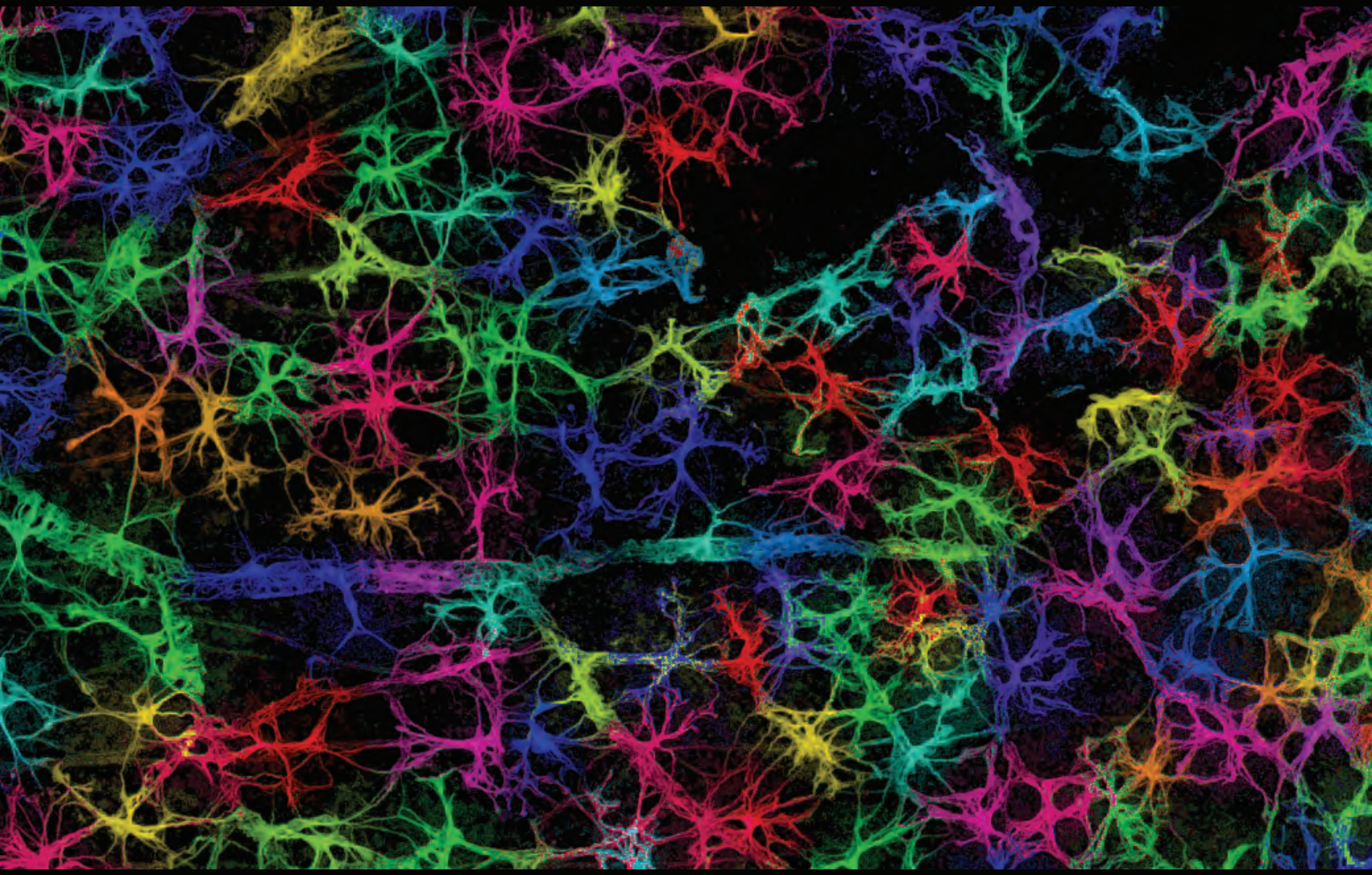
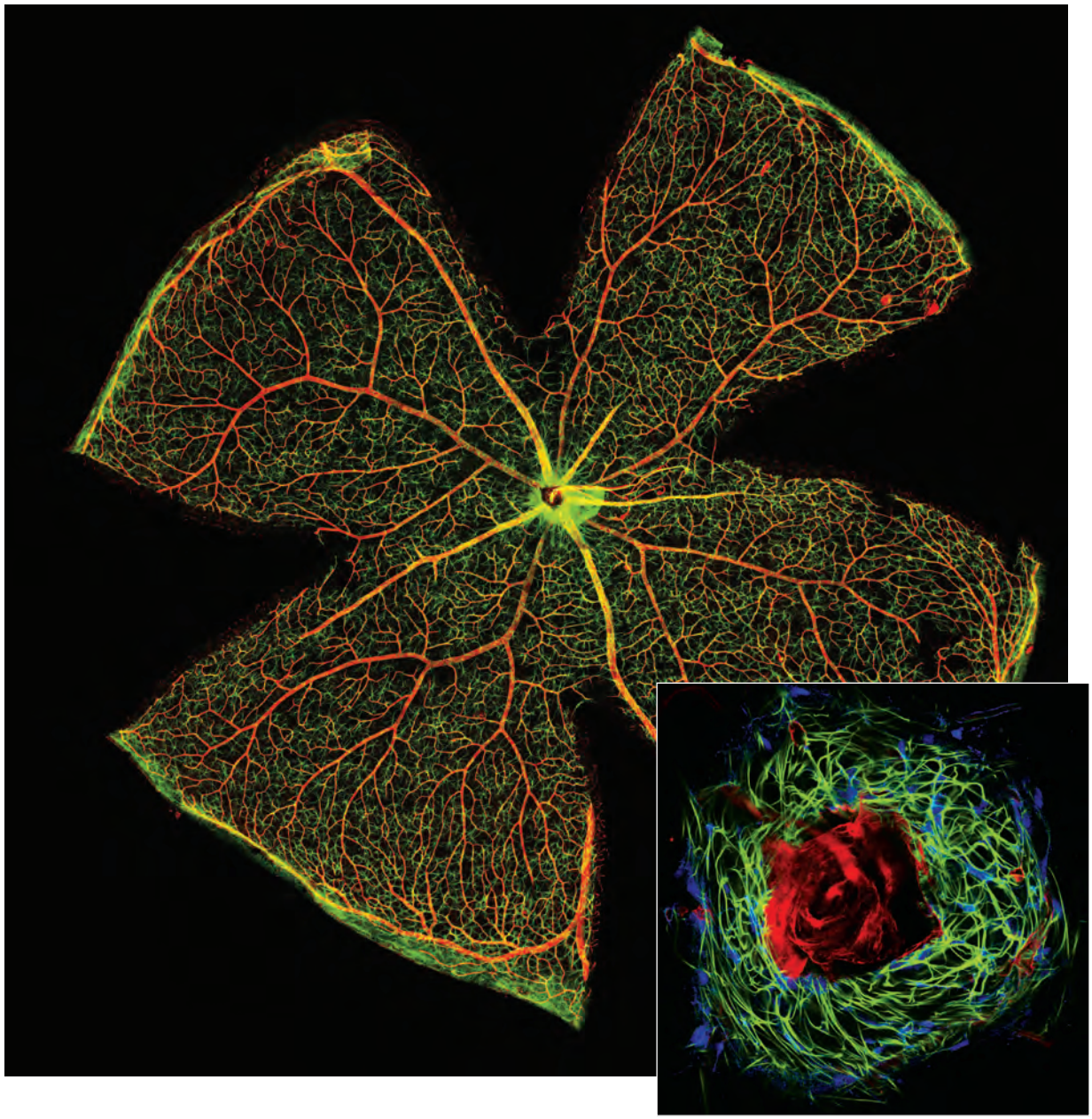
Astrocytes and Glaucomatous Neurodegeneration



A Report by the Lasker/IRRF Initiative for Innovation in Vision Science



Cover: Individual astrocytes segmented by assigning different hues to individual cells.

Frontispiece: The vitreal surface of a mouse retina showing the network of astrocytes and blood vessels. The astrocytes appear green; the retinal blood vessels are red. Around the optic nerve head (Inset), the astrocytes are especially dense and intertwined, such that individual cells or processes cannot be easily distinguished. In this image, astrocytes appear green, their nuclei blue, and the retinal blood vessels red.

Cover and frontispiece images of retinal astrocytes were provided by a collaborative group from the Neuroscience Research Institute and the Department of Computer Science, University of California, Santa Barbara, including: Gabe Luna, Mock Suwannatat, Rotem Raviv, Steven Fisher, Tobias Höllerer and Geoffrey Lewis.

The Albert and Mary Lasker Foundation and its programs are dedicated to the support of biomedical research toward conquering disease, improving human health and extending life. The Foundation's mission is to foster the prevention and treatment of disease and disabilities by honoring excellence in basic and clinical science, by educating the public, and by advocating for support of medical research. For more information about the Foundation, please visit www.laskerfoundation.org.

The International Retinal Research Foundation (IRRF) upholds a commitment to accelerate and sustain targeted research efforts into the diseases of the human eye, especially those affecting the retina and macula, to discover the causes, preventions, and cures of retinal and macular degenerative diseases and diabetic retinopathy. The IRRF will accomplish its mission by providing financial support of vision research directly, as well as through training fellowships, public awareness programs, and the promotion of the exchange of research findings. For more information about the IRRF, please visit www.irrfonline.org.

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The Initiative gratefully acknowledges the support of the Boards of Directors of the Albert and Mary Lasker Foundation and the International Retinal Research Foundation; and thanks the Project Chairman, John E. Dowling, Ph.D., Gordon and Llura Gund Professor of Neurosciences, Harvard University, for his leadership and direction; the Initiative's Steering Committee, for its guidance; the Project Administrator, Karen M. Wright and Assistant, Kate Chapman, for their critical and comprehensive administrative support; the workshop Discussion Leaders, for accurately capturing the key issues developed during workshop presentations and discussions in this document; and all participants, for their energy, expertise and lively discourse.

The Initiative gratefully acknowledges the Howard Hughes Medical Institute for its generous in-kind contribution by hosting the Initiative's plenary workshop at its Janelia Farm Research Campus in Ashburn, Virginia, and the gracious hospitality of the staff of the National Academy of Sciences' Eric Jonsson Center in Woods Hole, Massachusetts, where the two summer workshops were held.

For further information about the Initiative or for additional copies of this report, please contact: Karen M. Wright, Project Administrator at kwright@laskerfoundation.org or Kate Chapman, Project Assistant at kchapman@laskerfoundation.org.

Astrocytes and Glaucomatous Neurodegeneration

Summary Report
November 2010

Table of Contents

Astrocytes and Glaucomatous Neurodegeneration

Introduction

The Albert and Mary Lasker Foundation (Lasker) and the International Retinal Research Foundation (IRRF) entered into a ten-year collaborative research program on July 15, 2008 entitled the Initiative for Innovation in Vision Science (Initiative) designed to identify knowledge gaps in vision research and apply innovative solutions to develop and promote new clinical treatments and prevention of ocular diseases. The Initiative's long-term goal is accelerating discovery of sight-saving treatments and prevention of retinal degenerative diseases using novel scientific, engineering and technological approaches.

Under the guidance of a Joint Advisory Board (JAB) consisting of two members each from Lasker and the IRRF (Attachment 1), John E. Dowling Ph.D., Gordon and Llura Gund Professor of Neurosciences at Harvard University, agreed to serve as Project Chairman of the Initiative. Since recent evidence strongly implicates astrocytes as playing a causative role in certain central nervous system (CNS) disorders, especially amyotrophic lateral sclerosis (ALS) (see, for example, Science, 316: 353, 2007), an initial program to examine the role of astrocytes in degenerative diseases of the retina, including glaucomatous retinal ganglion cell (RGC) degeneration, was proposed and approved by the JAB as a first initiative.

