The Effect of Age on Optic Nerve Axon Counts, SDOCT Scan Quality, and Peripapillary Retinal Nerve Fiber Layer Thickness Measurements in Rhesus Monkeys

Brad Fortune¹✉, Juan Reynaud, Grant Cull, Claude F. Burgoyne, and Lin Wang

Discoveries in Sight Research Laboratories, Devers Eye Institute, and Legacy Research Institute, Legacy Health, Portland, OR

Correspondence: Brad Fortune, Discoveries in Sight Research Laboratories, Devers Eye Institute and Legacy Research Institute, 1225 NE Second Avenue, Portland, OR 97232, USA. e-mail: bfortune@deverseye.org

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Purpose: To evaluate the effect of age on optic nerve axon counts, spectral-domain optical coherence tomography (SDOCT) scan quality, and peripapillary retinal nerve fiber layer thickness (RNFLT) measurements in healthy monkey eyes.

Methods: In total, 83 healthy rhesus monkeys were included in this study (age range: 1.2–26.7 years). Peripapillary RNFLT was measured by SDOCT. An automated algorithm was used to count 100% of the axons and measure their cross-sectional area in postmortem optic nerve tissue samples ($N = 46$). Simulation experiments were done to determine the effects of optical changes on measurements of RNFLT. An objective, fully-automated method was used to measure the diameter of the major blood vessel profiles within each SDOCT B-scan.

Results: Peripapillary RNFLT was negatively correlated with age in cross-sectional analysis ($P < 0.01$). The best-fitting linear model was $\text{RNFLT}_{(\mu\text{m})} = -0.40 \times \text{age}_{(\text{years})} + 104.5 \mu\text{m}$ ($R^2 = 0.1$, $P < 0.01$). Age had very little influence on optic nerve axon count; the result of the best-fit linear model was $\text{axon count} = -1364 \times \text{Age}_{(\text{years})} + 1,210,284$ ($R^2 < 0.01$, $P = 0.74$). Older eyes lost the smallest diameter axons and/or axons had an increased diameter in the optic nerve of older animals. There was an inverse correlation between age and SDOCT scan quality ($R = -0.65$, $P < 0.0001$). Simulation experiments revealed that approximately 17% of the apparent cross-sectional rate of RNFLT loss is due to reduced scan quality associated with optical changes of the aging eye. Another 12% was due to thinning of the major blood vessels.

Conclusions: RNFLT declines by 4 μm per decade in healthy rhesus monkey eyes. This rate is approximately three times faster than loss of optic nerve axons. Approximately one-half of this difference is explained by optical degradation of the aging eye reducing SDOCT scan quality and thinning of the major blood vessels.

Translational Relevance: Current models used to predict retinal ganglion cell losses should be reconsidered.

Introduction

Previous studies of both human^{1–6} and nonhuman primate⁷ optic nerves have generally found that there are fewer axons in the eyes of older individuals. In one of the first of such studies, Dolman et al.¹ reported qualitatively that there seemed to be a diminished density of axons in the optic nerves of humans beyond 60 years of age. Then, using manual quantification techniques in a study of 16 human eyes, Balazsi et al.² reported a rate of decline of 5637 axons per year of age

(about 0.32% per year of the intercept). Shortly thereafter, Johnson et al.³ used a computerized image analysis technique to quantify axon numbers in 13 optic nerves from 11 human cadavers and reported a slightly higher rate of 7554 fewer axons per year of age (or $\sim -0.51\%$ of their intercept value). Mikelberg et al.⁵ also used a computerized technique to re-evaluate 12 of the optic nerves from their original study² of 16, limiting this newer study to only those samples with the most adequate tissue preservation, and found a rate similar to their earlier report (4909 fewer axons per year, or -0.37% of their intercept value). In a

larger study, Jonas et al.⁶ also found a similar rate of axon loss with increasing age (5426 fewer axons per year, or -0.5% of the intercept, $N = 22$). In contrast, Repka and Quigley⁴ applied strict inclusion criteria and found a substantially lower rate of loss (only 534 fewer axons per year, or $< -0.1\%$ of their intercept value, $N = 19$). Though the apparent rate of axon loss with increasing age was generally consistent across five of these six studies, it was significantly different from zero in only one of them,⁶ which underscores the fact that the rate derived from cross-sectional studies is generally shallow and difficult to determine given the wide range of variability across the human population at any age.⁴ This point was also made explicitly by Morrison and colleagues⁷ in their study of 28 rhesus monkeys. In that study, Morrison et al.⁷ estimated the rate of axon loss to be 4531 fewer axons per year (or -0.4% of their intercept value) from the monkey optic nerve, which also happened to be not significantly different from zero.⁷ Since the lifespan of the monkey is approximately one-third that of human, Morrison et al.⁷ calculated that the equivalent rate for human eyes would be approximately 1440 fewer axons per year of age, which is substantially lower than the rate reported by most of the other studies on human optic nerves (except Repka and Quigley⁴). Sandell and Peters⁸ also compared a group of six very old rhesus monkeys (average age of 31 years) with a group of seven young monkeys (average age of 7 years) and found a reduction of greater than 44% optic nerve axons in the older animals.

