Purpose: A reference atlas of optic nerve (ON) retinal ganglion cell (RGC) axons could facilitate studies of neuro-ophthalmic diseases by detecting subtle RGC axonal changes. Here we construct an RGC axonal atlas for normotensive eyes in Brown Norway rats, widely used in glaucoma research, and also develop/evaluate several novel metrics of axonal damage in hypertensive eyes.

Methods: Light micrographs of entire ON cross-sections from hypertensive and normotensive eyes were processed through a deep learning-based algorithm, AxoNet2.0, to determine axon morphological properties and were semiquantitatively scored using the Morrison grading scale (MGS) to provide a damage score independent of AxoNet2.0 outcomes. To construct atlases, ONs were conformally mapped onto an ON "template," and axonal morphometric data was computed for each region. We also developed damage metrics based on myelin morphometry.

Results: In normotensive eyes, average axon density was \(\sim 0.3 \text{ axons/}\mu\text{m}^2\) (i.e., \(\sim 80,000\) axons in an ON). We measured axoplasm diameter, eccentricity, cross-sectional area, and myelin g-ratio and thickness. Most morphological parameters exhibited a wide range of coefficients of variation (CoV); however, myelin thickness CoV was only \(\sim 2\%\) in normotensive eyes. In hypertensive eyes, increased myelin thickness correlated strongly with MGS \((P < 0.0001)\).

Conclusions: We present the first comprehensive normative RGC axon morphometric atlas for Brown Norway rat eyes. We suggest objective, repeatable damage metrics based on RGC axon myelin thickness for hypertensive eyes.

Translational Relevance: These tools can evaluate regional changes in RGCs and overall levels of damage in glaucoma studies using Brown Norway rats.
been examined to assess neuroprotective treatments for glaucoma\textsuperscript{10,11} and effectiveness of gene therapy in optic neuropathy.\textsuperscript{12} However, there are several limitations to the approaches used in these studies: (i) because extensive manual examination or human intervention was required, only small sub-regions across the optic nerve were assessed, which can lead to sampling errors and bias\textsuperscript{13}; and (ii) most studies examined only RGC axon count. Thus there remains a need to improve our understanding of normal and pathological axon appearance.

Here we used AxoNet 2.0, a deep learning-based tool for axon morphometric analysis,\textsuperscript{14} to comprehensively quantify axoplasm and myelin structure in Brown Norway rats, a strain widely used in models of glaucoma. More specifically, we analyze optic nerves from normotensive Brown Norway rat eyes to create a reference atlas of axonal structure. We then quantify axoplasm and myelin sheath morphometric changes in hypertensive eyes and correlate these quantifications with an existing subjective measure of axonal damage, the Morrison damage scale.\textsuperscript{15}

**Methods**

**Rat Optic Nerve Datasets**

We used adult Brown Norway rats with unilateral ocular hypertension induced by magnetic microsphere delivery into the anterior chamber. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Atlanta Veterans Affairs Medical Center and Georgia Institute of Technology and conformed to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Animal handling, tissue preparation and optic nerve imaging procedures are described in detail in the companion article.\textsuperscript{14} All rats in this study were previously used in studies related to neuroprotection in glaucoma induced by scleral stiffening,\textsuperscript{16–20} in which the hypertensive eyes were also exposed to a scleral stiffening agent or a control solution (Hanks’ balanced salt solution) delivered by retrobulbar injection. As noted in our companion article,\textsuperscript{14} the stiffening agents themselves led to axonal loss even in the absence of ocular hypertension.\textsuperscript{18–20} Thus, in the main body of this article, we consider eyes from only Hanks’ balanced salt solution–treated rats to focus on the effects of ocular hypertension. We define three cohorts of animals, denoted Cohorts IV to VI, to avoid confusion with the cohorts described in our companion article.\textsuperscript{14} In the Supplementary material, we also present selected data from animals that received both ocular hypertension and scleral stiffening (Supplementary Figs. S4, S5).

**Cohort IV: Normotensive Rat Eye Atlas Dataset**

Cohort IV was used to create a reference normotensive Brown Norway optic nerve atlas. We used only eyes that, for technical reasons, had optic nerve cross-sections that could be mapped onto a disc using the procedure described below. This cohort contained nine normotensive eyes, and we analyzed light micrographs of the entire optic nerve cross-section from these eyes.