A Steering Committee (SC) (Attachment 2) was established consisting of experts in diseases of the retina and glaucoma, as well as scientists with expertise in astrocytes, cells that have shown evidence of a toxic reactive response that may play a role in a number of diseases of the retina and glaucoma as well as CNS degenerations. The Steering Committee identified a group of bench and clinical scientists to bring together who have expertise in interdisciplinary fields and whose combined skills, knowledge and experience could produce innovative approaches to the significant hurdles impeding progress in the field.

Project Background

Glaucoma with or without elevated intraocular pressure (IOP) has been managed traditionally either medically or surgically, and in many cases visual field losses have been contained. However, in a certain percentage of patients, the loss of visual field continues. The latter conditions make it mandatory to study further the pathophysiology of glaucoma.

The optic nerve head (ONH) and lamina cribrosa[1] have long been implicated as a critical site in the initiation of glaucoma because of the sectorial nature of RGC loss in glaucoma, the evidence for an axonal transport defect at the ONH, and the many changes that occur in astrocytes at that location in glaucoma. Some of these astrocytic changes include evidence of alterations in astrocyte morphology, hypertrophy, reactive gliosis, and astrocyte migration away from the lamina cribrosal beams.[2]

In two workshops held during the summer of 2009 in Woods Hole, groups of interdisciplinary scientists focused on the following questions concerning astrocytes:

(1) Are astrocytes primary, contributing, or parallel to loss of ganglion cells and their axons in glaucoma?

(2) Are astrocytes neuroprotective or neurodestructive in glaucoma?

(3) Are there subtypes of astrocytes that are relevant to glaucoma?

(4) How are astrocytes in the ONH specialized to perform their specific functions?

(5) How do astrocytes change in response to age?

(6) What makes an astrocyte reactive, and what is the role of reactive astrocytes in glaucoma?

From these workshops, participants identified astrocytes as likely to be important contributors to glaucoma pathology in both positive and negative ways. The participants identified and refined the main questions which they determined to be unsolved, and important areas for further glaucoma research and which, by using modern day experimental tools, may now be experimentally addressed.

To tackle these questions, six areas related to glaucoma and the role of astrocytes in the disease were identified for further consideration. These were:

1) Astrocytes and other glial cells in glaucoma and other retinal diseases

2) Etiology of Glaucoma

3) Primary Physiological/Visual Defects in Glaucoma

4) Classification of Glaucomas

5) Animal Models of Glaucoma

6) Therapies

[1] In humans, the lamina cribrosa, which sits just below the ONH, consists of collagenous plates lined with astrocytes (see Figure 4, p. 26)

[2] The collagenous plates in a sense are holes and beams, with the beams being the material that criss-crosses the plates.

All members from the summer workshops as well as scientists with expertise that could fill additional knowledge gaps were invited to meet at the Howard Hughes Medical Institute's (HHMI) Janelia Farm Research Center on February 28-March 3, 2010 (Attachment 3). They examined these key areas in half-day targeted sessions and outlined a plan to focus on research that would surmount hurdles that have hindered progress in this field. Based on these sessions, the following represents a summary of the discussions along with promising areas of possible investigation that could profitably be explored. It needs to be noted, however, that because of the complex nature of the subject area, not all participants agree with all views and statements included in this document.

Key Questions and Critical Issues

Chapter 1. Roles of Astrocytes and Other Glial Cells in Glaucoma and Other Retinal Diseases

Discussion Leaders: Ben Barres and John Dowling

Two targeted sessions were devoted to astrocytes and their roles in glaucoma and other retinal diseases. Because the discussion and participants overlapped significantly in these two targeted sessions, a combined summary of the sessions is presented along with proposed areas for future research.

What is an astrocyte?

Astrocytes are neuroectodermally derived cells with processes that differ from neurons by being non-excitable. They are generally highly electrically coupled by gap junctions. Nearly all or all astrocytes have one or more processes that ensheath small blood vessels, and it is generally believed that they play a critical role in maintaining neuronal function. White matter astrocytes also ensheath nodes of Ranvier whereas gray matter astrocytes ensheath synapses. One astrocyte may ensheath thousands of synapses. Astrocytes are highly polarized with many proteins being found only on their end feet that ensheath blood vessels. The significance of this polarization is poorly understood, but it may be critical for their ability to deliver nutrients to neurons or to control ionic homeostasis. (see Figure 1).

What are the types of astrocytes normally found in the mammalian retina and optic nerve?

Astrocytes, in addition to Müller glia and microglia, are normally found in the retina, localized in the nerve fiber layer. In the ONH, however, our understanding of astrocyte types is much less clear. Some evidence suggests that the astrocytes there may be divided into at least two types. One type is called an optic nerve astrocyte that may be similar or identical to the bulk of astrocytes along the length of the optic nerve. In addition, there may be a specialized class of astrocyte called a lamina cribrosa-cyte that functions in generating the special extracellular matrix and laminar beams below the ONH. Possibly

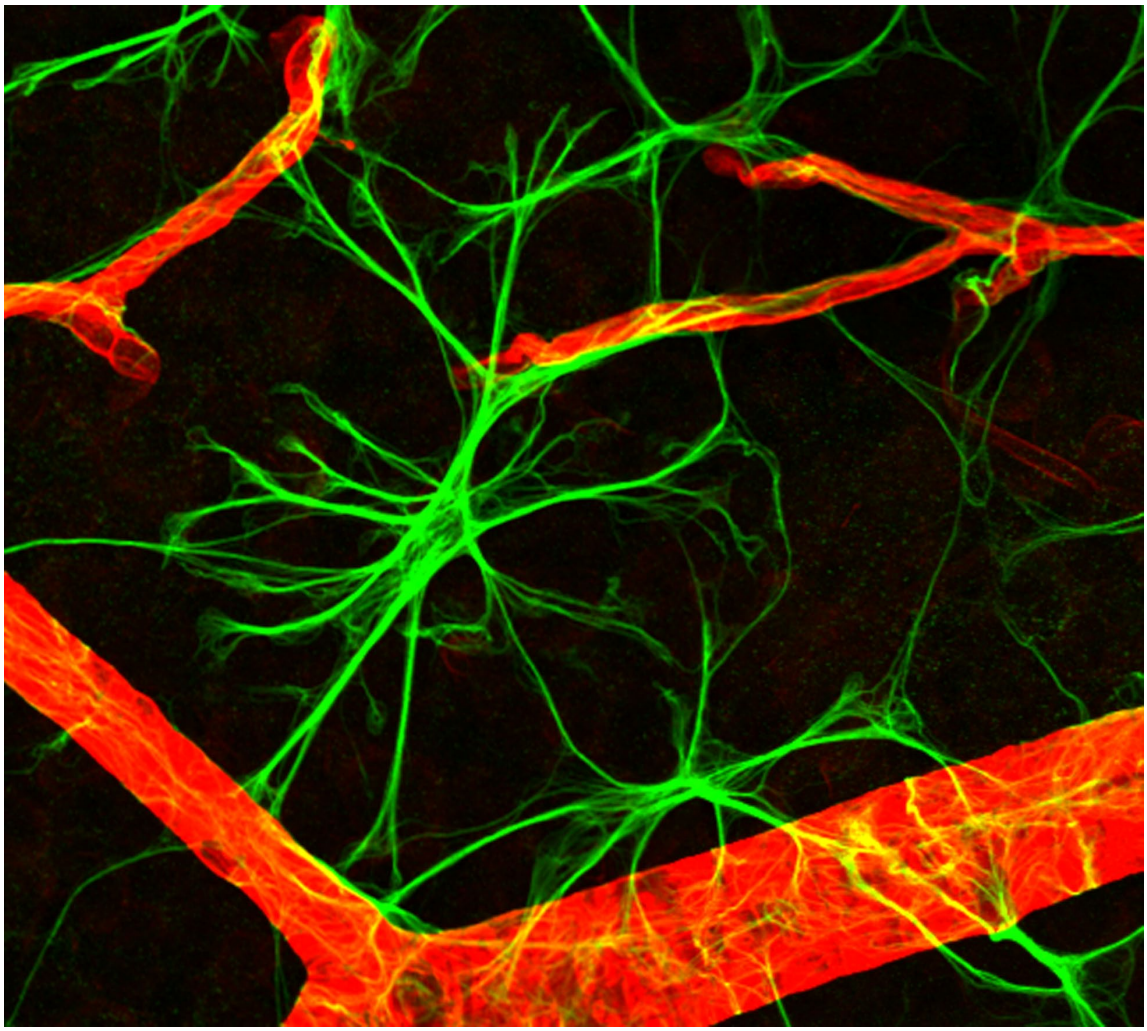

Figure 1. Normal astrocytes in the mouse retina. The astrocytes were stained with an anti-GFAP antibody and are green, whereas the blood vessels were stained with an anti-collagen IV antibody and are red. Note the astrocytic processes are in close association with the blood vessels. Micrograph courtesy of G. Luna, G. Lewis and S. Fisher.

there are also specialized astrocytes that serve an ensheathing function at the glia limitans of the ONH. There is evidence that astrocytes in the ONH are different from optic nerve astrocytes because they do not express the Ran-2 antigen (a membrane-tethered form of ceruloplasmin).

Research is needed to define better these types of astrocytes and to determine whether each class of retinal, ONH, and optic nerve astrocyte serves specialized functions that

may distinguish them. One approach that was identified as promising was to use the new "BAC-Trap" (bacterial artificial chromosome translating ribosome affinity purification) technique, to do gene profiling (Heiman et al., 2008). This method can be used to gene profile identified cell types of interest, even when there are only small numbers of them, without purifying or dissociating the retinal or ONH tissue. Another unanswered question is whether there are species differences in astrocytes, either in the types of astrocytes, their functions, or their gene expression. For instance, it is possible that mice lack the lamina cribrosa-cytes found in non-human primates and humans because rodents do not have the distinct collagenous plates of the lamina cribrosa, although otherwise the anatomy is similar. At least some evidence points to dramatic differences in the levels of expression of certain extracellular matrix genes. For instance, humans express 100-fold more thrombospondin, an extracellular matrix protein made by astrocytes, than do non-human primates and rodents. This may have important implications because thrombospondin is a strong stimulator of synapse formation and may have functions at blood vessels.

What are the normal functions of astrocytes and are they perturbed in glaucoma?

Although in general many functions of astrocytes are poorly understood, astrocytes have been demonstrated to have many functions. These include release of signals critical for the survival of neurons, storing glycogen and providing energy metabolites to neurons, ionic homeostasis, clearance of neurotransmitters, production of extracellular matrix and scar forming ability. Astrocytes also serve active roles in signaling synapse formation, synapse function, and synapse elimination. In culture, for instance, neurons display little ability to form synapses in the absence of glial cells. In the mature brain, they also secrete signals that regulate synaptic activity, although the general significance of this for neural circuit function is still poorly understood.

When neurons are electrically active, they release signals such as glutamate that stimulate nearby astrocytes to increase their intracellular calcium. This then propagates as a wave of elevated calcium from one astrocyte to the next throughout the glial syncytium. It is not known if this propagation occurs randomly or in specific glial circuits. The elevated calcium propagates to the ends of astrocyte processes. At blood vessels this triggers a release of signals from the astrocyte processes, such as ATP and prostaglandins, which induce vasodilation, thereby increasing blood flow. This mechanism is believed to couple local neuronal activity to an enhancement of local blood flow. Presumably this helps to provide the active neurons with enhanced energetic substrates and perhaps also helps to enhance clearance of metabolites. In addition, the enrichment of aquaporin-4 expression on the membranes of perivascular and subpial astrocyte end foot processes indicates that astrocytes may further interact with vascular cells in maintaining the blood-brain or blood-retinal barriers and regulating the osmotic microenvironment in the CNS and retina. It has recently been found that in certain metabolic conditions astrocytes

can also secrete substances that induce vasoconstriction and decrease blood flow. The nature of the conditions that stimulate vasodilation versus vasoconstriction are still poorly understood, as is the nature of the exact molecular identity of the signals released by astrocytes that control vasodilation and vasoconstriction. Furthermore, it is unclear whether astrocyte processes even contact blood vessels of the optic nerve in human and non-human primates. Studies with finer anatomical and imaging resolution may help answer this question.