One methodological aspect common to all these previous studies was their use of sampling techniques to estimate the total axon count: typically only 1% to 6% of each optic nerve cross-section was sampled and extrapolated to provide the estimate of total optic nerve axon count. Until recently, it was prohibitive to count every axon in large numbers of optic nerves, but it is known that partial sampling can result in errors when used to project to a total count.^{5,9} In particular, it is possible that changes associated with increasing age, such as altered axon diameter,^{3,4,7} could interact with a limited sampling method to produce a bias resulting in an erroneous estimate of the rate of age-related axon loss. In fact, using our recently developed and validated technique¹⁰ for counting axons in 100% of the optic nerve cross-sectional area, we found that there was no loss of axons with increasing age in a study of 32 healthy eyes from 27 rhesus monkeys.¹¹ This was potentially paradoxical since our initial data in 22 of those rhesus eyes indicated there was a slow, albeit insignificant, decline

with increasing age for retinal nerve fiber layer thickness (RNFLT) measurements made from spectral-domain optical coherence tomography (SDOCT) scans of the peripapillary retina ($-3.7 \mu\text{m}$ per decade, $R^2 = 0.07$, $P = 0.24$).¹¹ This slow rate of RNFLT loss with age was much like what has been reported widely in clinical studies of healthy humans,¹²⁻²⁵ which have nearly all concluded that the rate of loss of RNFLT with increasing age ($\sim 2 \mu\text{m}$ per decade) is consistent with age-dependent loss of optic nerve axons, despite the fact that only one histological study has definitively documented a rate of axon loss with age that is significantly different from zero.⁶ Indeed, very few of the clinical studies have critically evaluated this assumption. How can loss of approximately $2 \mu\text{m}$ per decade of RNFLT occur and be observed so consistently across clinical studies if there is no actual age-related loss of optic nerve axons?

Interestingly, in a study by Rao and colleagues,²⁶ the SDOCT scan signal index was found to be negatively associated with age but not the value of RNFLT as long as scan quality was considered simultaneously in their regression model. Since other studies had also found an association between RNFLT and scan quality,^{21,27-32} it is possible that the apparent effect of age on RNFLT is actually an artifact of age-related changes in the optical characteristics of the anterior eye (perhaps by altering the intensity profile along each A-scan and/or the image segmentation process). Indeed, Rao and colleagues²⁶ reported: "In our study, we found a significant negative relationship between age and most of the SD-OCT measurements in models without signal strength. Inclusion of signal strength in the model revealed an insignificant association between age and RNFLT measurements. The possibility that signal strength is a confounder in the association between age and RNFLT measurements should be considered."

Therefore, with the benefit of available, well-preserved, postmortem optic nerve tissue samples, as well as longitudinal SDOCT measurements of RNFLT in a large number of healthy nonhuman primate eyes, we sought in this study to evaluate the effect of age on optic nerve axon counts, SDOCT scan quality, and peripapillary RNFLT measurements.

Materials and Methods

Subjects

The subjects of this study were 83 rhesus macaque monkeys (*Macaca mulatta*), 66 female and 17 male

(Supplemental Table S1). Their average age ($\pm$ SD) was 10.6 ± 7.1 years, ranging 1.2 to 26.7 years. Their average weight was 6.4 ± 2.1 kg, ranging from 3.3 to 13.9 kg. All aspects of this study were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee (IACUC) at Legacy Health (USDA license 92-R-0002 and OLAW assurance A3234-01).

Anesthesia

All experimental procedures began with induction of general anesthesia using ketamine (15 mg/kg IM) in combination with either xylazine (0.8–1.5 mg/kg IM) or midazolam (0.2 mg/kg IM), along with a single subcutaneous injection of atropine sulphate (0.05 mg/kg). Animals were then intubated and breathed isoflurane gas (1%–2%; typically 1.25%) mixed with oxygen to maintain anesthesia during all imaging procedures. A clear, rigid, gas permeable contact lens filled with 0.5% carboxymethylcellulose solution was placed over the apex of each cornea. Heart rate and arterial oxyhemoglobin saturation were monitored continuously and maintained above 75 per minute and 95%, respectively. Body temperature was maintained at 37°C using a warming blanket.

