrating traumatic ocular injuries are not immediately attended to in the field, chronic retinal neuroinflammation can lead to progressive neuronal loss and contribute to visual loss.

Due to considerable concern in regard to the high frequency of traumatic brain injury causing visual dysfunction in humans, there are various rodent models of blast injury to the retina that demonstrate the effects of blast wave pressure on the retina. Studies demonstrate the susceptibility of the retina to the effects of low-level (120 ± 7 kPa) and high-level (≥180 kPa) blast wave pressure using models of varying blast intensity and duration, including, but not limited to, the use of 2,4,6-trinitrotoluene (TNT) with a penta-erythritol tetra-nitrate (PETN) booster [31], an air paintball gun targeted directly to the eyes of mice to deliver a puff of CO₂ (160-200 kPa) [163], and a compressed air-driven shock tube system [72]. Reports show that repeated exposure to blast wave pressure leads to macro- and microglial cell activation, an increase in biomarkers of inflammation and apoptosis, and overall changes in retinal histopathology, including thinning of the retina, detected up to 72h after exposure [30-32, 107, 164, 165]. Limited reports demonstrate prolonged effects of blast wave pressure. Our recently published study demonstrated that 30 days after exposure to successive bouts of blast wave pressure (300 kPa/43.5 psi), retinas of mice presented with activation of retinal Müller glia, astrocytes, and microglia, as well as photoreceptor cell loss [72]. Our work also describes accumulation of phosphorylated tau (Thr231 and Thr181) in retinal horizontal cells and Müller
glia of blast exposed mice, detected 30 days after blast exposure [72]. Chronic glial cell activation, persistent neuroinflammation, and phospho-tau accumulation have become increasingly recognized as key components of the pathological onset and progression of neurodegeneration following traumatic brain injury [41, 161, 166-169].

**Tauopathy in the retina**

In the healthy CNS, the microtubule-associated protein, tau, is essential to neuron health, however aggregation into neurofibrillary tangles or oligomers can lead to neurodegeneration [170]. Normally, tau is localized to axons to promote neuronal integrity and axonal support in the brain and the retina [171-173], however a stepwise process beginning with phosphorylation of specific sites, and later aggregation into tau-positive inclusions is shown to inhibit microtubule assembly and stabilization, leading to degeneration [174-178]. Tauopathies encompass many neurological entities, and some of the most commonly known tauopathies include AD, corticobasal degeneration, Pick’s disease, progressive supranuclear palsy, frontotemporal dementias with parkinsonism linked to chromosome 17 (FTDP-17), and chronic traumatic encephalopathy (CTE) [170]. Specific tauopathies vary in morphological appearance of the tau-containing inclusions and their relative distribution described in neurons [170], oligodendrocytes [170], astrocytes [72] and, to a lesser extent, in microglia [177]. Similar to its function in the brain, tau regulates axonal and cytoskeletal transport in the retina. Reports show tau expressed in developing retinal ganglion cell (RGC) axons, suggesting a role of tau in proper axon development and survival of RGCs [172]. To further support this role, studies also describe the endogenous association of tau with specific protein kinases present in the retina, cyclin-dependent kinase 5 (Cdk5) and Ca2+/calmodulin-dependent protein kinase II (CaMKII), both of which are highly expressed in neuronal axons. This interaction suggests
that retinal tau plays a role in regulating cytoskeletal assembly and promotes neurite outgrowth and migration [171, 179]. Phosphorylation of tau by these kinases may inhibit microtubule assembly, leading to the disruption of retinal cellular architecture [171, 179, 180]. Reports of retinal tauopathy describe accumulation of aggregated tau in the outer plexiform layer (horizontal cells), inner nuclear layer, inner plexiform layer, and nerve fiber layer of AMD, RP, and glaucoma patients [171, 181-183]. Additionally, based on immunohistochemical analysis of eyes from patients aged 49-87 years, Leger and colleagues reported a positive correlation between age and increased tau accumulation in RGCs and photoreceptor cells [184]. There is an abundance of evidence describing visual impairment in AD, and studies link the potential role of tau to the development of visual deficits (e.g., color perception, contrast sensitivity, peripheral vision) [185]. Neurofibrillary tangles (NFTs) containing phosphorylated tau have been observed in retinas of AD patients [186-189], specifically in RGCs and photoreceptors [190-192] and in transgenic mouse models of AD [193]. The most recent report describes that tau accumulation in the retina promotes neuronal dysfunction and precedes brain abnormalities in a mouse model of AD [191].

Chronic neuroinflammation due to TBI has been shown to promote abnormal tau phosphorylation and β-amyloid aggregate formation, linking it to increased risk (~2- to 4-fold increase) of neurodegenerative dementias, such as AD and CTE [168, 194, 195]. Neuropathology of tau hyperphosphorylation has been extensively reviewed with evidence suggesting that initial axonal injury after TBI promotes perturbation and dissociation of tau from microtubules driving its aberrant phosphorylation and aggregation [168, 196-198]. While the exact mechanisms via which TBI promotes tauopathies are not yet understood, studies show that chronic neuroinflammation may exacerbate the initial seeding of phospho-tau,
causing neuronal toxicity and apoptosis [196, 197]. Elevated levels of phosphorylated tau have been reported in pyramidal neurons of the hippocampus and Purkinje cells of the cerebellum two weeks after blast exposure [198] however, reports of retinal tau accumulation following blast injury are limited. Xu et al., report that repetitive mild TBI results in accelerated accumulation of phosphorylated tau in retinal ganglion cells of transgenic P301S (model of tauopathy) mice [199]. Additionally, we have recently reported that blast wave pressure results in the accumulation of phosphorylated tau in retinal horizontal cells and Müller glia. While many studies have focused on neurons as the host for misfolded tau accumulation, reports of glial tau-mediated mechanisms of toxicity and their adverse effects on neuronal physiology and function are limited and require further investigation.

**Retinal α-synuclein in Parkinson’s disease**

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by two prominent disease processes: progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc) in the midbrain that results in progressive motor deficits (bradykinesia, tremors, and rigidity), and the deposition of intraneuronal Lewy-bodies, that contain misfolded α-synuclein [200-202]. Encoded by the SNCA gene, α-synuclein is a highly conserved protein, that is expressed predominantly in pre-synaptic terminals of neurons in the mammalian central nervous system, however its physiological functions are still poorly understood. Recent evidence describes non-motor manifestations of PD (e.g., mood and sleep disorders, gastrointestinal deficits etc.) that occur years before the onset of the cardinal motor symptoms [203-206]. Among other non-motor phenomena, visual impairments (impaired visual acuity, perceptual disturbances, contrast sensitivity, deficits in color vision, etc.) have been most widely reported in PD [3-6], with ~80% of PD patients reporting some extent of
visual deficits [4, 7-9]. However, the extent of retinal involvement in the generation of these visual symptoms is unclear. The existence of a “preclinical” phase in PD is an emerging interest in the scientific community, particularly due to the potential for diagnosis earlier in the disease process than is currently possible. The strong similarities between the retina and the brain has led to an expansion of studies investigating retinal pathology in PD in an effort to identify biomarkers for early diagnosis, as well as for monitoring disease progression and the effect of therapeutic interventions as they become available (reviewed in [14]). Retinal pathology in PD and other synucleinopathies (dementia with Lewy bodies (DLB, and multiple system atrophy (MSA)) has been widely reported in patients, as well as animal models.

Visual deficits in PD have been classically attributed to dopaminergic deficiency [3, 4, 13, 204, 207-209]. A study of the rodent retina in 1963 demonstrated dopaminergic amacrine cells (A18) [210] that have since also been described in the inner nuclear layer of the human retina [211]. A18 amacrine cells in the retina are thought to modulate light adaptation and circadian rhythms [4, 212]. Early reports of visual deficits in PD patients are accompanied by reports of reduced tyrosine hydroxylase (TH, rate limiting enzyme in the synthesis of dopamine) deficiency [213]. Additionally, an examination of post-mortem Parkinsonian patients by Harnois and DiPaolo revealed that retinas from patients receiving levodopa (L-DOPA, precursor to dopamine) had higher dopamine concentrations versus those that were not receiving treatment [214]. Further studies on retinal dopaminergic deficiency and the extent to which this affects visual symptoms seen in PD are limited. Structural changes in the retina have also been reported in PD patients, including a post-mortem study that reported “swelling” of retinal ganglion cells and photoreceptor cells and intracellular inclusions in the outer plexiform layer of DLB patients [215]. Since the development of non-invasive tools to probe
the retina, an increasing number of studies have reported structural changes in retinas of patients with PD, DLB, and MSA. Optical coherence tomography (OCT) is a non-invasive tool that uses low coherence light (typically near-infrared) to capture high-resolution (3-5 microns) cross-sectional images by measuring backscatter and time delays from biological tissues, in this case the retina [216-218]. Electoretinography (ERG) and pattern ERG are tool that measures the latency and amplitude of the electrical activity generated by the retina as it responds to visual stimuli [219]. Using OCT, studies have reported thinning of the retinal nerve fiber layer in PD patients [220, 221] however, the functional implications of these structural changes are unknown. Studies have also reported alterations in PERG amplitudes and latencies in PD patients, that are reversed upon treatment with L-DOPA [222-225] but, these studies also require further investigation.

The earliest report of α-synuclein accumulation in the diseased retina describes α-synuclein aggregates localized to the inner retina (IPL and GCL) of eight postmortem PD patients [226]. Several studies have since reported the presence of α-synuclein [226, 227], or α-synuclein phosphorylated at serine-129 (pSer129) [228, 229] in the retina of PD patients. Phosphorylation of α-synuclein at serine 129 is one of the several post-translational modifications to α-synuclein known to occur in PD. However, over the past decade a growing body of evidence report dramatic accumulation of α-synuclein phosphorylated at serine 129 (herein referred to as α-synuclein (pSer129)) in the brains of PD patients, and in the brains of transgenic animal models [230-233]. Studies attribute this post-translational modification to the increased formation and self-propagation of α-synuclein aggregates, and Lewy body formation [230-234]. Ortuño-Lizaran et al. described intraneuronal α-synuclein (pSer129) deposits present in retinal ganglion cells of nine PD patients [229]. Beach et al. described
intraneuronal α-synuclein (pSer129) deposits in RGCs of only one PD patient, while 7/9 PD subjects and 1/3 DLB subjects had α-synuclein (pSer129) immunoreactivity in the RNFL [228]. Overall, studies examining retinas of PD patients provide evidence for the presence of α-synuclein (pSer129) immunoreactivity in the inner retina, specifically the RNFL, GCL, and IPL.

Evidence of α-synuclein deposits in PD patients led investigators to use existing animal models, both toxin-induced and genetic, to ask the following research questions: Are retinal changes seen in PD related to α-synuclein accumulation? Can retinal α-synuclein be visualized non-invasively in vivo? Can retinal α-synuclein burden be used as a biomarker for early/improved diagnosis? Classical toxin-induced rodent and primate models are those produced by 1-methyl-1,2,3,6-tetrahydropiridine (MPTP), rotenone, 6-hydroxydopamine (6-OHDA), and paraquat – shown to recapitulate microscopic, neurochemical and molecular hallmarks associated with PD [235-237]. In an early study, Bodis-Wollner and Tzelepi reported abnormal ERG responses and a decline in contrast sensitivity and visual acuity, in an MPTP-induced primate model [238]. Using OCT and detection of apoptosing retinal cells (DARC), Normando et al. described swelling of the retinal layers and apoptosis of RGCs in retinas of rats, 20 days after rotenone administration [11]. In a follow-up of this model, Normando et al. reported dopaminergic degeneration in the striatum and substantia nigra by day 60, becoming the first to demonstrate that retinal changes precede the classical pathological manifestations of PD in the brain [11]. Collectively, rodent and primate models of PD produced by rotenone, 6-OHDA, and MPTP have reported thinning of the inner retinal layers, photoreceptor loss, as well as decreased tyrosine hydroxylase immunoreactivity [95, 239-245].
Since the first genetic link of SNCA to PD in 1977, investigators have created numerous transgenic mouse models in which α-synuclein is overexpressed due to duplication or triplication of the SNCA gene or single nucleotide polymorphisms in the SNCA gene resulting in mutations (Ala53Thr, Ala30Pro, and Glu46Lys) linked to familial PD. Other transgenic rodent models of PD are those carrying LRRK2 mutations or knockout mice of DJ-1, PINK-1, and Parkin [246, 247]. These genetic mouse models recapitulate varying clinical aspects of PD including the slow and progressive accumulation of α-synuclein throughout the central nervous system, however, reports of α-synuclein expression in the retina are limited (reviewed in [248]). A live imaging study revealed accumulation of intraneuronal α-synuclein deposits in the GCL of α-synuclein:GFP mice in a transgenic (mThy1-α-syn) model of Parkinson’s disease [249]. Price et al. concluded that longitudinal live imaging of the retina in this mouse model may represent a useful, non-invasive tool to monitor α-synuclein burden in the retina, and to evaluate the therapeutic potential of α-synuclein targeting compounds [249].

A plethora of models have postulated the cell-to-cell propagation of pathological α-synuclein species throughout the CNS [234, 250-259], most notably those using inoculation of pre-formed α-synuclein fibrils/oligomers and/or α-synuclein expressing viral vectors to induce widespread accumulation of α-synuclein and progressive neurodegeneration [260-264]. These approaches have been pivotal in investigating the “prion-like propagation” of α-synuclein, specifically the ability of pathological α-synuclein to spread transcellularly and induce aggregation by templating protein misfolding [200, 234, 255, 256, 258, 259, 261, 262, 264-268]. However, reports on these models do not include investigation of the retina. In a recent report, we described retinal pathology in a transgenic mouse model (TgM83) expressing the human Ala53Thr α-synuclein mutations. We demonstrate that the accumulation of α-synuclein
(pSer129) and tau, activation of retinal glial cells, and loss of photoreceptor cells detected in retinas of 8-month-old transgenic mice is accelerated upon “seeding” or intracerebral inoculation with brain homogenate from clinically ill transgenic mice [73]. Compelling evidence for PD-related alterations in the retina, has propelled investigators to further explore the potential use of retinal α-synuclein as a diagnostic biomarker.

**Retinal PrPSc in transmissible spongiform encephalopathies**

Transmissible spongiform encephalopathies are a group of fatal and rapidly progressive protein-misfolding diseases associated with the aberrant folding and accumulation of prion protein that inevitably results in neurodegeneration [269-273]. The term ‘prion’ was coined by Stanley Prusiner, to describe a proteinaceous and infectious particle, as the transmissible disease-causing agent of TSEs [274]. These diseases can be sporadic, inherited, or infectious, and rely on an autocatalytic conversion of PrP<sup>C</sup>, that is primarily composed of α-helices and sensitive to cellular digestion, to PrP<sup>Sc</sup> that is largely made up of β-sheets and is resistant to proteases [275]. While primarily found in the CNS, PrP<sup>C</sup> has also been demonstrated in other tissues including heart, spleen, uterus, skeletal muscle, gastrointestinal tract, and species-dependent blood components [276-278]. Although there is a lack of consensus regarding the physiological role of PrP<sup>C</sup>, several biological functions have been proposed, including neuroprotection due to its antioxidant properties, antiapoptotic activity, transmembrane signaling, cell adhesion, and synaptic vesicle trafficking (reviewed in [279]). TSEs have been described in several species, including scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, transmissible mink encephalopathy (TME) in mink, and Kuru and Creutzfeldt Jakob disease (CJD) in humans. Structurally distinct conformers of PrP<sup>Sc</sup> give rise to strains within a species that exhibit and can be distinguished by specific disease
phenotypes, such as molecular profiles, clinical signs, incubation periods, and pathologies (e.g., vacuolation profiles in mouse models, neuroanatomical and cellular accumulation patterns of PrP\(^{Sc}\)) [270, 280-284]. Although distinct differences in regard to TSE pathogenesis can exist depending on a variety of factors (e.g., type of TSE, host species, strain, etc.) [282, 284-287], neuropathology common to all TSEs occur in a classical triad, namely spongiform change (vacuolation), gliosis (macro- and microglia), and neuronal loss [288].

Similar to other protein-misfolding diseases, the accumulation of PrP\(^{Sc}\) in the retinas of animals infected with TSEs has been widely demonstrated both in natural host species and animal models [289-304]. Accumulation of PrP\(^{Sc}\) has been classically demonstrated in retinas of sporadic or variant CJD patients [290, 291] accompanied by vacuolation, overall disruption of the synaptic (outer and inner plexiform) layers [305], and altered retinal function characterized using ERG [300, 301, 305-308]. Retinal accumulation of PrP\(^{Sc}\) has also been described in various murine models of TSEs [309-313], commonly reported with accompanied retinal disorganization [302], activation of retinal Müller glia and astrocytes [74, 122, 296, 297, 304], microglia [74, 122, 297], and in some cases neuronal loss [74]. A study describing the temporal separation of retinal pathologies in response to PrP\(^{Sc}\) reported that in a mouse model of scrapie PrP\(^{Sc}\) accumulation precedes activation of Müller glia and microglia, which is then followed by photoreceptor cell loss [74]. Additionally, Greenlee et al. reported that antemortem changes in retinal function and morphology, measured using ERG and OCT respectively, can be detected in BSE inoculated cattle up to 11 months prior to the appearance of any other clinical signs, highlighting the potential of the retina in TSE diagnosis [122]. A similar study demonstrated that functional changes develop in the retina of cattle inoculated with TME several months prior to unequivocal clinical signs in the animals [302]. Collectively,
these studies demonstrate that the retina is affected by TSEs prior to the emergence of traditional clinical signs, thus underlining the suitability of the retina to study prion disease pathogenesis, as well as the potential use of retinal imaging in prion disease diagnostics.

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CHAPTER 2. LASTING RETINAL INJURY IN A MOUSE MODEL OF BLAST-INDUCED TRAUMA

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Abstract

Traumatic brain injury (TBI) due to blast exposure is currently the most prevalent of war injuries. While secondary ocular blast injuries due to flying debris are more common, primary ocular blast exposure resulting from blast wave pressure (BWP) has been reported among survivors of explosions, but with limited understanding of the resulting retinal pathologies. Using a compressed air-driven shock tube system, adult male and female C57BL/6 mice were exposed to blast wave pressure of 300 kPa (43.5 psi) per day for three successive days, and euthanized 30 days post injury. We assessed retinal tissues using immunofluorescence for glial fibrillary acidic protein (GFAP), microglia specific proteins Iba1 and CD68, and phospho-PHF-tau (AT-270, AT-180). Primary blast wave pressure resulted in activation of Müller glia, loss of photoreceptor cells, and an increase in phospho-tau in retinal
neurons and glia. We found that 300 kPa blasts yielded no detectable cognitive or motor deficits, and no neurochemical or biochemical evidence of injury in the striatum or pre-frontal cortex, respectively. These changes were detected 30 days after blast exposure, suggesting the possibility of long-lasting retinal injury and neuronal inflammation after primary blast exposure.

**Key words:** blast wave pressure, traumatic brain injury, retinal pathology, Müller glia, photoreceptor cell loss, phospho-tau in neurons and glia, microglial activation

**Introduction**

The nature of twenty-first century military conflict has led to a dramatic increase in exposure of military personnel and civilians to blast wave pressure, leading to traumatic brain injury [1]. The retina is part of the central nervous system; as such, it is vulnerable to injuries similar to those that affect the brain [2]. Today, over 80% of military personnel suffering from traumatic brain injury also exhibit symptoms of visual dysfunction [3]. Primary ocular blast exposure resulting from blast wave pressure has been reported among survivors of explosions, but with limited understanding of the resulting retinal pathologies, therefore therapeutic interventions are currently out of reach [4]. Previous consolidation of data from the United States Department of Defense, shows a high percentage of visual field defects, photophobia, oculomotor dysfunction, and decrease in contrast sensitivity in service members, 45 to 60 days after blast-induced traumatic brain injury, supporting the need for a chronic blast wave pressure injury model [5]. Existing rodent models of blast injury show the susceptibility of the retina to the effects of low-level (120 ± 7 kPa) and high-level (≥180 kPa) blast wave pressure, including glial cell activation in the ganglion cell layer, inner nuclear layer and outer nuclear layer with an overall increase in biomarkers of inflammation and apoptosis [2, 4, 6]. Existing models
differ significantly in intensity and duration, however long-lasting effects of blast wave pressure have not been reported.

We conducted a study in which a compressed air-driven shock tube system was calibrated to deliver blast wave pressure of 300 kPa (43.5 psi) each day for three successive days to mice. Here we show that 30 days post exposure to successive blast wave pressure, the retinas of exposed mice present with glial cell activation, microglial activation, photoreceptor cell loss, and an increase in phosphorylated tau in the outer plexiform layer. Proximity of the eye to blast wave pressure had a substantial effect on the severity of these retinal responses. There was a notable difference in the response of the retina on the side of the mouse ipsilateral to blast exposure in comparison to the retina on the contralateral side. Behavioral parameters including the Morris Water Maze, Rotarod, and open-field activity, showed no deficits in cognitive or motor function. Neurochemical assessment of the striatum, as well as composition of astrocytes, microglia, and phosphorylated-tau within the pre-frontal cortex showed no indication of damage induced by blast wave pressure. To the best of our knowledge, this report is the first to compare neurological and retinal effects of blast injury. This study is also the first to show prolonged effects of blast wave pressure on specific retinal cell types. Specifically, we report Müller glia hypertrophy, microglial activation, photoreceptor cell loss, and an increase in phospho-tau in both retinal neurons and glial cells. This blast wave pressure model may provide insight into the underlying pathologic mechanisms and help to identify markers of long-lasting retinal injury due to blast exposure.
Materials and Methods

Ethics statement

This experiment was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC) and was approved by the Iowa State University Animal Care and Use Committee (protocol 4-11-7123-M).

