Heterogeneous Distribution of Axonal Cytoskeleton Proteins in the Human Optic Nerve


PURPOSE. Cytoskeleton proteins play a critical role in maintaining retinal ganglion cell structure, viability, and function. This study documents the distribution of cytoskeleton protein subunits in the various regions of the normal human optic nerve and identifies important relationships among mitochondria, myelin, and neurofilament proteins.

METHODS. Twenty-three optic nerves from human cadavers were used. Confocal microscopy was used to examine the distribution of neurofilament light, neurofilament medium, neurofilament heavy (phosphorylated and unphosphorylated), neurofilament heavy (phosphorylated only), actin, and microtubule associated protein (MAP)-1 along the sagittal plane of the optic nerve. Comparisons were made among superior, middle, and inferior regions and also among temporal, central, and nasal portions of the optic nerve. Colocalization of neurofilament light, mitochondrial cytochrome c oxidase (COX), and myelin was also performed.

RESULTS. There are significant differences in the pattern and distribution of neurofilament protein subunits, actin, and MAP-1 along the sagittal plane of the optic nerve. Cytoskeleton proteins and COX mitochondria are found in highest concentrations in the prelaminar and lamina cribrosa regions. COX and neurofilament light occur predominantly in unmyelinated nerve, with a significant decrease in concentration occurring on optic nerve myelination.

CONCLUSIONS. The heterogeneous distribution of cytoskeleton proteins along the sagittal plane may be an important functional adaptation that reflects the nonuniform nature of the physiological and structural environment of the optic nerve. The heterogeneous distribution of cytoskeleton proteins may also partly account for the asymmetric pattern of optic nerve damage after intraocular pressure elevation. (Invest Ophthalmol Vis Sci. 2009;50:2824-2838) DOI:10.1167/iovs.08-3206

Cytoskeletal proteins are highly concentrated within the intracellular compartment of neuronal cells and fulfill myriad important roles in maintaining cell viability and function. They are intrinsic determinants of cellular cytoarchitecture and preserve neuronal morphology by providing structural support to complex dendritic arborizations and axonal projections. With regard to neuronal homeostasis, cytoskeleton proteins also play a crucial role in regulating intracellular processes such as signal transduction, axonal transport, neuronal growth, synaptic plasticity, and neurotransmitter release. Preservation of the neuronal cytoskeleton is, therefore, critical to cell survival, and disruption of cytoskeleton protein elements can result in neurodegenerative disease.

Neurofilaments, microtubules, microtubule associated proteins (MAPs), and actin are the major constituents of the neuronal cytoskeleton. Neurofilaments are obligate heteropolymers composed of three subunits—neurofilament heavy (NFH), neurofilament medium (NFM), and neurofilament light (NFL)—in order of decreasing mass. Actin is expressed in β and γ isoforms in neurons, and several classes of MAP proteins contribute to the cytoskeleton scaffolding required to maintain the rigid architecture of axons and dendrites. The distribution of cytoskeleton protein subunits within neurons is not homogeneous but instead demonstrates a degree of heterogeneous compartmentalization that is specific to the function and regional demands of the axon, dendrite, cell body, and synapse. Local factors that are known to control cytoskeleton subunit expression within each of these neuronal compartments include mechanical forces acting on neuronal structures, mitochondrial activity, and myelin.

The architecture of the human retinal ganglion cell (RGC) is elaborate and consists of a cell body approximately 12 μm in diameter that is connected to its target synapse by an axon more than 50 mm long. The average diameter of the RGC axon is only 0.72 μm. In addition to its complex structure, RGC axons that constitute the optic nerve are unique because they traverse several physiological environments along their projected path. Within the eye, the pressure acting on RGC axons is equivalent to intraocular pressure (IOP), whereas the pressure acting on the same axons behind the optic disc is usually lower and is largely influenced by cerebrospinal fluid (CSF) pressure. The pressure difference between IOP and CSF pressure occurs across the lamina cribrosa, which is divided into anterior and posterior portions on the basis of distinct morphologic and cellular differences. In addition to being the site where RGC axons experience pressure gradients, the optic nerve head is also a region where axons undergo structural modifications. In the prelaminar and lamina cribrosa regions, RGC axons are unmyelinated and acquire a myelin sheath only after passing the posterior boundary of the lamina cribrosa. The optic nerve head is, therefore, an exceedingly important region in which RGC axons experience a range of structural and functional changes even under normal physiological conditions.

Optic nerve diseases are associated with cytoskeleton protein changes. Different reports have demonstrated optic nerve head cytoskeleton changes in glaucoma, acute IOP elevation, ocular hypertension, optic nerve crush, optic nerve transection, optic neuritis, and optic nerve stretch injury. These previous reports have provided evidence that the cytoskeleton response after neuronal injury is highly variable and is intimately related to the form of neuronal insult. Although the individual protein subunits affected and the temporal sequence

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of cytoskeletal change may vary in accordance with the injurious stimuli, a number of studies have demonstrated that regardless of the insult, cytoskeletal changes occur early and in the acute stages of cellular injury, before macroscopic and functional neuronal changes are evident.\textsuperscript{25,51} We have previously shown that cytoskeletal changes precede axonal transport alteration in the optic nerve head during acute IOP elevation.\textsuperscript{25} The neuronal cytoskeleton is a major contributor of retinal nerve fiber layer birefringence,\textsuperscript{52} and recent work by Fortune et al.\textsuperscript{31} has demonstrated that ERG changes and decreases in nerve fiber layer birefringence precede measurable nerve fiber layer thinning after optic nerve section, implying that cytoskeletal changes are an early marker of retinal ganglion cell axonal injury.