Peripapillary RNFL Thickness

Peripapillary RNFL thickness was measured using SDOCT (Spectralis; Heidelberg Engineering GmbH, Heidelberg, Germany) as previously described.^{33,34} For this study, the average peripapillary RNFL thickness was measured from a single circular B-scan consisting of 1536 A-scans. Nine to 16 individual sweeps were averaged in real time to comprise the final stored B-scan at each session. The position of the scan was centered on the optic nerve head at the first imaging session and all follow-up scans were acquired in this same location using the instrument's automatic active eye tracking software. A trained technician masked to the purpose of this study manually corrected the accuracy of the instrument's native automated layer segmentations when the algorithm had obviously erred from the inner and outer borders of the RNFL to an adjacent layer (such as a refractive element in the vitreous instead of the internal limiting membrane, or to the outer border of

the inner plexiform layer instead of the RNFL). SDOCT imaging was always performed under manometric IOP control (i.e., 30 minutes after setting IOP to 10 mm Hg in both eyes). Imaging was repeated in “baseline” sessions separated by approximately 1 to 2 weeks, after which unilateral experimental glaucoma was induced in each of these animals for separate studies. Only these baseline sessions were included in the cross-sectional portion of this study on RNFLT (see below): the average number of baseline imaging sessions was 5.4 ± 1.7 , ranging from 3 to 11 with a median of 5.0, resulting in a total of 421 sessions (842 total measures of RNFLT). The SDOCT quality score was greater than or equal to 20 dB for 97.5% of the 842 scans used in this study, greater than or equal to 30 dB for 58.3% of scans, and less than 15 dB for 0.5% of scans (median score was 31 dB).

Analysis and Statistics: Cross-Sectional Study

The average baseline value of peripapillary RNFLT was calculated for each eye for all animals that had a minimum of three baseline imaging sessions ($N = 78$; age ranging from 1.2–26.7 years, average age of 10.7 ± 7.3 years). The peripapillary RNFLT values of $N = 78$ right eyes were compared with the group of $N = 78$ left eyes and the effect of age was evaluated by linear and nonlinear regression in each group separately. All statistical analysis was performed using a commercial software package (Prism 5; GraphPad Software, Inc., La Jolla, CA).

Analysis and Statistics: Longitudinal Study

Linear regression was applied to derive an estimate of the longitudinal rate of RNFLT change for each control eye by deriving the slope of RNFLT versus time. In order to ensure robust results from ordinary least squares regression, we selected from the parent group of 83 animals only those control eyes that had greater than or equal to 8 longitudinal measurements of RNFLT spanning greater than or equal to 2-months duration. This resulted in a subgroup of $N = 68$ control eyes from animals spanning the age range 1.2 to 22.6 years (average age: 10.2 ± 5.8 years). This group of 68 control eyes used for the longitudinal analysis of RNFLT changes was followed for an average of 10 ± 7.4 months (range: 2–36 months; median: 8 months) past the end of the baseline period. This group had an average of 24.4 ± 13.8 RNFLT measurements (range: 9–72; median: 19) including their baseline measurements.

Optic Nerve Axon Counts

Optic nerve tissue samples were available for the healthy control eye of 46 of the 83 animals in this study. This group of 46 animals had an average age at death of 12.0 ± 6.2 years, ranging from 2.7 to 26.1 years. Complete axon counts from the retrobulbar optic nerve were obtained as previously described in detail.^{10,11} Animals were killed under deep anesthesia (either pentobarbital IV or isoflurane inhalation) and tissues were preserved in most cases by perfusion fixation with either 4% paraformaldehyde or 4% paraformaldehyde followed by 5% glutaraldehyde. In six animals, the eyes were immersion fixed in 4% paraformaldehyde immediately after enucleation. A 2- to 3-mm sample of each optic nerve, beginning 2-mm posterior to the globe, was cut with a vibrotome (VT 100S; Leica Microsystems GmbH, Wetzlar, Germany) into 0.5-mm thick transverse sections. Each of these thick optic nerve sections was postfixed in 4% osmium tetroxide and embedded in epoxy resin. Optic nerve cross sections (1- μ m thick) were then cut and stained with p-phenylenediamine and mounted on glass slides. The most complete and uniformly stained section from each optic nerve was then chosen for axon counting. Images covering 100% of the optic nerve cross section were automatically captured using an inverted light microscope (DM IRB; Leica) with an oil immersion $\times 100$ objective (PL Fluotar NA = 1.3; Leica) and custom software for X-Y-Z stage control (Applied Scientific Instrumentation, Inc., Eugene, OR) and image capture. Automated axon counting software was used to count all of the axons with normal morphological characteristics in each optic nerve cross-section. The total axon count for each optic nerve was represented by the sum of all counted axons across all images tiling its entire cross-sectional area; axons falling within the 10%-overlap area between adjacent tiles (10%) were counted only once. The cross-sectional area for each axon counted was recorded and binned into groups of $0.05 \mu\text{m}^2$.

SDOCT Scan Quality

Ordinary least squares linear regression was used to evaluate the association between scan quality score (dB) and age as well as between RNFLT and scan quality score. Multiple linear regression was also applied to evaluate both age and scan quality score as predictors of RNFLT with and without an interaction term.