Exposure to Blast Wave Pressure

Exposure to blast wave pressure was conducted using an open-ended shock tube as described by Shah, M.A. and colleagues [7]. In this model, a 0.3-m driver section is rapidly pressurized with compressed helium until it ruptures four, 0.35-mm-thick mylar membranes (burst pressure: ~9.9 MPa), propelling a shock wave down a 3-m, open-ended driven section (ID: 3.6 cm). Briefly, 20 adult male or female C57BL/6 mice were randomly divided into blast wave pressure (BWP) or sham-exposed groups. Ten mice were exposed to blast wave pressure of 300 kPa (peak pressure mean ±SD: 297±15 kPa; positive duration: 146±6 µsec) each day for three successive days, and euthanized at 30 days post injury. The remaining ten mice served as control. Mice were anesthetized with 4% isoflurane in 2 L/min oxygen for 90 seconds in the induction chamber, then supplemented with 2% isoflurane in 2 L/min oxygen via nose cone in blast animal holder until blast delivery (approximately 20 seconds). Gas anesthesia was withdrawn immediately before triggering blast. Blast winds accompanying intense blast overpressures can lead to substantial head acceleration generating severe or lethal injuries, therefore the animals were placed 45° lateral to the shock tube axis to avoid blast wind exposure. The body was constrained securely inside a padded metal holding tube to limit BWP exposure primarily to the head. The head was constrained to limit any movement, and prevent...
injury due to head rotation. Control mice received anesthesia and were constrained the same way, except for exposure to BWP. Mice we placed at a distance of 15 cm from the open end of the shock tube, with the right side of the head (ipsilateral eye) facing the blast wave (Fig 1). The angle and distance from the shock tube’s open end determined the peak overpressure and positive duration experienced by the mouse, both of which were measured by a pressure transducer located 1.75 cm below the mouse’s head. On average, recovery from the blast exposure and anesthesia, indicated by upright posture and ambulation, occurred after approximately 30 seconds.

**Histopathology and Immunohistochemistry**

Blast exposed and sham mice were anesthetized intraperitoneally with ketamine (200 mg/kg) and xylazine (20 mg/kg) followed by supplementation with isoflurane and perfused transcardially with 4% paraformaldehyde in 0.01 M phosphate-buffered saline. Ipsilateral globes were post-fixed in 4% PFA. After 24 hours, each lens was removed and globes were subjected to a sucrose gradient (10%, 20%, 30% all in 0.1 MPO₄ buffer), embedded in OCT and frozen using dry ice. Sagittal sections (9-µm-thick) of the ipsilateral retina were collected onto superfrost plus glass slides. Globes contralateral to the blast were postfixed in Bouin’s fixative for 24 hours, embedded in paraffin, and sectioned sagittally at 4-µm onto superfrost plus glass slides. Paraffin-embedded sections of the retina were rehydrated using xylene, followed by a decreasing ethanol concentration gradient (100%, 90% 70%), and a final wash with diH₂O. Heat-mediated antigen retrieval was performed using EDTA buffer (10 mM Trizma Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0) in an autoclave for 30 minutes. OCT-embedded sections of the retina were hydrated for 15 minutes using Tris-Buffered Saline with 0.05% Tween 20 (TBS-T). Paraffin and OCT-embedded tissues were incubated with
Background Buster (Innovex Biosciences Inc., Richmond, CA) for 1.5 hours. Primary antibodies against GFAP (1:500; Dako, Carpinteria, CA), Iba1 (1:500; Wako Chemicals USA, Inc. Richmond, VA), CD68 (1:100; Wako Chemicals, Richmond, VA), PHF-Tau Clone AT-270 (1:100; Thermo Fisher Scientific, Inc., Rockford, IL), PHF-Tau Clone AT-180 (1:100; Thermo Fisher Scientific, Inc., Rockford, IL), PHF-Tau Clone 39E10 (1:250, BioLegend), and Calbindin (1:1,000, Dako, Carpinteria, CA) were diluted in blocking solution containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 0.04% Triton X-100 (Thermo Fisher Scientific, Inc., Rockford, IL), and 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS and incubated for 48 hours at room temperature, then 24 hours at 4°C. Tissues were washed with TBS-T (6 x 5 minutes), and incubated with a secondary antibody cocktail including Cy™3 and/or Alexa Fluor 488–conjugated AffiniPure secondary antibodies (1:300; Jackson ImmunoResearch), and 40,6-diamidino-2-phenylindole, dilactate (DAPI, 1 µg/mL; Sigma-Aldrich) for 1.5 hours. Following another wash, slides were mounted with Vectashield HardSet antifade mounting medium (Vector Laboratories Inc., Burlingame, CA). Negative controls were processed in parallel by omission of the primary and/or secondary antibody.

Behavioral Studies

**Social Discrimination Test**

The social discrimination test (SDT) assesses the ability of a mouse to discriminate between its own bedding and the bedding of a mouse of the opposite sex. The test is performed inside a narrow rectangular (44.5 x 10.8 cm) chamber made of clear Plexiglas. A small container filled with bedding is fixed at either end of the chamber. Each container is sealed except for a 1-cm diameter hole on top to allow sniffing without disturbing the bedding. A 3-
min test session began by placing a mouse in the middle of the chamber facing his own bedding container. Movements inside the test chamber are video-tracked from above via webcam connected to ANY-maze software. The sniffing zone was defined by a circle 1 cm wider than the outside circumference of the bedding container, and time spent with head inside the sniffing zone was used to test for group differences.

**Morris Water Maze**

We assessed the impact of successive blast wave pressure exposure on learning and memory by subjecting mice to the Morris Water Maze (MWM) according to the rapid 2-day protocol described by Gulinello et al. with some modification. The apparatus comprised a round, 1.15-m diameter galvanized stock tank filled with water mixed with white tempera paint, allowing the movements of mice to be tracked by a webcam connected to ANY-Maze software. Several highly visible cues (references) were posted just outside the tank above each of four quadrants. Water temperature was maintained at 22-24°C. In this 2-day rapid MWM protocol, mice were subjected to one day of five 60-sec training trials pre-blast wave exposure and one day of five 60-sec training trials 30 days post exposure. During all five trials for both sessions, the 11.2-cm diameter platform (1% of pool area) was submerged 1-cm below the surface and remained in one fixed location. For Trial 1 only, the platform was made visible by a bright green vertical center post marking its location. Furthermore, each mouse was placed on the platform for 10 sec before initiating each trial. Every trial began with the mouse facing the tank wall, and trials ended with the mouse resting on the platform for 20 sec, having either reached the platform on their own or being placed there by the experimenter after the 60-sec trial ended. After each trial, mice were returned to their cages, which were placed on heating pads where they remained for 10-15 min, allowing enough time for their fur to dry before the
next trial began. We assessed maze performance by measuring the time taken to first reach the platform.

**Rotarod**

Locomotor coordination was measured on the accelerating rotarod (ACC, pc Rota Rod IV, Accuscan Instruments, Columbus, OH, USA), which could test four mice per run. At 30 days post blast wave pressure exposure, mice were placed on the 3.2-cm diameter rotarod as it rotated at 4 rpm. Then, after a 90-sec acclimation, the rotarod began accelerating to 20 rpm during a 1 min (16 rpm/min) period before ramping up to 20 rpm/min for an additional 2 min, thus reaching a maximum speed of 60 rpm after 3 min. By accelerating to high rpms in a relatively short time, we intended this to be a demanding motor coordination task to better discriminate subtle treatment effects while simultaneously minimizing the fatigue seen in prolonged constant speed trials. Latencies to fall are reported as the average of three trials, which were executed consecutively with a 20 sec gap (at 4 rpm) between acceleration trials. A trial ended when infra-red (IR) sensors below the rod registered a fall or when the experimenter triggered the sensors manually to terminate passive rotations. In the latter case, even though the trial was terminated, mice were left on the rotarod until the trial terminated for all mice.

**Open-field Test**

The spontaneous locomotor activity and thigmotaxis (wall-hugging or open-field anxiety) of mice exploring a novel open-field (OFT) was measured in a 40 x 40-cm (1600-cm$^2$) clear Plexiglas chamber that fit inside a VersaMax activity monitor (model VMM, Accuscan Instruments). The monitor is equipped with two 40 x 40-cm square arrays of IR beams. The IR LEDs within each array are 2.5 cm apart laterally, with the lowermost array monitoring the animal’s horizontal x-y position at 1.5 cm high, while the uppermost array at 10.5 cm high registered vertical movements. Horizontal and vertical activity scores represent
the number of IR beam breaks in the lower and upper rows of beams, respectively. The IR beam break data were acquired via the VersaMax Analyzer (model VMAUSB, Accuscan Instruments). We used VersaMap to define time spent in the center to be determined by beam breaks >10 cm (>4 IR beams) from walls. All VersaMax activity monitoring for pre- and post-treatment sessions lasted 12 min with only the last 10 min used for analysis. The first 2 min, representing within-session acclimation, were truncated.

**High Performance liquid chromatography (HPLC) analysis of striatal neurotransmitter levels**

Tissue concentrations of 1) dopamine (DA) and its two main metabolites 3,4-dihydroxyphenyl-acetic acid (DOPAC) and homovanillic acid (HVA), 2) serotonin (5-HT) and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA), and 3) norepinephrine (NE) were quantified using HPLC with electrochemical detection. After 30 days post blast wave exposure, samples from the striatum were prepared and quantified as described previously. Briefly, four female and four male mice randomly selected from each group were sacrificed via CO₂ inhalation followed by exsanguination by cardiac puncture. Target tissues dissected from the extracted brains were weighed and then immediately frozen on dry ice after suspending in 0.1 M perchloric acid solution containing 0.05% Na₂EDTA and 0.1% Na₂S₂O₅ before transferring them to -80°C. Tissue homogenates were centrifuged through 0.22-μm filters before diluting 1:9 in the phosphate-buffered acetonitrile mobile phase MD-TM (ESA Inc, Chelmsford, MA, USA). The primary analytes DA, 5-HT, NE and the metabolites DOPAC, HVA and 5-HIAA were separated isocratically by injecting 20 μl through a C-18 reversed-phase column (Microsorb-MV 100-3, 100 x 4.6 mm, 3-μm particles) using a flow rate of 0.6 ml/min on an HPLC system (UltiMate 3000, Dionex, Madison, WI, USA) coupled to an analytical auto-sampler (WPS-3000TSL, Dionex). The electrochemical detection system
consisted of a coulometric array detector (CoulArray 5600A, Dionex) with a guard cell (model 5020, Thermo Scientific, Chelmsford, MA) and an analytical cell (model 5014B, ESA Inc). The data acquisition and analysis were performed using CoulArray Data Station Software (ESA Inc).

**Western Blot Analysis of Brain Tissues**

Tissue homogenates were prepared using modified RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA) as previously described [8]. Briefly, pre-frontal cortex, ipsilateral to the blast was dissected and homogenized in modified RIPA. Protein concentrations were determined with the Bradford protein assay. Homogenates containing equal amounts of protein were separated on a 10-to-15% SDS-polyacrylamide gel. After separation, proteins were transferred to a nitrocellulose membrane, and non-specific binding sites were blocked by treating with LI-COR blocking buffer for 1 hour. The membranes were then incubated with primary antibodies directed against PHF-1 (rabbit monoclonal; 1:2000 dilution, a gift from the lab of Dr. Peter Davies, Albert Einstein College of Medicine), PHF-Tau Clone 39E10 (mouse monoclonal; 1:2000 dilution, BioLegend, San Diego, CA), Iba1 (Goat monoclonal; 1:1200 dilution, Abcam, Cambridge, MA), or GFAP (mouse monoclonal; 1:1200 dilution, Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. The primary antibody treatments were followed by treatment with IR800-conjugated anti-rabbit or Alexa Fluor 680-conjugated anti-mouse secondary antibody (1:5000, Abcam) for 1 h at room temperature. To confirm equal protein loading, blots were reprobed with β-actin antibody (1:15000 dilution, Abcam). Western blot images were captured with the Odyssey IR Imaging system (LI-COR) and data were analyzed using Odyssey 2.0 software.
Quantitative and Statistical Analysis

Fluorescence images were taken at 20x using a commercial upright microscope system (Zeiss AxioPlan 2 Microscope Imaging System; Oberkochen, Germany). Confocal images were captured using a Nikon A1R+ Resonant Scanning Confocal System with a Ti-E inverted microscope and laser lines 405, 488, 561 and 640 nm (Nikon Instruments Inc., Melville, New York). Three different sites of the retina were analyzed to gauge for differences in immunoreactivity: central retina at the thickest section of the retina adjacent to the optic nerve head, and two peripheral retinal locations on opposite sides of the retina within one 20x field of view from the peripheral margin. Micrographs were captured using a commercial photo-editing system (Adobe Photoshop and Adobe Illustrator [CC]; Adobe Systems). For quantification of GFAP, Iba1, AT-270, AT-180, and CD68 immunoreactivity, the percentage of the total image area thresholded (outer limiting membrane to inner limiting membrane of the retina) was analyzed using ImageJ (Rasband, W.S., ImageJ 1.49V, U. S. National Institutes of Health, Bethesda, Maryland). Sex vs. treatment interaction effect via two-way ANOVA was used to determine whether sexes should be pooled. For all behavioral, as well as histological studies the ratio of males to females were equal (n=5 males, 5 females). Preliminary analyses for behavioral and histological studies (p= <0.05), showed the two sexes did not respond differently to the same treatment (Table S2), therefore sexes were pooled. Pooled rotarod data was analyzed using an unpaired t-test. All other pooled behavioral data was analyzed using two-way ANOVA, and Tukey’s multiple comparisons test (post-hock). HPLC data was analyzed using an unpaired t-test. Quantified histological data was analyzed using two-way ANOVA, and Tukey’s multiple comparisons test (post-hock). Thickness of the outer nuclear layer (ONL) was measured by counts of cell bodies spanning the layer. For each animal (n=20),
the central retina within a cross section was used. Retinal thickness was analyzed using an unpaired t-test. Prism 6 for Windows (Graph Pad Software) was used for statistical analysis.

**Results**

**Blast wave pressure induces activation of retinal glial cells**

Müller glia are the principal glial cells of the retina with processes projecting radially through the thickness of the retina and provide limits at the outer and inner limiting membrane. In the healthy retina, GFAP in Müller glia is localized to the end feet at the retina’s inner limiting membrane [9]. In response to retinal injury or stress, thick GFAP immunoreactive processes span the retina from the inner limiting membrane to the external limiting membrane, concomitant with hypertrophied processes of Müller glia [10]. We assessed the distribution of GFAP immunoreactivity to detect activation of Müller glia after blast exposure. GFAP immunoreactivity in sham animals with retinas ipsilateral (Fig 2A) and contralateral (Fig S1A) to blast exposure, was localized to the Müller glia end feet, and astrocytes in the optic fiber layer. When quantified, the distribution of GFAP immunoreactivity in retinas of sham animals accounted for approximately 2.60 ± 0.39% (mean ± SE) of total area (Fig 2C; Fig S1C). Thirty days post blast exposure, the area of GFAP immunoreactivity in retinas ipsilateral to blast exposure was increased and consistently spanned the retina from the Müller glia end feet in the inner limiting membrane to the outer plexiform layer, with some apical processes visible in the outer nuclear layer (Fig 2B). There were no significant differences in the area of GFAP immunoreactivity between peripheral and central retinas of blast exposed mice (22.79 ± 3.19%, and 29.41 ± 2.35% of total area respectively; 5C). In contrast, GFAP immunoreactivity in retinas contralateral to blast exposure remained localized to the Müller glia end feet, with occasional processes extending past the optic fiber layer (Fig S1B). When quantified, the area
of GFAP immunoreactivity in retinas contralateral to blast exposure was 4.52 ± 0.77%, and 4.32 ± 0.74% of total area in peripheral and central retinas, respectively (Fig S1C).

**Successful blast exposure induces an inflammatory response in the retina**

The prevalence and morphology of retinal microglia in response to blast injury was assessed using Iba1 immunoreactivity. In sham animals, Iba1 immunoreactivity showed ramified microglia with thin irregular processes and small somata (occasionally lacking processes), within the inner and outer plexiform layers (Fig 3A). Iba1 immunoreactivity occupied 0.31 ± 0.06% of total area in peripheral retina, and 0.37 ± 0.11% of total area in central retina (Fig 3C). Iba1 immunoreactivity in the retinas ipsilateral to blast exposure showed an amoeboid-like morphology with thicker, and longer processes, and swollen cell bodies, all characteristics of activated microglia [11]. Iba1 immunoreactivity was localized to the inner and outer plexiform layers (Fig 3B). When quantified, Iba1 immunoreactivity accounted for 0.93 ± 0.19%, and 0.93 ± 0.18% of total area in peripheral and central retinas, respectively (Fig 3C). Iba1 immunoreactivity in retinas contralateral to blast exposure was not significantly different than retinas of sham animals, however morphological differences of microglia in retinas from blasted animals were noted (Fig S2A-C). The cell surface antigen CD68 is a common marker expressed on macrophages [12], therefore immunoreactivity for CD68 was used to examine blast exposed retinas for pro-inflammatory microglial activation. No CD68 immunoreactivity was detected in the retinas of sham exposed mice (Fig 3D; Fig. S2D). The retinas of exposed mice from the side ipsilateral to the blast were positive for CD68 immunoreactivity in the inner and outer plexiform layers (Fig 3E). When quantified, CD68 immunoreactivity was 1.22 ± 0.12%, and 1.16 ± 0.15% of total area in peripheral and central retinas, respectively (Fig 3F). In contrast, values for CD68 positive microglia in the retinas
contralateral to blast exposure were significantly lower than that of retinas ipsilateral to blast exposure, making up 0.32 ± 0.03%, and 0.30 ± 0.06% of total area in peripheral and central retinas, respectively (Fig S2D-F).

**Successive blast exposure results in an increase in phosphorylated tau species detected in retinal neurons and glia.**

Hyper-phosphorylated tau is a major component of neurofibrillary tangles involved in the pathology of Alzheimer’s disease and other neurodegenerative conditions [13]. Antibodies against AT-270, and AT-180 (detects paired helical filament with a phosphorylated threonine in position 181, and 231 respectively) were used to evaluate the presence of phosphorylated tau species in the retinas of animals exposed to blast wave pressure. There was very little AT-270, and AT-180 immunoreactivity in retinas of sham animals. AT-270 immunoreactivity comprised 0.04 ± 0.04% of total area in peripheral retinas, and 0.09 ± 0.07% of total area in central retinas, while AT-180 immunoreactivity made up 0.02 ± 0.01% of total area in peripheral retinas, and 0.03 ± 0.02% of total area in central retinas (Fig 4A, C, D, F). However, thirty days after successive blast exposure, retinas ipsilateral to blast exposure showed robust AT-270, and AT-180 immunoreactivity localized to the optic fiber layer, and outer plexiform layer (Fig 4B, E). When quantified, AT-270 immunoreactivity was approximately 6.34 ± 0.90% of total area in peripheral retinas, and 5.72 ± 0.84% in central retinas (Fig 4C). In contrast, values for AT-270 in the retinas contralateral to blast exposure were significantly lower than that of retinas ipsilateral to blast exposure (Fig S3). AT-180 immunoreactivity was approximately 3.67 ± 0.90% of total area in peripheral retinas, and 3.65 ± 0.53% in central retinas (Fig 4F). An antibody against total tau demonstrated there was no appreciable difference in total tau immunoreactivity when comparing retinas from blast animals to sham animals (Fig. S4). The outer plexiform layer contains numerous neuronal processes, including
Successive blast exposure causes a loss of photoreceptors

To determine if blast exposure resulted in changes in retinal morphology, we assessed the thickness of the outer nuclear layer, as measured by number of photoreceptor cell nuclei. Thirty days after blast exposure, the mean thickness of the outer nuclear layer of retinas ipsilateral to blast exposure, was significantly decreased from approximately 14 cell nuclei in sham retinas to 10 cell nuclei in retinas of blast exposed mice (Fig 7). Outer nuclear layer thickness in retinas contralateral to blast exposure, trended to decrease from 13 cell nuclei in sham animals to 11 cell nuclei, however the decrease did not reach significance. (Fig. S5).

Blast Wave Pressure does not Significantly Affect Cognitive or Motor Function.