Although cytoskeleton changes are known to occur in a multitude of optic nerve diseases, there has not yet been a detailed study of the neuronal cytoskeleton of the normal human optic nerve head. The variation in mitochondrial organelle, myelin protein content, and neural tissue pressure along the length of the human RGC axon could potentially influence the regional concentration of cytoskeleton protein subunits. This may help explain why the optic nerve head is injured in an asymmetric fashion in many diseases, including glaucoma, which is the second most common cause of blindness in the developed world.\textsuperscript{33,34} This report is a detailed study of the distribution of axonal cytoskeleton proteins in unmyelinated and myelinated portions of the human optic nerve. We compared the distribution of neurofilament subunits, actin, and MAP-1 proteins along the sagittal plane of the optic nerve. We made detailed comparisons among superior, middle, and inferior regions and temporal, central, and nasal regions of the optic nerve. Because critical physiological and structural axonal changes occur in the anterior optic nerve, we also performed a detailed analysis comparing the prelaminar, anterior lamina cribrosa, and posterior lamina cribrosa regions. Finally, we report the distribution of mitochondria along the sagittal plane of the optic nerve and colocalize the distribution of mitochondria, myelin, and neurofilament proteins to identify relationships among these three structures. The aim of performing this work was to improve our understanding regarding the organization of cytoskeleton proteins in the human optic nerve head and the roles they may play in physiological conditions and neurodegenerative disease. The findings from this study will add complementary histologic knowledge to previous reports that have measured changes in birefringence, an optical property thought to be a direct manifestation of cytoskeletal integrity, as a means of detecting cellular dysfunction in the early stages of ocular disease.\textsuperscript{31,35}

### METHODS

This study was approved by the human research ethics committee of the University of Western Australia. All human tissue was handled according to the tenets of the Declaration of Helsinki.

### Human Donor Eyes

Twenty-three human eyes from 14 donors were used for this study. All eyes were obtained from the Lions Eye Bank of Western Australia (Lions Eye Institute, Western Australia) after removal of corneal buttons for transplantation. Donor eyes used for this research had no documented history of eye disease. Demographic data and medical comorbidities of each optic nerve donor are presented in Table 1.

### Tissue Preparation

Before enucleation a scleral suture was placed at the 12 o'clock position to allow orientation of the optic nerve during tissue processing. When a stitch was not placed, the extraocular muscles and posi-
tion of the macula were used to orient the nerve. After the removal of corneal buttons, the eye was carefully dissected to expose the optic disc and the first centimeter of optic nerve. Tissue used for mitochondrial studies were immediately sectioned without any fixation. Tissue used for remaining studies was fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose before sectioning. Tissue for all studies was mounted in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Fine-Tek, Tokyo, Japan) and longitudinally sectioned into 12-μm specimens on a cryotome set at −30°C. To avoid the potential tilting of sections, the optic nerve was aligned parallel to the blade on the cryostat during sectioning. Longitudinal sections were cut along the sagittal plane beginning in the superior portion of each optic nerve and proceeding to the inferior part of the nerve. Specimens were numerically labeled as they were sectioned so that we could determine from which region of the optic nerve they were derived. Specimens were used for Van Giessen staining, cytochrome oxidase staining, or immunohistochemical labeling of cytoskeleton proteins and myelin. Slides used for myelin labeling were fixed for 10 minutes in 4% paraformaldehyde before staining. Donor eyes used for each of the different stains is presented in Table 1.

Staining Protocol

Van Giessen Stain. In all eyes, optic nerve sections adjacent to that used for immunohistochemical and cytochrome oxidase labeling were stained using the Van Giessen protocol.

Immunohistochemistry. Antibody labeling was used to study the distribution of cytoskeleton proteins and myelin within the optic nerve. The middle portion of the optic nerve in all 23 eyes was used to study the axonal cytoskeleton. In eight eyes from four patients, we also stained superior and inferior sections of the optic nerve so that we could determine regional differences in axonal cytoskeleton proteins. The middle portion of three optic nerves was dual stained with neurofilament light and myelin so that we could examine the distribution and relationship between these two proteins.

All slides used for immunohistochemistry were washed for 5 minutes in a wash solution composed of 0.01 M PBS and Tween 20 (Sigma-Aldrich, St. Louis, MO) before incubation with their primary antibody solution. All primary antibodies were made into solution with 1% goat serum (G9023; Sigma-Aldrich) and 1% bovine serum albumin (BSA). To improve permeability, 0.1% Triton X-100 (Sigma-Aldrich) was also used for all primary antibody incubations. Primary antibodies used in the present study were polyclonal antibody NFL directed against the neurofilament light subunit (1:500, AHP286; Serotec, Oxford, UK), polyclonal antibody NFM directed against the neurofilament medium subunit (1:200, AB1997; Chemicon International, Temecula, CA), monoclonal antibody NFH directed against the phosphorylated and nonphosphorylated neurofilament heavy subunit (1:400, N0142, clone N52; Sigma-Aldrich), monoclonal antibody NFHp directed against the phosphorylated neurofilament heavy subunit (1:200, N5389, clone NE14; Sigma-Aldrich), monoclonal anti-MAP1 antibody directed against MAP-1 (1:200, MAB362; Chemicon International), and polyclonal anti-MBP directed against myelin basic protein (1:500, MCA1848; Serotec, Oxford, UK). All primary antibodies were incubated for 24 hours except for anti-NFL, which was incubated for 48 hours.