**Cohorts V and VI: Novel Metrics of Axonal Damage**

An algorithm such as AxoNet 2.0 that rapidly and reliably extracts RGC axonal morphometric properties naturally leads to consideration of novel metrics to quantify RGC axonal damage. To evaluate such metrics, we analyzed light micrographs of the entire optic nerve cross-section from normotensive and hypertensive eyes representing a range of glaucomatous insult. Specifically, Cohort V consisted of 34 pairs of Brown-Norway rat eyes in which unilateral ocular hypertension had been induced.\textsuperscript{14} These eyes were previously used in other studies.\textsuperscript{16–21} Cohort VI was a subset of Cohort V, and consisted of 23 rats (33 eyes), again with unilateral microbead-induced ocular hypertension. In this cohort, we discarded eyes in which the optic nerve cross-section could not be mapped onto a disc, as was the situation for some eyes (n = 35) in Cohort IV.

**Axoplasm and Myelin Sheath Morphometric Measures**

The outputs of AxoNet 2.0 are described in detail in the companion manuscript.\textsuperscript{14} Here, for ease of reference, we present example axoplasm segmentations (Supplementary Fig. S1) and relist the derived morphometric properties for each axon:

- Axoplasm cross-sectional area (number of pixels lying within the axoplasm)
- Axoplasm cross-sectional convex area (number of pixels within the convex hull of the axoplasm)
- Axoplasm perimeter (number of pixels forming the border of the axoplasm)
Axoplasm diameter (smallest distance from the centroid of the axoplasm to the outer edge of the axoplasm). This metric was proposed by Quigley et al., who suggested that this measure of axoplasm diameter is less susceptible to distortion because of oblique sectioning of axons.

Axoplasm eccentricity, defined as the ratio of the focal distance divided by the major axis length of the ellipse defined by the bounding box of the axoplasm. The focal distance is defined as the distance between the focal points on the major axis. The focal points are two fixed points on the major axis whose sum of distances from any point on the ellipse is constant.

Myelin g-ratio (ratio between inner and outer diameters of the myelin sheath).

Myelin sheath thickness (smallest distance from inner to outer edge of myelin sheath).

Myelin sheath cross-sectional area (number of pixels within the myelin sheath).

Myelin sheath perimeter (number of pixels forming the outer border of the myelin sheath).

Construction of a Normative Brown-Norway Rat RGC Atlas

We used AxoNet 2.0 to analyze light micrographs of the entire optic nerve cross section from normotensive eyes (Cohort IV). First, all normal-appearing axons in each nerve were segmented, and axonal dimensions were extracted using AxoNet 2.0. Second, the same micrographs were conformally mapped to a predefined template that represented an idealized optic nerve cross-section. Third, axons were assigned to one of the 17 sectors of this template. Fourth, morphometric data were pooled within sectors and then across nerves to construct an atlas for each morphological metric. The following subsections provide more details about each of these steps.

Optic Nerve Template. ON cross-sections were mapped to a predefined, idealized optic nerve template created by subdividing a disc into 17 equal-area regions (Fig. 1). The template regions were aligned according to the anatomy of the right eye, i.e., superior at the top and nasal at the left. Note that the template does not have units such as millimeters or micrometers (i.e., there is no length scale for the template per se). Because optic nerve cross-sections have different shapes and sizes, mapping the micrographs to the template can only take into consideration the anatomical orientation of the nerve. Stated differently: the template allows the individual cross-sectional optic nerve micrographs to be consistently subdivided into regions. Computation of quantities that rely on knowledge of length scales, such as regional axon densities, is carried using each nerve’s original image after the subdivision has occurred.

Mapping Optic Nerve Micrographs to a Template.