Previous electron microscopic studies document that astrocytic processes are in close contact with blood vessels in the human ONH (Ye and Hernandez, 1995). In the prelaminar region of the human ONH, Anderson et al. (1967) pointed out that perivascular connective tissue at its outer surface is always clothed with astrocytes, whereas in the lamina, "astrocytes are reduced to a thin mantle that surrounds the nerve bundles, separating them from the laminar connective tissues and that around the central retinal vessels…and from the scleral collagen at the edge of the nerve canal." They further say "Capillaries are observed regularly in the laminar portion of the nerve" whereas "capillaries entering the nerve fascicles have a very thin layer of adventitial connective tissue, which in turn is surrounded by astrocytes." They also state "the postlaminar optic nerve astrocyte-vascular relationships are similar to the prelamina relationships, again emphasizing the special nature of the laminar region."

These findings raise many questions of importance to understanding glaucoma. Does the association of astrocyte processes with nodes of Ranvier or with blood vessels become altered or lost at the ONH in glaucoma? Could retinal or ONH astrocytes indirectly affect aqueous humor production and ocular hypertension through dysregulation of water-selective channels? Does axonal activity normally induce astrocytic calcium waves that control blood flow at the ONH? If so, does this function become uncoupled or perturbed in glaucoma? Also, are active axons deprived of needed blood flow, causing or contributing to the neurodegenerative process?

Pericytes are found at the ONH surrounding blood vessels, similar to what occurs throughout the body. Pericytes are contractile cells that help control vasoconstriction. Astrocytic processes have a close relationship with pericytes, being separated from them only by a basal lamina. When pericytes are damaged and die, astrocytic processes withdraw from blood vessels. Pericytes are highly vulnerable to death in certain medical conditions such as diabetes. There is some recent evidence that pericytes in the CNS may have a neuroectodermal origin, unlike most other pericytes in the body, which are mesodermally derived. If so, pericytes in the ONH may have unique functions at blood vessels or at the blood-nerve barrier. Recent studies have shown that loss of pericytes causes disruption of blood-immune barrier function in the CNS, leading to expression of certain cell adhesion molecules on CNS endothelial cells such as activated leukocyte cell adhesion molecule (ALCAM), and enabling immune cells such as lymphocytes to enter the CNS

parenchyma (Daneman et al., 2010). The question of whether pericytes are perturbed in the ONH in glaucoma is deserving of further study. Uveoscleral outflow of the aqueous at the ONH may also change the properties of optic nerve astrocytes in IOP-elevated eyes (Weinreb and Khaw, 2004) and deserves further study.

How do astrocytes detect mechanical forces at the ONH?

Because of the specialized nature of astrocyte morphology within the ONH, a question of importance to glaucoma that has so far received little study is whether mechanical forces, from increased IOP, eye movements, or other perturbations, cause direct tension and stretch on ONH astrocytes. New fluorescent indicators of mechanical stretch may potentially be usefully applied to address this question (Wang et al., 2005).

Another interesting question is whether mechanical stretch on ONH astrocytes induces astrocytic signaling. In culture, for instance, even slight mechanical forces have been shown to stimulate astrocytes to release ATP. If this were to occur at the ONH, ATP release could potentially stimulate microglial cells to migrate to the nerve head, activate astrocytes to become reactive, act on neurons, or alter blood flow. One idea for testing their potential significance in glaucoma would be to take advantage of a transgenic mouse line in which a dominant negative soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein has been expressed in astrocytes. These astrocytes are unable to release ATP (Pascual et al., 2005).

How else might mechanical forces at the ONH alter astrocyte function in glaucoma? A sufficient mechanical force might conceivably directly damage astrocytes and even result in their death. Astrocytes are often thought to be relatively resistant to cell death compared to neurons, but recent studies suggest that they may be as vulnerable (Cahoy et al., 2008; Foo and Barres, in preparation). Mechanical forces might be sufficient to trigger reactive gliosis (see below). Lastly, mechanical forces might perturb the vasodilatory role of astrocytes either by disrupting their vessel contacts, interfering with their gap junction coupling, or by perturbing other functions such as the control of ionic homeostasis.

How do ONH astrocytes change with age?

Because glaucoma susceptibility increases strongly with age, with most cases having their clinical onset between the ages of 50 and 60 years old, a possibility is that aging influences astrocyte phenotype at the ONH. This possibility deserves further study. There is some evidence that extracellular matrix material secreted by astrocytes may accumulate or become produced and secreted at a higher rate, thereby becoming thicker with age. This may stiffen them or decrease the extracellular space through which axons pass. Possibly this increased barrier may perturb delivery of nutrients to nearby axons. A related question is whether astrocyte precursor cells persist at the ONH and, if so, whether these precursors or more mature astrocytes undergo hyperplasia (proliferation) with age.

What is a reactive astrocyte?

Very early in any neurological perturbation or disease, astrocytes undergo a dramatic change in their phenotype. Their morphology becomes altered primarily by hypertrophy with thickened enlarged processes (see Figure 2). It is still debated to what extent this change is accompanied by hyperplasia of the astrocytes. In addition to the morphological changes, reactive astrocytosis is accompanied by dramatic alterations in their gene expression. Recent gene profiling studies have demonstrated that several hundred genes are upregulated by reactive astrocytes in the ischemic forebrain, some of them by hundreds of fold (Cahoy et al., 2004). The roles of many of these genes are unknown. Many cytoskeletal genes are highly upregulated, such as vimentin, glial fibrillary acidic protein (GFAP), and nestin, suggesting that reactive astrocytes may serve an important role in stiffening, encapsulating, and supporting damaged tissue. Potential neuronal survival factors are upregulated. In addition, many immune genes including cytokines and chemokines and complement cascade proteins are highly upregulated.

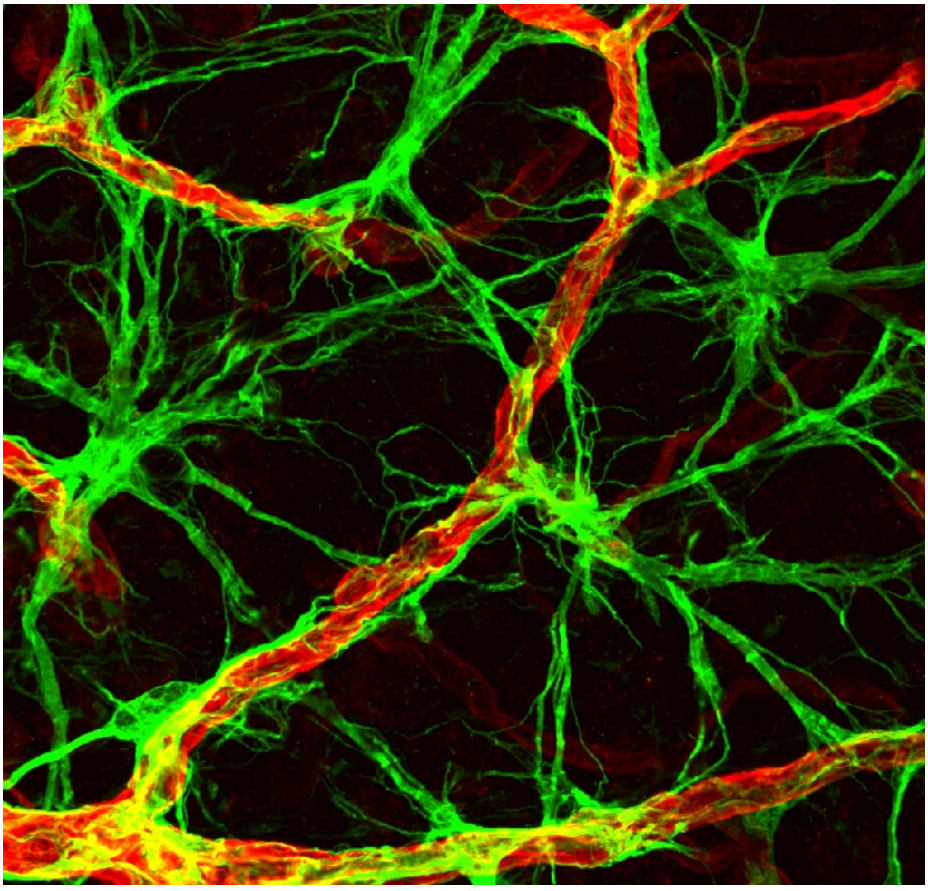

Figure 2. Reactive astrocytes in a mouse retina that was artificially detached from the back of the eye. Note the increased anti-GFAP expression (green) and "ragged" appearance of the cells as compared to the normal astrocytes shown in Figure 1. The blood vessels in red were stained with an anti-collagen IV antibody. Micrograph courtesy of G. Luna, G. Lewis and S. Fisher.

Some evidence suggests that reactive astrocytosis occurs in the glaucomatous ONH. However, because GFAP is normally highly expressed by optic nerve astrocytes, there is a great need for much more specific markers of reactive astrocytes that could be used to examine the retina, ONH and optic nerve to determine to what extent and where reactive gliosis occurs during the disease process. The BAC-Trap methodology, using the Aldh1L1-green fluorescent protein (GFP)-ribosome transgenic mouse, promises to be a powerful approach for addressing this question.

The nature of the inducing stimuli for triggering reactive gliosis is an important question for further study. Possibilities include loss of neurons or their axons, leakage of the blood-brain barrier, ischemia, immune cell signaling, and direct mechanical damage. It is unknown whether reactive gliosis is reversible. For instance, once the glaucomatous process has been initiated, does treatment to decrease IOP lessen reactive gliosis?

What are the roles of reactive astrocytes in glaucoma?

Assuming, as is currently believed, that the glaucomatous ONH contains reactive astrocytes, what is their function? First, they may play helpful roles. They may release neurotrophic signals that help axons and RGCs resist degeneration. They may provide enhanced mechanical support for degenerating tissue. They may fill in gaps left by the degeneration of axons or other cell types that have died, including astrocytes themselves. They may induce immune functions that are potentially helpful. In a mouse model of ALS, for instance, it has recently been shown that microglial cells transform into dendritic cells which signal lymphocytes, and that this helps promote survival of affected mice (Chiu et al., 2009). Lastly, reactive astrocytes are likely critical in sealing a damaged blood-brain barrier (Bush et al., 1999; Voskuhl et al., 2009).

However, reactive astrocytes may also potentially exacerbate the glaucomatous process. First, glial hypertrophy may strangulate axons, contributing to the loss of axonal transport observed in animal models of glaucoma at the ONH. Furthermore, reactive astrocytes overexpressing mutant superoxide dismutase (SOD) protein have been shown to have a direct toxic action on motor neurons in a mouse model of ALS (Nagai et al., 2007; Di Giorgio et al., 2007). When mutant astrocytes from these mice are cultured with wild type motor neurons, the astrocytes release an as yet unidentified toxic protein that causes neuronal cell death. Is this toxic signal also produced by astrocytes in glaucomatous optic nerves and if so, does it contribute to RGC death and axon loss? Because RGCs can be purified, it will be useful to determine whether these toxic astrocytes impair RGC viability in culture.

Moreover, other normal functions of astrocytes may be perturbed when astrocytes become reactive. In particular, there have not yet been studies that investigate whether reactive astrocytes are able to control blood flow normally, or whether there is osmotic im-

balance through dysregulation of its abundant aquaporin-4 expression when astrocytes are reactive. Some evidence suggests that the astrocyte's ability to clear neurotransmitters can become severely impaired, e.g., when reactive astrocytes downregulate their glutamate transporters (Pardo et al., 2006).