After primary antibody incubation all specimens were given four washes over 20 minutes in a 4°C wash solution. Slides were then incubated at room temperature with a secondary antibody for 6 hours. Secondary antibodies used included goat anti-mouse IgG (1:400, Alexa Fluor 488, A11001; Invitrogen, Eugene, OR), goat anti-mouse IgG (1:400, Alexa Fluor 546, A11003; Invitrogen), or goat anti-rabbit IgG (1:400, Alexa Fluor 488, A11008; Invitrogen). After secondary antibody incubation, all specimens were washed four times over 20 minutes in a 4°C wash solution. Slides were then mounted in glycerol and immediately viewed with the confocal microscope. For those eyes that were dual stained with a combination of anti-NFL and anti-MBP antibodies, the concentration of antibodies, incubation times, and wash protocol were identical with that described.

Previously described immunohistochemistry protocols were also used to study the actin cytoskeleton within optic nerves. Slides were first washed for 5 minutes in a 4°C wash solution. They were then incubated for 30 minutes with 0.5% Triton X-100 in PBS, after which they were preincubated for another 30 minutes with 3% BSA and 0.3% Triton X-100 in PBS solution. Slides were then incubated for 3 hours with Alexa Fluor 488-phalloidin (A12379; Invitrogen, Eugene, OR), which was made up in a solution of 3% BSA and 0.3% Triton X-100 to give a final concentration of 0.2 to 0.3 μM. After this, slides were washed four times in a 4°C wash solution before mounting in glycerol.

Diaminobenzidine Cytochrome Oxidase Histochemistry. Mitochondrial distribution within optic nerves was studied with diaminobenzidine cytochrome oxidase (DAB COX) histochemistry. The middle section of 10 optic nerves from seven donors was used for this part of the study. DAB COX histochemistry was used for this work followed the protocol outlined for the visual system previously described by Wong-Riley. In brief, sections were incubated at 37°C in the dark in a solution containing 50 mg 3,3′-diaminobenzidine hydrochloride (Sigma, St. Louis, MO), 30 mg cytochrome c (C7752; Sigma), and 4 g sucrose in 90 mL 0.1 M phosphate buffer (pH 7.4). COX activity was terminated by washing the slides three times in phosphate-buffered solution. Slides were then dehydrated in a graded ethanol series (50%, 75%, and 100%) followed by immersion in xylene. Slides were then coverslipped and allowed to dry overnight.

Microscopy and Image Acquisition

Light Microscopy. Van Giessen-stained slides were digitized using a high-resolution digital camera (DXM 1200; Nikon Corp., Tokyo, Japan) attached to a microscope (Eclipse E800; Nikon Corp.). Separate images of the optic nerve beginning in the prelaminar region and extending 3 mm behind the lamina cribrosa were captured using a 4× objective lens (Plan Apo, NA 0.75; Nikon) and were stitched together (version 8.0, Photoshop; Adobe Systems Inc., Mountain View, CA). Creating a montage of each Van Giessen-stained section allowed us to accurately determine optic nerve dimensions.

Images of mitochondria-stained slides were captured using a monochrome cooled charge-coupled device (CCD) camera (model CV12/1H; Photonic Science Ltd., Robertbridge, UK) attached to an upright microscope (BH-2; Olympus, Tokyo, Japan). Software (version 5.1; Image Pro Plus; Media Cybernetics, Silver Spring, MD) was used to drive the CCD camera and analyze the images. Previous reports have described the use of different interference filters to photographically increase DAB reaction product contrast. We used findings from this previous work and placed a filter that transmits 420 to 490 nm between the field diaphragm and the condenser to provide greater optical density for the DAB reaction product and to improve contrast between the DAB reaction product and nonstained tissue. Images of the entire prelaminar, lamina cribrosa, and proximal 3 mm of postlaminar tissue in each slide were captured in a dark room using a 10× objective (NA 0.30; Olympus). Individual images were then stitched together to create a montage of each slide.

Confocal Scanning Laser Microscopy. Digital images of immunohistochemical slides labeled with antibodies were captured using a confocal laser scanning microscope (MRC 1000/1024 UV; Bio-Rad, Hercules, CA) controlled by image acquisition software (Comos; Bio-Rad). Visualization of sections labeled with Alexa Fluor 488 secondary antibody was achieved by laser excitation at a 488-nm line from an argon laser with emission detected through a 522/35-nm bandpass filter. Visualization of sections labeled with Alexa Fluor 546 secondary antibody was achieved by laser excitation at a 543-nm line from a green helium neon laser with emissions detected through a 580/52-nm bandpass filter. When sections were labeled with multiple antibodies, images were captured sequentially using each laser line in turn. A 20× (NA 0.4; Nikon) dry lens was used to view all slides. Using a motorized stage and a macro written in MPL under image acquisition
software (Comos; Bio-Rad); a series of Z stacks was captured for each slide beginning in the prelaminar region and extending 3 mm behind the lamina cribrosa. Images were collected in grayscale (on a scale of 0–255). Each Z stack consisted of a depth of optical sections collected at 2-μm increments along the z plane. Each Z stack consisted of seven sequential images collected using Kalman averaging. Z stacks were then montaged using a plug-in in ImageJ software (version 1.36; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.htm). Creating a montage allowed us to study spatial changes in the prelaminar, lamina cribrosa, and postlaminar regions of the optic nerve in detail.

Image Analysis
Quantitation of all images was performed (Image Pro Plus, version 5.1; Media Cybernetics). All images for the manuscript were prepared using commercial software (Photoshop [version 8.0] and Illustrator CS2 [version 12.0]; Adobe Systems Inc., San Jose, CA). Confocal images in this manuscript were false colored using look-up tables available on ImageJ software.