In a follow-up experiment on the healthy control

eyes of $N = 8$ young animals (average age $\pm$ SD of 3.9 ± 0.4 years), the potential effects of reduced optical quality were evaluated by simulation using scattering filters inserted between the eye and Spectralis objective. The five imaging conditions in this experiment were as follows: (1) a standard peripapillary circular B-scan consisting of the average of $N = 100$ sweeps without any filters introduced; this scan was set as a “baseline” so that the position of all subsequent scans could be matched using the instrument’s eye tracking software, (2) repeat scan without any filters, average of $N = 16$ sweeps, (3) repeat scan without any filters, average of $N = 2$ sweeps, (4) repeat scan, $N = 16$ sweeps averaged, “Eighth White Diffusion” filter introduced (#252; LEE Filters, Burbank, CA), and (5) repeat scan, $N = 16$ sweeps averaged, “Quarter White Diffusion” filter introduced (#251; LEE Filters). For each condition the scan quality score and RNFLT measurement were recorded and evaluated using repeated measures analysis of variance (ANOVA, mixed model). The RNFLT value was derived from the SDOCT scans according to the same standard protocol described above (i.e., the instrument’s automated segmentation was always checked and edited if necessary by a trained, experienced technician). Technicians were masked to the specific purpose of each condition and to the broader goals of the experiment.

Major Blood Vessel Diameter Measurements

An objective, fully-automated method was used to measure the diameter of the major blood vessel profiles within each SDOCT peripapillary circular B-scan included in the cross sectional study of RNFLT. This method was based on measuring the width of the shadows cast by the blood vessels within the RNFL through the deeper retinal layers as follows. The average intensity along each A-scan was calculated for the portion lying between the posterior RNFL and Bruch’s membrane segmentations. Shadows were automatically detected on the basis of log intensity using a fuzzy classifier (see [Supplemental Figure S1](#)). For each scan, we calculated the average diameter both for all vessels larger than $50 \mu\text{m}$ and for the four largest vessels. Those values were then averaged for all baseline scans of each eye. Ordinary least squared linear regression was then used to determine the effect of age on major vessel diameter.

Results

Cross-Sectional Study

Peripapillary RNFLT ranged from 84.9 to 133.1 μm in the group of right eyes (with a mean value $\pm$ SD of $100.2 \pm 9.3 \mu\text{m}$) and from 85.5 to 135.0 μm in the group of left eyes (with a mean value $\pm$ SD of $100.2 \pm 9.6 \mu\text{m}$). Both distributions were positively skewed and leptokurtic ($P = 0.0002$ and 0.0003 , respectively, D'Agostino & Pearson omnibus normality test). There was no significant difference between the median values of right and left eye groups ($P = 0.84$, Wilcoxon matched-pairs rank sum test). Values of RNFLT were strongly correlated between the right and left eyes of each animal (Spearman $R = 0.97$, $P < 0.0001$).

Peripapillary RNFLT was negatively correlated with age in both right eyes (Spearman $R = -0.30$, $P = 0.004$) and left eyes (Spearman $R = -0.33$, $P = 0.001$). A linear model provided a significantly better description of the association between RNFLT and age as compared with either a segmented linear model (Extra sum-of-squares F -test: $F_{2,74} = 0.2$, $P = 0.81$ for right eyes, and $F_{2,74} = 0.2$, $P = 0.80$ for left eyes) or a quadratic model ($F_{1,75} = 0.2$, $P = 0.64$ for right eyes, and $F_{1,75} = 0.1$, $P = 0.71$ for left eyes). Figure 1 shows a scatter plot of RNFLT versus age with values for right eyes plotted as blue circles and left eyes plotted as green triangles. The best-fitting linear model for the group of right eyes was $\text{RNFLT}_{(\mu\text{m})} = -0.39 \times \text{age}_{(\text{years})} + 104.4 \mu\text{m}$ ($R^2 = 0.09$, $P = 0.006$) and for the group of left eyes was $\text{RNFLT}_{(\mu\text{m})} = -0.41 \times \text{age}_{(\text{years})} + 104.6 \mu\text{m}$ ($R^2 = 0.10$, $P = 0.005$). There was no significant difference between right and left eyes in their linear association with age ($F_{2,152} < 0.01$, $P = 0.99$): both the slope ($F_{1,152} < 0.01$, $P = 0.91$) and intercept ($F_{1,153} < 0.01$, $P = 0.98$) were identical for right and left eyes; thus, the pooled relationship between peripapillary RNFLT and age can be described by the equation $\text{RNFLT}_{(\mu\text{m})} = -0.40 \times \text{age}_{(\text{years})} + 104.5 \mu\text{m}$.