To provide context for retinal changes we report, following are results from assessment of cognitive and motor function as well as striatal neurotransmitter and GFAP and Iba-1 levels.
in the frontal cortex. To assess the effect of blast wave pressure on cognitive function, olfactory recognition memory and spatial learning we used the Social Discrimination Test (Fig 8A), and Morris Water Maze (Fig 8B) respectively. Cognitive function was assessed 30 days after exposure to blast wave pressure. Olfactory recognition memory refers to the ability to distinguish between familiar and unfamiliar stimuli, in this case the ability of adult male and female mice to discriminate between its own bedding and the bedding of a mouse of the opposite sex. Olfactory recognition was measured according to the time a mouse spent within the sniffing zone of its bedding. Preliminary results showed the two sexes did not respond differently to the same treatment, therefore sexes were pooled (data not shown). We show no significant difference in time spent in the sniff zone, between sham, and mice exposed to blast (Fig 8A). The Morris Water Maze was used to assess the effect of blast wave pressure on memory, by measuring latency to platform, or the time it took for a mouse to find and reach the platform following training sessions. Our data showed no significant difference in performance between sham, and blast-exposed groups (Fig 8B). A series of tests were performed to characterize the impact of blast wave pressure on spontaneous locomotion. Thirty days following blast exposure, mice were tested using the Versamax locomotor activity monitor and the Rotarod. No deficit in motor function was detected in blast exposed mice when compared to sham as seen with behavioral parameters including the Rotarod test (Fig 8C), open-field activity (Fig 8D), horizontal activity (Fig 8E), or vertical activity (Fig 8F). Together, our results suggest that blast wave pressure does not cause deficits in cognitive or motor function.
**Blast wave pressure does not cause damage to striatal or cortical neurons**

To further probe the effect of blast wave pressure on the brain, we measured striatal dopamine and its metabolites, DOPAC and HVA; serotonin and its metabolite, 5-HIAA; and norepinephrine by HPLC (Fig 9). Our results show no significant changes in striatal neurotransmitter levels between sham, and blast exposed groups. To assess for blast-induced pathology in the pre-frontal cortex, we measured protein levels of GFAP, Iba1, p-tau, and total tau. Western blot analysis revealed no significant differences in levels of Iba1, or phosphorylated tau in the pre-frontal cortex 30 days after exposure to blast wave pressure (Fig 10A, C, D), though, GFAP expression in the pre-frontal cortex was decreased 30 days after exposure to blast wave pressure (Fig 10A, B).

**Discussion**

This study demonstrates that three, daily successive exposures to 300 kPa (43.5 psi) blast wave pressure induces long-lasting glial cell activation, inflammation, neuronal loss, and an increase in phospho-tau in the mouse retina. Despite these retinal effects, no significant changes in cognitive or motor function were detected. This suggests that robust retinal pathology was not due to extreme severity of blast wave pressure. We show no significant changes in striatal neurotransmitters, and we did not detect significant differences in levels of Iba1, or phosphorylated-tau in the pre-frontal cortex. There was an observed decrease in GFAP in the pre-frontal cortex of these animals, which may be due to remodeling of the astrocyte network due to stress [15]. Thus, in this model, the retina may serve as a CNS compartment that is more vulnerable, and therefore may be an effective and more sensitive indicator of low-level injury due to blast wave pressure.
This work used a compressed air-driven shock tube system to expose mice to blast wave pressure of 300 kPa per day for three successive days. A compressed shock tube system provides an instantaneous rise in pressure, immediately followed by a decrease below the surrounding ambient pressure that occurs within two milliseconds, a range of time that closely reflects a real explosive blast [16]. Additionally, the mice were not exposed to the blast wind to prevent injury due to head rotation, thus ensuring that injury is due to neural tissue deformation resulting from the blast wave pressure. Our model provides for controlled investigation of the chronic effects of high-level blast wave pressure without the confounding effects of secondary blast injury due to flying debris, or injury due to head acceleration/deceleration.

The current study reports a change in distribution of GFAP immunoreactivity in Müller glia indicating activation in the retinas of blast-exposed mice. Activation of Müller glia was greater in retinas ipsilateral rather than contralateral with respect to the direction of the blast wave. Hypertrophy of Müller cells, indicated by an increase in the distribution of GFAP immunoreactivity is a widely reported pathology after exposure to blast wave pressure, however most are reports of acute trauma. A number of studies report an increased distribution of GFAP immunoreactivity in Müller cell processes seven days post exposure [2, 4, 17]. Increased distribution of GFAP throughout Müller glia is a ubiquitous response to stress observed in various retinal degenerative diseases [18]. Müller glia serve to maintain neuronal homeostasis including degradation of foreign material, therefore a stress response evident by swollen Müller end feet, and hypertrophied processes 30 days after blast exposure suggests a fundamental role in response to retinal trauma [18]. While glial cell activation immediately after retinal injury is important for the repair and protection of retinal neurons, studies show
that diabetic retinopathy and other retinal degenerative diseases may be exacerbated by reactive gliosis causing dysplasia and overall progression of neuronal degeneration [19, 20]. Due to this, insight into the regulation of this process may be necessary to determine the role of Müller glia in long lasting retinal injury due to successive blast wave pressure.

Our results using Iba1 immunoreactivity indicate activation of microglia with an amoeboid-like morphology and thick processes localized to the outer plexiform and inner plexiform layers of retinas exposed to blast. 30 days after blast exposure, Iba1 protein levels in the pre-frontal cortex were comparable to sham. Others have shown prevalence of microglia seven days after blast exposure, specifically in the pyramidal tract of the pons, and cerebellar white matter; as well as the optic tract. This reactivity, however, was no longer evident after two weeks [21]. The presence of reactive microglia in the retina with an amoeboid-like appearance, beginning at three days post blast exposure has also been reported [17]. Anti-CD68 was used to evaluate a pro-inflammatory response due to local proliferation, and enhanced phagocytosis [13]. CD68 immunoreactivity showed a prominent macrophagic response of microglia in the inner and outer plexiform layers of blast exposed retinas, compared to the absence of CD68 positive microglia in sham animals. Microglia comprise the majority of tissue macrophage population within the CNS, therefore activation represents a common pathomechanism in a variety of retinal degenerative diseases, often parallel to chronic inflammation and the onset of retinal cell death [12]. Without a strict regulatory mechanism to limit this immunologic cascade, microglial activation may significantly contribute to furthering retinal tissue damage [13, 22]. This may be the reason for the presence of activated microglia 30 days after exposure to blast wave pressure. Further studies to assess the functional
phenotype of microglial activation, whether classical or alternative, are necessary to evaluate the specific role of microglia in long lasting retinal injury due to blast wave pressure.

We report increased tau phosphorylation (Thr231, and Thr181) in retinas of blast exposed mice. Phosphorylation of Thr231 is shown to reduce levels of acetylated tubulin, inhibiting microtubule assembly and stabilization [23]. Accumulation of neurofibrillary tangles is a hallmark feature of neurodegenerative disorders, all of which present with neuronal tau-positive inclusions as a predominant feature [24, 25]. In the healthy brain, tau localizes to axons to promote neuronal integrity and axonal transport, however a stepwise process including phosphorylation of specific sites, and later aggregation into neurofibrillary tangles, or oligomers can lead to neurodegeneration [26]. Most tauopathies are characterized by the abnormal accumulation of tau in both neurons and glial cells in contrast to the healthy brain where tau expression is minimal in glial cells. Glial tau phosphorylation occurs most often in oligodendrocytes, astrocytes, and to a lesser extent in microglia [25, 27, 28]. Studies in blast models demonstrate elevated levels of phosphorylated tau expressed by pyramidal neurons of the hippocampus and Purkinje and basket cells of the cerebellum two weeks after blast exposure, but the presence of phosphorylated tau species in the retina have not been investigated [29]. Huber et al., report elevated levels of cleaved tau in the brain 30 days after a single blast exposure event, therefore setting the stage for chronic neurofibrillary tangle formation [29]. Similarly, as part of the CNS, the retina is also susceptible to such damage. As previously stated, Müller glia undergo activation in response to retinal damage, which exacerbates pathology, therefore an increase in tau phosphorylation in Müller glia may also be a response to stress. Furthermore, while glial activation has proven protective in some contexts, glial cells are often implicated as secondary effectors of toxicity through the release of pro-
inflammatory cytokines [30, 31]. Perhaps the increase of phospho-tau in Müller glia contributes to the pathological response to stress. Retinal horizontal cells are a class of interneurons that regulate input and output between photoreceptor cells and bipolar cells via feedback and feedforward inhibition [32]. As horizontal cells modulate signal transmission from photoreceptors to bipolar cells, continued degeneration of horizontal cells due to tau accumulation may cause disruption of this signal, and contribute to retinal dysfunction [33]. Gupta et al. show a significant increase in tau protein (isoform AT8, phosphorylated at serine 202 and threonine 205) colocalized with parvalbumin in horizontal cells, within the posterior retina of surgical glaucoma specimens [34]. The significance of phospho-tau in Müller glia and horizontal cells in response to retinal damage caused by blast wave pressure requires further investigation.

We report a decrease in retinal thickness, specifically of the outer nuclear layer of the retinas of mice ipsilateral to blast exposure. By 30 days after blast exposure, the thickness of the outer nuclear layer decreased by approximately 28.5%. Since the outer nuclear layer contains cell bodies of photoreceptor cells, rods and cones, a decrease in thickness indicates photoreceptor cell death. Previous studies show decreased axon density and minimal degeneration ten months after blast injury, however, data on the loss of specific cell types and the thickness of retinal layers were not reported [16]. We also report that blast wave pressure of 300 kPa (43.5 psi) per day for three successive days does not induce deficits in cognitive function including olfactory recognition or spatial memory, evidenced by normal performance in the Social Discrimination test, and the Morris Water Maze. Consistent with this, the mice exhibited normal function on Rotarod, as well as in motor parameters in open field. Prior work has reported that mild traumatic brain injury (TBI), caused by repeated overpressure of 50 –
60 psi, results in open-field anxiety, as well as motor deficits at 1-2 weeks post exposure [21, 35]. Mild TBI caused by 50-60 psi blasts in mice induced sustained cognitive and motor deficits in mice, coupled with mild neuronal loss and diffuse axonal injury, similar to pathology observed in human mild TBI [35]. Additionally, Gullotti et al. conclude that 415±41 kPa (60±6 psi) is necessary to generate a significant behavioral deficit in terms of traumatic brain injury severity and survivability [36]. This study demonstrates no detectable changes in striatal neurotransmitters that regulate general cognitive and motor ability. Together, this data suggests that even with three daily successive exposures, the overpressure used in this study (43.5 psi), was not enough to generate prolonged cognitive or motor deficits.

Due to considerable concern in regard to the high frequency of traumatic brain injury causing visual dysfunction in humans, there are various existing rodent models of blast injury to the retina, but reports demonstrating long-lasting retinal injury are sparse. In this approach, blast wave pressure was targeted to the right side of the head, with the mice positioned to avoid blast wind-induced head rotational injuries that are likely severe or lethal at the high blast overpressures tested. One research group has characterized a model in which a total of 5 kg of 2,4,6-trinitrotoluene (TNT) with a penta-erythritol tetra-nitrate (PETN) booster was detonated to deliver high blast wave pressure of 180 KPa, and 480 KPa, while another group used an air paintball gun to directly target the eyes of mice with a puff of CO$_2$ (160-200 kPa), leading to corneal edema, and deficit in visual acuity [4, 37]. Repeated exposure to blast wave pressure originating from TNT (~120 ± 7 kPa) has been shown to induce glial cell activation in the retina, specifically in the ganglion cell layer and inner nuclear layer, inflammation, and compromised vascular permeability detected four to 72 hours after exposure, however prolonged or long term effects using either models were not demonstrated [4].
We demonstrate long-lasting effects of blast wave pressure on specific retinal cell types. Upregulation of GFAP by Müller glia is a robust indicator of retinal stress that may contribute to and exacerbate prolonged retinal pathology due to blast injury. We show elevated species of phospho-tau in horizontal cells and in regions of activated Müller cells. Horizontal cells contribute to the ganglion cell receptive field surround, which is essential for contrast sensitivity. Our results suggest that phospho-tau accumulation, as well as corresponding glial cell activation, may set the stage for decreased contrast sensitivity commonly reported by service members. Additionally, we show detectable photoreceptor cell loss 30 days after blast exposure, indicating a need for early intervention to counter primary blast effects on the retina. This mouse model of blast-induced retinal trauma provides insight into chronic retinal pathologies caused by blast wave pressure, without injury to the brain. Our work will aid in efforts not only to understand and potentially prevent retinal damage due to blast exposure, but also to assess the magnitude of exposure and identify individuals who may need or benefit from proactive treatments as they become available.

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Figure 1. Schematic representation of blast exposure. Exposure to blast wave pressure was conducted as described by Shah and colleagues. Animals were placed 38° lateral to the shock tube axis, 15 cm from the Mylar membrane, with the right side of the head (ipsilateral eye) facing the blast wave. Control mice were restrained the same way, but were not exposed to blast wave.

Figure 2. Distribution of GFAP in retinas ipsilateral to blast exposure, and quantification of the area of GFAP immunoreactivity. GFAP is localized to the OFL of retinas of sham mice (A). Thirty days following blast exposure, the distribution of GFAP immunoreactivity increased; spanning the retina from the OFL to the ONL (B). Bar graphs show a significant increase in the percentage area of GFAP immunoreactivity in peripheral and central retina after blast exposure (C). Data are expressed as mean ± SEM of 10 mice per group. Abbreviations: OFL = optic fiber layer; GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer. Scale bars = 20 μm. ** = p < .01; **** = p < .0001
Figure 3. Distribution of Iba1 and CD68 in retinas ipsilateral to blast exposure, and quantification of microglial activation. There are distinct morphological differences between microglia of control and blast-exposed retinas. Microglia of sham retinas are ramified with small cell bodies, and thin processes (A; arrows). Microglia of blast exposed retinas are amoeboid with swollen soma, and long thick processes (B; arrows). Microglia of both control and blast exposed retinas are localized to the OPL, and IPL. High magnification (63x) images of microglial morphology inset upper right corner (A, B). CD68 immunoreactivity shows activated microglia localized to the OPL and IPL (E), compared to the lack of activated microglia in sham retinas (D). Bar graphs show a significant increase in area of Iba1, and CD68 immunofluorescence in peripheral and central retina after blast exposure (C, F). Abbreviations: GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; Scale bars = 20 μm. Data are expressed as mean ± SEM of 10 mice per group. * = p < 0.05; **** = p < .0001
Figure 4. Expression and distribution of AT-270, and AT-180 in retinas ipsilateral to blast exposure, and quantification of immunoreactivity. Thirty days after successive blast exposure, retinas ipsilateral to the blast showed intense AT-270, and AT-180 immunoreactivity for phosphorylated tau epitopes was localized to the OPL, and OFL (B, E), while no AT-270 or AT-180 immunoreactivity was detectable in sham retinas (A, D). Phosphorylated tau in blast-exposed retinas is also occasionally localized to the IPL, with sparse immunoreactivity (B, E). Bar graphs show an increase in total fluorescence area for both phosphorylated tau epitopes in both peripheral and central retina after blast exposure (C, F). Abbreviations: OFL = optic fiber layer; GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; Scale bars = 20 μm. Data are expressed as mean ± SEM of 10 mice per group. *** = p < .001; **** = p < .0001
Figure 5. Colocalization of phosphorylated tau and Calbindin in retinas ipsilateral to blast exposure. Double labeling with AT-270 and calbindin shows colocalization of the two proteins in the OPL (A-C). Confocal imaging (40x) confirms colocalization in the OPL, suggesting that phosphorylated tau (pThr 181) is present in horizontal cells (D-F). Double labeling with AT-180 and calbindin also shows colocalization (G-I). Abbreviations: GCL = ganglion cell; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; Scale bars = 20 μm (A-C, G-I); Scale bars = 40 μm (D-F).
Figure 6. Colocalization of phosphorylated tau and GFAP in retinas ipsilateral to blast exposure. Double labeling with AT-270 and GFAP shows colocalization of the two proteins in the OPL and OFL (A-I). Confocal imaging (40x) confirms co-localization in the OPL (D-F) and OFL (G-I), suggesting that phosphorylated tau (pThr 181) is present in Müller glia. Double labeling with AT-180 (pThr231) and GFAP shows colocalization, primarily in the OPL (J-L). Abbreviations: OFL = optic fiber layer; GCL = ganglion cell; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; Scale bars = 20 μm (A-C, G-I); Scale bars = 40 μm (D-F).

Figure 7. Measurement of retinal thickness. Successive blast exposure causes a decrease in retinal thickness of retinas ipsilateral to blast exposure. Thickness of outer nuclear layer (ONL) is expressed as number of cell bodies spanning the thickness of the layer. Mean retinal thickness of sham retinas is 13.83±.31 nuclei. In retinas ipsilateral to blast exposure, mean retinal thickness is 10.33±.84 nuclei. Data are expressed as mean ± SEM of 10 mice per group. ** = p < .01
Figure 8. Successive blast exposure does not affect cognitive or motor function. Performance parameters using the VersaMax infrared computerized activity monitoring system, and Morris Water Maze (MWM) were used as a measure of cognitive function (A, B). The social discrimination test assesses olfactory recognition memory (A), while the MWM assesses for overall memory retention (B). Motor function was measured using the Rotarod, and VersaMax infrared computerized activity monitoring system. VersaMax data showing latency to fall on Rotarod (C), center time in VersaMax open-field (D), horizontal activity (E), and vertical activity (F). Center time in VersaMax open-field (D) is also a correlate of open-field anxiety. Analysis of spontaneous locomotor activity shows no significant difference between sham, and mice exposed to blast wave pressure. Data are expressed as mean ± SEM of 10 mice per group.
Figure 9. Blast wave pressure does not deplete striatal neurotransmitter levels. Thirty days after blast exposure, levels of striatal dopamine, norepinephrine, serotonin and its metabolite levels were measured using high-performance liquid chromatography (HPLC). Compared to the striata of control mice, exposure to blast does not significantly alter levels of striatal dopamine (DA), 3,4-dihydroxyphenyl-acetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), 5-hydroxytryptamine (5-HT, serotonin), or 5-hydroxyendoleacetic acid (5-HIAA) as measured by HPLC. Data are expressed as mean [ng/mg] ± SEM of 4 mice per group.
Figure 10. Western blot analysis of GFAP, Iba1, and pTau protein expression levels in the Prefrontal cortex. GFAP (50 kDA), Iba1 (15 kDA), pTau (50 kDA), Tau (50 kDA), and Actin (37 kDA) immunoreactive bands (A). Representative bar graphs showing quantitative densitometric analysis of GFAP/Actin ± SEM (B); Iba1 ± SEM (C); p-Tau/Tau ratio ± SEM (D) in prefrontal cortex of 6 mice per group. * = p < 0.05
Figure S1. Expression and distribution of GFAP in retinas contralateral to blast exposure, and quantification of GFAP immunoreactivity. GFAP immunoreactivity in retinas of sham and blast exposed mice is localized to the OFL. (A, B). Immunoreactivity in blast exposed mice shows apical processes of Müller glia in the OPL (B). Bar graphs show an increase in fluorescence in peripheral and central retina after blast exposure (C).

Abbreviations: GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; Scale bars = 20 μm. Data are expressed as mean ± SEM of 10 mice per group. * = p < 0.05
Figure S2. Expression and distribution of Iba1 and CD68 in retinas contralateral to blast exposure, and quantification of microglial activation. Microglia of sham exposed retinas have ramified, thin processes (A; arrows). Central retinas of blast exposed mice show an increase in Iba1 immunoreactivity. Microglia of blast exposed mice have thicker cell bodies, and fewer processes (B; arrows). High magnification (63x) images of microglial morphology inset upper right corner (A, B). Bar graphs show in increase in Iba1 immunoreactivity in central retina after blast exposure (C). There is no CD68 immunoreactivity in sham retinas (D), however blast exposed retinas show CD68 immunoreactivity in the inner and outer plexiform layers (E). Bar graph shows a significant increase in CD68 immunoreactivity in peripheral and central retina after blast exposure (F). Abbreviations: GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; Scale bars = 20 μm. Data are expressed as mean ± SEM of 10 mice per group. * = p < 0.05; **** = p < .0001
**Figure S3.** Expression and distribution of AT-270 in retinas contralateral to blast exposure, and quantification of immunoreactivity. Minimal AT-270 immunoreactivity in control retinas localized to OPL, and OFL (A). Phosphorylated tau in blast-exposed retinas is localized to the OPL, and OFL with strong intensity, and weak in the IPL (B). Bar graphs show an increase in fluorescence in peripheral and central retina after blast exposure (C). Abbreviations: OFL = optic fiber layer; GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; Scale bars = 20 μm. Data are expressed as mean ± SEM of 10 mice per group. *** = p < 0.001; **** = p < .0001

**Figure S4.** Expression and distribution of total tau in retinas ipsilateral to blast exposure. Total tau immunoreactivity localized to OPL and IPL in both control, and blast-exposed retinas (A, B). Abbreviations: GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer.
Figure S5: Measurement of retinal thickness. Thickness of outer nuclear layer (ONL) is expressed as number of cell bodies spanning the thickness of the layer. Mean retinal thickness of sham retinas is 13±6.3 nuclei. In retinas contralateral to blast exposure, mean retinal thickness is trending to decrease (11.33±5.6 nuclei), but does not reach significance. Data are expressed as mean ± SEM of 10 mice per group.
CHAPTER 3. ACCELERATED ACCUMULATION OF RETINAL \( \alpha \)-SYNUCLEIN (pSer129) AND TAU, NEUROINFLAMMATION AND AUTOPHAGIC DYSREGULATION IN A SEEDED MOUSE MODEL OF PARKINSON’S DISEASE.