Segmented optic nerve micrographs and the corresponding axon morphology output files were individually processed in MATLAB (v. R2019b) using the following steps:

- The segmented optic nerve micrograph was down-sampled to one-quarter the original size using the “imresize” function, due to memory and processing constraints.
- A two-dimensional triangular mesh that overlay the optic nerve cross-section was generated, using the “meshgrid” and “delaunay” functions.
- The image with the overlain mesh was conformally mapped to a unit disk using functions developed by Yueh et al. This mapping is an angle-preserving transformation. We refer to this mapped image as the “conformed micrograph.”
- The conformed micrograph was displayed in a graphical user interface to allow manual selection of the inferior pole and rotation of the micrograph such that the inferior pole lay at the bottom. In brief, an Euler rotation matrix, $R$ was generated

$$R = \begin{bmatrix} \cos(\theta) & -\sin(\theta) \\ -\sin(\theta) & \cos(\theta) \end{bmatrix}$$
where \( \theta \) was the clockwise angle between the selected point and the location of the inferior pole of the template. Then, a simple affine transformation was performed by multiplying the rotation matrix with the Cartesian coordinates of the image. The conformed micrograph was also flipped along the vertical axis if the nerve was obtained from the right eye by multiplying the \( x \) coordinates of each pixel in the image by \(-1\).

The coordinates of the conformed micrograph were mapped to the 17 different regions of the template. Then, each axon was assigned to one of the 17 regions based on the location of its centroid in the mapped domain.

**Pooling Data for Atlas Construction.** After assigning each axon to one of the 17 regions, we computed descriptive statistics (mean, standard deviation and coefficient of variation) for each morphometric property and for each region over the population of normotensive eyes as follows:

- For each morphological property that was computed for individual axons, such as axoplasm cross-sectional area and eccentricity, the morphometric data from all axons within a region was averaged over that region. For each morphometric outcome, this produced a single value per region which was then used to produce region-specific descriptive statistics over the population of eyes.
- For properties that were defined by region, e.g., axon density, we directly computed descriptive statistics over the population of eyes for each region.

**Evaluation of Novel Metrics of Axonal Damage**

Using Cohorts V and VI, we constructed and tested several metrics that we hypothesized might be sensitive indicators of axonal damage. To correlate these metrics with an independent measure of axonal damage, we semi-quantitatively scored the ONs from normotensive and hypertensive eyes using the Morrison grading scale. Nerves were scored from 1 (normal) to 5 (extreme damage) by five experienced scorers using a consistent rubric. Any nerve where there was a significant disagreement (\( \geq 2 \) grading points between scorers) was discussed by all scorers, after which each scorer had the opportunity to adjust their score (although adjustment was not required). The final score for each nerve was the average of the scores from the five scorers. For ease of visualization in some graphs shown below, we rounded the average score to the nearest integral value. The number of animals per hypertensive eye damage score in Cohort V was five, seven, nine, and 13 for rounded scores of 2 to 5, respectively. The corresponding numbers of eyes in Cohort VI, including both hypertensive and normotensive eyes, were nine, six, two, six, and 10 for rounded scores of 1 to 5, respectively.

We considered the following four axonal damage metrics:

**Myelin Sheath Thickness.** The myelin sheath thickens and unravels during Wallerian degeneration. Furthermore, normotensive eyes showed very consistent RGC axonal myelin sheath thickness (see Results), suggesting that myelin sheath thickening could potentially be a sensitive measure of early axonal damage. Thus we explored the potential of “damage metrics” based on RGC myelin sheath thickness. We analyzed 34 pairs of Brown-Norway rat eyes (Cohort V), determining the following 3 quantities:

- The pairwise difference in the mean myelin sheath thickness, defined as the mean myelin sheath thickness in the hypertensive eye minus the mean myelin sheath thickness in the contralateral control eye.
- The myelin sheath thickness averaged over all normal-appearing axons in the entire optic nerve. This was computed for each control eye and separately for each hypertensive eye.
- The myelin sheath g-ratio (myelin sheath inner diameter divided by outer diameter) averaged over all normal-appearing axons in the entire optic nerve. This too was computed for each control eye and separately for each hypertensive eye.