Optic Nerve Axon Counts

Consistent with our previous report on a smaller group of animals,¹¹ age had very little influence on optic nerve axon count; the result of the best fit linear model was axon count = $-1364 \times \text{Age}_{(\text{years})} + 1,210,284$ ($R^2 < 0.01$, $P = 0.74$; Fig. 1B). Thus, the best estimate from this data set was a loss of only 1.1% of the intercept value per decade. Assuming an

average axon diameter within the RNFL of 1.0 μm ,³⁵ as well as an average linear to visual angle conversion for the macaque eye of 248 $\mu\text{m}/\text{deg}$ ($0.86\times$ scaling relative to the average human eye, thus, an SDOCT peripapillary circle scan circumference of 9.37 mm), this estimate of 1364 axons lost per year of age converts to an estimate of RNFLT loss of $-1.19 \mu\text{m}$ per decade, which is lower than the rate of $-4.0 \mu\text{m}$ per decade observed for cross sectional data. Conversely, the observed rate of $-4.0 \mu\text{m}$ per decade for RNFLT loss converts to an average axon diameter within the RNFL of 1.87 μm , which is toward the upper end of the range of previous observations.³⁵ Thus, the rate of RNFLT loss with increasing age observed in this cross sectional data set is faster than the age related loss of optic nerve axons, assuming there is no decrease in axon diameter or preferential loss of the thickest axons with increasing age.

In order to evaluate the effect of age on axon size, we first binned the cohort into three groups: younger (age 5.8 ± 2.2 , $N = 16$), middle aged (age 11.6 ± 1.2 , $N = 15$), and older (age 19.1 ± 4.1 , $N = 15$), then used ANOVA to examine whether the frequency distribution of axon size differed across age groups. We observed a significant interaction between age group and axon size ($P < 0.0001$), such that the number of thin axons ($\leq 0.3\text{-}\mu\text{m}^2$ cross-sectional area, or diameter $\leq 0.62 \mu\text{m}$) was larger in young eyes than in middle aged or older eyes and larger in middle aged than in older eyes ($P < 0.01$ for all). Older eyes had a greater frequency than either young or middle aged eyes for axons with a cross-sectional area between 0.3 and $0.55 \mu\text{m}^2$ (diameter between 0.62 and $0.84 \mu\text{m}$, $P < 0.01$ for all). There were no significant differences between age groups for axons with a cross-sectional area greater than or equal to $0.6 \mu\text{m}^2$ ($0.87\text{-}\mu\text{m}$ diameter), though there was a trend toward greater frequency of these thicker axons with increasing age. Using linear regression, we found that mean axon diameter also increased with age ($R^2 = 0.22$, $P = 0.001$). In fact, despite the decreasing total number of axons with increasing age, the total axonal cross-sectional area actually increased significantly with age ($R^2 = 0.12$, $P = 0.02$). Collectively, this pattern can be interpreted as older eyes having lost the smallest diameter axons and/or that the thinnest axons have an increased diameter in the optic nerve of older animals. The findings of increased axon diameter observed in the optic nerve of older animals does not necessarily mean that the axons will also be thicker within the RNFL. Nevertheless, the results of this axon size analysis make it even more difficult to

Figure 1. (A) Peripapillary RNFL thickness plotted versus age for the right eyes (blue circles) and left eyes (green triangles) of $N = 78$ monkeys. The blue line shows the best-fitting linear model for the group of right eyes: $\text{RNFLT}_{(\mu\text{m})} = -0.39 \times \text{age}_{(\text{years})} + 104.4 \mu\text{m}$ ($R^2 = 0.09$, $P = 0.006$) and the green line shows the best-fitting linear model for the group of left eyes: $\text{RNFLT}_{(\mu\text{m})} = -0.41 \times \text{age}_{(\text{years})} + 104.6 \mu\text{m}$ ($R^2 = 0.10$, $P = 0.005$). (B) Optic nerve axon count plotted versus age for healthy control eyes ($N = 46$). The line shows the best fitting linear model: $\text{axon count} = -1364 \times \text{age}_{(\text{years})} + 1,210,284$ ($R^2 < 0.01$, $P = 0.74$). (C) Average SDOCT scan quality score (SQS) plotted versus age for $N = 78$ monkeys. The line shows the best-fitting linear model: $\text{SQS}_{(\text{dB})} = -0.27 \times \text{age}_{(\text{years})} + 33.6 \text{ dB}$ ($R^2 = 0.42$, $P < 0.0001$). (D) Average RNFLT plotted versus signal-to-noise ratio (SNR, i.e., linearized SQS) for $N = 78$ monkeys. The line shows the best-fitting linear model: $\text{RNFLT}_{(\mu\text{m})} = 0.005 \times \text{SNR} + 92.8$ ($R^2 = 0.25$, $P < 0.0001$).

explain the observed rate of RNFLT loss with increasing age on the basis of axon loss alone.