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**Abstract**

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by accumulation of misfolded \( \alpha \)-synuclein within the central nervous system (CNS). Visual problems in PD patients are common, although retinal pathology associated with PD is not well understood. The purpose of this study was to investigate retinal pathology in a transgenic mouse model (TgM83) expressing the human A53T \( \alpha \)-synuclein mutation and assess the effect of \( \alpha \)-synuclein “seeding” on the development of retinal pathology. Two-month-old TgM83
mice were intracerebrally inoculated with brain homogenate from old (12-18 months) TgM83 mice. Retinas were then analyzed at 5 months of age. We analyzed retinas from 5-month-old and 8-month-old uninoculated healthy TgM83 mice, and old (12-18 months) mice that were euthanized following the development of clinical signs. Retinas of B6C3H mice (genetic background of the TgM83 mouse) served as control. We used immunohistochemistry and western blot analysis to detect accumulation of α-synuclein, \( \text{pTau}^{\text{Thr231}} \), inflammation, changes in macroautophagy, and cell death. Raman spectroscopy was used to test the potential to differentiate between retinal tissues of healthy mice and diseased mice. This work demonstrates retinal changes associated with the A53T mutation. Retinas of non-inoculated TgM83 mice had accumulation of α-synuclein, “pre-tangle” tau, activation of retinal glial cells, and photoreceptor cell loss by 8 months of age. The development of these changes is accelerated by inoculation with brain homogenate from clinically ill TgM83 mice. Compared to non-inoculated 5-month-old TgM83 mice, retinas of inoculated 5-month-old mice had increased accumulation of α-synuclein (pSer129) and \( \text{pTau}^{\text{Thr231}} \) proteins, upregulated microglial activation, and dysregulated macroautophagy. Raman spectroscopic analysis was able to discriminate between healthy and diseased mice. This study describes retinal pathology resulting from the A53T mutation. We show that seeding with brain homogenates from old TgM83 mice accelerates retinal pathology. We demonstrate that Raman spectroscopy can be used to accurately identify a diseased retina based on its biochemical profile, and that α-synuclein accumulation may contribute to accumulation of \( \text{pTau}^{\text{Thr231}} \) proteins, neuroinflammation, metabolic dysregulation, and photoreceptor cell death. Our work provides insight into retinal changes associated with Parkinson’s disease, and may contribute to a better understanding of visual symptoms experienced by patients.
Key Words: Retina in Parkinson’s Disease, photoreceptor cell loss, tau in retina, microglial activation in retina, Müller glial activation, misfolded α-synuclein, human A53T mutated α-synuclein, autophagy in PD retina, Raman Spectroscopy.

Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by two key disease processes: progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc) in the midbrain that leads to deficits in voluntary movement, and the accumulation of intraneuronal Lewy-bodies, containing misfolded α-synuclein [1]. Prominent non-motor phenomena, specifically visual disturbances, are a considerable cause of morbidity in Parkinson’s disease [2, 3]. There is evidence of visual dysfunction at several levels of the visual pathway in patients with PD, including morphological and electrophysiological indication of disruption of retinal structure and function [2, 4, 5]. However, the direct effect of misfolded α-synuclein on the retina is not well understood, and molecular biomarkers of PD progression in the retina are lacking.

Here, we use the TgM83 mouse model to investigate retinal pathology due to a human α-synuclein (A53T) gene mutation. We also use this model to study the effect of α-synuclein seeding in the brain, on development of pathology in the retina. Often referred to as “prion-like propagation”, the transport of misfolded proteins from one central nervous system structure (CNS) seeds protein misfolding and propagation in another [6-11]. This is consistent with the Braak staging of Parkinson’s disease, which describes a progressive and non-random process with specific neuronal types giving rise to the development of Lewy pathology in a specific pattern (i.e. caudo-rostral progression of disease from the lower brainstem, followed by the midbrain, forebrain, and cerebral cortex) and as disease progresses, lesion severity in
vulnerable brain regions increases [12]. A growing body of \textit{in vitro} and \textit{in vivo} evidence describes the ability of pathological α-synuclein to spread transcellularly and induce aggregation by templating protein misfolding [6, 7, 9, 10, 13, 14]. An \textit{in vivo} study shows that seeding with an inoculum derived from the brain of clinically ill mice, results in acceleration of α-synuclein-associated disease and shortening of survival time [7]. Disease acceleration is associated with an insoluble form of α-synuclein phosphorylated on serine 129 [7]. Among several existing post-translational modifications to α-synuclein known to occur in PD, phosphorylation at Ser129 (herein referred to as pSer129), has been reported to enhance α-synuclein toxicity both \textit{in vitro} and \textit{in vivo} [15-17]. Although α-synuclein is not an infectious agent, studies attribute the enhanced toxicity of pSer129 to increased formation and self-propagation of α-synuclein aggregates [6]. In this study, we use a mouse model of α-synuclein seeding to study the effect of α-synuclein spreading in the CNS, specifically on development and progression of retinal pathology. Herein, we report the time course of specific retinal changes associated with the A53T mutation, including: accumulation of pSer129 and ‘pre-tangle tau protein in the retina, dysregulation of macroautophagy in response to the increase in misfolded proteins, and loss of photoreceptors. We demonstrate accelerated development of pathologic biomarkers in mice inoculated with brain homogenate from clinically ill TgM83 mice.

We also explored the use of Raman spectroscopy to identify disease-specific molecular signatures in retinal tissues from α-synuclein A53T mutant mice. Raman spectroscopy is a sensitive and nondestructive technique that provides rapid biochemical characterization of tissues for diverse applications [18]. The Raman effect occurs when an incident monochromatic light, emitted by a laser, scatters when it passes through a substance. The
scattered light is inelastic, meaning it contains additional frequencies that are different than that of the incident frequency. These frequencies (i.e. emitted photons) are captured by a Raman spectrophotometer, and the resulting spectrum reveals fingerprint regions specific to molecules’ chemical bonds and configurations. Raman spectra collected from samples are used to train and test a classifier to accurately discriminate between diseased and healthy tissues according to changes in their molecular structure. Several neurodegenerative diseases result from misfolding and accumulation of specific proteins (e.g. α-synuclein and tau in Parkinson’s disease, tau and beta-amyloid in Alzheimer’s disease, and prion proteins in transmissible spongiform encephalopathies) that change the chemical composition of tissues. Here, Raman spectroscopy was used to differentiate between the biochemical fingerprints of retinal tissues from healthy mice and mice with the α-synuclein A53T mutation. The classifier was able to discriminate between retinal tissues of control, healthy, and diseased mice with high accuracy, even between samples that appeared histologically similar.

To the best of our knowledge, this report is the first to demonstrate retinal pathology resulting from the A53T mutation, and that seeding with brain homogenate from clinically ill mice accelerates retinal pathology. This work implies a direct effect of α-synuclein toxicity on the retina, causing chronic neuroinflammation, metabolic dysregulation and cell death. Additionally, this study describes the use of Raman spectroscopic analysis for the differentiation of control retinas and those affected by α-synuclein associated pathology, with high specificity. Here, we describe an in vivo model that provides insight into how Parkinson’s disease affects the retina, and how α-synuclein related changes may contribute to visual disturbances experienced by patients.
Methods

Animals

Retinal tissues were provided by Thierry Baron (Anses, Laboratoire de Lyon, Unité Maladies Neurodégénératives, Lyon, France). Briefly, 2-month-old homozygous TgM83 mice were subjected to intracerebral ventricular inoculation (ICV) with 20 µL of 1% (wt/vol in glucose 5%) brain homogenates obtained from half of the brain of clinically ill TgM83 mice (12-18 months), and euthanized three months after IC inoculation, corresponding to a pre-clinical stage [7]. Clinically-ill TgM83 mice were euthanized following the development of motor clinical signs of disease, in this case paralysis of the hind limbs. Care and housing of mice, as well as ethical approval is described as per Mougenot et al., 2011. Eye globes were postfixied in Bouin’s fixative for 24 h, embedded in paraffin, and sectioned sagitally at 4-µm onto superfrost plus glass slides. We analyzed retinas of homozygous TgM83 mice at 5, 8 (clinical illness), and 12-18 (clinical illness) months of age; inoculated TgM83 mice at 5 months of age; and B6C3H mice (genetic background of the TgM83 mouse model) at 5 and 8 months of age (Figure 1).

Immunohistochemistry

Paraffin-embedded sections (4 µm) of the retina were rehydrated using xylene, followed by a decreasing ethanol concentration gradient (100%, 90% 70%), and a final wash with diH2O. Heat-mediated antigen retrieval was performed using EDTA buffer (10 mM Trizma Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0), or Citrate buffer (ScyTek Laboratories, Logan, UT) in an autoclave for 30 min. Tissues were incubated with Background Buster (Innovex Biosciences Inc., Richmond, CA) for 2 h. Primary antibodies against α-synuclein (1:1000, BD Biosciences, San Jose, CA, 610786), α-synuclein p129S (1:500;
Abcam, Cambridge, MA, ab51253), tyrosine hydroxylase (1:50, Santa Cruz Biotechnology, Dallas, TX), glial fibrillary acidic protein (GFAP; 1:500; Dako, Carpinteria, CA), CD11b (1:500; Abcam), CD68 (1:100; Abnova, Taiwan), PHF-Tau Clone AT-180 (1:100; Thermo Fisher Scientific, Inc., Rockford, IL), calbindin (1:2000, Swant, Inc., Switzerland), LC3 (1:1000, Cell Signaling, Danvers, Massachusetts), or caspase-3 (1:50, Santa Cruz Biotechnology) were diluted in blocking solution containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 0.05% Triton X-100 (Thermo Fisher Scientific, Inc., Rockford, IL) and 5% normal serum (Jackson ImmunoResearch, West Grove, PA) in Tris-Buffered saline (TBS), and incubated for 48 h at room temperature. Tissues were washed with TBS-T (6 x 5 min) and incubated with a secondary antibody cocktail, including Cy³ and/or Alexa Fluor 488–conjugated AffiniPure secondary antibodies (1:250; Jackson ImmunoResearch) and 40,6-diamidino-2-phenylindole, dilactate (DAPI, 4 µg/mL; Sigma-Aldrich) for 1.5 h. Following another wash, slides were mounted with Vectashield antifade mounting medium (Vector Laboratories Inc., Burlingame, CA). Immunoreactivity for α-synuclein, α-synuclein p129S, tyrosine hydroxylase, and Caspase-3 was developed using Envision þ Dual Link System-horseradish peroxidase (Dako, Carpinteria, CA) with diaminobenzidine (Vector Labs, Peterborough, UK), and slides were counterstained with hematoxylin. Negative controls were processed in parallel by omission of the primary and/or secondary antibody.

**Fluorescent Microscopy and Statistical Analysis**

Fluorescence and confocal images were taken at 20x and 60x, using a Nikon A1R+ Resonant Scanning Confocal System with a Ti-E inverted microscope and laser lines 405, 488, 561 and 640 nm (Nikon Instruments Inc., Melville, New York). Micrographs were created using a commercial photo-editing system (Adobe Photoshop and Adobe Illustrator [CC];
Adobe Systems). For quantification of GFAP, CD11b, AT-180, and CD68 immunoreactivity, the percentage of the total image area thresholded (outer limiting membrane to inner limiting membrane of the retina) was analyzed using ImageJ (Rasband, W.S., ImageJ 1.49V, U. S. National Institutes of Health, Bethesda, Maryland). Quantified histological, and western blot data from three separate replicates was analyzed using a two-way ANOVA, with a Tukey’s multiple comparisons test (post-hock). Thickness of the outer nuclear layer (ONL) was measured by counting of cell bodies spanning the layer. For each mouse, the central retina, near the optic disc, within a cross section was used for analysis. Retinal thickness was analyzed using an unpaired t-test. Prism 6 for Windows (Graph Pad Software) was used for statistical analysis.

**Protein Extraction from Paraffin Embedded Tissues for Western Blot Analysis**

Bouin’s fixed paraffin-embedded sections (100μm total, 10 x 10μm sections) of the retina were collected into 1.5 mL microcentrifuge tubes. Sections were deparaffinized and rehydrated using xylene, followed by a decreasing ethanol concentration gradient (100%, 95%, 70%). Then, 100 μL of lysis buffer containing protease and phosphatase inhibitor cocktail was added to each sample, and samples were incubated on ice for 5 min. Samples were then incubated at 100°C for 20 min, then again at 80°C for 20 min using a thermomixer with agitation (750 rpm). Samples were then centrifuged for 15 min (14,000g at 4°C). Supernatant was collected, and samples were kept overnight at -20°C. Tissues containing equal amounts of protein were separated on 4-20% SDS-polyacrylamide gels. After separation, proteins were transferred to polyvinylidene difluoride (PVDF) membranes, and non-specific binding sites were blocked by treating with 5% non-fat dry milk in 0.5% TBS-T for 1 h. The membranes were then incubated with primary antibodies directed against α-synuclein (1:5000, BD
91 Biosciences, San Jose, CA), α-synuclein p129S (1:1000; Abcam, Cambridge, MA), pAMPK<sup>T172</sup> (1:1000, Cell Signaling, Danvers, Massachusetts), AMPK (1:1000, Cell Signaling), pULK1<sup>S757</sup> (1:1000, Cell Signaling), ULK1 (1:1000, Cell Signaling), and LC3 (1:5000, Cell Signaling) overnight at 4°C. The primary antibody treatments were followed by treatment with HRP-linked, anti-rabbit or anti-mouse secondary antibody (1:5000, Cell Signaling) for 1 h at room temperature. Western blot images were captured with the ChemiDoc<sup>TM</sup> XRS Imaging System (Bio-Rad), and images were analyzed using Image Lab<sup>TM</sup> 6.0 Software (Bio-Rad). All groups are normalized to 5-month-old B6C3H mice (genetic background of the TgM83 mouse model), providing a representative of a fold-change from the control, therefore y-axis units are arbitrary.

**Raman Spectroscopy**

Paraffin-embedded sections (4μm) of the retina were placed on gold-aluminum coated microscope slides. Raman measurements were performed using a DXR dispersive Raman microscope (Thermo Scientific, Inc., Madison, Wisconsin) with a 532nm, 14 mW excitation laser with 50 μm pinhole at ambient temperature and collected with a 20s exposure time from 110 to 3527 cm<sup>-1</sup> at a resolution of 1 cm<sup>-1</sup>. Retinal ganglion cells were resolved with a 60x objective. Twenty spectra were collected from twenty different retinal ganglion cells at different locations and an average spectrum was then calculated for that cell to be used as one RGC spectrum in subsequent analysis (20 spectra from each retinal section, for a total of 400 spectra from each group). Raman peaks that exhibited the most significant changes between control and diseased tissue samples were identified. All spectra were baseline corrected, smoothed, and normalized to reduce baseline variability and background noise at the region between 110 and 3527cm<sup>-1</sup>, using an iterative correction algorithm [18] coded with R. After
preprocessing, a principal component analysis (PCA) was used to reduce dimensionality. Multi-class classification was generated using a support vector machine (SVM) [19]. Cross-validation was applied by randomly choosing 75% of the spectral data to build the classification model, using the remaining 25% of the spectra to test and validate the classifier. Average classification accuracy was calculated from 10 random replications of the discriminant process.

Results

Retinas of TgM83 mice exhibit α-synuclein accumulation.

In Parkinson’s disease, α-synuclein aggregates are highly enriched in α-synuclein that has been phosphorylated at serine 129 (pSer129), which has been reported to enhance the tendency of α-synuclein to aggregate and disrupt normal function in vitro and in vivo [20, 21]. To investigate whether α-synuclein pSer129 is present in retinas of transgenic mice, we performed immunohistochemistry and western blot analysis. In addition to probing for a specific pathogenic form of α-synuclein, we used immunohistochemistry and western blot analysis to determine levels and pattern of total α-synuclein immunoreactivity in retinas of transgenic mice. There was no pSer129 or total α-synuclein immunoreactivity detected in control retinas (Figure 2A, B). In retinas of all 5-month-old transgenic mice, pSer129 immunoreactivity was evident only in the outer nuclear layer (Figure 2C). Compared to total α-synuclein, pSer129 immunoreactivity was sparse, with two to three patches of labeling per retinal section of all mice (Figure 2C, D). Retinas of inoculated 5-month-old transgenic mice demonstrated upregulated pSer129 immunoreactivity, compared to 5-month-old non-inoculated mice (Figure 2C, E). In retinas of inoculated mice, pSer129 immunoreactivity was detected throughout the outer segments of photoreceptor cells, outer nuclear layer, outer plexiform layer, and inner nuclear layer, demonstrating accelerated accumulation of α-
synuclein in the seeded model (Figure 2C, E). Compared to control, retinas of 8-month-old and clinically ill transgenic mice had α-synuclein labeling similar to that of inoculated 5-month-old mice (Figure 2G-L). In retinas of 8-month-old mice, pSer129 immunoreactivity was only evident in the outer nuclear layer, compared to total α-synuclein immunoreactivity that was also present in the inner retina (Figure 2I, J). Retinas of clinically ill mice had similar distribution of pSer129 immunoreactivity throughout the outer and inner retina (Figure 2K, L).

In all micrographs, p129S and total α-synuclein immunoreactivity was more robust in the outer retina, compared to the inner retina. Western blot analysis of p129S and total α-synuclein protein levels produced similar results. Retinas of 8-month-old, clinically ill, and inoculated 5-month-old mice, had significantly more α-synuclein (~2 fold compared to control and 5-month-old transgenic mice; Figure 2M, N). Our results indicated no significant difference in pSer129 accumulation among 8-month-old transgenic, clinically ill, or inoculated 5-month-old mice, suggesting that seeding of α-synuclein from the brain to the retina results in accelerated accumulation of the misfolded protein.

**Activation of Müller glia in response to α-synuclein accumulation.**

To determine activation of Müller glia in response to misfolded protein accumulation, we assessed the distribution of GFAP immunoreactivity using immunohistochemistry. Müller glia are the principal glial component of the retina, responding to injury by increasing GFAP expression in processes radially through the thickness of the retina [22]. GFAP immunoreactivity in retinas of control mice was localized to the Müller glia end feet and astrocytes in the optic fiber layer (Figure 3A, D). Retinas of 5-month-old non-inoculated, and 5-month-old inoculated, and 8-month-old transgenic mice had comparable GFAP immunoreactive processes spanning the retina from the optic fiber layer to the outer plexiform
layer (Figure 3B, C, E). Retinas of clinically ill mice had robust Müller glial activation with hypertrophied Müller glia end feet, and GFAP immunoreactive processes detectable in the outer nuclear layer (Figure 3F). When quantified, the area of GFAP immunoreactivity accounted for $13.99 \pm 0.86\%$ (TgM83 5m), $13.32 \pm 1.19\%$ (inoculated TgM83 5m), $10.40 \pm 1.21\%$ (TgM83 8m), or $23.41 \pm 2.17\%$ (clinical TgM83 12-18m) of total area (Figure 3G). Activation of Müller glia was first detected in retinas of 5-month-old TgM83 mice and was significantly increased in retinas of 8-month-old and clinically ill mice. Compared to non-inoculated mice, retinas of 5-month-old inoculated mice did not have increased GFAP, suggesting that α-synuclein seeding did not have a detectable effect on activation of Müller glia.

**TgM83 mice exhibit pTau$^{\text{Thr231}}$ accumulation in retinal neurons and Müller glia**

To determine whether the α-synuclein (A53T) gene mutation leads to accumulation of tau, an antibody against AT-180 (pThr231) was used for an immunohistochemical analysis. Tau protein plays a critical role in microtubule assembly, axonal transport, and neurite outgrowth, and is a major component of neurofibrillary tangles (NFTs) found in Alzheimer’s disease (AD) and Parkinson’s disease (PD) [23]. Phosphorylated tau (pTau$^{\text{Thr231}}$) immunoreactivity was first appreciable in retinas of 5-month-old transgenic mice, with no pTau$^{\text{Thr231}}$ immunoreactivity detected in retinas of corresponding control mice (Figure 4A, B). Compared to non-inoculated mice, retinas of 5-month-old inoculated mice had greater pTau$^{\text{Thr231}}$ immunoreactivity (~7-fold higher), localized to the outer plexiform layer, ganglion cell layer, and optic fiber layer (Figure 4C, E). Compared to retinas of age-matched control mice, those of 8-month-old transgenic mice had upregulated pTau$^{\text{Thr231}}$ immunoreactivity (Figure 4D, E), although significantly less than retinas of inoculated, and clinically ill
transgenic mice (Figure 4C, F). When quantified, phosphorylated tau immunoreactivity comprised 1.11 ± 0.26% (TgM83 5m), 8.02 ± 0.82% (inoculated TgM83 5m), 2.63 ± 0.54% (TgM83 8m), or 6.71 ± 1.53% (clinical TgM83 12-18m) of total area (Figure 4G). The outer plexiform layer contains neuronal processes of horizontal cells that synapse with terminal regions of rods and cones (rod spherules and cone pedicles). Double immunofluorescence and confocal microscopy was used to confirm colocalization of pTau<sup>Thr231</sup> with calbindin immunoreactive processes in the outer plexiform layer (Figure 4H-J). Retinas of 5-month-old inoculated mice showed colocalization of immunofluorescence for calbindin and AT-180 (pThr231) in the outer plexiform layer. Since the pattern of AT-180 immunoreactivity in the outer plexiform layer was also similar to processes of activated Müller glia, double labeling with GFAP and AT-180 was used to confirm co-localization of GFAP and AT-180 in the outer plexiform layer. Retinas of 5-month-old inoculated mice had colocalization of immunofluorescence for GFAP and AT-180 (pThr231) in the outer plexiform layer. This data describes accumulation of phosphorylated tau in processes of neurons and retinal Müller glia, first detected at 5 months of age. This was coincident with the first detection of pSer129 in retinas of 5-month-old transgenic mice. Parallel to pSer129 immunoreactivity, pTau<sup>Thr231</sup> was upregulated at 8 months and in clinically ill mice. Finally, we show significant upregulation of pTau<sup>Thr231</sup> immunoreactivity in retinas of inoculated mice, compared to retinas of non-inoculated, suggesting accelerated tau phosphorylation in response to α-synuclein seeding.