**Preferential Loss of Large RGC Axons.** It is known that large-diameter RGC axons are lost preferentially in glaucoma in primates, a finding we were able to replicate in Brown-Norway rats. We therefore investigated the utility of a damage metric based on the ratio of large to small axons between hypertensive and control eyes in Cohort V. Based on previous results, axons were counted as “large” if their axoplasmic diameter was greater than or equal to 0.8 μm and “small” if their axoplasmic diameter was less than or equal to 0.5 μm. These thresholds were determined based on manual inspection of the distribution of axons sizes in our rats. For each pair of eyes, we determined the ratio of “large” to “small” axons within a nerve, \( X \), defined as

\[
X = \frac{\sum_{i}^{\text{large}} R_i}{\sum_{i}^{\text{small}} R_i}.
\]
Finally, for each animal, we computed a “large-to-small metric”, $L2S$, by dividing $X$ for the hypertensive eye by $X$ for the contralateral control eye, with values less than one indicating preferential loss of large axons in the hypertensive eye. The rationale for creating this metric was the hope that it could sensitively detect RGC axonal loss without relying directly on total axon counts, which can vary substantially from animal to animal.

The four axonal damage metrics and their association with the Morrison damage score were analyzed via a series of regression models. Outcome measures independently calculated for each eye (myelin sheath thickness, myelin sheath g-ratio) were analyzed via generalized linear mixed models. These models included whether the eyes were hypertensive (treatment), the effect of rounded score at each level of treatment (treatment:rounded_score), and the difference between the rounded and real scores (score deviance) as a continuous covariate. Two eyes were obtained from the same animal, accounted for via a random intercept term ($1|id$). Thus the final model used was outcome $\sim$ treatment + treatment:rounded_score + score deviance + ($1|id$). As these outcomes are strictly positive, they were assumed to have Gamma error distributions, and a log link function was used to relate the linear predictors to the mean outcome $\mu$, $\mu=\exp(\beta_0+\beta_1X)$. This model structure led to well dispersed and normal residuals, as judged by the Kolmogorov–Smirnov Test and Pearson Chi-squared test on simulated residuals.

By nesting rounded score within treatment, the model’s fit on this dataset permit hypothesis testing between controls and different levels of damaged hypertensive eyes. It also allows for the estimation of the effect of damage score on outcomes, but only in hypertensive eyes, not in normotensive eyes. We opted for this approach as it more accurately models the experimental design than the full model, where eyes with damage scores between 2–5 were almost entirely within the hypertensive group. Further estimation of how damage score relates to outcomes were thus limited to the hypertensive eyes.

The effect of score on outcome measures within the hypertensive eye only (myelin sheath thickness, myelin sheath g-ratio) and outcome measures that were calculated on a per animal basis (interocular difference in myelin sheath thickness, $L2S$ metric) were analyzed using simple regression models (outcome $\sim$ score). A Gamma error structure and the log link function were used again in all outcomes except interocular difference in myelin sheath thickness, on which an ordinary linear regression was done.

Regression models were calculated using the lme4 (22) package and post-hoc analyses were run using the emmeans (23) package in R (4.1.1). Family error rate of the post-hoc comparisons was controlled via the multivariate $t$ correction.

**Results**

**Axonal Atlas for Normotensive Eyes**

As expected, axons were essentially uniformly spatially distributed across the optic nerve, although we observed a slightly higher axon density near the nasal quadrant in comparison to the temporal quadrant (Fig. 2). The mean axon density was $0.30 \pm 0.08$ axons/μm$^2$, corresponding to $\sim$80,000 axons in a typical rat optic nerve, and the mean axon diameter was $0.69 \pm 0.01$ μm (Fig. 2). This mean diameter is consistent with previous work: $0.80 \mu$m (mean; standard deviation not reported) in albino rats and $0.73 \pm 0.32 \mu$m (mean $\pm$ standard deviation) in grey rats. Axon density negatively correlated with axon diameter ($r = −0.262$, Table).

The average axoplasm eccentricity, axoplasm perimeter, axoplasm cross-sectional area and axoplasm convex area were $0.72 \pm 0.005$, $4.35 \pm 0.06 \mu$m, $1.48 \pm 0.04 \mu$m$^2$, and $1.66 \pm 0.05 \mu$m$^2$ respectively (mean $\pm$ standard deviation; Fig. 3). Therefore axoplasm solidity, defined as the ratio between axonal cross-sectional area and convex area, was $\sim1$, a value consistent with the definition of healthy RGC axons (i.e., those having a homogeneous light-colored axoplasm resembling relatively simple shapes such as ellipses or circles with little/no protrusions). Axoplasm cross-sectional area and convex area were weakly positively correlated with axoplasm diameter ($r = 0.277$, 0.056; Table), as expected, whereas axoplasm perimeter did not correlate with axoplasm diameter ($r = −0.152$).