For quantitative analysis, immunohistochemical and mitochondria-labeled sections were divided into prelaminar, anterior lamina cribrosa, posterior lamina cribrosa, and postlaminar regions with the aid of Van Giessen-stained slides that were acquired from adjacent sections of optic nerve. Van Giessen-stained slides were also used to determine peripheral prelaminar thickness and lamina cribrosa thickness for each eye. Previous authors have performed detailed histologic studies of the human optic nerve head, and we used descriptions from these previous reports to divide each optic nerve image in our study into different laminar regions (Fig. 1).

- Prelaminar region—The most anterior portion of the optic nerve head is identified by the sparse and irregular arrangement of glial cell nuclei and by the paucity of connective tissue structures in this region.
- Anterior lamina cribrosa region—This is situated adjacent to the choroid. Transversely oriented glial cell nuclei in this region form distinctive columnar structures that follow the course of nerve fibers.
- Posterior lamina cribrosa region—This is usually thicker than the anterior lamina cribrosa. Dense connective tissue sheets bridge the diameter of the scleral canal in this region. The fenestrated collagen sheets of the posterior lamina cribrosa are continuous with the connective tissue fibers of the sclera and form narrow openings for the transmission of axonal bundles.
- Postlaminar region—Axonal fibers in this region are divided into fascicles by connective tissue sheaths that run parallel to the direction of nerve bundles. At the periphery of the optic nerve, connective tissue sheaths are continuous with the pia mater sheath of the meninges. Adjacent connective tissue sheaths are sometimes seen to be crossed over by fibrous bands in this region.

Measurement of Cytoskeleton Proteins in the Optic Nerve. The methodology used to divide optic nerves for quantitative analysis is illustrated in Figure 2. Confocal microscope images of...
cytoskeleton proteins were analyzed in gray scale (0–255 pixel intensity) with the average projection of each montaged Z stack used for analysis. Confocal images of cytoskeleton proteins from the superior, middle, and inferior sections of the optic nerve were analyzed in the same manner (Figs. 2A, B). Before analysis each confocal image was divided into temporal, central, and nasal regions by dividing the inner neural canal into thirds and into prelaminar, anterior lamina cribrosa, and posterior lamina cribrosa regions, a proximal 400-μm region of immediate postlaminar tissue, a second region of postlaminar tissue that commenced 1 mm behind the lamina cribrosa and extended 400 μm posteriorly, and a third region of postlaminar tissue that commenced 2 mm behind the lamina cribrosa and extended 400 μm posteriorly (Figs. 2D, E). Thus, 18 regions were analyzed within each confocal microscope image. A Van Giessen-stained image of a section

**Figure 2.** Three-dimensional schematic diagram illustrating the regions of human optic nerve examined for cytoskeleton protein studies (A). Sections along the sagittal plane from the superior, middle, and inferior regions of the optic nerve were studied (B). Each section was divided into prelaminar, anterior lamina cribrosa (ALc), posterior lamina cribrosa (PLc), and immediate postlaminar regions, a region 1 mm behind the posterior boundary of the lamina cribrosa, and a region 2 mm behind the posterior boundary of the lamina cribrosa before analysis. Temporal, central, and nasal measurements from each of the superior, middle, and inferior sections allowed us to make comparisons among nine regions in the coronal plane (superotemporal, superocentral, superonasal, midtemporal, midcentral, midnasal, inferotemporal, inferocentral, and inferonasal) within each of the regions in the sagittal plane (C). Van Giessen-stained sections (D) were used to divide confocal microscope images (E) for analysis.
adjacent to that for cytoskeleton labeling was used to demarcate each of the laminar regions on the confocal image. After the division of each confocal image into 18 regions, we used a quantitative histogram function to calculate the average pixel intensity per 1 μm² in each of the 18 regions. This was determined by using a sample window of a constant size and sampling an equal number of random points within each region. Only neural tissue was sampled. The mean pixel intensity in each region was then used for statistical analysis. To standardize measurements within each image, we expressed the mean pixel intensity value within each of the 18 regions as a percentage of the mean pixel intensity value within the prelaminar region of that image. This value was defined as the standardized cytoskeletal antibody intensity. Because we had taken measurements from superior, middle, and inferior sections and from temporal, central, and nasal regions of nerve, we were able to make comparisons among nine segments in the coronal plane (superotemporal, superocentral, superonasal, midtemporal, mid-central, midnasal, infrotemporal, inferocentral, inferonasal) in each of plane (superotemporal, superocentral, superonasal, midtemporal, mid-central, midnasal, infrotemporal, inferocentral, inferonasal) in each of the prelaminar, lamina cribrosa, immediate postlaminar, 1-mm postlaminar, and 2-mm postlaminar regions (Fig. 2C).

Quantitation of the DAB COX Reaction. Before quantitation of cytochrome c oxidase activity in the optic nerve head, we divided each COX-stained montage into prelaminar, anterior lamina cribrosa, and posterior lamina cribrosa regions, a proximal 400-μm region of immediate postlaminar tissue, a second region of postlaminar tissue that commenced 1 mm behind the lamina cribrosa and extended 400 μm posteriorly, and a third region of postlaminar tissue that commenced 2 mm behind the lamina cribrosa and extended 400 μm posteriorly. Thus, six regions were analyzed for each section of optic nerve. Van Giesen-stained images were used to delineate each of the laminar regions. With the use of a quantitative histogram function, average pixel intensity per 1 μm² in each of the six regions was calculated by using a sample window of a constant size and sampling an equal number of random points. These values were averaged, and then pixel intensity value for each region was used for statistical analysis. To standardize measurements within each image, we expressed the mean pixel intensity value within each of the six regions along the sagittal as a percentage of the mean pixel intensity value within the prelaminar region of that image. This value was defined as the standardized DAB COX intensity. A darker DAB reaction product and, hence, a greater amount of mitochondrial stain resulted in a lower pixel intensity value during quantitative analysis. We used the inverse of the ratio between the measured value and prelaminar mean in all graphical representations so that pixel intensity value was positively correlated with the amount of mitochondrial stain.