**Microglial activation parallels retinal degeneration due to accumulation of pSer129.**

To assess microglial activation, we performed immunohistochemistry using antibodies against CD11b and CD68. Upregulation of CD11b, the β-integrin marker of microglia, and macrophage antigen CD68 is associated with microglial activation in neuroinflammation [24-
In retinas of control mice, CD11b immunoreactive microglia had small somata with thin processes, sparsely dispersed through the outer and inner plexiform layers (Figure 5A, D). CD11b immunoreactivity in retinas of 5- and 8-month-old control mice occupied 0.23 ± 0.03% and 0.20 ± 0.03% of total area, respectively (Figure 5A, D, G). Compared to control retinas, CD11b immunoreactivity of transgenic mice at 5 months of age occupied 0.68 ± 0.06% of total area (Figure 5A, B, G). Activated microglia were detected first in retinas of 8-month-old mice, shown by an increase in CD11b immunoreactivity and a change to an amoeboid like morphology (Figure 5D, E). Retinas of inoculated 5-month-old mice, and clinically ill mice had significantly upregulated CD11b immunoreactivity, with large microglia often detected in the photoreceptor cell layer and outer segments (Figure C, F). CD11b immunoreactivity occupied 1.05 ± 0.06%, and 1.48 ± 0.203%, and 1.67 ± 0.03% of total area, respectively (Figures 5G). A similar trend was observed in CD68 immunoreactivity, with activated and phagocytic microglia localized to the inner retina of transgenic mice (Figure 5H-N). Similar to CD11b, upregulated CD68 immunoreactivity was first evident in retinas of 8-month-old transgenic mice, occupying 1.35 ± 0.30% of total area (Figure 5K, L). CD68 immunoreactivity was similar in retinas of clinically ill mice and those inoculated with brain homogenate, occupying 2.45 ± 0.01% and 2.31 ± 0.03% of total area, respectively (Figure 5J, M, N). Results showed accelerated activation of CD11b+/CD68+ microglia in retinas of 5-month-old inoculated mice, similar to microglial activation seen in retinas of clinically ill mice.

**Dysregulation in retinal autophagy due to an increase in misfolded proteins.**

To assess the effect of accumulation of misfolded proteins on autophagy in the retina, we performed western blot and immunohistochemical analyses of proteins involved in initiation of autophagy and vesicle formation (Figure 6A). In Parkinson’s disease, an increase
in autophagy is a proper response during clearing of bulk cytoplasmic contents, such as intracellular α-synuclein, however a decrease below basal levels indicates autophagic dysfunction [27, 28]. Conversely, a sustained increase of autophagosomes may also indicate an overload of a system that is incapable of clearing unwanted proteins [27, 28]. To determine changes in the initiation of autophagy, we examined the activation status of major autophagy-initiation proteins, pAMPK<sup>T172</sup> and pULK1<sup>S757</sup>, by western blot analysis. There was significant upregulation of pAMPK<sup>T172</sup> protein levels in retinas of 5-month-old and 8-month-old transgenic mice, and a trending decrease in retinas of 5-month-old inoculated and clinically ill transgenic mice as compared to retinas of control B6C3H mice (Figure 6B, C). Inversely, pULK1<sup>S757</sup> protein levels were significantly lower in retinas of 5-month-old and 8-month-old transgenic mice, however they were upregulated in retinas of 5-month-old inoculated and clinically ill transgenic mice (Figure 6B, D). To assess vesicle formation, we conducted western blot analysis and immunohistochemistry of autophagosome marker, LC3. Similar to pAMPK<sup>T172</sup> protein levels, LC3 II protein levels were upregulated in retinas of 5-month-old and 8-month-old transgenic mice and downregulated in retinas of 5-month-old inoculated and clinically ill transgenic mice, compared to retinas of control mice (Figure 6B, E). Immunohistochemistry results showed diffuse LC3 immunoreactivity in the outer and inner retina, suggesting an active basal level of autophagy ongoing under normal conditions (Figure 6F). In retinas of 5-month-old transgenic mice, we saw significantly more LC3-immunoreactive autophagosomes, primarily localized to the outer nuclear layer and outer plexiform layer (Figure 6G). In retinas of inoculated 5-month-old mice, we saw depletion of LC3 throughout the entire retina (Figure 6H-J). This was similar to what was seen in retinas of 8-month-old and clinically ill mice (Figure 6H, I). Taken together, these results show changes
in macroautophagy associated with increased accumulation of pSer129 detected at clinical illness, or due to α-synuclein seeding. At earlier timepoints, there was an increase in autophagic activity, suggested by the increase in pAMPK^{T172} and LC3 II, and a decrease in pULK1^{S757}. However, in retinas of clinically ill mice and inoculated mice seeded with α-synuclein, we see autophagy similar to that of control mice, which suggests that an overloading of the autophagic system, by excessive α-synuclein accumulation, may lead to its ultimate dysfunction.

**Photoreceptor cell death in α-synuclein associated retinopathy.**

To determine if there was photoreceptor cell loss, we assessed the thickness of the outer nuclear layer, as measured by number of photoreceptor cell nuclei. The mean thickness of the outer nuclear layer of age-matched control B6C3H mice were 14.50 ± 0.22 and 13.00 ± 0.36 cell nuclei, respectively (Figure 7A). Outer nuclear layer thickness was comparable between 5-month-old non-inoculated and inoculated transgenic mice, with 13.40 ± 0.40 and 13.2 ± 0.37 cell nuclei, respectively (Figure 7A). Photoreceptor cell loss was evident in retinas of 8-month-old and clinically ill transgenic mice (Figure 7A). Mean outer nuclear layer thickness in retinas of 8-month-old transgenic mice was 8.50 ± 0.50 cell nuclei (35% decrease compared to age-matched control), while outer nuclear layer thickness in retinas of clinically ill mice was 5.50 ± 0.50 cell nuclei (70% decrease compared to retinas of control mice; Figure 7A). Caspase-3 is an important mediator of neuronal apoptosis and plays a pivotal role in pathologic death in the CNS due to Parkinson’s Disease. To determine if caspase-3 activation preceded photoreceptor cell degeneration due to the A53T mutation, we used cleaved anti-caspase-3 as an indicator of apoptosis. Retinas of B6C3H mice had no detectable caspase-3 labeling, while those of 8-month-old and clinically ill mice had caspase-3 immunoreactivity in both the outer nuclear layer and outer segments of photoreceptor cells (Figure 7B-D). Detectable caspase-3
immunoreactivity coincided with retinal outer nuclear layer thinning, suggesting the potential role of caspase-3 in A53T mutation induced photoreceptor cell death.

**A53T mouse model of synucleinopathy does not show changes in retinal tyrosine hydroxylase immunoreactivity.**

Tyrosine hydroxylase (TH) catalyzes the formation of L-DOPA in the biosynthesis of dopamine, therefore loss of TH activity followed by a loss of the protein is classically contributed to dopamine deficiency and phenotypic expression in PD [29]. To determine whether TH-expressing (dopaminergic) cells in the retina were affected due to the A53T mutation, immunohistochemistry was performed using anti-tyrosine hydroxylase. TH immunoreactivity was comparable in all retinas, with approximately one to four amacrine cell bodies in the inner nuclear layer of each retinal section and thick dendritic processes in the IPL (Figure 7E-J). Results showed no changes in TH-immunoreactive amacrine cells.

**Raman spectroscopic analysis can differentiate between spectral signatures of normal and diseased retinas.**

To detect biochemical differences in the retinal ganglion cell layers of healthy and diseased mice, we measured 20 spectra from 20 cells within the ganglion cell layer of 6 groups of mice, for a total of 400 spectra from each group. Ganglion cells were used for analysis because they are the innermost (closest to the lens) cellular layer of the retina. The Raman spectra reflects the molecular make up of a specific sample, with contributions from functional groups of major macromolecules that make up cells, i.e., proteins, RNAs, and DNAs. Fifteen of the 20 spectra were used to train a classifier, and 5 were used to test the classifier. The mean classification accuracy ± SD, was then calculated to determine how well the classifier could distinguish between two pairs of samples (Table 1). Our data demonstrates a high classification accuracy between retinal ganglion cells of control (B6C3H 5 months and 8 months) and
transgenic mice (TgM83 5 months and 8 months), 89.33 ± 5.43% and 92.00 ± 4.73%, respectively. We also observed high classification accuracies between retinal ganglion cells of retinas that were histologically similar [i.e. retinal ganglion cells of inoculated 5-month-old mice (Inoc. TgM83 5m) and clinically ill mice (TgM83 clinical) were accurately distinguishable 75.00 ± 3.23% of the time, and retinal ganglion cells of 8-month-old mice (TgM83 8m) and clinically ill mice were accurately distinguishable 90.00 ± 6.0% of the time]. The lowest classification accuracy (75.00 ± 3.23%) was observed when comparing tissues that were the most histologically similar, specifically retinas of clinically ill and inoculated mice. These results suggest that Raman spectroscopy can effectively discriminate between retinal ganglion cells of healthy and diseased mice and is sensitive enough to differentiate between retinas that are otherwise histologically indistinguishable when analyzing α-synuclein immunoreactivity, pTau^{Thr231} immunoreactivity, and microglial activation.

**Table 1:** Mean classification accuracies ± SD for retinal ganglion cells between a paired set of samples.

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**Discussion**

**Accumulation of pSer129 is accelerated in retinas of seeded mice.**

Our results demonstrate that pSer129, the principal protein modification in α-synuclein critical in the pathogenesis of Parkinson’s disease, is expressed widely in retinas of transgenic mice (TgM83). In human patients, the earliest report of α-synuclein accumulation in the retina describes α-synuclein aggregates localized to the inner retina of postmortem PD patients [30].
Additionally, a live imaging study revealed accumulation of α-synuclein deposits in retinal ganglion cells of α-synuclein::GFP mice in a model of Parkinson’s disease [31]. A recent study described accumulation of phosphorylated α-synuclein deposits in the inner retina of human PD and Lewy body disease subjects, similar to Lewy bodies and Lewy neurites that occur in the brain [32]. Our work describes outer and inner retinal accumulation of pSer129 due to the α-synuclein (A53T) gene mutation, which has not yet been reported in humans. We see detectable accumulation of pSer129 at 5 months of age, localized to the photoreceptor cell layer. Accumulation of pSer129 in the inner retina is detected in clinically ill mice and 5-month-old mice seeded with α-synuclein. Similar localization is observed with total α-synuclein, detected at 5 months of age in the photoreceptor cell layer, then in the inner retina at 8 months of age. Our results also show that seeding accelerates the disease process in the retina, as the pattern and magnitude of pSer129 protein accumulation was almost indistinguishable between inoculated 5-month-old mice and clinically ill mice. To gauge the effects of injection-related neuroinflammation, several studies report that inoculation of brain homogenate from healthy TgM83 mice fails to accelerate synucleinopathy [7, 14, 33].

We show, for the first time, that inoculation of brain homogenate of affected mice into young transgenic mice causes a prominent acceleration of retinal pSer129 accumulation, consistent with a “prion-like” propagation of α-synuclein highlighted in many works [6, 7, 9, 10, 13, 34]. Mougenot et al. report accumulation of diffuse α-synuclein (pSer129) inclusions in brains of TgM83 mice inoculated with brain homogenates from clinically ill TgM83 mice, as well as early occurrence of clinical signs and smaller survival times [7]. An increasing amount of \textit{in vitro} and \textit{in vivo} evidence argues that neurodegenerative diseases, including Parkinson’s disease, Alzheimer’s disease, multiple system atrophy (MSA), and progressive
supranuclear palsy (PSP), are in fact prion-like diseases, in which misfolded proteins (e.g. α-synuclein and tau) undergo self-propagation. Although, it is important to note that for example, α-synuclein prions in PD are distinct from prions in other synucleinopathies [9, 10, 35-37], therefore may behave differently depending on disease.

**Microglial and Müller glial activation differs in response to α-synuclein seeding.**

The first detection of pSer129 in the retina was accompanied by up-regulation of GFAP in Müller glia, perhaps an early indicator of retinal stress in response to the presence of α-synuclein. GFAP immunoreactivity was significantly higher in retinas of 8-month-old and clinically ill mice, and parallel to increased pSer129 accumulation at those time points. Activation of Müller glia is a ubiquitous response observed in many retinal diseases [38], however this observation has not been previously reported in α-synuclein related retinopathy. Consistent with these results, we have previously described activation of Müller glia that was coincident with the earliest detection of retinal misfolded prion protein in a mouse model of misfolded protein transport and accumulation [8]. Interestingly, GFAP immunoreactivity in retinas of 5-month-old inoculated mice and age-matched non-inoculated mice were comparable, suggesting that α-synuclein “seeding” did not have a detectable effect on activation of Müller glia. However, altered microglial morphology and retinal neuroinflammation demonstrated by the microglia specific markers, CD11b and CD68, were better indicators of disease acceleration. A significant increase in CD11b immunoreactivity was first detected in retinas of 8-month-old transgenic mice, concurrent with robust CD68 immunoreactivity localized to the outer and inner retina. Both CD11b and CD68 immunoreactivity was higher in retinas of clinically ill mice and retinas of 5-month-old inoculated mice, suggesting acceleration of retinal neuroinflammation in response to α-
synuclein accumulation. These results were also supported by changes in microglial morphology. We observed reactive microglia with large cell bodies and retracted processes with an amoeboid like appearance in retinas of clinically-ill and 5-month-old inoculated mice, while retinas of non-inoculated 5-month-old mice had microglia with small cell bodies and long bipolar processes indicative of a ramified, non-activated state. The contribution of microglia-mediated neuroinflammation to retinal degenerative diseases is widely known [39], however our work describes dynamic changes in cell surface markers and morphology of microglia in response to α-synuclein accumulation that has not been previously reported in the retina. Numerous studies using transgenic α-synuclein animal models link α-synuclein exposure to MHCII-dependent microglia-mediated neuroinflammation, including inflammatory cytokine release, increased oxidative and nitrosative stress, and subsequent dopaminergic cell death [40, 41]. Although chronic microglial activation is a detrimental contributor to neurodegenerative diseases, such as Parkinson’s disease and Alzheimer’s disease (reviewed in [42]), clearance and degradation of α-synuclein is a pivotal measure taken by microglia to not only prevent the spread of α-synuclein, but perhaps actively avert α-synuclein internalization and deposition into neurons [43, 44]. Acceleration of microglial activation in seeded mice may correspond to the phagocytic nature and superior scavenging capabilities of microglia that are absent in Müller glia, suggesting that microglial activation may be a more sensitive indicator of retinal disease progression.

**Increased accumulation of pTau^{Thr231} in the retina coincides with accumulation of pSer129.**

A hallmark feature of neurodegenerative diseases, including Parkinson’s disease (PD) and Alzheimer’s disease (AD), is the accumulation of hyper-phosphorylated tau in the form of neurofibrillary tangles in both neurons and glial cells [45]. One study described pTau^{Thr231}
accumulation in retinal ganglion cells in a mouse model of Alzheimer’s disease (AD), suggesting a prominent role for aberrant tau in retinal neuron dysfunction and visual defects experienced by patients [46]. We have previously reported the accumulation of pTau\textsuperscript{Thr231} in retinal neurons and glia in a mouse model of traumatic brain injury [47]. Elevated levels of hyperphosphorylated tau have been previously reported in the striata of adult A53T mutant mice [48, 49]. Another study described the colocalization of tau oligomers with astrocytes and microglia in retinas of Alzheimer’s disease (AD) and frontotemporal lobar dementia (FTLD) patients, suggesting a detrimental relationship between the spread of tau oligomers and neuroinflammation [50]. Thus far, the accumulation of tau in the retina has not been described in a model of Parkinson’s disease.

Our work demonstrates p-tau\textsuperscript{Thr231} accumulation in processes of retinal neurons and Müller glia, appreciable at 5 months of age. Phosphorylation of tau on Thr231, described as “pre-tangle” tau, has been shown to inhibit tau’s ability to bind and stabilize microtubules [51]. Interestingly, the first detection of phosphorylated tau coincides with the first detection of α-synuclein (pSer129). Just as with pSer129, we see significantly higher pTau\textsuperscript{Thr231} immunoreactivity in retinas of inoculated mice, suggesting accelerated accumulation of phosphorylated tau in response to α-synuclein seeding. The role of tau in the pathogenesis of PD is presently unknown, however evidence suggests a stimulatory effect of α-synuclein on tau phosphorylation [52]. Due to the co-occurrence of tau and α-synuclein filamentous amyloid inclusions in human neurodegenerative diseases, \textit{in-vivo} studies suggest that the interaction between α-synuclein and tau is synergistic and can promote hyper-phosphorylation and fibrillization in the TgM83 mouse model [53]. Studies propose that the interaction between α-synuclein and tau leads to specific and sequential phosphorylation events of the tau protein.
resulting in loss of function (decreased microtubule binding), as well as gain of function (abnormal tau-tau interactions), leading to tau dysfunction and accumulation into neurofibrillary tangles [23, 54-56]. Moreover, an in vitro study demonstrates that misfolded α-synuclein displays a self-replicating, as well as a cross-seeding ability (i.e. aggregated α-synuclein can trigger misfolding of cellular prion protein) [13]. Another in-vitro study reports that tau fibrils can induce intracellular tangle-like aggregates, suggesting seeding activity of misfolded tau as a potential mechanism of tau propagation in-vivo [57].

Our work is the first to describe accumulation of “pre-tangle” tau in retinal neurons and glia, due to the A53T α-synuclein gene mutation. Also, this work provides evidence that α-synuclein seeding may contribute to, and possibly accelerate the accumulation of pTauThr231 proteins in the retina. Although compelling, the mechanisms underlying the interaction of α-synuclein (pSer129) and tau require further investigation.

**A decrease in autophagy is coincident with increased accumulation of α-synuclein, and pTauThr231.**

This work describes changes in retinal macroautophagy associated with the pathogenic A53T mutant α-synuclein, that have not been previously reported. Macroautophagy is the only known intracellular mechanism potentially capable of degrading bulk protein aggregates, although in mammals, all of the proteins involved and the precise sequence of events are still not completely established (reviewed in [58]). Briefly illustrated in Figure 6, the initiation stage involves activation of ULK1 (a serine-threonine kinase), regulated by both mTOR and AMPK. Activation of mTOR leads to the enzymatic inactivation of ULK1, by downstream phosphorylation at serine 757. Conversely, when AMPK is activated by phosphorylation at Throneine 172 and mTOR is inhibited, due to nutrient deficit or stress, ULK1 is
dephosphorylated at Serine 757 and thereby activated allowing for autophagy to progress. Subsequently, lipidation of LC3 I to form LC3 II, and binding of specific adaptor proteins such as p62, contributes to autophagosome formation. Finally, fully formed autophagosomes fuse with lysosomes for degradation and recycling of cellular contents including organelles and proteins. Our results demonstrate two opposing events in the autophagic pathway: *upregulation* of the initiation of autophagy, suggested by the increase in pAMPK$^{T172}$ and LC3 II, along with the decrease in pULK1$^{S757}$ at “early” timepoints (retinas of 5-month-old and 8 months old mice); *downregulation* of autophagy at the “later” timepoint (retinas of clinically ill mice). Retinas of inoculated or “seeded” mice were almost indistinguishable from those of clinically ill mice, perhaps suggesting impaired autophagy due to an overload of the system by misfolded α-synuclein accumulation. Here, the upregulation event first detected in retinas of 5-month-old transgenic mice, coincides with the first detection of pSer129 and pTau$^{Thr231}$ accumulation, a proper response during clearing of cytoplasmic contents. Later, downregulation of autophagy seen in retinas of clinically ill and seeded mice, could suggest impaired autophagy due to an overload of misfolded proteins, α-synuclein and pTau$^{Thr231}$. In neurodegenerative diseases such as PD, AD, and Huntington’s disease, autophagy is impaired (reviewed in [58]). Consistent with our results, the general hallmark effect of CNS disease on autophagy is the accumulation of autophagosome-like structures. However, whether autophagosome accumulation occurs due to an impairment in the degradation of autophagosomes, or due to an increased need for the clearance of unwanted intracellular contents, is not yet known. Specifically, in PD associated mutations A53T and A30P, a study demonstrated that α-synuclein inhibits chaperone-mediated autophagy (CMA) by binding to its receptor and preventing not only degradation of itself, but that of other autophagy substrates.
It is also unknown whether abnormalities in the autophagy pathway are caused by mutant or misfolded proteins, or whether deficits in the degradation of misfolded proteins are yet another factor that leads to protein aggregation. The complex intricacies of the autophagic pathway in neurodegenerative diseases are far from being understood, however data presented in this study suggests it may play a role in the cellular response to misfolded proteins including α-synuclein (pSer129) and pTau^{Thr231}.

**Loss of retinal neurons in α-synuclein associated disease.**

This study reports loss of photoreceptors first detected in retinas of 8-month-old transgenic mice, corresponding to widespread distribution of pSer129 in the outer nuclear layer. The first detection of pSer129 immunoreactivity in retinas of 5-month-old mice precedes retinal thinning detected in retinas of 8-month-old mice. This observation is supported by studies that demonstrate an increase in misfolded proteins before onset of pathological changes [60]. Loss of photoreceptors also coincided with cleaved caspase-3 immunoreactivity, detectable in the outer nuclear layer and the outer segments of photoreceptor cells, which may suggest photoreceptor apoptosis. Photoreceptor cell loss in response to the accumulation of misfolded proteins has been previously reported in a seeded rodent model of scrapie, and similar to our observation, loss of retinal neurons did not occur until late in the disease long after the first detection of misfolded prion protein [8]. Besides scrapie, the vulnerability of photoreceptor cells to the accumulation of misfolded proteins has also been shown in other models of transmissible spongiform encephalopathies (TSEs), including Creutzfeldt-Jacob disease [61] and transmissible mink encephalopathy [62], however photoreceptor cell death in response to α-synuclein accumulation has not been previously reported. Visual deficits in PD are often attributed to dopaminergic deficiency [3], however our results demonstrate no
detectable changes in tyrosine hydroxylase immunoreactivity in dopaminergic amacrine cells. While there is evidence of reduced tyrosine hydroxylase immunoreactivity in retinas of PD patients, this observation was made in five patients and has not been since examined [63]. Retinal dopamine deficiency in PD has not been since explored. Further studies are necessary to delineate the effect of α-synuclein on retinal dopamine, and whether this contributes to visual dysfunction in PD.