The average myelin sheath cross-sectional area, perimeter, thickness and g-ratio were $1.14 \pm 0.02 \mu$m$^2$, $9.85 \pm 0.13 \mu$m, $0.37 \pm 0.007 \mu$m, and $0.64 \pm .006$, respectively (mean $\pm$ standard deviation; Fig. 4). This was expected, because healthy axons generally have thin myelin sheaths ($\sim2$-fold difference between axoplasm diameter and myelin sheath thickness). Myelin sheath morphological parameters exhibited a coefficient of variation of only $\sim2\%$ whereas other metrics had larger coefficients of variation. Myelin sheath perimeter and
cross-sectional area were positively correlated with myelin sheath thickness ($r = 0.436$ and $0.713$, respectively; Table), whereas g-ratio was negatively correlated with myelin sheath thickness ($r = -0.841$, Table), as expected.

Importantly, we note that some of these dimensions are slightly larger than reported in our companion article (e.g., mean axoplasm cross-sectional area in this work was $1.48 \mu m^2$ vs. $1.13 \mu m^2$ in the companion article). As discussed in the companion article, we believe that this is due to bias introduced by treatment of “edge axons” when morphometrically analyzing axons from individual subimages of an optic nerve, and therefore morphometric statistics obtained from analyses on entire optic nerves are preferred over analyses on subimages.

**Myelin Sheath Thickness as an Indicator of Axonal Damage**

Because of the low coefficients of variation exhibited by myelin sheath thickness in normotensive eyes and the tendency of the myelin sheath to thicken during Wallerian degeneration, we sought to explore the potential of metrics related to myelin sheath thickness to detect optic nerve damage. We therefore analyzed 34 pairs of Brown-Norway rat eyes with unilateral ocular hypertension (Cohort V), computing the pairwise difference in mean myelin sheath thickness (hypertensive eye $-$ normotensive eye). We observed larger mean myelin sheath thicknesses in hypertensive eyes with larger Morrison damage scores, especially for Morrison scores of 3 to 5 (Figs. 5A, 6A). The situation with grade 2 nerves was less clear, in part because there were only a few pairs of eyes in which the hypertensive eye had a damage score of 2. Although pairwise interocular difference in myelin sheath thickness changed with damage score, the outcomes were not fully separable between rounded damage scores because of the overlap in values at each score (Fig. 5A). However, regression analysis between the pairwise difference in mean myelin sheath thickness and damage score showed a strong correlation ($P < 0.0001$, $R^2 = 0.65$; Fig. 5B), suggesting that the pairwise interocular difference may be able to estimate the degree of damage inflicted on the hypertensive eye.
We also note that there were several pairs of eyes demonstrating a negative pairwise difference between hypertensive and normotensive eyes (Fig. 5A). Further analysis revealed that this was due to a small number of normotensive eyes that had rounded damage scores greater than 1 (see below). Eliminating such animals led to all animals having a positive pairwise difference in mean myelin sheath thickness (Supplementary Fig. S2).

Another way to analyze this data is to consider the mean myelin sheath thickness for each individual eye, rather than computing a pairwise difference in mean myelin sheath thickness. Such an approach again demonstrated that the mean myelin sheath thickness increased with damage score (Fig. 6). The mean myelin sheath thickness was able to differentiate between the damage score 3 ($P < 0.001$), damage score 4 ($P < 0.001$), and damage score 5 ($P < 0.001$) groups with respect to the control group (Fig. 6A). Additionally, mean myelin sheath thickness alone was sufficient to model the damage score ($P < 0.001$; Fig. 6B).

Finally, consistent with the above observations, we observed a decrease in myelin sheath g-ratio with damage score (Fig. 7). Similar to trends of mean myelin sheath thickness with Morrison damage score, mean myelin sheath g-ratio was able to differentiate between the damage score 3 ($P < 0.001$), damage score 4 ($P < 0.001$) and damage score 5 ($P < 0.001$) groups with respect to the control group (Fig. 7A). Mean myelin sheath g-ratio also correlated well with the damage score ($P < 0.001$; Fig. 7B). However, we again note that visual inspection indicates there is overlap between cohorts (i.e., that cohorts are not cleanly separable into rounded damage scores via one or more linear, univariate functions). Further inspection, with classification models, would be required to examine the stratification capabilities of myelin sheath morphometrics.