Colocalization of COX, MBP, and NFL. In three eyes we dual stained the middle section of the optic nerve with antibodies to NFL and myelin basic protein. A section adjacent to that used for dual labeling was also stained with DAB COX. This allowed us to study the relationship and distribution of myelin, mitochondria, and neurofilament proteins in each of the laminar regions.

Statistical Analysis

All statistical testing was performed using commercial software (SigmaStat, version 3.1; SPSS, Chicago, IL). Kolmogorov-Smirnov testing was performed on all data before analysis to determine whether data were normally distributed. Normally distributed data were analyzed with ANOVA and post hoc factor comparison performed with a paired Student’s t-test and Bonferroni correction. Non-normally distributed data were analyzed with ANOVA on ranks with the Tukey test used for post hoc paired analysis. Results are expressed as mean ± SE. Two analyses were performed on cytoskeleton and mitochondria data.

The first analysis was performed to determine gross regional differences in cytoskeleton protein and mitochondrial stain among the prelaminar, lamina cribrosa, immediate postlaminar, 1-mm postlaminar, and 2-mm postlaminar segments of the optic nerve. ANOVA was used to assess the effect of individual donor eye, eye (right or left), optic disc side (temporal, central, nasal), section of optic disc (superior, middle, inferior), and optic disc region along the sagittal plane on the parameters standardized cytoskeletal antibody intensity (a separate analysis was performed for each of the six antibodies) and standardized DAB COX intensity.

The second analysis was performed to determine detailed differences within the prelaminar, anterior, and posterior lamina cribrosa regions with regard to cytoskeleton protein and mitochondrial distribution. Critical changes are known to occur in the anterior portion of the optic nerve, and we performed the second analysis to correlate regional cytoskeleton and mitochondrial variation in the anterior optic nerve with previously reported glial, axonal, and connective tissue changes. ANOVA was used for the second analysis with cytoskeleton protein and mitochondria data from prelaminar, anterior lamina cribrosa, and posterior lamina cribrosa regions only.

Results

Eye Donors

The mean age of donors was 46.0 ± 4.2 years (age range, 22–68 years). We examined 11 right eyes and 12 left eyes from a total of 12 male and 2 female donors. Average postmortem time before eyes were enucleated was 11.0 ± 1.0 hours. Mean peripheral prelaminar thickness from all eyes was 714.4 ± 39.4 μm, and mean lamina cribrosa thickness was 540.7 ± 26.4 μm.

Cytoskeleton Antibody Staining

NFH. The intensity of NFH was visibly greatest in the prelaminar and lamina cribrosa regions of the optic nerve head, with a gradual decrease in intensity of stain along the distal course of the nerve (Fig. 3A). Statistical analysis revealed a significant difference in the intensity of stain among the different laminar regions (three-way ANOVA, P < 0.001; Fig. 4A). Subanalysis revealed that intensity was greatest in the prelaminar region and lowest in the region 2 mm distal from the lamina cribrosa. There was no significant difference between 1-mm postlaminar and 2-mm postlaminar regions (P = 0.428). The intensity in the immediate postlaminar region was significantly different from the lamina cribrosa and the 1-mm postlaminar segment (both P < 0.001). We did not find a significant difference between right or left eyes (three-way ANOVA, P = 0.166). There was no significant difference among temporal, central, and nasal nerve bundles (three-way ANOVA, P = 0.096; Fig. 5A). The intensity of stain was different among superior, middle, and inferior sections (three-way ANOVA, P = 0.008; Fig. 5A), with inferior sections having greater stain than middle and superior sections (both P < 0.045; Fig. 5A).

When we compared NFH antibody intensity among the prelaminar region, anterior lamina cribrosa region, and posterior lamina cribrosa region we did not find any differences (three-way ANOVA, P = 0.167; Figs. 6A, 7A). When we analyzed each of the six regions along the sagittal plane of the optic nerve separately, we did not find a significant difference among the nine segments in the coronal plane in any region of nerve (two-way ANOVA; all P > 0.051).

NFHp. NFHp stain was greatest in the prelaminar and lamina cribrosa regions of the optic nerve, with a decrease in stain intensity in the postlaminar regions (Figs. 3B, 4B). There was no difference in stain intensity between the 1-mm and 2-mm postlaminar segments (P = 0.925; Fig. 4B), but the immediate postlaminar region was significantly different from all other regions (all P < 0.003; Fig. 4B). There was a difference among superior, middle, and inferior sections (three-way ANOVA, P = 0.001; Fig. 5B), with superior sections having greater stain than inferior and middle sections (both P < 0.006). There was also a difference among temporal, central, and nasal regions of nerve (three-way ANOVA, P = 0.004; Fig. 5B), with nasal sections having greater stain than the central
the greatest stain visible in the prelaminar region (Fig. 3C). All regions along the proximal-distal course of the optic nerve were significantly different from each other (three-way ANOVA, \( P < 0.001 \); Fig. 4C) apart from the 1-mm versus 2-mm postlaminar segments (\( P = 0.989 \)). Intensity of stain was significantly different among prelaminar, anterior lamina cribrosa, and posterior lamina cribrosa regions (three-way ANOVA, all \( P < 0.002 \); Figs. 6C, 7C). There was no difference among superior, middle, and inferior sections of optic nerve (three-way ANOVA, \( P = 0.164 \); Fig. 5C) or among temporal, central, and nasal regions of the nerve (three-way ANOVA, \( P = 0.272 \); Fig. 5C). When we analyzed each of the six regions along the sagittal plane of the optic nerve separately, we found a significant difference among the nine segments in the coronal plane in the prelaminar and lamina cribrosa regions (two-way ANOVA, both \( P < 0.049 \)).