Longitudinal Study

The mean rate of RNFLT change over time was $+0.63 \pm 3.4 \mu\text{m}/\text{y}$ with an interquartile range (IQR) from -1.26 to $2.19 \mu\text{m}/\text{y}$ and a median rate of $+0.25$

$\mu\text{m}/\text{y}$ ($N = 68$), which was not significantly different from zero ($P = 0.23$, Wilcoxon rank sum test). The longitudinal rate of RNFLT change was unrelated to age ($R^2 < 0.01$, $P = 0.66$). Consistent with this result based on applying linear regression to the entire sequence of measurements for each control eye is the fact that the final measurement obtained in each

sequence, which was a median of 8 months later, was not significantly different from the baseline average in this group of 68 control eyes ($P = 0.77$, matched-pairs t -test). The final measurement of RNFLT expressed relative to the baseline average for each individual eye ranged from 0.95 to 1.08 (median of 1.00) with an average value of 1.00 ± 0.036 . This was indistinguishable from the hypothetical value of 1.0 ($P = 0.67$, one-sample t -test, under the assumption that there had been no change during the period from baseline to the final measurement). The final baseline-relative value of RNFLT was also unrelated to age ($R^2 < 0.01$, $P = 0.87$). The lower bound of the 95% confidence interval (CI) of the mean longitudinal rate was $-0.19 \mu\text{m}/\text{y}$, which was half the rate observed in the cross-sectional study ($-0.40 \mu\text{m}/\text{y}$), and which did not overlap with the upper bound of the cross-sectional rate estimate ($-0.21 \mu\text{m}/\text{y}$). Thus, taken together, the results of the longitudinal analysis do not provide consistent evidence of declining RNFLT with increasing age.

However, the power to detect a very small amount of age-related RNFLT change was limited for the longitudinal study. For example, given longitudinal measurement noise of $\pm 7\%$ ^{33,34,36} and $N = 68$ eyes, the power to detect an average RNFLT loss of $1 \mu\text{m}$ at the final longitudinal follow-up was 76%, while the power to detect $0.33\text{-}\mu\text{m}$ loss (equivalent to the cross-sectional rate of $-0.4 \mu\text{m}/\text{y}$ for the average longitudinal duration of 10 months) was limited to just 23%. Thus, while the longitudinal results offer some evidence of stability in control eyes, they are also consistent with the slow rate of decline ($0.4 \mu\text{m}/\text{y}$) observed in the cross-sectional study (i.e., the longitudinal results offer some evidence that the cross-sectional rate is not likely to be much faster than observed here).

SDOCT Scan Quality

Given that the cross-sectional estimate of age-related loss of RNFLT exceeded what might be explained by the loss of axons alone and also exceeded the estimate derived from longitudinal analysis, we sought to determine whether other effects of age, or variables that were highly correlated with age, were influencing the cross-sectional estimate of declining RNFLT. For example, other investigators have reported that OCT scan quality is reduced in older healthy human eyes²⁶ and that reduced OCT scan quality is associated with thinner measurements of RNFLT^{21,27-30} and image segmentation errors.³⁷

Consistent with these earlier reports, we found an

inverse correlation between age and scan quality score (Fig. 1C, Pearson $R = -0.65$, $P < 0.0001$). We also found a significant association between RNFLT and linearized scan quality score (i.e., signal-to-noise ratio [SNR] $R^2 = 0.25$, $P < 0.0001$, Fig. 1D; for Spectralis SDOCT scans, the scan quality score in dB is a transform of SNR according to the equation: scan quality score = $10 \times \log_{10}[\text{SNR}]$, personal e-mail communication with Heidelberg Engineering, September 2013). When age and scan quality score were both used as predictors in a multiple linear regression model to explain RNFLT, only scan quality score was a significant predictor ($P = 0.008$ for scan quality score, $P = 0.44$ for age). When an interaction term was added to the model, it was not significant and again, only scan quality score was a significant predictor ($P = 0.01$ for scan quality score, $P = 0.27$ for age, $P = 0.21$ for interaction).

These results could be explained by one possibility whereby scan quality is a powerful surrogate of age, but otherwise does not directly influence measurements of RNFLT. For example, if increasing age causes both a decline in the number of optic nerve axons and a corresponding, proportional loss of RNFLT along with alterations in the eye's optics that result in lower scan quality, then the scan quality could be strongly correlated with RNFLT, but otherwise have no direct bearing on the measurement of RNFLT. Conversely, it is possible that scan quality exerts a strong independent influence on measurements of RNFLT. For example, it is possible that the reduced optical quality of the aging eye alters the faithfulness with which the scan represents the anatomy (the true signal) such that a bias toward thinner measurements of RNFLT is introduced for scans with lower quality, perhaps by affecting image segmentation algorithms (automated or manual procedures). To address this question, we carried out the simulation experiment described in the Methods section. In a group of healthy eyes of younger animals ($N = 8$), we first recorded a reference scan consisting of 100 sweeps averaged along the standard peripapillary circular path. Then we reduced the number of sweeps averaged in subsequent scan conditions to 16 and 2, which should reduce the SNR of the average images upon which the segmentation algorithm operates by $2.5\times$ and $7\times$, respectively. Then we repeated the 16-sweep recording after introducing simulated cataracts (mild and moderate) between the eye and instrument objective.