**Raman spectroscopy can detect molecular changes in Parkinson’s disease related retinopathy.**

In this study, we explored the potential of using Raman spectroscopy for differentiating retinal tissues of transgenic mice from those of healthy controls. Since our histological data demonstrated pathologic changes in retinas of transgenic mice, it was reasonable to hypothesize a difference in the molecular makeup of affected ganglion cells. For the first time in this study, spectroscopic markers associated with changes in α-synuclein related intracellular protein compositions in retinal ganglion cells were identified with high accuracy. Raman spectroscopy has been an invaluable tool facilitating several biomedical applications, including diagnostics and therapeutic development. For example, Raman spectroscopy is slowly making its way into clinics as a non-invasive way to characterize different cancers, by collecting biochemical information from *in situ* samples of tumors [64, 65]. Raman spectroscopy has also been successfully used for the evaluation of glaucomatous retinal changes in canine tissues [18]. Taken together with our results, Raman spectroscopic screening could potentially be used for early characterization of pathologic retinal changes due to a variety of neurodegenerative disorders. If further optimized and refined, and in combination with *in vivo* imaging techniques, Raman spectroscopy may be implemented to develop effective and safe early-diagnostic modalities for PD patients with deficits in vision.
Conclusions

In this study, we report retinal changes associated with the α-synuclein A53T mutation, as well as describe the effect of α-synuclein “seeding” on the development of retinal pathology. We demonstrate that pSer129 is first detected in retinas of transgenic mice at 5 months of age, then widely expressed in the outer and inner retina. This finding is consistent with the first detection of pTauThr231, and activation of retinal Müller glia and microglia. We report that microglial activation seems to be a more sensitive indicator of retinal stress, as we see a robust increase in microglial activation, but not Müller glia, due to seeding. We show, for the first time, that seeding with brain homogenate from clinically ill mice, causes a prominent acceleration of retinal pSer129 and pTauThr231 accumulation. This finding is consistent with other works describing the self-propagating ability of misfolded proteins. Our work also suggests that seeding with brain homogenate from clinically ill mice leads to the dysfunction of protein degradation, and collapse of protein quality control systems leading to neuroinflammation and further accumulation of misfolded proteins. An increase in pSer129 and pTauThr231 accumulation may lead to dysregulation in retinal autophagy and cell death, however mechanistic studies are necessary to delineate direct effect of prion-like α-synuclein propagation from the detrimental effects of general cellular dysfunction specific to protein degradation. Finally, we provide evidence that Raman spectroscopy can be used to differentiate between retinal tissues from control, healthy, and diseased mice with high accuracy, even among retinas that appear histologically similar. If further refined, successful implementation of this non-invasive and rapid technique can potentially replace histology as the current gold standard of synucleinopathy diagnosis, perhaps earlier in the disease process than is currently
possible. Taken together, our work sheds light on the pathological events of Parkinson’s disease in the retina that have not been previously reported.

Acknowledgements

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References


Figure 1. Experimental Design. Retinas of transgenic mice (TgM83) at 5, 8, and 12-18 months of age, and age-matched controls (B6C3H) were analyzed using immunohistochemistry and western blotting. Additionally, retinas of 2-month-old TgM83 mice were inoculated with brain homogenate from clinically ill (12-18-month-old) TgM83 mice, and retinal tissues were analyzed 3 months later.
Figure 2. Accumulation of α-synuclein. (A, B) Retinas of B6C3H mice had no phospho-α-synuclein (p129S) immunoreactivity. (C-F) Retinas of transgenic mice showed a similar trend, with sparse perinuclear and extracellular phospho α-synuclein (p129S) immunoreactivity at 5 months of age, evident in the inner retina at the stage of clinical disease. (E) Retinas of inoculated mice showed increased α-synuclein (p129S) immunoreactivity, localized to ONL as well as the inner retina. (G, H) Retinas of B6C3H mice had no α-synuclein immunoreactivity. (I) Perinuclear and extracellular α-synuclein accumulation first evident at 5 months of age, localized to ONL. (J-L) As disease progressed, α-synuclein accumulation increased in intensity, distributed throughout the inner retina. (K) Retinas of inoculated TgM83 mice are comparable to retinas of clinically ill TgM83 mice, with α-synuclein localized to ONL and inner retina. **Abbreviations**: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. **Insets**: High magnification images of α-synuclein immunoreactivity. **Scale bars**: 40 µm; insets 15 µm. (M) α-synuclein (pS129) (15 kDa), α-synuclein (15 kDa), immunoreactive bands. (N) Representative bar graph showing quantitative densitometric analysis of α-synuclein (pS129)/α-synuclein ± SD **P < 0.01 vs. B6C3H and TgM83 (5m); ***P < 0.001 vs. B6C3H and TgM83 (5m).
Figure 3. Activation of Müller glia. (A, D) GFAP is localized to end feet of Müller glia in retinas of control mice. (B, C, E, F) Distribution of GFAP immunoreactivity is increased, spanning the entire thickness of the retina. (G) Bar graphs show significant increase in GFAP immunoreactivity in retinas of transgenic mice. Data are expressed as mean ± SD. 

**Abbreviations:** GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. 

**Scale bars:** 20 µm. ****P < 0.0001 versus B6C3H (5m); +++P < 0.001 versus B6C3H (8m); ++++P < 0.0001 versus B6C3H (8m); †† †† † P < 0.0001.
Figure 4. Accumulation of phosphorylated tau. (A, D) No pTau$_{\text{Thr}231}$ immunoreactivity was detectable in retinas of control mice. (B) pTau$_{\text{Thr}231}$ immunoreactivity was first appreciable in retinas of transgenic mice at 5 months of age, localized to OPL and GCL. (C, E, F) Perinuclear pTau$_{\text{Thr}231}$ accumulation is increased as disease progressed. (C) pTau$_{\text{Thr}231}$ in retinas of inoculated TgM83 mice was comparable to retinas of TgM83 mice at clinical stage of disease. (G) Bar graphs show percent area of fluorescence for pTau$_{\text{Thr}231}$. Data are expressed as mean ± SD. (H-M) Confocal microscopy shows colocalization of GFAP and AT180 and of calbindin and AT180, suggesting that tau (pThr231) is present in Müller glial processes, and calbindin immunoreactive horizontal cells and photoreceptor cell terminals, respectively. **Abbreviations:** GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. **Inset:** High magnification image of perinuclear pTau$_{\text{Thr}231}$ localized in GCL. **Scale bars:** A-F 25 µm; H-M 15 µm; C inset 5 µm. ****P < 0.0001 versus B6C3H (5m); +P < 0.05 versus B6C3H (8m); +++P < 0.0001 versus B6C3H (8m); ††P < 0.01; ††††P < 0.0001.
Figure 5. Microglial activation. (A, B) Microglia of B6C3H and TgM83 (5m) are quiescent, with long processes and small cell bodies, localized primarily to the plexiform layers. (C-F) Robust microglial activation is evident in retinas of TgM83 mice (8m, inoculated, and clinical). Activated microglia have large cell bodies, thick or retracted processes, and appear to invade nuclear layers, and the outer segments of photoreceptor cells. (H-M) CD68 immunoreactivity shows activated and phagocytic microglia localized to the inner retina, compared to the lack of activated microglia in control retinas. (J, M). Microglial activation in inoculated TgM83 mice seems accelerated, as it is comparable to that of clinical disease. (G, N) Bar graphs show percent area of fluorescence for CD11b and CD68. Data are expressed as mean ± SD. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Insets: High magnification images of changes in microglial morphology due to activation. Scale bars: 20 µm; insets 5 µm. (G) ****P < 0.0001 versus B6C3H (5m); +P < 0.05 versus B6C3H (8m); +++P < 0.001 versus B6C3H (8m); †P < 0.05; †††P < 0.001. (N) ****P < 0.0001 versus B6C3H (5m); +++P < 0.001 versus B6C3H (8m); ††††P < 0.0001.
Figure 6. Dysregulation of autophagy. (A) Schematic diagram of autophagic progression, broken down into the general stages, including induction, autophagosome formation, and degradation. (B) Western blot analysis of pAMPK\(^{T172}\), pULK1\(^{S757}\), and LC3 expression levels in the retina. pAMPK\(^{T172}\) (64 kDA), AMPK (64 kDA), pULK1\(^{S757}\) (150 kDA), ULK1 (150 kDA), and LC3 (14,16 kDA) immunoreactive bands. (C-E) Representative bar graphs showing quantitative densitometric analysis of pAMPK\(^{T172}/\text{AMPK} \pm \text{SD (I)}\); pULK1\(^{S757}/\text{ULK1} \pm \text{SD (J)}\); LC3 II/total protein \(\pm \text{SD (K)}\) in retinas of all mice. *\(P < 0.05\); **\(P < 0.01\); ****\(P < 0.0001\).

(F) Diffuse LC3 immunoreactivity in retinas of control mice is representative of basal autophagy (G) Increase in LC3-immunoreactive autophagosomes in ONL and OPL evident in retinas of TgM83 (5m) mice is suggestive of normal autophagic response to disease. (H-J) Retinas of TgM83 (8m), Inoculated TgM83 (5m), and clinically ill mice show an appreciable decrease in LC3-immunoreactive puncta, similar to control retinas with basal autophagy.

**Abbreviations**: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. **Scale bars**: 40 µm. Data are expressed as means ± SD. *\(P < 0.05\) versus B6C3H (5m); ††††\(P < 0.0001\) versus B6C3H (8m); †P < 0.05; †††††P < 0.0001.
**Figure 7. Neuronal loss.** (A) A53T mutation causes progressive photoreceptor cell death evident first at 8 months of age. Photoreceptor cell loss is also evident in retinas of inoculated TgM83 (5m). (B-D) No caspase-3 labeling is seen in retinas of B6C3H mice, while caspase-3 immunoreactive photoreceptor cells are evident in retinas of 8-month-old and clinically ill mice, indicating apoptotic cell death. (E-J) Tyrosine hydroxylase immunoreactivity was comparable among all mouse groups. **Abbreviations:** GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. **Scale bars:** 40 µm. Data are expressed as means ± SD. *P < 0.05 versus B6C3H (5m); ++++P < 0.0001 versus B6C3H (8m); ‡P < 0.05; ‡‡‡‡P < 0.0001
CHAPTER 4. INCUBATION PERIODS OF CLASSICAL AND ATYPICAL BOVINE SPONGIFORM ENCEPHALOPATHIES REFLECT AN INVERSE RELATIONSHIP BETWEEN PrP\textsuperscript{Sc} – ASSOCIATED NEUROINFLAMMATION AND AUTOPHAGY.

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Abstract

Neurodegenerative protein misfolding disorders result from aberrant folding and accumulation of specific proteins. Transmissibility distinguishes transmissible spongiform encephalopathies (TSEs) from other protein misfolding diseases. TSEs strains variations can influence disease phenotypes such as host susceptibility, biochemical and immunohistochemical profiles, and incubation periods. Bovine spongiform encephalopathy (BSE) is a TSE that occurs in cattle and can be subdivided into three different strains: classical BSE, atypical high-type, and low-type BSE. Both H-type and L-type BSEs, have shorter incubation periods and, therefore, an accelerated disease progression when compared to classical BSE. Currently, there is a lack of knowledge about the factors that influence disease
progression making this a key challenge for the development of therapies for protein misfolding diseases. Due to the similarities between TSEs and other protein misfolding diseases, TSEs can be used to understand other proteinopathies. In this study, we used the differences between classical and atypical BSE as a model to identify the molecular factors associated with disease progression. The NLRP3 inflammasome is a critical component of the innate immune system that leads to release of IL-1β (Interlukin-1β), an important regulator of neuroinflammation in many protein misfolding diseases. Macroautophagy is an intracellular mechanism that plays an essential role in protein clearance and homeostasis. In this study, we use the retina as a model to investigate the relationship between disease incubation period, PrPSc accumulation, neuroinflammation, and changes in macroautophagy. We demonstrate that atypical BSEs, characterized by shorter incubation periods, present with greater accumulation of PrPSc, glial-cell activation, NLRP3 inflammasome activation, and decreased autophagy. Our work suggests a relationship between disease time course, neuroinflammation, and the autophagic stress response, that has not been previously reported. This work may help identify novel therapeutic biomarkers that can delay, reverse, or even prevent the progression of protein-misfolding diseases.

**Keywords:** autophagy, bovine spongiform encephalopathy, incubation period, NLRP3 inflammasome, prion disease, retina.

**Introduction**

Many neurodegenerative diseases, including Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease (HD), and transmissible spongiform encephalopathies (TSEs) result from protein misfolding followed by the subsequent aggregation of these proteins into abnormal, toxic species that accumulate in tissues [1-3]. A key feature that distinguishes
prion diseases from other protein misfolding diseases is the transmissibility of TSE agents. While the causative agent of all TSEs is PrP\textsuperscript{Sc}, the pathological isoform of the normal cellular prion protein (PrP\textsuperscript{C}), different strains of TSEs within a species may be distinguished by different pathogenesis and disease phenotypes. These include distinct immunohistological characteristics (i.e., lesion profiles, neuroanatomical deposition patterns of PrP\textsuperscript{Sc}), and clinical signs and incubation periods [2, 4-8]. Specifically, BSE can be subdivided into classical BSE, colloquially known as mad cow disease, and sporadic/atypical BSE [4]. Atypical BSE can be further subdivided into H-type and L-type, with the H- and L- designations based on a higher or lower molecular mass of the unglycosylated PrP\textsuperscript{Sc} isoform, when compared to classical BSE [4]. Both H-type and L-type BSE, herein referred to as BSE-H and BSE-L, have shorter incubation periods after experimental intracranial inoculation and, therefore, an accelerated disease progression when compared to classical BSE. One of the key challenges for drug discovery and development of therapeutic approaches is a lack of well-validated disease biomarkers due to a limited understanding of the molecular and cellular factors that interact with the misfolded proteins and influence how rapidly a disease progresses [1]. Due to the striking parallels of TSEs to the non-infectious protein misfolding diseases (i.e., PD, AD, and HD), prion diseases can be used to study and understand other proteinopathies [1]. In this study, we exploited the differences between classical and atypical BSEs to identify the molecular factors or determinants that are associated with the slower progression of classical BSE, versus the more rapid progression of atypical BSEs. Specifically, we use the strain-dependent variation in incubation periods between classical and atypical BSEs as a controlled framework to examine the relationship between a diverse set of immunological characteristics and incubation periods.
PrP\textsuperscript{Sc} accumulates in retinas of TSE infected animals [9-16], and antemortem changes in retinal function and morphology are detectable in BSE inoculated cattle up to 11 months prior to the appearance of any other clinical signs of disease suggesting that the retina is an excellent experimental model to investigate disease pathogenesis. We use the retina as an experimental model to investigate the effect of PrP\textsuperscript{Sc} accumulation and specific neuroinflammatory processes - including glial cell activation, NLRP3 inflammasome activation, and macroautophagy - on disease incubation periods. The NLRP3 inflammasome is a multiprotein complex that is an important component of innate immunity and a known contributor to the progression of several protein misfolding diseases (i.e., TSEs, PD, and AD) [17, 18]. Macroautophagy, often referred to as autophagy, is a highly conserved metabolic process responsible for the clearance of bulk cytoplasmic components, including protein aggregates. Several studies indicate that misfolded proteins provoke microglia-driven NLRP3 inflammasome activation that has been reported to be associated with autophagic dysfunction (i.e., a decline in autophagic processes) [17, 19-23]. However, the collective effect of inflammasome activation and autophagy on disease progression and incubation periods is unknown.

In this study, we report that disease incubation period is inversely correlated to PrP\textsuperscript{Sc} accumulation, glial-cell activation, and NLRP3 inflammasome activation in the retina, brainstem, and thalamus. Specifically, cattle inoculated with atypical BSE-H and BSE-L, characterized by shorter incubation periods, compared to classical BSE, had greater PrP\textsuperscript{Sc} deposition, robust activation of Müller glia, astrocytes, and microglia, and microglia-driven NLRP3 inflammasome activation. We show that incubation period reflects a negative correlation between PrP\textsuperscript{Sc}-associated NLRP3 inflammasome activation and autophagy.
Specifically, we describe opposing events in the autophagic pathway: upregulation of autophagy detected in cattle inoculated with classical BSE; and a decline in autophagy in cattle inoculated with BSE-H or BSE-L that paralleled greater PrP\textsuperscript{Sc} accumulation, neuroinflammation and shorter incubation periods. For the first time, we demonstrate an association between incubation period and several molecular disease processes (i.e., accumulation of misfolded proteins, glial cell activation, NLRP3 inflammasome activation, and macroautophagy) \textit{in vivo}, that can further our understanding of the factors that influence disease progression. This study provides a better knowledge of the molecular basis of accelerated disease progression in different strains of prion disease. Due to the similarity of TSEs with other protein misfolding diseases, this work could have important implications for understanding the interplay between misfolded proteins and the cellular responses \textit{in vivo}, and identifying biomarkers that can be targeted for clinical trials.

**Materials and Methods**

**Ethics statement**

This experiment was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, DC) and the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, Champaign, IL). The protocol was approved by the Institutional Animal Care and Use Committee at the National Animal Disease Center (protocol number: 3985) and Iowa State University (protocol number: 7154).
Animals and inoculum

Adult Holstein steers (3.8–4.5 years old) were inoculated intracranially with 1 ml of a 10% (wt./vol) brain homogenate as previously described [24]. Twelve cattle were inoculated with the agent of classical BSE, nine cattle were inoculated with the agent of BSE-H, and ten cattle were inoculated with the agent of BSE-L (referred to as inoculated with classical BSE, BSE-H, or BSE-L, respectively). In this study, cattle referred to as BSE-L include: French L-type BSE (n=2), Canadian L-type BSE (n=2), Italian L-type BSE (BASE) (n=1), and TME (n=5) [25]. The classical BSE inoculum was from a case diagnosed in the U.S. in 2003 and the high-type BSE (BSE-H) inoculum a case diagnosed in the US in 2004 [26]. Control cattle for this study included one sham-inoculated animal from this study, one sham-inoculated animal from a TME to cattle transmission study, and three sham-inoculated animals from a CWD to cattle transmission study [27, 28]. The inoculated cattle were observed daily by animal care staff at NADC, and examined regularly by investigators to determine the onset of clinical disease. Cattle were euthanized at the onset of unequivocal signs of clinical disease, but were not allowed to develop severe, end-stage disease. Upon euthanasia, all cattle were confirmed positive by western blot or enzyme immunoassay as directed (IDEXX HerdChek BSE-Scrapie Antigen ELISA test kit, Westbrook, ME).

Immunohistochemistry and Western blot analysis

Paraffin-embedded sections (4 μm) were rehydrated using xylene, followed by a decreasing ethanol concentration gradient (100%, 90%, 70%), and a final wash with diH₂O. Heat-mediated antigen retrieval was performed using EDTA buffer (10 mM Trizma Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0), or citrate buffer (ScyTek Laboratories, Logan, UT) in an autoclave for 30 min. Tissues were incubated with Background Buster (Innovex
Biosciences Inc., Richmond, CA) for 1.5 h. Primary antibodies against glial fibrillary acidic protein (GFAP) (1:1000, Dako, Carpinteria, CA), Iba1 (1:2000, Abcam, Cambridge, UK), NLRP3 (1:250, Adipogen, San Diego, USDA), ASC (1:500, Adipogen), caspase-1 (1:1000, Adipogen), IL-1β (1:1000, Abcam), LC3A/B (1:2000, Cell Signaling, Danvers, Massachusetts), or caspase-3 (Santa Cruz Biotechnology) were diluted in blocking solution containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 0.05% Triton X-100 (Thermo Fisher Scientific, Inc., Rockford, IL) and 5% normal serum (Jackson ImmunoResearch, West Grove, PA) in Tris-Buffered saline (TBS), and incubated for 48 h at room temperature. Tissues were washed with TBS-T (6 x 5 min) and incubated with a secondary antibody cocktail, including Cy3 and/or Alexa Fluor 488–conjugated AffiniPure secondary antibodies (1:250; Jackson ImmunoResearch) and 4’,6-diamidino-2-phenylindole, dilactate (DAPI, 4 μg/mL; Sigma-Aldrich) for 1.5 h. Following another wash, slides were mounted with Vectashield antifade mounting medium (Vector Laboratories Inc., Burlingame, CA). Negative controls were processed in parallel by omission of the primary and/or secondary antibody. For detection of PrPSc, slides were stained by an automated immunohistochemical method, described previously [12, 24]. The primary antibody used was F99/97.6.1 (O’Rourke; Pullman, Washington) at a concentration of 5 μg/ml.