How can the role of reactive gliosis in glaucoma be tested? Gene profiling can be used to determine the signaling pathways that induce reactive astrocytes in the ONH. This approach has been used in other parts of the CNS, e.g., to implicate the Janus tyrosine kinase/signal transducer and activator of transcription 3 (JAK/STAT3) and nuclear factor-kappa B (NFKB) pathways (Sofroniew 2009). Other astrocyte signaling pathways are also likely involved. Mutant mice, in which specific signaling pathways are impaired, such as the STAT3 conditional knockout mice, will be a powerful tool for addressing this question (Herrmann et al., 2008). These mice can now be crossed to mouse models of glaucoma such as the DBA/2J (an inherited mouse model of glaucoma, see Chapter 5). However, it is likely that simply preventing the entire reactive gliosis process will be detrimental in glaucoma, as has been shown in other mouse disease models. Rather, research is needed to determine the specific astrocyte signaling pathways or proteins that are damaging and ablate those specifically. These experiments should lead to new drug targets for treating glaucoma.

Lastly, this raises a critical question about understanding the significance of reactive gliosis in glaucoma. Does glaucoma progress because of a primary axon degeneration process in which reactive gliosis is simply a secondary consequence? Or instead, does reactive gliosis drive the disease progression by driving axon loss? One experimental approach to get at this would be to investigate whether Wallerian degeneration--slow (WldS) rodents which exhibit robust protection of distal axons from various injury stimuli--are resistant to gliosis in experimental glaucoma.

What is the role of reactive astrocytes in activating the classical complement cascade?

Reactive astrocytes in the retina or ONH may produce proteins of the complement cascade, similar to reactive astrocytes in other parts of the CNS. A study of reactive Müller glial cells found increased complement protein expression (Kuehn et al., 2006). This is a critical question to address in glaucoma because the complement protein, C1q, the initiator protein of the classical complement cascade, is up to 100-fold upregulated in neurodegenerative diseases such as Alzheimer's and ALS, and is also upregulated early in the course of rodent and human glaucoma (Stevens et al., 2007; Stasi et al., 2006). Recent work has implicated the classical complement cascade as active at retinal synapses in the DBA/2J mouse model of glaucoma (Howell et al., 2007). Because this cascade has been shown to help mediate normal synapse elimination by RGCs during visual system development, this has led to the hypothesis that this cascade also mediates synapse

loss in glaucoma (see Figure 3). These studies have so far found that complement protein deposition in the inner plexiform layer occurs prior to significant loss of RGCs. These findings strongly suggest that a normal mechanism of developmental synapse elimination is reactivated in the mature visual system by the glaucomatous process and that this synapse elimination process may well drive the entire glaucomatous neurodegeneration process. Presumably the compromise of optic nerve axons at the ONH triggers the reactive glial process which in turn triggers activation of the complement system. Although activation of the complement cascade is likely not the initial trigger of glaucoma, it may be a common part of the downstream pathophysiological process that leads to neurodegeneration. If so, blockers of this cascade may inhibit glaucomatous degeneration, loss of visual function, and enable normal synapse reparative processes to occur.

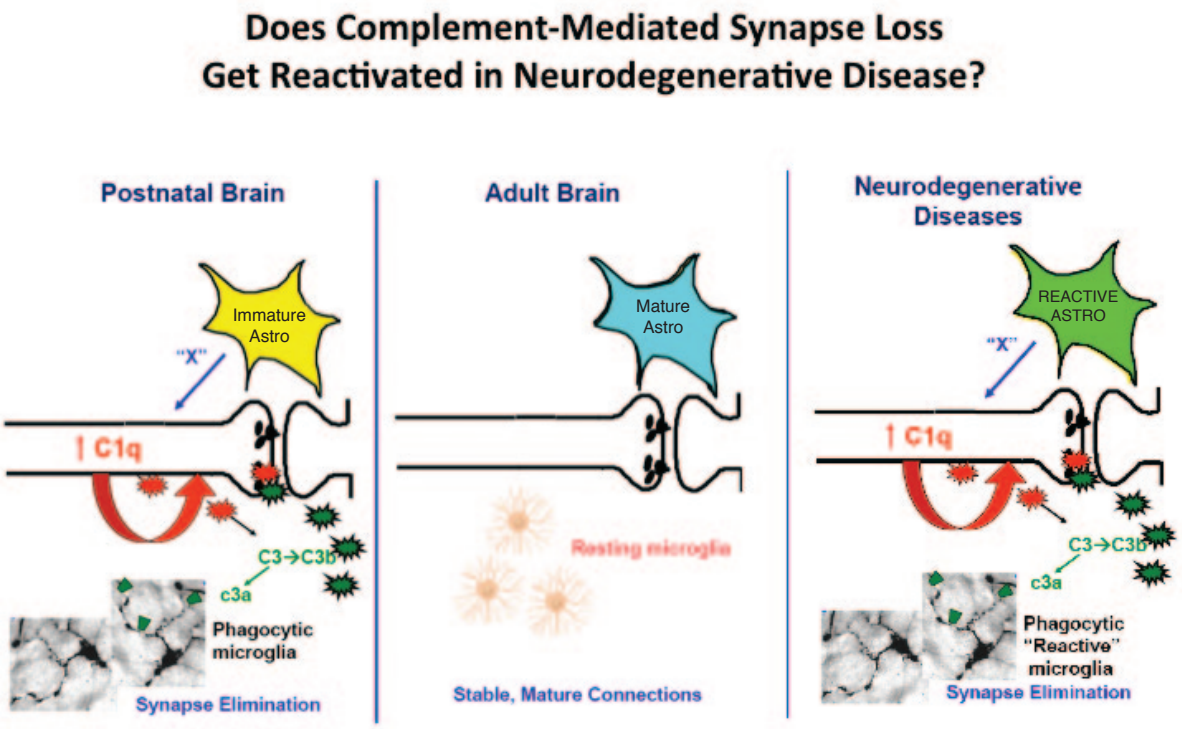

Figure 3. The complement protein C1q is upregulated early in the course of glaucoma, where it is localized to synapses that are destined to degenerate. These data suggest that the complement cascade drives the neurodegenerative process in glaucoma and potentially in other neurodegenerative diseases as well. Figure courtesy of B. Barres.

These observations raise important questions for further research. What is the identity of the reactive glial signal that induces C1q expression? What is the source of the C1q protein? C1q expression in RGCs is induced by developing astrocytes, but gene profiling studies show that most secreted C1q may be released by microglia, which have high levels of C1q mRNA. Once secreted, how does C1q bind to synapses, and what is the synaptic C1q receptor? Most importantly, will C1q or C3 deficiency protect DBA/2J mice

or other mouse models of glaucoma from neurodegeneration and visual impairment? Because C1q and C3 deficient mice are already available, they can be used to test this question in various mouse models of glaucoma.

Another question is why axonal compromise would lead RGC to eliminate their synapses? In ALS, when some axons degenerate, the remaining axons that have survived now make new synapses on the denervated muscle cells (leading to the large motor units and fasciculations that are one hallmark of this disease). Potentially an analogous reorganization process is occurring in glaucoma within the retina. To test this possibility, GFP reporter mice in which a small subset of amacrine cells or bipolar cells that normally synapse on RGCs could be crossed with the DBA/2J mouse model to determine if these presynaptic neurons now make an increased number of synaptic contacts with surviving RGCs. It is unclear yet whether synapse loss also occurs in rodent models of glaucoma at the tectal or lateral geniciulate terminations, or whether retinal synapse loss occurs early in the course of human glaucoma. The question of whether retinal synapses are being lost in the retinal CNS target areas is particularly critical because disconnection of axons from their synaptic connections could be a factor driving RGC axon loss.

The need for better eye banks and more banked, better preserved glaucomatous tissue will be critical for answering this question. Lastly, if there is a massive loss of retinal synapses early in the DBA/2J mouse model, this would indicate that synapses other than with RGCs must be involved, because the latter is only a small portion of synapses within the inner plexiform layer. Research is needed into why this happens in this model, whether there is innocent bystander damage of neighboring synapses, and whether this contributes to the visual impairments occurring in human glaucoma. If synapse loss occurs early in the DBA/2J model of glaucoma, is this also true in human glaucoma? New methods to image synapse density in the inner plexiform layer of the retina in humans may be a helpful new tool in diagnosing and treating the disease.

What is the role of microglia in glaucoma?

There is evidence of microglial activation in glaucoma, but it is uncertain when microglial activation occurs. Some recent studies suggest that microglial activation may be a very early event (Bosco et al., 2008; Steele et al., 2006). If so, it may be useful to develop new imaging methods for diagnosing glaucoma. The roles of microglia in glaucoma merit much more study. Microglia are the primary source of complement protein C1q within the CNS as well as other complement components. Does microglial activation occur before, coincident, or after reactive astrocytosis? Does reactive astrocytosis induce microglial activation or vice versa? Do microglial cells promote repair or do they cause damage? Using new tools to ablate and manipulate microglia may answer such questions.

Proposed Studies

1) Use the BAC-Trap technique to gene profile ONH astrocytes and compare them to already elucidated gene profiles for other types of astrocytes. Specific markers of ONH astrocytes can then be identified and their specific ONH localization determined.

2) Profile astrocyte heterogeneity and regional specificity at different domains in the retina, ONH and optic nerve, and identify molecular signatures of astrocytes in these regions as well as markers of reactive astrocytes that contribute to (or ameliorate) disease pathology.

3) Use molecular markers identified through gene profiling to functionally elucidate the role of astrocytes as primary, secondary, or contributing cell types in glaucoma through inducible modulation or ablation of astrocytes at specific domains in the retina and optic nerve.

4) Study the relationship between astrocytic/microglial reactivation and functional changes in such critical processes as immune signaling, regulation of vascular flow and blood brain barrier maintenance.

5) Use novel imaging modalities to characterize the morphological and cellular changes occurring in different cell types, including astrocytes, at ONH in glaucomatous conditions and with age.

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Chapter 2. Etiology of Glaucoma

Discussion leaders: Richard Masland and Len Levin

The notion that there are two distinct populations of glaucoma patients, those with elevated IOP levels and those with normal IOP levels, may have blurred our understanding of glaucoma etiology. During this targeted session, data presented demonstrated that although many glaucoma patients do have IOPs within normal range, IOP in glaucoma patients fits a skewed normal distribution rather than a bimodal one (Gillespie et al., 2003). The presentation of these data underscores a prevailing theme of the discussion: whether glaucoma results from several sequences of events occurring in parallel or is the consequence of one simple sequence of events. Session participants agreed that the possibility remains that the disease can be explained by a simple sequence of events and that even if disease pathogenesis does turn out to be multifactorial, there may be a final common pathway, the initial steps of which could be targeted by one or a very limited number of therapies. Devising strategies for blocking the initial steps of this pathway may be preferable to tackling each risk factor separately.

What are the hallmarks of glaucomatous disease that can guide our understanding of glaucoma etiology?

The discussion during this session was guided by the consensus that an increased susceptibility to IOP (whether "elevated" or not) is critical to the pathogenesis of glaucoma and that the hallmark of glaucomatous disease is the development of nerve fiber bundle pattern visual field defects (paralleled by sector) that do not cross the nasal horizontal meridian (corresponding to the temporal horizontal meridian of the visual field) (Yalvac et al., 2009). Based on this consensus, session participants agreed that the injury causing these defects must occur at or immediately anterior to the lamina cribrosa. The latter possibility was based on discussion of whether the separation of the superior and inferior arcuate fibers anterior to the lamina cribrosa was a correlate of the rarity of visual field defects progressing over the nasal horizontal meridian. One plausible exception to this rule was raised based on a scenario where there could be an initial injury to a small cluster of RGC somas, death of their axons, and then a bystander effect of adjacent axons in or near the ONH. There is currently insufficient evidence for or against this mechanism. The rest of the session was devoted to discussion of mechanisms at the ONH that would correlate with an increased susceptibility to a given level of IOP and the production of the characteristic structural and functional deficits observed in glaucoma.

What is the evidence for direct axonal injury in glaucoma?