Scan quality was successfully manipulated as intended in this experiment: Figure 2A shows that

Figure 2. (A) SDOCT scan quality score plotted for each of the five experimental conditions: baseline average of 100 sweeps (BL, Ave = 100), followed by two conditions with reduced signal averaging (Ave = 16 and Ave = 2 sweeps), followed by simulated mild (eighth diffusion) and moderate (quarter diffusion) cataracts. Box plots represent the median and interquartile range and horizontal hash marks represent the extremes of each distribution of values. (B) RNFLT plotted for each of the follow-up scan conditions as percent change from baseline values (mean baseline RNFLT $\pm$ SD was $104.6 \pm 8.7 \mu\text{m}$, $N = 8$ eyes of 8 young animals).

scan quality score was significantly reduced from the baseline condition mean value of 37.1 ± 3.6 dB to 24.8 ± 4.8 dB under the “mild” cataract simulation (eighth diffusion filter, $P < 0.0001$) and became significantly worse under the “moderate” cataract simulation (quarter diffusion filter, 14.9 ± 5.6 dB, $P < 0.0001$). As expected, there was no significant change from baseline in the underlying SNR for the

recordings obtained with reduced sweep averaging (note, the Spectralis reports the SNR averaged over the duration of the recording, not the SNR apparent in the final stored image, which theoretically would have been reduced to ~ 33 and ~ 29 dB, respectively). Figure 2B shows that RNFLT measurements were significantly affected by reduced SNR ($P = 0.02$, Friedman nonparametric ANOVA for repeated measures), particularly when induced by the simulated moderate cataract. The median change from baseline RNFLT for the four follow-up conditions, were -1.0% , -1.4% , -1.9% , and -2.8% , respectively ($P = 0.08$, 0.01 , 0.02 , 0.008 , respectively, Wilcoxon rank sum test, one-sample t -test). Thus, the mild cataract condition, which decreased SNR in this group of young eyes to approximately 25 dB, close to the average observed for the oldest animals in the cross sectional analysis, resulted in a RNFLT reduction of approximately 2% (95% CI: -1% to -3%). The actual RNFLT loss observed over this same age span in the cross-sectional analysis was approximately 12%, so the artifact due to reduced optical quality of the aging eye contributes approximately one-sixth, perhaps as much as one-quarter, of the total aging effect. Figure 3 shows an individual example of the simulation experiment results.

Major Blood Vessel Diameter Measurements

An objective, fully automated segmentation technique reliably detected the shadows cast by blood vessels through the deeper layers of the retina (see Supplemental Figure S1). There were an average of 7.8 ± 1.5 vessels detected in each eye with a diameter wider than $50 \mu\text{m}$ (IQR: 7.6 – 8.8). Their average diameter across all eyes was $88.7 \pm 8.3 \mu\text{m}$ (IQR: 88.3 – $92.5 \mu\text{m}$). Assuming round profiles, the total cross-sectional area of major vessels within each B-scan had an average value of $47,669 \pm 8572 \mu\text{m}^2$ (IQR: $46,831$ – $52,807 \mu\text{m}^2$), which corresponds to $5.5 \pm 0.9\%$ (IQR: 5.3 – 6.0%) of the total RNFL cross-sectional area. Given that this detection method reflects the vessel lumen diameter, two pixels were added to each radius ($11.3 \pm 0.6 \mu\text{m}$) to account for the vessel wall thickness,³⁸ which resulted in a total cross sectional vessel area of $75,293 \pm 13,266 \mu\text{m}^2$ (IQR: $73,939$ – $82,281 \mu\text{m}^2$), or $8.7 \pm 1.4\%$ of the total RNFL cross-sectional area, consistent with values previously reported for 12° circular peripapillary B-scans in rhesus monkey eyes.³⁹ The average diameter of the four largest vessel profiles in each eye was $109.8 \pm 10.7 \mu\text{m}$ (IQR: 109.3 – $115.3 \mu\text{m}$).