The protein extraction method for western blot analysis has been previously described in Mammadova et al., 2019 [29]. The primary antibodies directed against LC3II (1:2500, Cell Signaling), pULK1\textsuperscript{S757} (1:1000, Cell Signaling), and ULK1 (1:1500, Cell Signaling) were incubated overnight at 4°C. All groups are normalized to control and/or mock-treated animals, providing a representative of a fold-change from the control, therefore y-axis units are arbitrary.
Fluorescent microscopy and statistical analysis

Fluorescence and confocal images were taken at 20x and 60x, using a Nikon A1R+ Resonant Scanning Confocal System with a Ti-E inverted microscope and laser lines 405, 488, 561 and 640 nm (Nikon Instruments Inc., Melville, New York). Micrographs were created using a commercial photo-editing system (Adobe Photoshop and Adobe Illustrator [CC]; Adobe Systems). For quantification of GFAP, Iba1, NLRP3, and ASC immunoreactivity, the percentage of the total image area thresholded (outer limiting membrane to inner limiting membrane of the central retina) was analyzed using ImageJ (Rasband, W.S., ImageJ 1.49V, U. S. National Institutes of Health, Bethesda, Maryland). Equivalent regions of each brain structure (i.e., brainstem at the level of the obex, thalamus (interthalamic nuclei), and caudate nuclei) were used for the creation of micrographs and quantification using ImageJ. Quantified histological data was analyzed using a two-way ANOVA, with a Tukey’s multiple comparisons test (post-hock). Prism 6 for Windows (Graph Pad Software) was used for statistical analysis.

Results

To investigate the relationship between incubation period and disease associated pathology, we conducted a controlled comparison of tissues from cattle experimentally inoculated with classical and atypical BSEs (BSE-H and BSE-L). Incubation period for classical BSE, BSE-H or BSE-L is reported as the time from intracranial inoculation to the time when unequivocal signs of clinical disease are present. Clinical signs of disease included abnormalities in gate and/or stance, moderate to severe ataxia, and hyperreaction to stimuli (i.e. noise, movement). Cattle inoculated with atypical BSEs had significantly shorter incubation periods compared to cattle inoculated with classical BSE (Fig. 1). The average incubation period for cattle inoculated with classical BSE, BSE-H, or BSE-L was 683.7 ± 45.2
dpi, 513.7 ± 4.8 dpi, and 472.8 ± 11.9 dpi respectively (Fig.1). The differences in incubation period were statistically significant (p<0.0001). The migration pattern of PrP^Sc on western blot was analyzed to verify that the cattle inoculated with classical or atypical BSEs retained a classical, high-type, or low-type migration pattern as previously described [10, 25].

**Increased PrP^Sc deposition coincides with shorter incubation periods of BSE-H and BSE-L**

To investigate the relationship between PrP^Sc deposition and incubation period, PrP^Sc immunoreactivity was assessed in retinas of cattle inoculated with classical BSE, BSE-H, and BSE-L. Retinas from cattle inoculated with classical BSE had punctate or granular PrP^Sc localized primarily to the synaptic layers (IPL and OPL in Fig. 2a, b). The distribution of PrP^Sc was more extensive in retinas of cattle inoculated with BSE-H or BSE-L (Fig. 2c, d). PrP^Sc immunoreactivity was much more intense and uniform in the synaptic layers (Fig. 2c, d). Globular deposits of PrP^Sc were evident in the outer segments of photoreceptor cells and dispersed throughout the nuclear layers with robust intracellular PrP^Sc immunoreactivity evident within cells located in the ganglion cell layer (Fig. 2c). Compared to retinas of cattle inoculated with BSE-H, PrP^Sc was rarely detected in the outer segments of photoreceptor cells, outer nuclear layer, and ganglion cell layer of BSE-L inoculated cattle (Fig. 2c, d). These results demonstrate that increased retinal PrP^Sc accumulation correlates with shorter incubation periods, characteristic of atypical BSEs. To investigate the relationship between PrP^Sc deposition and incubation period in the brain, PrP^Sc immunoreactivity was assessed in the hypoglossal nuclei of the brainstem at the level of the obex, interthalamic nuclei, and caudate nuclei of cattle inoculated with classical BSE, BSE-H, and BSE-L. Compared to control, the brainstems at the level of the obex of cattle inoculated with classical BSE had granular and aggregated PrP^Sc deposits (Fig. 2e, f). Compared to cattle with classical BSE, PrP^Sc
immunoreactivity in the brainstems of cattle inoculated with BSE-H or BSE-L was more intense (Fig. 2g, h). A similar trend in PrP\(^{\text{Sc}}\) deposition was seen in the thalami of these cattle (Fig. 2i-l). Conversely, PrP\(^{\text{Sc}}\) immunoreactivity in the caudate nuclei of cattle inoculated with classical BSE, BSE-H, or BSE-L was similar in the deposition pattern (i.e., aggregated and plaque-like PrP\(^{\text{Sc}}\) deposits) and magnitude (Fig. 2m-p). Strain-dependent differences in incubation time of classical, H-type, and L-type BSE did not parallel differences in the amount of PrP\(^{\text{Sc}}\) accumulation in the caudate nuclei. Our results demonstrate that increased PrP\(^{\text{Sc}}\) accumulation in some brain regions (i.e., brainstem and thalamus) correlates with shorter incubation times of atypical BSEs.

**Glial cell activation parallels PrP\(^{\text{Sc}}\) accumulation**

Activation of Müller glia and microglia is a widely reported retinal response during progression of TSEs, like many protein misfolding diseases [9, 10, 12, 30]. To assess the activation of these principal retinal glial cells, we measured the distribution of GFAP and Iba1 immunoreactivity, respectively. GFAP immunoreactivity in quiescent Müller glia is constrained to their end feet. However, under conditions of retinal injury or stress, GFAP expression is increased and widely distributed throughout the radial processes of Müller glia that span from the inner limiting membrane to the outer limiting membrane [31]. GFAP immunoreactivity in the retinas of control cattle was localized to the Müller glial end feet and astrocytes in the optic fiber layer (Fig. 3a). Compared to control cattle, the retinas of cattle inoculated with classical BSE had increased GFAP immunoreactivity, observed in the radial processes spanning the retina from the optic fiber layer to the outer plexiform layer (Fig. 3b). When quantified, there was a significant difference in GFAP immunoreactivity between the retinas from cattle inoculated with sham and classical BSE (p>0.0001) (Fig. 3i). Compared to
the retinas of cattle inoculated with classical BSE, the retinas from cattle inoculated with BSE-H or BSE-L had robust GFAP immunoreactivity spanning the retina from the optic fiber layer, with hypertrophied Müller glial end feet and activated astrocytes, to the outer limiting membrane (Fig. 3b-d). When quantified, GFAP immunoreactivity in retinas of cattle inoculated with BSE-H and BSE-L, was significantly greater than that of classical BSE (p<0.01, and p<0.05 respectively) (Fig. 3i).

An antibody against Iba1, microglia/macrophage specific calcium binding protein, was used to assess activation of microglia in the retinas of all cattle. Assessment of Iba1 immunoreactivity enables the analysis of cell shape and morphologic changes of microglia and provides information as to their function (i.e., routine surveillance or phagocytosis). Compared to control, retinas of cattle inoculated with classical BSE had Iba1 immunoreactive microglia with small somata and thin/long processes, sparsely dispersed through the synaptic layers (Fig. 3e, f). Quantification of Iba1 immunoreactivity showed no significant difference between retinas of control cattle and those inoculated with classical BSE (Fig. 3j). When compared to retinas from control and classical BSE inoculated cattle, the retinas from cattle inoculated with BSE-H or BSE-L had a marked increase in the distribution of Iba1 immunoreactive microglia and a corresponding change to an amoeboid-like morphology (Fig. 3e-h). Iba1 immunoreactivity in the retinas from cattle inoculated with atypical BSEs was widely distributed throughout all retinal layers with substantially more in synaptic layers and also was detected in the neuronal layers and the outer segments (Fig. 3g, h). When quantified, Iba1 immunoreactivity in retinas of cattle inoculated with BSE-H and BSE-L, was significantly greater than that of classical BSE (p<0.0001) (Fig. 3j).
To assess the activation of astrocytes and microglia in the brain (i.e., brainstem at the level of the obex, thalamus, and caudate nucleus) we measured the distribution and magnitude of GFAP and Iba1 immunoreactivity, respectively (Supp. Fig. 1 and Supp. Fig. 2). Compared to control, all three brain regions of cattle inoculated with classical BSE had significantly more GFAP immunoreactivity (Supp. Fig. 1m-o). Compared to classical BSE cattle, GFAP immunoreactivity in the brainstem and thalamus of cattle inoculated with BSE-H or BSE-L was stronger (Supp. Fig. 1m, n). Conversely, GFAP immunoreactivity in the caudate nuclei of cattle inoculated with classical BSE, BSE-H, or BSE-L was comparable in pattern and magnitude (Supp. Fig. 1o). Similar to the retina, Iba1 immunoreactivity in all three brain regions of cattle inoculated with classical BSE, was not significantly different than control (Supp. Fig. 2m-o). Compared to classical BSE cattle, Iba1 immunoreactivity in the brainstem and thalamus of cattle inoculated with BSE-H or BSE-L was significantly higher (Supp. Fig. 2m - o). Similar to PrP\textsuperscript{Sc}, and GFAP immunoreactivity, Iba1 immunoreactivity in the caudate nuclei of cattle inoculated with classical BSE, BSE-H, or BSE-L was comparable (Supp. Fig. 2o).

Taken together, these results demonstrate that compared to classical BSE, retinas of BSE-H or BSE-L inoculated cattle had robust activation of Müller glia, astrocytes, and microglia. These observations correlated with intense PrP\textsuperscript{Sc} accumulation, also detected in retinas of BSE-H or BSE-L cattle. Similar differences between classical and atypical BSEs were observed in the brainstem and thalamus. However, activation of astrocytes and microglia in the caudate nucleus was comparable in cattle inoculated with all three strains of BSE and paralleled levels of PrP\textsuperscript{Sc} accumulation in the tissue. Thus, our results collectively suggest a
relationship between shorter incubation times, greater accumulation of PrP^{Sc}, and neuroinflammation.

**Activation of the NLRP3 inflammasome parallels the shorter incubation times of atypical BSE-H and BSE-L**

Contribution of NLRP3 inflammasome activation to the progression of protein misfolding diseases has been extensively described in TSEs, PD, and AD (reviewed in [18]). To probe the relationship between incubation time and NLRP3 inflammasome activation in BSE, we assessed the expression of key inflammasome components: NACHT, LRR and PYD domains-containing protein 3 (NLRP3), the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC), active caspase-1, and active IL-1β (Fig. 4). NLRP3 immunoreactivity in the retinas of control cattle was negligible (Fig. 4a). Retinas from cattle inoculated with classical BSE had increased NRLP3 immunoreactivity, and similar to Iba1 immunoreactivity, it was localized to the synaptic layers (Fig. 4b). Retinas of cattle inoculated with atypical BSEs, had robust NLRP3 immunoreactivity, with approximately a two-fold increase compared to classical BSE (Fig. 4c-e). Double immunofluorescence and confocal microscopy confirmed that NLRP3 was almost exclusively localized to Iba1+ microglia, consistent with previous reports linking the detrimental effects of chronic microgliosis to inflammasome activation [17, 19, 32]. Immunohistochemical analysis of ASC immunoreactivity, yielded similar results (Fig. 4i-m). In contrast to NLRP3 expression, however, negligible multifocal ASC immunoreactivity was observed in the retinas from cattle inoculated with classical BSE (p > .05 versus control) (Fig. 4i, j, m). Retinas from cattle inoculated with atypical BSEs had significantly more ASC immunoreactivity (~eight-fold increase), compared to the retinas from cattle in the sham or classical BSE inoculated groups (Fig. 4k-m). Assembly of the NLRP3 inflammasome triggers cleavage of procaspase-1 into
active caspase-1 that converts the cytokine precursor pro-IL-1β into active IL-1β, a key proinflammatory mediator [33]. Western blot analysis of downstream inflammasome markers, active caspase-1 and IL-1β, showed minimal protein expression in retinas from cattle in either the sham or classical BSE inoculation groups (p > 0.05) (Fig. 4n-p). In comparison, the retinas from cattle inoculated with atypical BSEs had a significant increase in both caspase-1, and IL-1β protein expression (Fig. 4n-p).

Similar results were observed in the brainstem at the level of the obex, thalamus, and caudate nucleus (Supp. Fig. 3 and Supp. Fig. 4). Consistent with results from the retina, double immunofluorescence and confocal microscopy confirmed that NLRP3 was localized to Iba1+ microglia in the obex (Supp. Fig. 3a-c), thalamus (data not shown), and caudate nucleus (data not shown). The obex and thalami from cattle inoculated with BSE-H or BSE-L had significantly more NLRP3 (Supp. Fig. 3d, e) and ASC (Supp. Fig. 3g, h) immunoreactivity compared to cattle inoculated with sham or classical BSE. Levels of NLRP3 and ASC immunolabeling in the caudate nucleus was comparable in cattle inoculated with either classical BSE, BSE-H, or BSE-L. The obex, and thalami from cattle inoculated with BSE-H and BSE-L, also had higher expression of caspase-1 (Supp. Fig. 4a, b, d) and IL-1β (Supp. Fig. 4a, c, e), compared to cattle inoculated with sham or classical BSE. Protein expression levels of caspase-1 (Supp. Fig. 4a, f) and IL-1β (Supp. Fig. 4a, g) in the caudate nucleus was comparable in classical BSE, BSE-H, and BSE-L. These results demonstrate increased microglia-driven NALP3 inflammasome activation in cattle inoculated with atypical BSEs, which parallels increased PrPSc accumulation and shorter disease incubation times.
**Autophagic progression is impaired in atypical BSE-H and BSE-L**

In protein misfolding diseases, an increase in autophagy is a proper response during clearing of bulk cytoplasmic contents. A decrease below basal levels may indicate autophagic dysfunction due to an overload of a system incapable of degrading proteins [34-37]. To assess the relationship between incubation time and autophagy in the retina, we performed immunohistochemical and western blot analysis of proteins involved in the initiation of autophagy and vesicle formation (Fig. 5). To determine changes in vesicle formation, we analyzed distribution, and protein expression of the autophagosome marker, LC3. Retinas from cattle from the sham and classical BSE inoculation groups had diffuse LC3 immunoreactivity localized to the outer segments and synaptic layers consistent with a basal level of autophagy under normal conditions (Fig. 5a). Retinas from cattle inoculated with classical BSE had robust, multifocal LC3-immunoreactivity primarily in the synaptic layers similar to the pattern of PrPSc immunoreactivity in these cattle (Fig. 5b). In contrast, retinas from cattle inoculated with atypical BSE-H and BSE-L, had inappreciable and diffuse LC3 immunoreactivity, comparable to retinas from control cattle (Fig. 5c, d). Western blot analysis of LC3-II protein expression revealed similar results. Compared to control, level of LC3-II protein expression was ~2-fold higher in retinas from classical BSE cattle (p>0.001), and comparable in retinas of BSE-H (p>0.05) and BSE-L (p>0.05) cattle (Fig. 5e, f).

To determine changes in the initiation of autophagy, we examined expression levels of the key autophagy-initiation protein, pULK1$^{S757}$. Briefly, the initiation stage involves activation of ULK1 (a serine-threonine kinase), regulated by both mTOR and AMPK. Activation of mTOR leads to the enzymatic inactivation of ULK1, by downstream phosphorylation at serine 757. Conversely, when AMPK is activated, ULK1 is dephosphorylated at Serine 757 and thereby activated allowing for autophagy to advance [38].
Compared to control, protein levels of pULK1$^{S757}$ were significantly lower in retinas from cattle inoculated with classical BSE (Fig. 5e, g). Protein levels of pULK1$^{S757}$ in retinas from cattle inoculated with BSE-H and BSE-L, were comparable to basal levels detected in retinas from sham-inoculated cattle (Fig. 5e, g). Again, similar results were observed in the brainstem, thalamus, and caudate nucleus (Supp. Fig. 5). The brainstem, and thalami from cattle inoculated with BSE-H and BSE-L, had significantly higher expression of LC3II (Supp. Fig. 5a, b, d), and lower expression of pULK1$^{S757}$ (Supp. Fig. 5a, c, e). However, protein expression levels of LC3II (Supp. Fig. 5a and f), and pULK1$^{S757}$ (Supp. Fig. 5a and g) in the caudate nucleus were similar in tissues from cattle inoculated with classical BSE, BSE-H, or BSE-L. These results indicate an inverse relationship between incubation period and autophagy, suggesting that that autophagic dysfunction may contribute to increased PrP$^{Sc}$ accumulation in atypical BSEs, leading to an accelerated disease phenotype and shorter incubation periods.

Taken together, these results describe a negative correlation between disease incubation period and PrP$^{Sc}$ accumulation in the retina and in the brain. We report a positive correlation between retinal PrP$^{Sc}$ accumulation and NRLP3 inflammasome activation (Fig. 6a). Finally, we describe a negative correlation between incubation period and autophagy (Fig. 6b). Our work suggests a reciprocal regulation between PrP$^{Sc}$-associated neuroinflammation and autophagy in vivo that reflects differences in incubation period and disease progression.

**Discussion**

**Disease incubation period is inversely correlated to PrP$^{Sc}$ accumulation, glial-cell activation, and NALP3 inflammasome activation**

In this study, we report a correlation between strain-dependent differences in disease incubation period and accumulation of PrP$^{Sc}$ in the retina, brainstem, and thalamus. Specifically, cattle inoculated with the agents of atypical BSE-H or BSE-L had shorter
incubation periods and greater PrP\textsuperscript{Sc} deposition compared to cattle inoculated with classical BSE demonstrating a negative correlation between incubation period and disease pathology. Prion disease strains can be differentiated by incubation periods, neuroanatomical and cellular deposition patterns of PrP\textsuperscript{Sc}, and other molecular properties [5-7, 39]. Distinct prion strains, within a species, have been shown to exhibit different patterns and levels of PrP\textsuperscript{Sc} accumulation in the brain independent of incubation period. For example, PrP\textsuperscript{Sc} deposition in classical scrapie in sheep occurs primarily in the medulla oblongata rather than the cortices of the cerebellum and the cerebrum as seen in atypical sheep scrapie [4, 7, 40]. Additionally, spongiform lesions and PrP\textsuperscript{Sc} immunoreactivity in the brains from cattle inoculated with classical BSE are distinct from that of cattle with atypical BSE-H. In natural and experimental cases of classical BSE, spongiform lesions are distributed consistently throughout the brain [41], whereas in BSE-H, spongiform lesions and PrP\textsuperscript{Sc} immunoreactivity are more intense in the brainstem and midbrain, increasing in intensity caudally, from the frontal cortex to the occipital cortex [13, 30, 41]. Moreover, patterns of PrP\textsuperscript{Sc} immunoreactivity (i.e., intraglial, stellate, intraneuronal, etc.) in the brains from BSE-H and classical BSE affected cattle, vary depending upon region [13, 30]. While strain-dependent patterns of PrP\textsuperscript{Sc} accumulation have been widely explored, studies examining the relationship between strain-dependent incubation period and magnitude of PrP\textsuperscript{Sc} accumulation are lacking.

Historically, glial activation has been assumed to be merely a response to pathophysiological events in protein-misfolding diseases, however, more recent studies have established key contributions of glial cells to neuroinflammation and neurodegenerative disease pathogenesis (reviewed in [42]). This work describes a positive correlation between glial cell activation and PrP\textsuperscript{Sc} accumulation that is reflected in disease incubation period.
Compared to cattle with classical BSE, the retinas from cattle inoculated with atypical BSEs (BSE-H and BSE-L) had robust activation of Müller glia that paralleled intense PrP\textsuperscript{Sc} accumulation and shorter incubation periods. Müller glia, which are normally quiescent, are ubiquitously activated in response to a wide range of pathogenic stimuli, exerting protective and toxic effects on retinal neurons. Although activation of Müller glia has been previously reported in BSE [10], and other prion diseases [9], the relationship between incubation period and Müller glial activation in the retina has not been previously described. Examination of brain regions demonstrated that compared to classical BSE the brainstems and thalami from cattle inoculated with atypical BSEs had more intense activation of astrocytes that paralleled PrP\textsuperscript{Sc} accumulation. However, levels of astrocyte activation in caudate nuclei were comparable in classical and atypical BSEs. The close association of activated astrocytes and PrP\textsuperscript{Sc} deposits is well documented in prion disease further supporting our results [43]. Although astrogliosis is known as a hallmark of prion disease, the role of astrocytes in prion disease pathogenesis has not been elucidated. A correlative study suggested a relationship between the differential activation of astrocytes and PrP\textsuperscript{Sc} deposition in human cerebellar samples of CJD [44]. Moreover, in agreement with similar findings from several \textit{in vitro} studies, a co-localization study suggests that astrocytes drive and can sustain prion propagation [45]. While these results may collectively suggest a negative correlation between the magnitude of astrocyte activation and incubation period, to the best of our knowledge, this report is the first to show this correlation.