Direct physical injury to axons from region-specific compression, either against the sclera as axons turn to enter the ONH or against beams of the lamina cribrosa, could conceiv-

ably produce the stereotyped pattern of visual field deficits seen in glaucoma. Age-related stiffening of the sclera and remodeling of the pores of the lamina cribrosa could affect the magnitude of compression. Pulsatile increases in IOP due to eye movements and other perturbations could accentuate the physical injury to RGC axons caused by these structures. In addition, decreased ocular elasticity due to scleral stiffening might increase the amplitude of these pulsatile increases in IOP. Although changes in the lamina cribrosa have been observed in glaucoma patients, it is still unknown if these changes precede the onset of RGC loss and if they occur in all cases of glaucoma. Session participants agreed that a systematic screening of glaucoma patients for lamina cribrosa remodeling would be a helpful step toward elucidating the role of the lamina cribrosa in the disease. Systematic research into the prevalence and nature of glaucoma among individuals with connective tissue disorders might also shed light on the relative importance of scleral and laminar rigidity in the disease (Cordeiro et al., 1999). Despite the appeal of lamina cribrosa remodeling as a proposed mechanism, the fact that sectorial loss of RGCs is observed in mouse models of glaucoma, where there is no extracellular matrix component of the lamina cribrosa, suggests that other factors may be responsible. Although the DBA/2J mouse lacks a rigid lamina cribrosa, the nerve is nonetheless surrounded by the rigid structure of the sclera, which could be the site of mechanical damage. Participants agreed on the continued value of studying animal models in which the physical landscape of the ONH differs from the human, in order to gain insights into the role of specific anatomic features in disease pathogenesis. In addition, there is a strong need for a small animal model in a species that has a collagenous lamina, such as the tree shrew (Albon et al., 2005), and perhaps others as yet undiscovered.

What is the evidence for the role of reactive astrocytes in glaucoma?

Session participants discussed an alternative scenario, in which RGC axons are injured indirectly by an astrocyte-mediated mechanism. Astrocytes, in response to a perceived threat, could retract their processes and in effect abandon their supportive role and instead elaborate a glial scar. Ideas about perceived threats that could trigger astrocyte reactivity included astrocyte stretching induced by age-related laminar remodeling and a shift in astrocyte barosensitivity. It should be possible to test the role of many intrinsic properties of ONH astrocytes through genetic manipulation of these cells in a mouse model of glaucoma. The finding in mice that the same astrocyte can span the entire ONH (Sun et al., 2009) makes it difficult to imagine how an astrocyte-based mechanism would produce the sectorial patterns of RGC loss seen in glaucoma. Regional hypertrophy of a subset of astrocyte processes was raised as one potential explanation. The group also considered the possibility that formation of a glial scar may be a downstream event resulting from a physiologically appropriate astrocytic response to injured RGC axons or localized ischemia. In this scenario, the astrocytic response would be secondary to RGC axonal damage, rather than the reverse.

What is the evidence for the role of axoplasmic transport block in glaucoma?

Direct or astrocyte-mediated compression of optic nerve axons could lead to RGC death through block of axoplasmic transport and consequent soma deprivation of target-derived growth factors. Experiments investigating the effects of axoplasmic transport block are often confounded by concomitant ischemia produced by the methods used to block transport. Session participants agreed that studies to clarify whether axoplasmic transport block alone leads to RGC loss would be helpful (Agarwal et al., 2009). The effect of blocking axoplasmic flow on astrocytes at the ONH is not known and would be useful to investigate. The idea that blockade of axoplasmic transport is critical to glaucoma pathophysiology is called into question by the observation that patients suffering from slow-growing tumors compressing the optic chiasm frequently recover vision following resection of their tumors, despite possible long-standing block of axoplasmic transport (Butlers et al., 2009). However, it remains to be directly demonstrated that blockade of axonal transport is responsible for these patients' pre-surgical blindness. In summary, it is not clear why axons at the lamina cribrosa have a less reversible response to compression in glaucoma.

What other mechanisms might underlie glaucoma?

ONH ischemia was discussed as a potential etiology for glaucoma. Session participants agreed that while ischemia certainly appears to play a role in susceptibility to glaucoma, there is insufficient evidence for vascular factors that act independently of IOP. Furthermore, no vascular supply to the ONH corresponds to the sectorial deficits that characterize glaucoma. However, this is an area that has been incompletely investigated, because the vasculature of the ONH varies between humans and laboratory animals – and even between human individuals. Improvements in imaging techniques should permit better studies of the effect of IOP on blood flow through the lamina cribrosa (Flammer et al., 2002). It is interesting that diffuse fluorescein leakage around the nerve head has been reported in glaucoma patients (Arends et al., 2005): the significance of this observation is not clear. The significance of disc hemorrhages in disease pathogenesis was discussed. Microglial activation was discussed, and it was thought that if it were demonstrated in non-inflammatory models of glaucoma, its etiological role could be considered more carefully. Finally, participants agreed on the value of a large-scale effort to sequence the genomes of glaucoma patients in search of novel candidate genes.

Proposed Studies

1) Undertake clinical studies to assess the prevalence and nature of glaucoma among individuals with connective tissue disorders on the relative importance of scleral and laminar rigidity in the disease.

2) Conduct studies to clarify whether axoplasmic transport block alone leads to RGC loss.

3) Develop a small animal glaucoma model in a species that has a collagenous lamina (Morrison et al., 2008).

4) Study the susceptibility to glaucoma of transgenic animals in which astrocyte function/reactivity has been altered.

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Chapter 3. Primary Physiological/Visual Defects in Glaucoma

Discussion Leaders: Len Levin and Richard Masland

With the goal of establishing protocols for early disease detection and prevention, participants in this session discussed the early events in glaucoma that are currently or may one day be detectable in the clinic, the strengths and weaknesses of current mainstays of disease detection, and ideas for the development of new tests.

Are there diffuse threshold changes in early glaucoma?

The question of whether diffuse photopic or scotopic threshold changes occur in early glaucoma has been debated in the literature. Discussion participants concluded that there is currently insufficient evidence that changes of this nature occur in human patients at an amplitude and consistency which would justify routine testing. In cases where diffuse scotopic sensitivity loss was observed in animal models of glaucoma, participants discussed the possibility that artificial elevation of IOP to non-physiological levels in these animals may cause damage to retinal cells other than RGCs. A recent study, for example, suggested that AII amacrine cells that convey the rod signal from the rod bipolar cells to cone bipolar cell terminals are compromised early in a mouse model with elevated IOP, resulting in a substantial loss of rod sensitivity. In one colony of DBA/2J mice that have a very severe phenotype compared to others, diffuse contrast sensitivity loss has been reported prior to the development of elevated IOP (Schuettauf et al., 2007). This report of functional deficits independent of an increase in IOP was viewed as at odds with theories that elevation in IOP is upstream of retinal damage.

The role of perimetry in glaucoma detection

White-on-white perimetry testing is one of the current mainstays of glaucoma detection, along with optic disc examination and IOP measurement. However, perimetry is relatively insensitive to early glaucomatous optic nerve damage. Studies suggesting preferential loss of magnocellular- and koniocellular-projecting RGCs in early glaucoma were discussed, and the role of increased intrinsic susceptibility, decreased redundancy, or anatomical distribution of these RGC subtypes were considered. Session participants discussed the use of short wavelength and motion-sensitive perimetry as screening tools. Participants concluded that while the results of short wavelength perimetry are confounded by optical changes in the ageing eye, motion-sensitive perimetry appears to be a promising detection method and should be further researched (Verdon-Roe GM et al., 2006). An example of motion-sensitive perimetry is the Moorfields MDT: http://www.moorfieldsmdt.co.uk/

The value of IOP measurement in glaucoma detection

While it is clear that IOP plays an important role in glaucoma pathophysiology, the mechanisms by which IOP contributes to disease are currently unclear. Continuous IOP measurements taken in the non-human primate demonstrated that frequent minor movements such as saccades and blinking cause significant fluctuations in IOP (Strouthidis et al., 2008). While the relative importance of elevated baseline IOP versus increased amplitude of IOP fluctuations (for example as a result of age-related scleral stiffening) is not yet understood, these data point to the inability of a single IOP measurement to fully capture the state of what is clearly a dynamic intraocular system. Session participants agreed that the ability to continuously measure IOP in patients with glaucoma is an important step in understanding the disease and may prove to be of diagnostic value. Finally, real-time IOP measurement would allow a critical variable to be controlled for, making it possible for other changes in this system to be studied against a less noisy background.

Can detection of remodeling of the lamina cribrosa be used for glaucoma assessment?

Changes in the appearance of the lamina cribrosa have been visualized using confocal scanning laser ophthalmoscopy (CSLO) in glaucoma patients (Fontana et al., 1998). To date, it is unclear if these changes precede visual loss, if they occur early enough to be useful in disease detection, and if there are cases of glaucoma in which these laminar changes do not occur. The discussion participants agreed that an unbiased screening study examining the lamina cribrosa of glaucoma patients would be useful.

Can detection of microglial activation be used for glaucoma assessment?

Whether or not microglia play an important role in the pathophysiology of glaucoma, they may act as sensing cells in early glaucoma, and assessing their state of activation could serve as an early indicator of disease. Discussion participants expressed concern that changes in microglia currently observed in animal models of the disease may be an artifact of inflammation in those models. Session attendees concluded that before detection of microglial activation is pursued as a clinical testing method, changes in these cells need to be studied in a non-inflammatory model of the disease.

New tests that might be useful for detecting glaucoma or informing treatment decisions in patients with diagnosed disease

The session concluded with a discussion of new testing methods that might be useful in the detection and care of patients with glaucoma. An important step in advancing glaucoma treatment is to establish clinical windows during which interventions can slow, stop, or reverse the disease process. For example, a test allowing one to differentiate

a “sick” RGC from a cell injured past the point of recovery would allow clinicians to position their patients more accurately along the timeline of disease progression and thereby make more informed treatment decisions. Such a test would require the development of safe compounds for real-time imaging of activity in the human retina. Examples discussed included calcium-sensitive dyes (May et al., 2003), voltage-sensitive dyes, and markers of RGC metabolism, necrosis and apoptosis. The session participants also expressed interest in developing markers of activation of other cell types in the retina, such as astrocytes, endothelial cells and microglia, contingent on demonstration that their activation indeed correlates with disease in patients with glaucoma. Finally, session participants felt that tests for measuring physiologic properties of the retina such as retinal oxygenation and blood flow to the retina and ONH would be useful, if not for diagnostic purposes, then at least for achieving a better understanding of glaucoma pathophysiology.

Proposed Studies

1) Evaluate motion-sensitive perimetry as a promising detection method for early glaucoma.

2) Measure IOP continuously in patients with glaucoma. Further, real-time IOP measurement would allow a critical variable to be controlled for, making it possible for other changes in this system to be studied against a less noisy background.

3) Conduct an unbiased screening study examining the lamina cribrosa of glaucoma patients.

4) Examine changes in microglial cells in a non-inflammatory model of the disease.

5) A series of possible new testing methods were suggested and are outlined in the above paragraph.

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Chapter 4. Classification of Glaucomas

Discussion Leaders: Elke Lütjen-Drecoll, Simon John and Gareth Howell

The discussion largely focused on the limitations to studying glaucoma in humans, not the least of which is the restricted access to human tissues at present. The group brainstormed a number of strategies for obtaining human eyes, and agreed that the development of a centralized biobank to procure and store these tissues for research purposes is essential. The European Glaucoma Society GlaucoGENE project (described below) was discussed, and the group agreed that it could inform the creation of such a program in the U.S and beyond.

What are the differences in the morphology of the anterior segment of the eye in human glaucomas?

The glaucomas are defined by differences in the history and morphology of the anterior eye segment, and include the open-angle glaucomas, e.g., primary open angle glaucoma (POAG), steroid-induced glaucoma, pseudoexfoliation glaucoma (PEXG), pigmentary glaucoma, and the closed-angle glaucomas. Investigation of human donor eyes has allowed for the ultrastructural study of the anterior eye segment. Importantly, in each of the open-angle glaucomas, there is an increase in extracellular material (ECM) in the conventional outflow pathways, especially at the inner wall of Schlemm's canal (SC) (see Figure 4). This increased ECM is thought to contribute to increased IOP. However, the morphology, and hence the pathogenesis of formation of this material, differs between glaucoma subtypes (Tektas and Lütjen-Drecoll, 2009).