It has been previously published that contralateral (normotensive) eyes can show effects because of hypertension in the ipsilateral eye, and we were therefore concerned that our normotensive eyes might not represent a truly naïve cohort. Indeed, several of our normotensive eyes showed Morrison damage scores of 2 or above (when rounded to the nearest integer; most, but not all, of these animals had normotensive eye damage scores slightly greater than 1.5). We therefore reasoned that pairs of eyes in which the hypertensive eye showed large Morrison damage scores might show larger morphometric changes in the normotensive eye. To test this hypothesis, we therefore stratified mean myelin sheath thickness and g-ratio in the normotensive eye by the damage score in the contralateral hypertensive eye (see coloring of points in the control eyes in Fig. 6A and Fig. 7A). To our surprise, we did not

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**Table.** Spearman Rank-Order Correlation Analysis Between 17 Regions of Optic Nerve Template for All 10 Morphometric Outcomes

<table>
<thead>
<tr>
<th>Axon Density</th>
<th>Axoplasm Diameter</th>
<th>Axoplasmic Cross-sectional Area</th>
<th>Axoplasmic Convex Area</th>
<th>Axoplasm Eccentricity</th>
<th>Axoplasm Perimeter</th>
<th>Myelin Sheath G-Ratio</th>
<th>Myelin Sheath Thickness</th>
<th>Myelin Sheath Cross-sectional Area</th>
<th>Myelin Sheath Perimeter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.262</td>
<td>0.277</td>
<td>0.922</td>
<td>0.955</td>
<td>0.649</td>
<td>0.632</td>
<td>0.664</td>
<td>0.696</td>
<td>0.951</td>
<td>0.954</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01. The tabulated quantity is Spearman's rank correlation coefficient, ρ, computed using morphometric values in the axonal atlas.
Figure 3. Normative atlases displaying mean (A, D, G, J), standard deviations (B, E, H, K) and coefficient of variation (C, F, I, L) for axoplasm cross-sectional area (A, B, C), axoplasm cross-sectional convex area (D, E, F), axoplasm perimeter (G, H, I), and axoplasm eccentricity (J, K, L) for nine normotensive Brown-Norway rat eyes (Cohort IV). For explanation of abbreviations, see Figure 1.
Figure 4. Normative atlases displaying mean (A, D, G, J), standard deviations (B, E, H, K) and coefficient of variation (C, F, I, L) for myelin g-ratio (A, B, C), myelin sheath thickness (D, E, F), myelin sheath cross-sectional area (G, H, I), myelin sheath perimeter (J, K, L), and myelin sheath eccentricity (M, N, O) for nine normotensive Brown-Norway rat eyes (Cohort IV). For explanation of abbreviations, see Figure 1.
To gain further insight into the correlation between increased myelin thickness and Morrison damage score, we plotted the distribution of myelin thicknesses in eyes of different damage scores (Fig. 8). It is evident that there is a rightward shift in the distribution of myelin sheath thickness with increasing damage score, in addition to a large number of axons with sheaths of c. 0.6 microns in the higher damage score eyes.
Morphometric Analysis of RGC Axons in Rats

Figure 7. Mean myelin sheath g-ratio for control and hypertensive eyes for 34 Brown-Norway rats (Cohort V) between discrete Morrison damage scores (rounded to the nearest integral value) (A) and continuous Morrison damage scores of the hypertensive eyes only (B). Values of groups in panel A were (mean ± SD): 0.625 ± 0.034 for control eyes, 0.623 ± 0.013 for damage score 2 eyes, 0.571 ± 0.015 for damage score 3 eyes, 0.537 ± 0.038 for damage score 4 eyes, and 0.489 ± 0.031 for damage score 5 eyes (**P < 0.001). In panel B, the solid line represents the mean prediction of the model, dashed lines represent the lower and upper bounds of the 95% confidence band. Line of best fit: log(mean myelin sheath g-ratio) = −0.30 ± 0.08*damage score. Refer to Figure 5 for explanation of other graph elements.