Here, we demonstrate an association between incubation period, PrP\textsuperscript{Sc} accumulation, and microglial activation \textit{in vivo}. Our results show that compared to classical BSE, retinas from cattle inoculated with atypical BSEs, had altered microglial morphology and robust activation
that also paralleled intense PrP Sc accumulation and shorter incubation periods. We see a similar correlation in the brainstem and thalamus, but not in the caudate nucleus. The molecular mechanisms underlying the microglial response to PrP Sc are still largely unknown. A previous study reported that the depletion of microglia resulted in enhanced PrP Sc accumulation and augmented prion infectivity suggesting a neuroprotective role [46]. Several time course studies reported that the onset of microglial activation occurs at an early pre-clinical phase of prion disease, before spongiform change and/or neuronal loss, indicating that microglia may contribute to PrP Sc-induced neurodegeneration [9, 47-49]. The current study shows that the distribution and magnitude of microglial response positively correlates with PrP Sc deposition indicating a negative correlation between incubation period and microglial activation that has not been previously reported in prion diseases. Several murine models of prion disease describe the role of microglia in prion pathogenesis highlighting shortened or prolonged disease incubation period and augmented PrP Sc accumulation as a common effect of modulating microglia-related molecules (i.e., toll-like receptors, cytokines and chemokines, complement systems, etc.) (reviewed in [50]). The detrimental effects of microglial activation in prion disease also were investigated in the context of NALP3 inflammasome activation [17, 18]. The NALP3 inflammasome is a multiprotein complex that has been shown to be activated by PrP fibrils leading to the secretion of IL-1β and other proinflammatory factors [17, 19-21]. Our results demonstrate robust microglia-driven NLRP3 inflammasome activation in the retinas from cattle inoculated with BSE-H or BSE-L as evidenced by a significant upregulation of key inflammasome proteins (i.e., NLRP, ASC, active-caspase-1, and active-IL-1β). Retinas from cattle inoculated with classical BSE had minimal NLRP3 and ASC immunoreactivity, while protein expression levels of downstream inflammatory mediators, caspase-1 and IL-1β,
were comparable to controls. While previous research has identified several independent mechanisms at play in regard to NALP3 inflammasome activation in response to PrP fibrils, this is the first report that describes the correlation between incubation period, PrP<sup>Sc</sup> accumulation, and NLRP3 inflammasome activation in prion disease [17, 18, 51].

**Incubation period reflects a negative correlation between PrP<sup>Sc</sup>-associated NALP3 inflammasome activation and autophagy**

Our work describes contrasting events in the autophagic pathway: upregulation of autophagy in the retinas from cattle inoculated with classical BSE indicated by the increase in LC3II, decrease in pULK1<sup>S757</sup>, and downregulation of autophagy in the retinas from cattle inoculated with BSE-H and BSE-L. Recently, it was reported that the inhibition of autophagy enhances release of active IL-1β, and inversely, gene silencing of the NALP3 inflammasome promotes autophagy in an *in vitro* prion model [22]. In the current study, upregulation of autophagy in classical BSE, paralleled longer incubation periods (~640 dpi). Conversely, downregulation of autophagy correlated with robust PrP<sup>Sc</sup> accumulation, NLRP3 inflammasome activation, and shorter incubation periods (~490 dpi). The neuroprotective role of autophagy is suggested by several studies demonstrating that the upregulation of autophagic flux can clear misfolded and aggregated intracellular proteins and suppress neuroinflammation via the degradation of IL-1β [35, 52-59]. While numerous studies show the upregulation of autophagosomes in response to misfolded proteins in neurodegenerative diseases (i.e. prion disease, PD, and AD), whether this event is a proper response to accumulation of misfolded proteins or due to an impairment in the degradation of autophagosomes, is not known [29]. Moreover, it is unknown whether an impairment in the degradation of misfolded proteins is yet another factor that leads to protein aggregation or whether autophagic dysfunction is driven by misfolded proteins. For the first time, this work demonstrates a negative correlation between
neuroinflammation and autophagy in vivo that is strain-dependent and is reflected in disease incubation period.

**A unique framework for understanding factors that influence disease progression**

A hallmark of prion diseases and other neurodegenerative proteinopathies is the formation of misfolded protein aggregates that cause neuroinflammation and cellular toxicity that contribute to cellular collapse. Therapeutic options that are being explored target different steps in the synthesis, processing, and degradation of proteins implicated in neurodegenerative diseases. A major challenge that impacts the development of therapies is the incomplete knowledge of factors that influence disease progression. The strain-dependent variation in incubation periods between classical and atypical BSEs provides a controlled platform to investigate the molecular underpinnings that lead to an accelerated disease progression in atypical BSEs as compared to classical BSE. In this study, we describe the relationship between incubation period, PrP\(^{Sc}\) accumulation, microglia-driven NLRP3 inflammasome activation, and autophagy that has not been previously reported. Targeting potential mechanisms of several NLRP3 inflammasome inhibitors has been increasingly regarded as an effective approach to developing therapies for PD, AD, and HD [60-64]. Moreover, inhibition of macroautophagy is widely shown to be a contributing factor in several proteinopathies, therefore, upregulation of autophagy has also been regarded as a valuable therapeutic strategy (reviewed in [65]). The roles of gliosis, the NLRP3 inflammasome, and protein clearance via macroautophagy all have been individually studied in the pathogenesis and progression of several protein misfolding diseases including prion disease, PD, AD, and HD [60-64]. However, the collective effect of these processes on incubation period is reported here for the first time. This study provides a unique framework for understanding an interconnected system
in which the progression of protein misfolding disease is affected by the magnitude of misfolded proteins in the tissue, the corresponding neuroinflammatory response, and the protein-clearance system.

Conclusions

Collectively, this study describes several key correlations between incubation period, PrPSc accumulation, neuroinflammation, and the autophagic stress response, the mechanisms of disease, that have not been previously reported. We demonstrate that the retinas from cattle inoculated with atypical BSEs (BSE-H and BSE-L), characterized with shorter incubation periods compared to classical BSE, have robust PrPSc accumulation. Increased PrPSc accumulation in these cattle corresponds to significantly increased retinal glial cell activation and microglia-driven NRLP3 inflammasome activation that also is a reflected in a shorter incubation period. Finally, we report that increased PrPSc accumulation and NRLP3 activation are correlated with the down-regulation of autophagy and shorter incubation periods. Our findings support and further clarify a previously reported mechanism describing the interconnection of neuroinflammation and autophagy in response to prion disease. For the first time, we describe the relationship between strain-dependent misfolded protein accumulation, neuroinflammation, and autophagy in vivo that may be predictors of disease incubation period. These results should advance our understanding of the interaction between disease pathogenesis and incubation period and help identify new potential therapeutic targets for intervention.

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References


Fig. 1 Incubation periods of classical and atypical BSEs. Percent survival graph showing the average incubation period defined as the number of days from inoculation to the onset of unequivocal clinical signs for cattle inoculated with the classical BSE (n=12), BSE-H (n=9), or BSE-L (n=10) agents. **Abbreviations**: dpi, days post inoculation.
Fig. 2 Accumulation of PrP<sup>Sc</sup> in tissues from cattle with classical BSE, BSE-H, or BSE-L. a The retinas from negative control cattle had no PrP<sup>Sc</sup> immunoreactivity. b The retinas from cattle inoculated with classical BSE had PrP<sup>Sc</sup> immunoreactivity localized to the OPL and IPL. c, d The retinas from cattle inoculated with BSE-H or BSE-L had increased PrP<sup>Sc</sup> immunoreactivity in all retinal layers. e-h Brainstem at the level of the obex (hypoglossal nucleus) from cattle inoculated with BSE-H or BSE-L had more PrP<sup>Sc</sup> immunoreactivity compared to cattle inoculated with classical BSE. i-l Thalamus (interthalamic nuclei) from cattle inoculated with BSE-H or BSE-L had more PrP<sup>Sc</sup> immunoreactivity compared to that from cattle inoculated with classical BSE. m-p PrP<sup>Sc</sup> immunoreactivity in the caudate nuclei from cattle inoculated with the BSE-H or BSE-L agents was comparable to classical BSE. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segments. Scale bars: 40 µm.
Fig. 3 Activation of retinal glial cells. a GFAP is localized to end feet of Müller glia in retinas of control cattle. b Distribution of GFAP immunoreactivity is increased in the retinas of cattle with classical BSE. Immunoreactivity spans the retina from the GCL to the OPL. c, d The retinas from cattle inoculated with BSE-H or BSE-L have significantly increased GFAP immunoreactivity spanning the entire retinal thickness. e, f Iba-1 immunolabeled microglia in the retinas from cattle inoculated with classical BSE are quiescent with long processes and small cell bodies that are localized primarily to the plexiform layers. g, h Robust microglial activation is evident in retinas of BSE-H or BSE-L inoculated cattle. Iba1 positive microglia have larger cell bodies, thicker/retracted processes, and are localized to the synaptic and nuclear layers. i, j Bar graphs show significant increase in GFAP and Iba1 immunoreactivity in retinas of cattle inoculated with classical BSE, BSE-H, and BSE-L. Data are expressed as
mean ± SEM. **Abbreviations**: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segments. **Scale bars**: 40 µm. ****P < 0.0001 versus control; ####P < 0.0001 versus classical BSE; ##P < 0.01 versus classical BSE; #P < 0.05 versus classical BSE.
Fig. 4 NALP3 inflammasome activation. a-m Immunohistochemical analysis of key inflammasome components that are upregulated in cattle inoculated with BSE-H or BSE-L compared to cattle inoculated with classical BSE: a-h NLRP3 and i-m ASC. e, m Bar graphs show percent area of fluorescence for NLRP3 and ASC. Data are expressed as mean ± SEM. f-h Colocalization of Iba1+ microglia and NLRP3 confirming presence of NLRP3 inflammasome in activated, hypertrophic microglia. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segments. Scale bars: 40 µm. ****P < 0.0001 versus control; ####P < 0.0001 versus classical BSE. n Western blot analysis of cleaved caspase-1 (p20) and cleaved IL-1β (p17) expression levels in the retina. o, p Representative bar graphs showing quantitative densitometric analysis of cleaved caspase-1 (p20)/GAPDH ± SEM, and cleaved IL-1β/GAPDH (p17) ± SEM in retinas of cattle inoculated with sham, classical BSE, BSE-H, and BSE-L. ****P < 0.0001 versus control; ####P < 0.0001 versus classical BSE.

Fig. 5 Strain-dependent changes in macroautophagy. a Diffuse LC3 immunoreactivity in the retinas from negative control cattle is representative of basal autophagy. b Increase in LC3-immunoreactive autophagosomes in outer segments of photoreceptor cells, outer and inner plexiform layers, and ganglion cell layer evident in retinas from cattle inoculated with classical BSE is suggestive of autophagic response to misfolded protein (PrPSc) accumulation. c, d Retinas from cattle inoculated with BSE-H or BSE-L have lack of LC3-immunoreactive puncta similar to control retinas. Scale bars: 40 µm. Data are expressed as means ± SEM. e Western blot analysis of LC3II, pULK1 S757, and ULK1 expression levels in the retina. f-h Representative bar graphs showing quantitative densitometric analysis of LC3 II/GAPDH ± SEM, p62/GAPDH ± SEM, and pULK1 S757/ULK1 ± SEM. *P < 0.05 versus control; ***P < 0.001 versus control; ####P < 0.001 versus classical BSE.
Fig. 6 Reciprocal regulation between neuroinflammation and autophagy is reflected in incubation periods. **a** Graph represents a negative correlation between disease incubation period and neuroinflammation (represented by % fluorescence of NLRP3 immunoreactivity, also shown in Fig. 4) in retinas from cattle inoculated with classical BSE, BSE-H, or BSE-L. **b** Graph represents a negative correlation between disease incubation period and autophagy (represented by LC3II protein expression, also shown in Fig. 5) in retinas from cattle inoculated with classical BSE, BSE-H, or BSE-L.
Supp. Fig. 1 Activation of astrocytes in the brainstem, thalamus, and caudate nucleus. a-h Immunohistochemical analysis of GFAP shows increased activation of astrocytes in the brainstem and thalamus of BSE-H or BSE-L inoculated cattle, compared to those inoculated with classical BSE. i-l GFAP immunoreactivity in the caudate nucleus is similar in classical BSE, BSE-H, or BSE-L inoculated cattle. m-o Representative bar graphs show quantification of mean GFAP immunoreactivity in the brainstem, thalamus, and caudate nucleus of classical BSE, BSE-H, or BSE-L inoculated cattle. Data are expressed as mean ± SEM. ****P < 0.0001 versus control; ***P < 0.001 versus control; **P < 0.01 versus control; *P < 0.05 versus control; #P < 0.05 versus classical BSE.
Supp. Fig. 2 Activation of microglia in brainstem, thalamus, and caudate nucleus. a-h Immunohistochemical analysis of Iba1 shows increased activation of microglia in brainstem and thalamus of BSE-H or BSE-L inoculated cattle, compared to those with classical BSE. i-l Iba1 immunoreactivity in the caudate nucleus is similar in classical BSE, BSE-H, or BSE-L inoculated cattle. m-o Representative bar graphs show quantification of mean Iba1 immunoreactivity in the brainstem, thalamus, and caudate nucleus of classical BSE, BSE-H, or BSE-L inoculated cattle. Data are expressed as mean ± SEM. ***P < 0.001 versus control; **P < 0.01 versus control; ###P < 0.001 versus classical BSE; #P < 0.05 versus classical BSE.
Supp. Fig. 3 NALP3 inflammasome activation: analysis of NLRP and ASC immunoreactivity in the brainstem, thalamus, and caudate nucleus. 

a-c Colocalization of Iba1+ microglia and NLRP3 confirm the presence of NLRP3 inflammasome in activated, hypertrophic microglia in brainstem from a steer with BSE-H. Scale bars: 40 µm.

d-f Bar graphs show percent area of fluorescence for NLRP3 is upregulated in the brainstems and thalami, but not in the caudate nuclei of cattle inoculated with BSE-H or BSE-L when compared to cattle inoculated with classical BSE.

g-i Bar graphs show percent area of fluorescence for ASC is upregulated in the brainstems and thalami, but not in the caudate nuclei, of cattle inoculated with BSE-H or BSE-L, compared to cattle inoculated with classical BSE. Data are expressed as mean ± SEM. ****P < 0.0001 versus control; ***P < 0.001 versus control; **P < 0.01 versus control; ###P < 0.001 versus classical BSE; ##P < 0.01 versus classical BSE; #P < 0.05 versus classical BSE.
Supp. Fig. 4 NALP3 inflammasome activation: western blot analysis of caspase-1 and IL-1β protein expression in brainstem, thalamus, and caudate nucleus. a Western blot analysis shows higher protein expression levels of cleaved caspase-1 (p20) and cleaved IL-1β (p17) in the brainstems and thalami, but not in the caudate nuclei of cattle inoculated with BSE-H or BSE-L when compared to cattle inoculated with classical BSE. b-g Representative bar graphs showing quantitative densitometric analysis of cleaved caspase-1 (p20)/GAPDH ± SEM and cleaved IL-1β/GAPDH (p17) ± SEM. ****P < 0.0001 versus control; ***P < 0.001 versus control; **P < 0.01 versus control; *P < 0.05 versus control; ####P < 0.0001 versus classical BSE; ###P < 0.001 versus classical BSE; #P < 0.05 versus classical BSE.
Supp. Fig. 5 Strain-dependent changes in autophagy: analysis of LC3II, pULK1\textsuperscript{S757}, and ULK1 expression levels in brainstem, thalamus, and caudate nucleus. a Western blot analysis shows higher protein expression levels of LC3II and pULK1\textsuperscript{S757} in the brainstems and thalami, but not in the caudate nuclei of cattle inoculated with BSE-H and BSE-L when compared to cattle inoculated with classical BSE. b-g Representative bar graphs showing quantitative densitometric analysis of LC3 II/GAPDH ± SEM and pULK1\textsuperscript{S757}/ULK1 ± SEM. ****P < 0.0001 versus control; ***P < 0.001 versus control; **P < 0.01 versus control; *P < 0.05 versus control; ####P < 0.0001 versus classical BSE; ###P < 0.001 versus classical BSE; ##P < 0.01 versus classical BSE; #P < 0.05 versus classical BSE.
CHAPTER 5. CONCLUSIONS

The studies conducted in this dissertation demonstrate the retinal immune response to injury and protein misfolding. Additionally, this work defines novel pathological landmarks to describe the progression of retinal injury and protein-misfolding diseases in vivo. In chapter two, we investigated the effect of high-level blast wave pressure on the retina in a controlled setting. Modern military conflict has dramatically increased the occurrence of ocular injury due to exposure to blast wave pressure [1]. While traumatic brain injury is a well-recognized consequence of blast wave pressure, it is underappreciated that more than 80% of military personnel experiencing TBI also exhibit visual dysfunction [2]. Currently, diagnosis and therapeutic interventions for penetrating eye injuries from fragmentation are readily available, however, non-penetrating ocular injuries due to blast wave pressure are often not apparent. Due to this, underlying retinal changes are poorly understood. In this study, using a compressed air-driven shock tube system, mice were exposed to successive bouts of 300 kPa (43.5 psi) - equivalent to 3-times atmospheric pressure – without the confounding effects of secondary blast injury (e.g., head acceleration or deceleration, penetrating trauma due to flying debris, etc.). After 30 days, the mice were subjected to neurological assessments to probe for cognitive and motor function, and subsequent analysis of the retina and the brain. This study demonstrated several long-lasting pathological changes in the retina, that was not detected in the brain. Specifically, we reported activation of Müller glia and microglia, photoreceptor cell loss, and accumulation of phosphorylated tau species in retinal neurons and Müller glia – 30 days after exposure to blast. However, analysis of striatal neurotransmitter levels and markers of neuroinflammation in the pre-frontal cortex revealed no remarkable changes. Additionally, we reported no changes in cognitive or motor function. This work was the first to compare
ocular and neurological effects of primary blast injury and demonstrate early indicators of retinal injury due to blast wave pressure. Finally, this study describes a model in which the retina may serve as a CNS compartment that is more vulnerable and, therefore, may be a more sensitive and effective indicator of injury due to blast wave pressure.

In chapter 3, we investigated retinal changes associated with the α-synuclein A53T mutation, and the effect of α-synuclein “seeding” on the retina. Visual disturbances are one of the more prominent non-motor phenomena in Parkinson’s disease and represent a considerable cause of comorbidity [3-5]. There is morphologic and electrophysiologic evidence of disruption in retinal structure and function at several levels of the visual pathway in patients with synucleinopathies [4, 6, 7]. However, the direct effect of misfolded α-synuclein on the retina is not well understood, and indicators of disease progression in the retina are lacking. Our results demonstrate that α-synuclein (pSer129), the primary post-translational modification in α-synuclein critical in the pathogenesis of PD, is widely expressed in the outer and inner retinas of transgenic mice (TgM83). This finding corresponded with the first detection of tau (pThr231), and activation of retinal Müller glia and microglia in retinas of transgenic mice. Furthermore, we demonstrated that inoculation of brain homogenate of clinically ill mice into young mice causes an acceleration of retinal pathology. Specifically, retinas of inoculated mice had more prominent α-synuclein (pSer129) and tau (pThr231) accumulation, and more robust activation of microglia. Our study also demonstrates that seeding with brain homogenate from old affected mice may lead to retinal autophagic dysfunction, potential collapse of protein quality control systems resulting in further accumulation of misfolded proteins, neuroinflammation, and subsequent retinal damage. Specifically, we describe two contrasting changes in retinal macroautophagy: upregulation of
autophagy-related proteins in young mice and depletion or downregulation of autophagy-related proteins in clinically ill mice, and young mice that were inoculated with brain homogenate from clinically ill mice. Perhaps this suggests an impairment in the retinal protein degradation system due to an overload of misfolded α-synuclein. Finally, this study provides evidence that Raman spectroscopy can be used to distinguish between retinal tissues from control, healthy, and diseased mice with high accuracy, even among retinas that appear histologically similar. If further advanced, this non-invasive and rapid technique can be successfully implemented in the early identification of pathological retinal changes and potentially replace microscopic examination as the current gold standard of synucleinopathy diagnosis. Collectively, this work provides insight on the pathological events of PD in the retina that have not been previously reported, describes indicators of disease progression in the retina, and describes a method that can be used to detect molecular changes in PD related retinopathy.

In the final research chapter 4, we described a unique framework for understanding factors that influence progression of neurodegeneration in protein-misfolding diseases. Briefly, we utilized the strain-dependent variation in incubation periods between classical and atypical BSEs as a controlled platform to investigate indicators of accelerated disease progression in atypical BSEs (H-type and L-type) as compared to classical BSE. We demonstrate that retinas from cattle inoculated with atypical BSEs (BSE-H and BSE-L), characterized with shorter incubation periods compared to classical BSE, have more PrP^Sc accumulation throughout the retina. Increased PrP^Sc accumulation in these cattle corresponds to more robust retinal glial cell activation, and microglia-driven NLRP3 inflammasome activation. Finally, we report that increased PrP^Sc accumulation and neuroinflammation are correlated with the down-regulation
of autophagy when compared to retinas of cattle inoculated with an agent of classical BSE. Overall, this study describes several important correlations between incubation period, PrP<sup>Sc</sup> accumulation, neuroinflammation, and the autophagic stress response that have not been previously reported. A hallmark of proteinopathies is the formation of misfolded protein aggregates that cause neuroinflammation and subsequent neurodegeneration, and although therapeutic options that are being explored target different steps in the synthesis, processing, and degradation of proteins that are implicated in neurodegenerative diseases - a key challenge that remains is the incomplete knowledge of factors that influence disease progression. This study provides insight on the relationship between disease incubation time, neuroinflammation, and autophagy in vivo that may be indicators of disease progression and applicable to the development of therapies for a variety of misfolding protein disorders.

Collectively, the studies conducted in this dissertation describe distinct retinal changes associated with neurodegenerative insults or diseases. Currently, there is an ongoing pursuit for well-validated biomarkers for diagnosis and monitoring of neurodegenerative disease progression. Investigators describe the limitations of brain imaging such as the increasing costs of MRI and PET scans or through skull fluorescence imaging and other existing diagnostics, such as the invasive nature of accessing CSF fluid [8, 9]. Meanwhile, advances in retinal imaging techniques, such as OCT and Raman spectroscopy, have allowed for non-invasive assessment of disease-associated or experimental retinal changes [10, 11]. Many studies provide compelling evidence for retinal alterations associated with neurodegenerative insults, suggested to be present, and possibly detectable before typical cerebral manifestations. Therefore, there is an urgency to further investigate novel imaging techniques that can enable visualization as well as quantification of disease-related biomarkers.
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