**NFL.** The intensity of NFL stain was greater in the right eye (three-way ANOVA, \( P < 0.001 \)). Similar to the other neurofilament antibodies, the intensity was greatest in the prelaminar and lamina cribrosa regions of the optic nerve head (Fig. 3D). Statistical analysis revealed that all regions along the course of the optic nerve were different from each other (three-way ANOVA, \( P < 0.001 \); Fig. 4D) apart from the 1-mm versus 2-mm postlaminar segments of the optic nerve (\( P = 0.158 \)). The intensity of NFL antibody was significantly different between the prelaminar and lamina cribrosa regions (three-way ANOVA, \( P < 0.001 \); Figs. 6D, 7D), with the posterior lamina cribrosa region greater than the anterior lamina cribrosa (\( P < 0.001 \)) and the prelaminar cribrosa (\( P = 0.039 \)) regions. There was no difference among central, temporal, and nasal regions of the optic nerve head (three-way ANOVA, \( P = 0.060 \); Fig. 5D) or among superior, middle, and inferior sections of the optic nerve head (three-way ANOVA, \( P = 0.066 \); Fig. 5D). When we analyzed each of the six regions along the sagittal plane of the optic nerve separately, we did not find a significant difference among the nine segments in the coronal plane in the lamina cribrosa and postlaminar regions (two-way ANOVA, all \( P > 0.268 \)).

**Microtubule Associated Protein-1.** Axonal staining with MAP-1 antibody was strongest in the optic nerve head, with a gradual decrease in the intensity of stain along the course of the optic nerve (Fig. 3E). We observed some MAP-1 staining in nonneural tissue within the optic nerve head. This was most evident in connective tissue that composed the laminar plates between nerve bundles (Figs. 3E, 6E). Previous work has shown that antibodies against MAPs have some cross-reactivity to the epitopes of collagen proteins.\(^3\) Within neural tissue, the intensity of MAP-1 stain was significantly different between regions along the course of the nerve (three-way ANOVA, \( P < 0.001 \); Fig. 4E) with the exception of the 1-mm versus 2-mm postlaminar segment (\( P = 0.990 \)). When we compared the different laminar regions, we found that the intensity of stain in the prelaminar region was significantly greater than in the posterior lamina cribrosa region (\( P = 0.040 \); Figs. 6E, 7E), but there was no difference between the prelaminar region and the anterior lamina cribrosa region (\( P = 0.973 \)). The intensity of stain was greater in the right eye than in the left eye (three-way ANOVA, \( P = 0.003 \)). The intensity of stain among the various sides of the optic nerve was different (three-way ANOVA, \( P < 0.001 \); Fig. 5E), with the temporal side having more stain than the central portion of nerve (\( P = 0.014 \)). There was also a significant difference among superior, middle, and inferior sections (three-way ANOVA, \( P < 0.001 \); Fig. 5E), with the inferior portion of nerve having more stain than the superior (\( P = 0.040 \)) and middle portions (\( P < 0.001 \)). When we analyzed each of the six regions along the sagittal plane of the optic nerve separately, we did not find a significant difference among the nine segments in the coronal plane in any region of nerve (two-way ANOVA, all \( P > 0.072 \)).

**NFM.** The intensity of NFM antibody was greater in the right eye than in the left eye (three-way ANOVA, \( P < 0.001 \)). Intensity decreased along the course of the optic nerve, with

**Figure 3.** Montaged confocal microscope images of human optic nerves stained with antibodies to (A) NFH, (B) NFHp, (C) NFM, (D) NFL, (E) MAP-1, and (F) actin. For all cytoskeleton antibodies, the greatest intensity of stain was seen in the anterior portion of the optic nerve. *Dotted lines:* boundaries of the lamina cribrosa. Scale bar, 200 \( \mu \)m.

Comparison between prelaminar and lamina cribrosa regions revealed a difference (three-way ANOVA, \( P < 0.001 \)), with post hoc analysis revealing that the intensity of stain in the anterior lamina cribrosa region was less than in the posterior lamina cribrosa (\( P < 0.001 \)) and the prelaminar regions (\( P = 0.013 \); Figs. 6B, 7B). When we analyzed each of the six regions along the sagittal plane of the optic nerve separately, we did not find a significant difference among the nine segments in the coronal plane in any region of nerve (two-way ANOVA, all \( P > 0.067 \)).