Figure 8. Distribution of myelin sheath thicknesses as determined by AxoNet 2.0 in pooled cohorts of normotensive (control) eyes and hypertensive eyes with discrete Morrison damage scores (rounded to the nearest integral value) of 2–5. For each curve, all axons of all eyes within a given category (normotensive eyes, hypertensive eyes with rounded damage scores of 2, 3, 4, and 5) were pooled and used to compute histograms. The histograms were created using 80 equal-width bins between 0 to 1.5 μm (i.e., a bin width of 0.01875 μm).

Preferential Loss of Large Axons as an Indicator of Damage

Motivated by the observation that large axons are preferentially lost in hypertensive eyes, we next considered an alternate measure of axonal loss/damage. We created a “large-to-small” loss metric, \( L_{2S} \), which combined information about the large axon loss rate between paired hypertensive and contralateral control eyes in a way that was relatively independent of total axon counts. Values of \( L_{2S} \) less than one indicate a preferential loss of large axons in the hypertensive eye versus the contralateral control eye. We observed a complex non-monotonic dependence of the \( L_{2S} \) metric on Morrison damage score (Fig. 9A), although mean values were \( \leq 1 \) for all damage scores, consistent with preferential loss of large axons in hypertensive eyes. This non-monotonic behavior persisted even when eliminating pairs of eyes for which the normotensive eye had a rounded Morrison score greater than one (Supplementary Fig. S3). Focusing only on pairs where the hypertensive eye showed more damage (damage scores 3–5), there was a decrease in \( L_{2S} \) with increasing
Figure 9. L2S values (i.e., the ratio of large to small axons [hypertensive/control] vs. degree of damage [n = 34 pairs of eyes, Cohort V]) between discrete Morrison damage scores (rounded to the nearest integral value) (A) and continuous Morrison damage scores (B). As described in the companion article, there is a preferential loss of large axons across all degrees of damage (i.e., all mean ratios are less than 1). Values of groups in panel A (mean ± SD) were: Damage score 2 = 0.54 ± 0.39, Damage score 3 = 0.90 ± 0.50, Damage score 4 = 0.66 ± 0.27, and Damage score 5 = 0.59 ± 0.65. Panel B shows that the L2S metric did not correlate with Morrison damage score. Refer to Figure 5 for explanation of other graph elements.

damage, as expected. However, we again note there was a significant spread in the L2S metric within a given damage score. Additionally, we did not observe a significant correlation between the L2S score and damage score (Fig. 9B).

Atlas of Myelin Sheath Thickness in Hypertensive Eyes

To determine whether there was preferential myelin sheath thickening in certain regions of hypertensive eyes, we also created a myelin sheath thickness atlas for optic nerves having a range of Morrison damage scores from Cohort VI (Fig. 10). Surprisingly, although there was a clear increase in myelin thickness with Morrison damage score consistent with Figures 5, 6, and 7, we did not observe any specific subregions that showed clear preferential changes in myelin thickness.

Discussion and Conclusion

The detection and objective quantification of retinal ganglion cell axonal damage/changes is challenging. Here, we leveraged deep learning-based software, AxoNet 2.0,14 to address this problem in two ways. First, we created a “reference” axonal atlas from light micrographs of optic nerve cross-sections of normotensive Brown-Norway rat eyes to help understand normal axonal morphology and its inherent variability. Second, we developed several novel metrics of RGC axonal damage based on axonal morphometry. The reference atlas displayed trends consistent with previous work based on manual assessment of ONs of normotensive eyes; for example, nerves displayed relatively spatially uniform axonal densities and axonal sizes. Notably, the thickness of the myelin sheath, as assessed by various metrics, was very consistent between eyes and was also fairly spatially uniform.

We also developed several novel metrics based on RGC axon morphometry, testing their ability to detect optic nerve damage. These metrics exploited morphometric measures that exhibited low coefficient of variation in normotensive eyes, specifically those related to myelin sheath thickness. We also developed a metric designed to quantify the preferential loss of large axons known to occur in hypertensive eyes. We observed that myelin sheath thickness and g-ratio strongly correlated with optic nerve damage score (Figs. 5, 6, 7) consistent with observations in the axonal atlas for hypertensive eyes (Fig. 10). Conversely, the L2S metric was less useful in this regard, and we recommend that the L2S metric not be further used.