The association of elevated IOP and optic neuropathy

Although studies of the pathophysiology of increased IOP in human subjects are limited, many reports show that pressure changes are sufficient to induce optic nerve neuropathy in animal models of glaucoma. However, the fact that glaucomatous optic nerve changes may occur in the absence of notably high IOPs in both humans and rodents implicates differences in susceptibility of optic nerve structures to pressure changes.

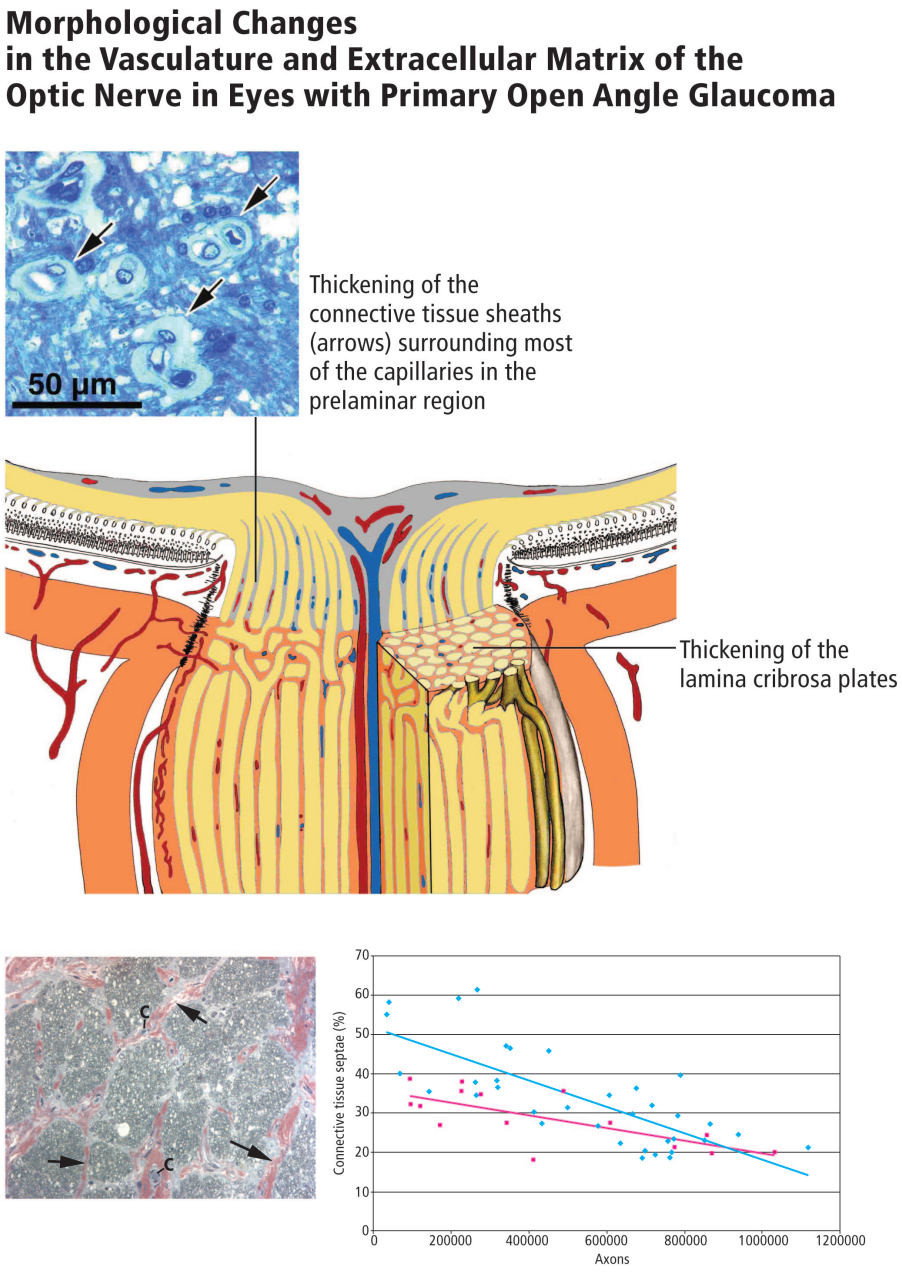

Figure 4. Thickening of connective tissue septae (arrows) surrounding the capillaries (C) in the postlaminar region and decrease in capillary density. (from Gottanka et al., 2005). Figure courtesy of E. Lütjen-Drecoll.

Potentially susceptible structures include the following (Anderson 1970; Morrison et al., 1989; Quigley 1985; Hernandez and Pena 1997; Hernandez and Ye 1993):

a. **Prelaminar region.** This region includes only non-myelinated nerve fibers, surrounded by astrocytes. It is possible that changes in astrocytes could damage these fibers.

b. **Transitional zone.** The non-myelinated fibers of the prelaminar region become myelinated by oligodendrocytes as they exit the laminar region, a transition zone that could exhibit increased susceptibility to IOP changes.

c. **Connective tissues.** Differences in age-related changes of the connective tissue of the lamina and the suspension tissue for the nerve could be responsible for differences in mechanical damage to the nerve by increased IOP.

d. **Blood supply.** Arterial capillaries derive only from the periphery of the optic nerve, while venous capillaries are present only in the center (Hayreh 2001; Hayreh 2001; Tektas et al., 2010; Onda et al., 1995). This arrangement could lead to less oxygen tension in the central optic nerve, although the typical distribution of nerve fiber damage in glaucoma suggests that the blood supply in the prelaminar region is not primarily involved. Interestingly, in the postlaminar nerve, capillaries are only present in connective tissue septae, not in the nerve fiber bundles. Astrocytes surround these nerve fiber bundles. Whether astrocytes in the postlaminar region of the nerve have contacts with the capillaries and whether there are changes in glaucomatous nerves is not yet known (see below).

Are there differences in the morphology of optic nerve neuropathies in different kinds of open-angle glaucoma?

Although little is known about such differences, recent studies found significant differences in the postlaminar regions between POAG and PEXG (Gottanka et al., 1997; Gottanka et al., 1997; Gottanka 2005). For example, in POAG, connective tissue septae are thicker than in PEXG or age-matched controls, thereby expanding the distance between the capillary lumen and nerve fiber bundles. There is a difference between transforming growth factor beta 2 (TGFβ_2) concentrations in the aqueous humor and presumably vitreous body between the two. In 50 percent of POAG eyes but not in PEXG, TGFβ_2 is significantly increased. Treatment of optic nerve astrocytes with TGFβ_2 shows that the cells are stimulated to produce increased amounts of ECM. The increased amounts of ECM in the trabecular meshwork (TM) and in the connective tissue septae of the nerve might be due to the elevated TGFβ_2 levels. Additionally, there may be a disconnection between astrocytes and these capillaries. Capillary density (number per area) is decreased in POAG but not in PEXG. These differences indicate that factors in addition to IOP might be causative for the pathogenesis of the nerve changes in POAG.

Future directions

Participants strongly agreed on the importance of studying glaucoma in human eyes and patients to better understand the pathophysiology of disease, to test hypotheses developed from various animal models of glaucoma, and to identify subgroups and

susceptibilities for diverse populations. Future directions include searching for further aqueous humor and other factors that might cause an increase in IOP in POAG eyes or that might make nerves more susceptible to IOP-induced changes. For this purpose it would be necessary to perform genetic studies in humans and collect aqueous humor, blood, and other tissues to identify pathophysiological changes.

Collecting donor eyes for further study of morphological changes is also critical and allows for comparisons of imaging with immunohistochemical and ultrastructural data.

Limitations for the prosecution of such studies include access to human patients and tissues. Although some ophthalmic diseases have developed adequate resources to obtain human eyes and tissues, glaucoma researchers have not yet done this to an equivalent degree. Participants believe this could be due to (1) characteristics of the disease, such as its late onset or low morbidity/mortality (2) complex social difficulties which preclude access to human tissues in general, and (3) funding sources. The discussion led to considerable debate as to how to develop a network of hospitals and researchers that could provide human specimens.

The GlaucoGENE of the European Glaucoma Society project began out of the identified need to better understand the pathogenic basis for glaucoma and to develop ultimately relevant therapeutics (Founti et al., 2009). Building on existing biobank structures, GlaucoGENE seeks to develop more standardized and accurate tissue collection and phenotyping, by emphasizing detailed phenotyping of quantitative traits, such as biometry, psychophysics, and imaging. The project aims to collect data from probands, especially those with POAG, OHT, PEXG, PDG, and PAC (G), as well as first degree relatives and controls. Standardization across participating centres is coordinated by having facility heads meet to discuss standard operating procedures with technicians from each centre across the European Union. Funding for contributing centres is based on the level of detail of phenotyping provided. The feasibility study funding was provided by the European Glaucoma Society, which cost an estimated 80,000 Euros for about 60 eyes' worth of data. The program is currently being expanded, though it promises to provide a unique and standardized approach for biobanking that could be applied more broadly.

Proposed Studies

1) Examine various forms of glaucoma genetically.
2) Examine aqueous humor, blood and other tissues for pathophysiological changes in various glaucomas.
3) Examine in detail morphological changes that occur in various glaucomas.
4) Develop an eye and tissue bank for glaucomatous eyes.

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Chapter 5. Animal Models of Glaucoma

Discussion Leaders: Simon John, Gareth Howell and Elke Lütjen-Drecoll

The animal models session participants discussed and evaluated existing animal models used in the study of glaucoma. The group agreed that animal models provide essential insights into the mechanisms of glaucoma, and will prove useful for testing potential treatments for human glaucoma. The group also established that while useful models exist, developing a fuller array of well-characterized and complementary glaucoma models will be beneficial. Discussions focused on those models most relevant to the neurobiology of glaucoma. Although rodents and non-human primates are likely the most accessible, the groups briefly discussed other models such as sheep, cows, and dogs, as summarized below.

Are animal models providing an understanding of glial cells in glaucoma?

Though there are no definitive answers, clues are emerging regarding the role of glial cells in glaucoma. Many different types of glial cells exist in the retina and optic nerve, including astrocytes, microglia, Müller glia, and oligodendrocytes. Importantly, studies demonstrate that the lamina of the ONH is rich in astrocytes. In humans, the lamina cribrosa consists of collagenous plates lined with astrocytes. Researchers hypothesize that these plates may be important for causing pressure-induced damage directly to axons or indirectly by compressing blood vessels. However, at the equivalent region in mice and rats, the network of astrocytes exists in the absence of collagenous plates. Interestingly, in DBA/2J mice, RGC axonal damage occurs at the 'glial' lamina despite the absence of these plates, indicating that these plates are not necessary for axonal damage. This has also been observed in experimental glaucoma in rats (Morrison et al., 1995, Howell et al., 2007). These data suggest that astrocytes within the lamina may play a role in the pathogenesis of glaucoma. Recently, other findings implicate microglia in glaucoma, although laboratories have reached little consensus about the potentially beneficial or detrimental roles of activated microglia in animal models of glaucoma.

The key features of glaucoma necessary in an animal model

The group discussed key features of glaucoma most important for animal models. These features include: (i) regional loss of RGCs, (ii) an insult to axons in the ONH, and (iii) RGCs being the primary cell type lost, with other cells of the retina remaining relatively intact. However, animal models that do not satisfy all of these criteria can still provide important mechanistic insights. The most widely used models to date involve elevation of IOP, a key risk factor for glaucoma. However, models with the above features of glaucoma without high IOP will prove valuable in understanding other risk factors. While optic

nerve remodeling and/or pressure-induced excavation are key features of human glaucoma, the group did not feel these features are essential for an animal model, though the presence of these features would add confidence to the relevance of the model for human glaucoma.