NFM. The intensity of NFM antibody was greater in the right eye in than in the left eye (three-way ANOVA, \( P < 0.001 \)). Intensity decreased along the course of the optic nerve, with...
FIGURE 4. Quantitative analysis of cytoskeleton protein distribution along the course of the optic nerve. Mean standardized pixel intensity (± SE) within each region of the optic nerve for (A) NFH, (B) NFHp, (C) NFM, (D) NFL, (E) MAP-1, and (F) actin are presented. A spline curve was used to connect data points. Pixel intensity values at points along the nerve were normalized and expressed as a percentage of intensity of the prelaminar value. There was no difference in the intensity of stain between the 1-mm and 2-mm postlaminar segments for any antibody (three-way ANOVA, \( P > 0.05 \)). Intensity in the lamina cribrosa region for each antibody is the mean of anterior lamina cribrosa and posterior lamina cribrosa measurements. Mean prelaminar and lamina cribrosa thicknesses were used to determine distances from the inner limiting membrane.
Actin. Actin staining was different between the right and left eyes, with the right eyes having a greater amount of stain (three-way ANOVA, \( P = 0.049 \)). The greatest amount of stain was seen in the lamina cribrosa region of the optic nerve head (Fig. 3F). In addition to staining of neural tissue, we observed actin staining of laminar plates (Fig. 6F). On statistical analysis, the intensity of stain in the different laminar regions of the optic nerve was significantly different (three-way ANOVA, \( P < 0.001 \); Fig. 4F). The intensity of stain in the lamina cribrosa was greater than in all other regions (all \( P < 0.001 \)). The intensity of stain was greater in the posterior lamina cribrosa region than in the anterior lamina cribrosa (\( P < 0.001 \); Fig. 7F) and the...
prelaminar ($P < 0.001$; Fig. 7F) regions. There was no difference between the prelaminar and immediate postlaminar segments ($P = 0.792$) and between the 1-mm and 2-mm postlaminar segments ($P = 0.732$). All other regions were significantly different from each other. There was no difference among superior, middle, and inferior sections of the optic nerve (three-way ANOVA, $P = 0.604$; Fig. 5F). When the temporal, central, and nasal regions of the optic nerve head were examined, we found no significant difference (three-way ANOVA, $P = 0.425$; Fig. 5F). When we analyzed each of the six regions along the sagittal plane of the optic nerve separately, we did not find a significant difference among the nine segments in the coronal plane in any region of nerve (two-way ANOVA, all $P > 0.245$).

Mitochondrial Staining

In all 10 human eyes, the greatest amount of mitochondrial stain was visible in the prelaminar and lamina cribrosa regions of the optic nerve head (Figs. 8A, 8B). Postlaminar regions had significantly less stain than the anterior optic nerve head. On quantitative analysis there was a significant difference in the amount of mitochondrial stain along the length of the optic nerve (two-way ANOVA, $P < 0.001$; Fig. 8C). Post hoc analysis revealed that there was no difference among prelaminar, anterior lamina cribrosa, and posterior lamina cribrosa regions (two-way ANOVA, $P = 0.600$; Fig. 8D); however, these three regions had significantly greater levels of mitochondrial stain than all postlaminar regions (all $P < 0.001$). Although there was a gradual decrease in the amount of mitochondrial stain along the distal course of the postlaminar nerve, there was no significant statistical difference among the immediate postlaminar segment, 1-mm distal postlaminar segment, and 2-mm distal postlaminar segment (all $P > 0.351$; Fig. 8C).

Colocalization of COX, MBP, and NFL

In all three eyes studied, the intensity of NFL proteins was closely correlated with the intensity of mitochondrial stain (Figs. 9A, 9B). NFL staining was greatest in regions where there
were greater amounts of DAB COX stain. Myelination of the human optic nerve began behind the posterior boundary of the lamina cribrosa; however, the onset of myelination was not uniform across the optic nerve (Fig. 9C). Myelination began sharply after the posterior lamina cribrosa boundary in most regions; however, in some regions, the optic nerve was unmyelinated for a variable distance after the posterior laminar boundary, before myelin staining was seen.

NFL and mitochondrial staining were inversely related to myelin staining, demonstrating a dramatic decline in stain after the onset of optic nerve myelination (Fig. 9D). In unmyelinated regions of postlaminar optic nerve, we observed a significantly higher level of NFL and mitochondrial stain than in regions of myelinated postlaminar nerve (Fig. 9D).

**DISCUSSION**

We used a large cohort of normal human optic nerves and examined the distribution of NFH, NFHp, NFM, NFL, actin, and MAP-1 proteins in the prelaminar region, anterior lamina cribrosa (LC), and posterior LC regions. Mean standardized pixel intensity (±SE) in each region for antibodies (A) NFH, (B) NFHp, (C) NFM, (D) NFL, (E) MAP-1, and (F) actin are presented. Differences in stain between the prelaminar and lamina cribrosa regions were found for all antibodies except NFH. **Significant difference with **P < 0.05 as determined by three-way ANOVA with post hoc testing.
cribrosa regions, where more ATP is required for electrical conduction. A decrease in the concentration of NFL and mitochondria correlated closely with the onset of optic nerve myelination. Increasing the concentration of mitochondria and cytoskeleton proteins may therefore be a physiological adaptive mechanism that compensates for the absence of myelin and increases the speed of electrical conduction by increasing axonal diameter and energy supplies within unmyelinated regions of the optic nerve head.

Each one of the cytoskeleton protein subunits plays an important role in strengthening axonal structure and determining axonal caliber.49 NFM increases axonal diameter by forming cross-bridges between adjacent neurofilament proteins and regulating inter-neurofilament spacing.51,52 NFL, however, reduces inter-neurofilament distances, and previous work has demonstrated that an increase in NFL is associated with a decrease in axonal caliber.49 A decrease in NFM proteins associated with a reciprocal increase in NFL proteins between the prelaminar and posterior lamina cribrosa regions may account for the gradual decrease in RGC axonal diameter as it travels from the prelaminar region to the posterior lamina cribrosa region of the optic nerve head.45 The role NFH plays in determining axonal caliber remains controversial. Studies in transgenic mice have shown that NFH can increase or have no effect on axonal diameter.53,54 We have shown that NFH is homogeneously distributed in the prelaminar and lamina cribrosa regions, supporting data from the former study53 and implying that NFH does not play an important role in influencing axonal caliber in the human optic nerve head. We have previously shown that the pressure gradient acting on optic nerve axons is greatest at the lamina cribrosa.17,18 Axonal cytoarchitecture is known to be shaped locally by external compressive forces, and actin proteins play an important role in tolerating cytoplasmic shear stress and in maintaining cellular architecture during states of elevated neural tissue pressure.12,55 This may be one explanation for an increase in actin protein concentration in the anterior and posterior lamina cribrosa regions, in which the pressure gradient acting on axons is known to be greater than on other portions of the optic nerve.17