We have benchmarked AxoNet 2.0’s axon counting performance against manual counts for mice ($R^2 = 0.97$ between automated vs. manual counts) and non-human primates ($R^2 = 0.98$), and have commented on AxoNet 2.0’s segmentation performance in these animals.14 We believe that the methods described in the present article could be translated to other animal models of optic neuropathies (e.g., mice). Although metrics based on myelin sheath thickness were strongly correlated with an independent measure of optic nerve damage (Morrison damage score), they...
Figure 10. Atlases displaying mean (left panels), standard deviations (center panels), and coefficient of variation (right panels) for myelin sheath thickness (in μm) for different Morris damage scores in 33 rat eyes (Cohort VI). No regional differences in myelin thickening were apparent. For explanation of abbreviations, see Figure 1.
were unable to unambiguously stratify nerves into different Morrison damage score levels. However, it must be recalled that there is not an unambiguous (gold standard) metric for optic nerve damage, and the Morrison damage score has some degree of subjectivity associated with it, even for experienced individuals. Conversely, our metrics based on myelin sheath thickness are objective but may be able to more sensitively detect subtle levels of damage, depending on the extent and history of axonal loss. Combining these metrics would be a fruitful area for future research.

We also point out that our algorithm was designed to detect “normal-appearing” axons, for reasons described in our companion article. Thus the deep-learning algorithm and the damage metrics presented in this work should not include information from damaged axons. It is therefore intriguing that we observed an association between myelin thickening and nerve damage score, suggesting the presence of subtle myelin changes even in ostensibly normal-appearing axons from damaged nerves. This is evident in Figure 8: ignoring the peak in myelin sheath thickness around 0.6 μm, there is a clear rightward shift in the distribution of myelin thickness with increasing damage score. This shift is consistent with the development of early myelin changes occurring in a damaged nerve even if the axon itself appears “normal.” The peak around 0.6 μm in Figure 8 is harder to interpret but may indicate more damaged axons that the algorithm nonetheless classified as normal-appearing.

Unilateral ocular hypertension in mice has been shown to lead to optic nerve head glial changes and axonal transport deficits in the normotensive contralateral eye, yet we did not observe evidence of greater damage in the contralateral normotensive eye when the hypertensive eye had a higher Morrison damage score. We believe that this is due to the morphometric outcomes we considered, again recalling that we focused on normal-appearing axons. It does suggest that the use of normotensive eyes from pairs in which the contralateral eye was hypertensive did not bias our results with respect to “normal-appearing” axons (i.e., in the normotensive reference atlas, normal-appearing axons were not influenced by contralateral damage and, hence, likely reflects the situation of normal-appearing axons in optic nerves from naive animals, although confirming this suggestion would require further study using true naive control animals). Of course, we cannot comment on damaged axons because we did not quantify them with AxoNet 2.0.

However, because most studies of glaucoma in animal models will use unilateral ocular hypertension, we suggest that the normotensive atlas that we present is appropriate for most experimental studies of glaucoma. There also exists the possibility of contralateral variation in normotensive optic nerves, which may influence contralateral comparison of damage metrics. We are aware of only one study that briefly mentioned differences in axon counts between contralateral normotensive eyes from three human specimens, but no statistical analysis was performed. Furthermore, we are aware of no studies comparing our morphometric outcome measures between contralateral normotensive eyes. It would be interesting to evaluate and compare these metrics on normotensive eyes obtained from truly naive animals, although we expect such differences to be small because our morphometric outcome measures are obtained by averaging over many axons within a nerve.

We conclude by noting several technical limitations of the reference axonal atlases. Because the atlases are based optic nerve segmentation by AxoNet 2.0, they are also subject to the limitations of this software. Furthermore, there are unavoidable issues of tissue preparation and image acquisition artifacts that can affect the atlas, such as obliquely cut sections and blurred image regions. Another limitation was the use of a relatively simple template registration technique, which restricted us to the use of near-circular optic nerve cross-sections to preserve anatomic mapping. In the future, we suggest incorporating more advanced mapping techniques such as landmark-based nonlinear registration, feature-based image deformation and even artificial intelligence algorithms to develop a more robust pipeline that can create atlases using ONs of any cross-sectional shape.

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