Existing models and their advantages and limitations

Generally, models are characterized as either inherited or induced (Howell et al., 2008). Inherited models have the advantage that they can show an age-related, chronic form of glaucoma that may more closely model human glaucomas than induced models. However, these features also make them challenging to use, both in complexity and the cost of experiments. Inducible models, in which IOP is artificially elevated, are potentially more controllable, although considerable variability can still occur. Despite this, they can be less expensive to use than inherited models. Overall, the group agreed that regardless of the model used, large sample sizes are necessary. Current studies often use small numbers of animal subjects, making it difficult to know if conclusions are real or robust. Using adequate sample sizes is most realistic with rodent models, given their relatively small size and limited housing costs. Furthermore, the group discussed the importance of validating glaucoma-relevant mechanisms across models to strengthen findings and to guide the selection of processes that could be further examined in clinical trials.

Current animal models include:

1. Mice

Mice provide a powerful well-developed and proven system for dissecting molecular mechanisms of various diseases. There are various advantages and disadvantages for specific models.

Inherited Models

DBA/2J is the best-characterized inherited and later-onset mouse model of glaucoma (Anderson et al., 2006; Howell et al., 2007; Inman and Horner, 2007; Jakobs et al., 2005; Morrison et al., 2008; Panagis et al., 2010; Schlamp et al., 2006; Soto et al., 2008). It shows features of human glaucoma including regional patterns of RGC loss, an axon insult in the ONH, IOP elevation and optic nerve excavation (see Figure 5). The remaining layers of the retina remain intact in many but not all colonies. However, colony-specific differences exist. For instance, some investigators report differences in the age of onset of glaucoma phenotypes (Steele et al., 2006; Bosco et al., 2008; Buckingham et al., 2008). Some suggest that environmental differences between colonies may account for the observed differences. Therefore, it is important to characterize adequately the glaucoma profile and overall phenotype in each colony. Given the variation in onset and severity of glaucoma observed in DBA/2J mice–a feature that mimics human glaucoma–it

is important to study large numbers of animals. For potential neuroprotective treatments, some advise that a minimum of 40-60 eyes should be assessed, though it may be necessary to examine 100 eyes for treatments that have small but important effects. *In vivo* imaging of the retina and optic nerve is difficult in DBA/2J mice due to media abnormalities and iris atrophy (Libby et al., 2005). Some investigators also report very early retinal changes in at least some colonies of DBA/2J mice (Steele et al., 2006).

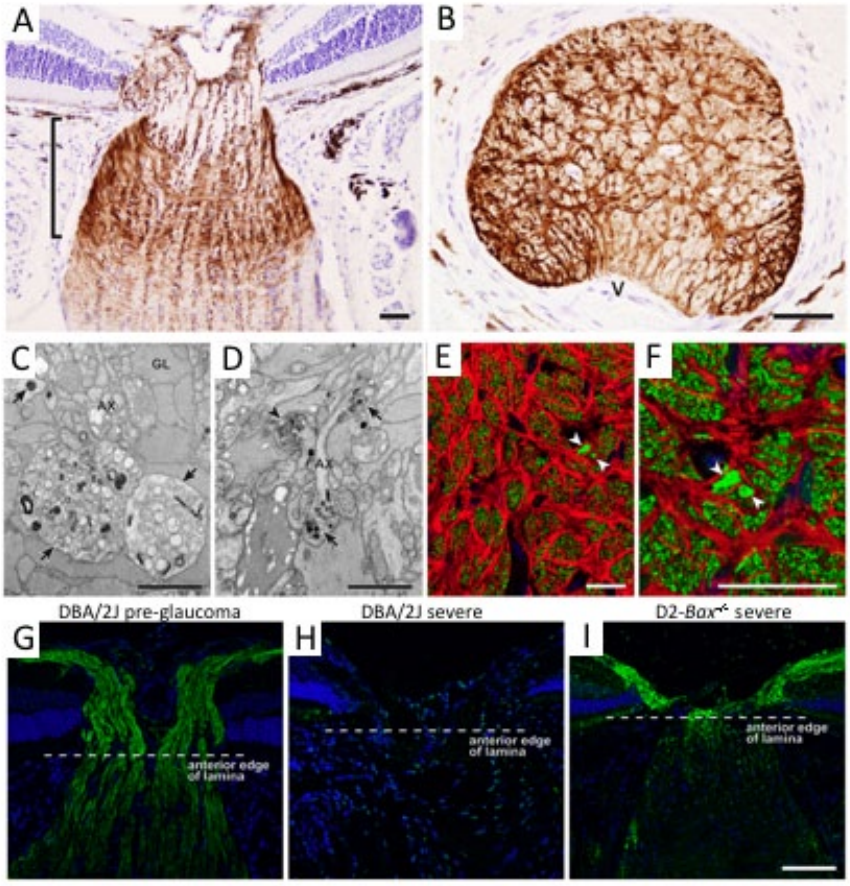

Figure 5. The ONH is an important site in DBA/2J glaucoma. (**A-B**) Aged DBA/2J mice have a robust glial lamina (stained with GFAP, a marker for astrocytes). In contrast to humans, the lamina does not contain collagenous plates. Despite this difference, the lamina in DBA/2J mice is still an important site for glaucoma. (**C-F**) Firstly, early damage to RGC axons occurs at the glial lamina. At the glial lamina, dystrophic neurites (arrows and arrowheads) are readily detected in DBA/2J mice with no significant RGC axon loss compared to controls. Dystrophic neurites consist of swollen and damaged axon segments containing an accumulation of organelles including swollen mitochondria (D, arrow head). Due to the accumulation and organization of axonal contents, dystrophic neurites are also detected using antibodies against neurofilament (**E-F** green, arrowheads) (red, GFAP). (**G-I**) Secondly, axons survive up to the lamina in BAX (BCL-2-associated X protein) -deficient mice with severe glaucoma. In the peripheral nervous system, direct and focal axon injury can result in degeneration of the entire length of the distal portion of the axon that is separated from the cell body by the lesion. In contrast, the proximal portion of the axon that is attached to the cell body can survive up to the proximity of the axon insult, as long as the cell body survives. In BAX-deficient DBA/2J mice, RGC somata survive indefinitely (Libby et al., 2005b) and so the proximal axon can be used to identify the location of an insult to RGC axons. In a young DBA/2J mouse (G), the axons clearly gather in the nerve fiber layer at the inner edge of the optic nerve and continue to pass through to the lamina. In a 10-mo-old DBA/2J mouse with severe axon loss behind the eye (H), axons are completely missing from the nerve fiber layer and entire optic nerve. In BAX deficient DBA/2J mice, proximal axons survive from the RGC bodies to the proximity of the anterior edge of the glial lamina (I).There is an abrupt loss of axons at the lamina. These data, along with further controls in the original study, provide strong experimental evidence for an insult to axons within or very close to the lamina in this glaucoma. Neurofilament-labeled axons (green) in longitudinal sections of the optic nerve are shown. (A-B) Bars, 50 mm (C-D) Bars, 2 mm. (E-F) Bars 20 mm. (G-I) Bars, 75 mm. Adapted from Howell et al, 2007. ©The Rockefeller University Press. The Journal of Cell Biology, 2007, 179:1523–1537 (used with permission).

Induced Models

In laser-induced models of glaucoma, researchers use lasers to damage the drainage structures and blood vessels at the limbus to elevate IOP (Chang et al., 1999; Gross et al., 2003; Grozdanic et al., 2003; Mabuchi et al., 2003). Currently, laboratories use different protocols that vary in the degree of laser burns applied around the limbus, which may have significant influence on experimental outcomes. There is concern about a greater vascular or ischemic contribution than in typical human glaucoma and about the degree of damage to the eye. Laser-induced models in various species are useful and are discussed further below. However, due to the thin ocular wall and small eyes of mice, many participants felt that mouse eyes often do not tolerate this method well, resulting in substantial damage that may impact experimental outcomes. In various laboratories, IOP elevation following laser treatment is highly variable and not consistently elevated but progressive cell death still occurs. It is not clear how much of this cell death is due to IOP elevation or other factors resulting from laser treatment, especially in mice.

Calkins and colleagues recently reported the development of an inducible bead model for mice with approximately 20 percent of RGCs lost after a modest elevation (Nakazawa et al., 2006). Polystyrene beads are injected into the anterior chamber to elevate IOP. Other laboratories are using similar protocols. However, this model is new and, as such, not yet working in many laboratories, and it exhibits variability among operators. Injecting beads to elevate IOP is a worthwhile area of development, but further work is required to assess the full utility of this model for the study of glaucoma, such as the patterns and specificity of affected cell types and if RGC axons are damaged in the ONH. These models require more control and uniformity if they are to be used for genetic experiments (e.g., to identify genes that modify glaucoma susceptibility).

2. Rats

Rats are valuable models for the evaluation of some glaucoma phenotypes and are generally larger and easier to handle than mice. However, they are not as easy to manipulate genetically as mice, though the use of genetic rat models is growing. As with mice, rats have a lamina without collagenous plates.

Inducible Models

In the Morrison model of glaucoma, sustained IOP elevation is produced using an injection of hypotonic saline solution into the drainage structures of the eye (Sappington et al., 2010). This results in axonal degeneration and, presumably, RGC loss that is correlated to the degree and duration of IOP elevation. It is not as widely used as other models, given the technical challenges of the procedure. However, the group felt that this is a very valuable animal model.

The laser induced-models discussed previously are available in multiple species including rat. Like the mouse model, problems with inter-animal variations and trauma due to the use of the laser are apparent. Likewise, inducible bead models of glaucoma can be used.

3. Non-human primates

Non-human primate studies are frequently necessary prior to clinical trials. IOP is elevated primarily using laser treatment (Morrison et al., 1997; Pederson and Gaasterland, 1984; Agarwal et al., 1991). Primates are important for all preclinical biomedical research as they are the closest evolutionarily to humans. However, this also makes many researchers reluctant to work with them. Primates are expensive to use due to housing and the length of time that experiments take. Therefore, they are less suitable for identifying molecular mechanisms involved in glaucoma or for initially testing potential treatments, and more suitable for bridging the gap between rodents and humans in translational research.

Developing new animal models for glaucoma

Despite the current and future impact of existing models, new models need to be developed. Efforts continue to develop new inherited models in mice. These models would ideally complement DBA/2J mice, with shorter onset and simpler genetics of IOP elevation. Currently, an N-ethyl-N-nitrosourea (ENU) mutagenesis screen is identifying and characterizing new inherited models in mice.

A useful approach to generate new models will be to “humanize” mice. In this approach, mice are engineered to contain human genes. This could be used to assess known human glaucoma mutations in the mouse. While this approach holds promise for the future, it has proven difficult for some genes, including the myocilin, trabecular meshwork inducible glucocorticoid response (*MYOC*) gene, at least in part due to differences in the encoded protein between mice and humans (Agarwal et al., 1991; Gould et al., 2006; Senatorov et al., 2006). Humanizing mice will also be important for assessing the roles of specific pathways and processes. For example, complement is implicated in various models of glaucoma but, like the *MYOC* gene, the encoded proteins differ between species. Generating mice with human versions of complement cascade genes will prove valuable for studying the role of this cascade in glaucoma and assessing treatments.

Given the importance of the lamina in glaucoma, it is necessary to assess models in species that have differing architecture of the ONH. In particular, a larger animal with collagenous plates in the lamina, such as pig, could provide valuable insight into the contribution of this region in glaucoma. Furthermore, the US Food and Drug Administration (FDA) accepts safety and efficacy data generated in the pig for clinical trials. A second species of interest is the rabbit, which has intraretinal myelinated RGC axons and a notable absence of a glial lamina. Experiments in rabbits could aid in assessing the

importance of unmyelinated RGC axons in the astrocyte-rich lamina in glaucoma. However, rabbits differ from humans in other ways, such as a dissimilar retinal vasculature that could confound these experiments. Ongoing studies are assessing a laser-induced model in the pig and a bead model in the rabbit. Other emerging models include the mouse lemur, the tree shrew and the zebrafish, and their continued development is important. For example, the tree shrew has a lamina with collagenous plates and is closely related to primates. Zebrafish, on the other hand, provide for facile genetic screens. Overall, the group agreed that the development of new animal models for the study of glaucoma and potential treatments is important.