Glial cell and connective tissue components vary in proportion along the optic nerve and demonstrate a distinctly separate morphologic appearance in the prelaminar, anterior lamina cribrosa, and posterior lamina cribrosa regions.19,39,40 Glial cells and connective tissue elements are intimately linked to cytoskeleton proteins,46 and the heterogeneous distribution of

**FIGURE 8.** Mitochondrial distribution along the human optic nerve was studied using DAB COX stain. Van Giessen-stained images (A) were used to divide DAB COX images (B) into different laminar regions. Mitochondrial stain was visibly greater in the anterior portion of the optic nerve. Quantitative analysis revealed that the amount of stain was significantly greater in the prelaminar and lamina cribrosa regions than in all postlaminar regions (C). A spline curve has been used to connect data points. Pixel intensity values at points along the nerve were normalized and expressed as a percentage of intensity of the prelaminar value. The inverse of pixel intensity was used for graphs so that pixel intensity value was positively correlated with the amount of mitochondrial stain. There was no difference in amount of stain among prelaminar, anterior lamina cribrosa, and posterior lamina cribrosa regions (D). Dotted lines: boundaries of the lamina cribrosa. Scale bar, 200 μm.
these structures is thought to contribute to the asymmetric pattern of optic nerve damage after IOP elevation. Previous work has shown that the earliest changes to axonal transport after an increase in IOP occurs in the superior region of the optic disc. The decrease in the concentration of MAP-1, coupled with an increase in concentration of NFHp at the superior portion of the optic disc, may provide a structural explanation for axonal transport vulnerability in this location.

Although there was marked heterogeneity of stain along the sagittal plane of the optic nerve, there was a relatively homogenous distribution of stain along the coronal plane for most cytoskeleton proteins. NFM, which is a major determinant of axonal diameter, was the only neurofilament protein that demonstrated an uneven distribution of stain among the superior, inferior, middle and temporal, nasal, and central portions of the lamina cribrosa region. This may be reflective of the difference in laminar pore size and axonal diameters in this region.

The postmortem time interval for tissue used in this report ranged from 5 to 16.5 hours. We did not observe a remarkable difference in the pattern and quality of cytoskeletal antibody stain among eyes that were exposed to different postmortem interval times. Our observation was similar to previous work demonstrating that most proteins in the human brain are stable within the postmortem interval range of 5 to 21 hours. Our standardized quantitative analysis, however, demonstrated a consistent difference between right and left eyes for some cytoskeletal components. In this report we were unable to clarify the reason for greater NFM, NFL, MAP-1, and actin staining in the right eye. The optic nerve is injured in an asymmetric fashion in many ocular conditions, including glaucoma, and the increased susceptibility of the ipsilateral optic nerve to injury/protection compared with the contralateral optic nerve may be related to the asymmetric distribution of cytoskeletal elements among nerves. This hypothesis will have to be clarified with further work. Within the human retina the intensity of staining of some cytoskeletal proteins is also asymmetric, with the degree of NFHp staining known to be related to axon size and axon bundle location. The purpose of the present report was to investigate differences in cytoskeletal

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**Figure 9.** Study of the distribution and relationship of neurofilament light (NFL), mitochondrial cytochrome oxidase (COX), and myelin (MBP) in the human optic nerve. Distribution of NFL (A) and COX (B) demonstrated a similar pattern and was most concentrated in the prelamellar and lamina cribrosa regions. Optic nerve myelination (C) began behind the lamina cribrosa, but not all portions of the nerve acquired a myelin sheath immediately after the posterior boundary. The intensity of NFL stain and the onset of myelination demonstrated a reciprocal relationship, with unmyelinated regions of nerve demonstrating a greater amount of NFL stain. Dotted lines: boundaries of the lamina cribrosa. Scale bar, 500 μm.
antibody staining among the major quadrants of the optic nerve head. Our methodology was not designed to specifically study the variation in cytoskeletal stain among axonal populations of different diameters. Histomorphometric studies, however, have previously subdivided the human optic nerve along the coronal plane and have determined the mean axonal diameter in each region of the optic nerve head.\textsuperscript{16,67} Results from these previous studies can be correlated with our results on cytoskeletal antibody stain pattern along the coronal plane to approximate any relationship between cytoskeleton antibody stain intensity and axonal diameter.

This detailed report of the human optic nerve demonstrates that the distribution of cytoskeleton protein subunits is significantly heterogeneous and maintains close relationships with mitochondria, myelin, laminar structures, and environmental tissue pressures. These findings are relevant because they may help improve our understanding regarding glaucomatous optic nerve pathogenesis and may help explain why selective subpopulations of RGC axons have an increased susceptibility to injury after IOP elevation. Although experimental work has demonstrated that significant RGC cytoskeleton changes are induced after an acute rise in IOP,\textsuperscript{23} in a clinical setting, the same pressure elevation does not commonly result in macroscopic optic disc changes and visual field defects that can be diagnosed at the bedside. Clinically evaluating the neuronal cytoskeleton would be a valuable tool in the early detection of RGC axonal injury. Recent developments in ocular imaging have demonstrated that it may be possible to detect early cytoskeleton changes on the basis of their optical properties and birefringence.\textsuperscript{31,35} With further work, correlation among histologic data regarding optic nerve head cytoskeleton proteins and results from axonal imaging techniques may provide a useful clinical avenue for noninvasively detecting early optic nerve axonal injury.

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