Proposed Studies

Standard scientific practice is to share reagents and detailed methods after publication. However, technical difficulties and cost can preclude such widespread access. Importantly, when exact techniques are not shared and available to all, it can be difficult to compare and understand experiments. Furthermore, if others do not validate techniques, there can be a degree of distrust of data. Mechanisms to promote sharing and cross-validation will benefit the field. Such existing resources include:

1) IOP assessment methods.

2) Axon counting methods.

3) Databases of glaucoma-relevant information (such as gene expression studies and retina/brain atlases). As much as possible, efforts should be made to incorporate data from animal models into online resources.

In addition to sharing existing resources, the development of new resources will prove valuable. These include:

1) Develop and evaluate new animal models (see above).

2) Improve genetic resources for various model animals, i.e., mice, rats, zebrafish, etc.

3) Conduct studies in mice by gene targeting technologies (cyclization recombination (Cre) lines and conditional knockouts) on diverse genetic backgrounds and panels of cell-specific fluorescent proteins to mark glaucoma-relevant cell types in different colors.

4) Develop new methods for elevating IOP. Adenoviral gene transfer is a promising avenue being explored, i.e., intraocular injections of TGF-β_2-transducing virus can elevate IOP in both rats and mice.

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Chapter 6. Therapies

Discussion Leader: Alan Laties

Clinical trials have long shown that treatments that lower IOP can lessen the initial loss of the visual field (Kass 1994), the progressive loss of the visual field (Leske et al., 1999; Nouri-Mahdavi et al., 2004), and the loss of the visual field in normal tension glaucoma (Lichter et al., 2001) by up to 60 percent. Indeed, such agents remain the mainstay treatment for glaucoma. A variety of other therapies have been tried with limited results, a number of which were discussed in this session. In addition, there was substantial discussion regarding clinical trials and their limitations.

Neuroprotection

Studies on non-IOP-lowering treatments are few, and there is a dearth of published data in the peer-reviewed literature. Two parallel long-term Phase III clinical trials of oral memantine, which aimed to directly protect the optic nerve rather than to reduce IOP, failed to reach their primary outcome measures (Pyott 2008). Due to ethical considerations, all subjects continued therapy to lower IOP. The primary end point was based on visual fields although secondary end points included optic disc analysis and psychophysical tests. The results of the study are not yet published, with only press releases briefly outlining the results from the maker of the drug. There could have been a number of reasons as to why the trials failed to demonstrate efficacy: the study design of the trial may not have been optimal, there may have been inadequate preliminary data on the two dosages used, there may have been inadequate preclinical data to show that the drug was effective, or the need to keep patients on IOP-lowering therapy may have diluted any effect. Some discussants believed that a small pilot study would have been informative.

A clinical trial on the non-IOP-related effects of brimonidine, an α_2 adrenergic agonist, on visual field progression (Gandolfi et al., 2004), showed that the test group receiving brimonidine had less visual field deterioration than those treated with 360º laser trabeculoplasty. However, this has only been presented in abstract form, and was not published in the peer-reviewed literature. Brimonidine's primary mechanism of action is the activation of the α_2 adrenoceptors in the ciliary body decreasing cyclic adenosine monophosphate (cAMP) levels, thus decreasing aqueous humor production (http://www.eyes.org/common/attachments/articles/alpha_agonists.pdf). However, various pathways have been suggested for its neuroprotective effects including via neurotrophin deprivation and effects on NMDA receptor signaling.

The Low-Pressure Glaucoma Treatment Study was a medium-sized, double-masked clinical trial comparing the course of low-tension glaucoma patients randomized to IOP reduction with topical twice daily brimonidine tartrate 0.2% versus twice daily timolol

maleate 0.5%. Despite publication of the trial design, the results have not yet been published.

Glaucoma clinical trials also suffer from general problems. Both recruitment and the variability of treatment and response at the individual level pose challenges. Researchers have to assume that individual subjects adhere to the requirements of the trial, but inevitably, conclusions drawn based on the population are affected by the aberrant subject, their compliance, and heterogeneity that confounds study factors.

Auto-Antibodies and Inflammation in Glaucoma

Growing evidence obtained from clinical and experimental studies over the last decade strongly suggest the involvement of the immune system in glaucoma. Paradoxically, the role of the immune system in glaucoma has been described as both neuroprotective and neurodestructive. A balance between beneficial immunity and harmful autoimmune neurodegeneration may ultimately determine the fate of RGCs in response to various stressors in glaucomatous eyes. Based on clinical data in humans, it has been proposed that one form of glaucoma may be an autoimmune neuropathy, in which an individual's immune response facilitates a somatic and/or axonal degeneration of RGCs by the very system which normally serves to protect it against tissue stress (Garbe et al., 1997).

Schwartz and colleagues advocated the use of glatiramer acetate, Copolymer-1; Cop-1, as a treatment to reduce the T-cell mediated response in glaucoma, showing that vaccination with Cop-1 leads to a significant reduction in elevated IOP-induced RGC death in a rat model of ocular hypertension (Cheung et al., 2008). A high correspondence of autoantibody patterns found in glaucoma study populations from different continents provided evidence that serum autoantibody patterns may be useful biomarkers for glaucoma detection or for determining prognosis in future studies by means of pattern-matching algorithms (Grus et al., 2006).

In a preclinical study, the cytokine tumor necrosis factor-α (TNF-α) was shown to provide an essential, although indirect, link between ocular hypertension (OH) and RGC loss *in vivo* (Tezel and Wax 2007). Blocking TNF- α signaling or inflammation was identified as potentially helpful in treating glaucoma. Increased IOP in the mice resulted in TNF-α release.

Another recent study by Di Polo indicated that a non-cell-autonomous mechanism involving signaling events in neighboring Müller glia plays a decisive role in retinal neuron death *in vivo* (Sieving et al., 2006). This contradicts the traditional view of Müller glia as playing a neuroprotective role by releasing neurotrophic and antioxidant factors early after injury in the CNS. The observation that molecular events in Müller glia determine the fate of retinal neurons shifts scientific understanding of excitotoxic damage. Excitotoxicity usually refers to excess glutamate binding to cell-surface NMDA receptors on neu-

rons, triggering massive Ca2+ influx and activating proapoptotic signaling cascades. However, the NMDA receptor antagonist memantine failed in glaucoma clinical trials (see above) and NMDA antagonists have consistently failed in trials of stroke, suggesting that other mechanisms contribute to the devastating excitotoxic damage *in vivo.* In Di Polo's study, loss-of-function experiments demonstrated that a non-cell-autonomous mechanism accounts for over sixty percent of the excitotoxic neuronal loss in the retina. The conclusion is that the blockade of glutamate receptors may not ameliorate disease progression unless other major damage-inducing players, e.g., glia-derived TNF-α, are also inhibited.

Other Neuroprotectives in the Treatment of Glaucoma

In addition to the treatment approaches mentioned above, a number of other potential therapies in glaucoma were discussed.

Ciliary neurotrophic factor (CNTF), a natural neuroprotective protein, has been shown in human clinical trials to protect against the loss of photoreceptors. One Phase I trial, indicated that CNTF is safe for the human retina, even with severely compromised photoreceptors (Neufeld et al., 1999). In addition, the inhibition of nitric-oxide synthase 2 by aminoguanidine provides neuroprotection of RGCs in one model of chronic glaucoma (Neufeld et al., 1999), but not another (Pang et al., 2005). Small molecules can mimic neurotrophins and neurotrophins are possibly additive (Kato and Lindsay 1994).

Using drugs that have already been shown to be safe in other clinical applications appears to be a good strategy. Candidate neuroprotectives include: tacrolimous, erythropoietin, minocycline, beta secretase inhibitor, beta-amyloid antibodies, and free radical scavengers. The clinical effects of each, however, need to be studied in glaucoma clinical trials.

Considerations for Therapy Testing

One of the drawbacks in assessment of IOP in glaucoma patients is that a one-time measurement in the clinic may not reflect fluctuations and absolute control outside that time. IOP telemetry offers several new possibilities in a manner similar to 24-hour blood pressure monitoring. New approaches include lens implantation and contact lenses, though none yet have reached large-scale clinical trials.

The gold standard of visual field testing has proved inadequate for neuroprotective trials. However, imaging advances show promise (Cordeiro 2009). Nerve fibers that already show damage are more likely to be in the vicinity of nerve fibers that exhibit new damage. ONH topographic measurements with the Heidelberg Retinal Tomograph (HRT) look at phenomena that need further validation. Optical Coherence Tomography (OCT) may offer a tool to assess still-living ganglion cells (Chambers 1976). However, at pres-

ent there is no structural measure which corresponds to functional activity. Change may be evident in one before the other or simultaneously.

The involvement of RGC apoptosis may be advantageous to deducing the deeper mechanisms of glaucoma. Use of intravenous fluorescent-labeled annexin V in glaucoma is currently being assessed in a Phase I clinical trial (Cordeiro et al., 2010). Another potential parameter is strain at the optic nerve. This needs to be assessed, especially at the peri-papillary sclera, where the strain is highest (Burgoyne and Downs 2008).

The Ideal Clinical Trial

For human trials, large numbers of glaucoma patients are necessary, but this becomes costly. Subjects should be examined frequently, and imaging done as often as possible with both structural and functional measures. Future clinical trials need to distinguish between what occurs in the optic nerve axons and what happens to the soma and its dendrites. This has been a point of contention, and both aspects need to be investigated.

Proposed Studies

1) Encourage the National Eye Institute (NEI) and similar agencies to develop pilot instruments, study sessions, psychophysical measurement improvements and delineation of mechanisms related to the various experimental models.

2) Test multiple animal models of different species in order to establish with strong significance a treatment that can then be tested on humans. If a trial is based on only one model, its conclusions may not be generalizable. Use of different rodent models should be encouraged. Monkey and pig eyes are closer to the size of the human eye and should be explored for preclinical studies. In order to develop treatments to pass FDA approval, a large animal model is necessary. Costs can be prohibitive - a monkey study with just a few subjects can cost $1 million.

3) Improve psychophysical measures of clinical outcomes through the addition of eye tracking to visual field assessment. New functional tests for glaucoma need to be developed including ring perimetry. Conclusive work needs to progress in the analysis of visual field loss for a reliably sensitive indicator – use of trend versus event analysis needs to be definitively assessed. Any new measurement must be robust in order to maximize the likelihood that the FDA will approve an apparently effective drug. The scientific community should adopt common standards with which to proceed.

4) Assess and validate imaging. The FDA should recognize that both structural and functional endpoints should be considered in glaucoma. A clear unmet need is the ability to measure and assess the reversibility of injury to the ganglion cell. Additional needs include: cell-surface markers that diagnose impaired, but not dead, cells; fluorescent marker for targets such as Stat3; and improvements in imaging modalities such as OCT that can define cellular changes. Finally, efficacious neuroprotectives without side effects would be needed for treatment of asymptomatic disease.

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Concluding Remarks

Glaucoma remains an intractable condition. Whereas certain risk factors are clear – high IOP and age – exactly how these risk factors contribute to the disease is by no means clear. Most believe the disease relates to changes occurring initially at the ONH, but what precisely these changes are and what initiates them is quite mysterious.

One impetus for this initiative was the recent findings implicating reactive astrocytes as the cause of certain neurodegenerative diseases, particularly ALS. Astrocytes – perhaps of several types – are present in and around the ONH and in the ganglion cell layer. Since changes in the ONH are clearly implicated in the etiology of glaucoma and the ganglion cells are the primary cell type lost in glaucoma, careful consideration of astrocytes as playing a key role in glaucoma is clearly warranted and was the main focus of the initiative.

As perhaps might be expected, no firm conclusions regarding the role of astrocytes in glaucoma were reached. However, much useful information concerning these fascinating cells and their possible roles – both positive and negative – was exchanged. A number of potential studies to elucidate further the role of astrocytes and other glial cells in retinal function and glaucoma were proposed as well as studies to further our understanding, diagnosis and treatment of glaucoma were suggested.

We hope this report helps point the way toward an understanding and eventual cure of this devastating disease.

# # # #

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INDEX

A

B

INDEX

INDEX

I

L

M

N

INDEX