There was a significant decline with age for both

Figure 3. Individual example of simulation experiment results. The SDOCT scan recorded for each of the five experimental conditions is shown in the *left column* of panels ([A] baseline average of 100 sweeps; [B] average of 16 sweeps; [C] average of two sweeps; [D] average of 16 sweeps with mild cataract simulation; [E] average of 16 sweeps with moderate cataract simulation) without image segmentations. The SQS is shown in the lower *right corner* of each panel. The *right column* of panels (A'–E') shows the same B-scan images with segmentations included; the value of RNFLT is shown in the lower *right corner* of those panels.

the average diameter of all vessels wider than 50 μm (Pearson $R = -0.29$; $P = 0.0002$; diameter [μm] = $92.2 \mu\text{m} - 0.33 \times \text{age} [\text{years}]$; [Supplemental Figure S1](#)) and the average of the four thickest vessels in each eye (Pearson $R = -0.22$; $P = 0.005$; diameter [μm] = $113.3 \mu\text{m} - 0.33 \times \text{age} [\text{years}]$, not shown). There was no significant change with age for the number of detected vessels ($P = 0.10$) or the percent of total RNFL cross-sectional area made up by vessel profiles ($P = 0.57$). These results indicate that age-related thinning of the major retinal vessels contributes directly to the apparent age-related decline of RNFLT, the former accounting for 11.6% of the latter.

Discussion

The results of this study provide an estimate for the rate of RNFLT decline with age in rhesus monkeys that is comparable with other, similar cross-sectional clinical studies in humans. The life span of rhesus monkeys is approximately 2.5 to 3.5 times shorter than humans, so the rate observed in

this study, $-4.0 \mu\text{m}$ per decade, converts to a human equivalent rate of -1.1 to $-1.6 \mu\text{m}$ per decade, which is slightly lower than the rate of about $-2 \mu\text{m}$ per decade reported in most cross-sectional clinical studies in humans.¹²⁻²⁵

Of note was that a simple, direct geometric relationship between axon count and RNFLT was insufficient to reconcile their respective rates of decline with age as derived from cross-sectional data. The rate of RNFLT loss was approximately 3 times faster than that predicted by axon loss, which is opposite to the relationship predicted by previous models.^{40,41} The previous modeling studies began with the premise that the age-related rate of axon loss evident in the published literature seemed higher than the rate of loss of RNFLT when both were expressed as a percentage of their linear intercept (i.e., extrapolated values at birth).^{40,41} Thus, in order to reconcile the apparent rate difference, along with the rate of retinal ganglion cell loss estimated from age-related loss of visual sensitivity, these investigators postulated that there must be an age-related progressive increase in the nonneural portion of RNFLT (i.e.,

a progressive decrease in the density of axons within the RNFL, which becomes partially offset by an increase of nonneural tissue, presumably glia).^{40,41}

One aspect of the rationale for the model developed by Hawerth and Wheat,^{40,41} specifically, that the age-related loss of retinal ganglion cell numbers predicted from visual sensitivity is steeper than predicted by loss of RNFLT, relies on the unlikely assumption that age-related loss of visual sensitivity is due solely to loss of retinal ganglion cells. The other aspect of their rationale was that the rate of axon loss was thought to be faster than the rate of RNFLT decline. In contrast to this premise, here we find that the rate of axon loss (1364 fewer axons for each monkey year of age) predicts a rate of RNFLT loss that is approximately three times slower than the rate actually observed in the cross-sectional data ($-0.4 \mu\text{m}$ per year).

The rate of axon loss found in this study is slower than all but one⁴ of the previous estimates for human or monkey,^{2-5,7,8} which is likely due to the key methodological difference, whereby we counted 100% of the axons in each optic nerve but the earlier studies sampled only 1% to 6% of the axons in each optic nerve. Projecting total counts on the basis of a limited sampling strategy is likely to result in underestimates for older eyes if there is a decrease in axon density with age that is in any way inhomogeneous across the optic nerve, such as reported by Sandell and Peters⁸ (Supplemental Figure 2). The older monkeys studied by Sandell and Peters⁸ were also limited to very old animals (average age of 31 years, human equivalent age of 93 years), which may mean their results represent accelerated loss of axons beyond the age of the oldest animal in our study (27 years). Alternatively, projection underestimates could also result from selective loss of larger diameter fibers and/or shrinkage. However, we found evidence of the opposite phenomenon in this study, consistent with the tendency reported in four other studies.^{3-5,7} This study was also based on a sample ($N=46$ control eyes of 46 monkeys) that is approximately 2 to 4 times larger than previous studies.^{2-5,7} Thus, the evidence reported here suggests that the age-related rate of optic nerve axon loss reported in earlier studies that sampled only a small portion of the optic nerve are likely over-estimates (compared with Repka and Quigley).⁴ Moreover, the data from the present study are not consistent with a model whereby the axon density within the RNFL declines with age. In contrast, the cross-sectional data indicate that the rate of RNFLT loss is actually an over-estimate of

axon loss, suggesting that factors other than axon loss contribute to the apparent loss of RNFLT with age.

Consistent with these findings, Kim and colleagues⁴² studied whole-mount retinæ and found no loss of RGCs in old rhesus monkeys (age 24.4–27.8 years, $N=6$) as compared with young monkeys (age 4.8–12.4 years, $N=7$). In a similar study of human retinæ, Curcio and Drucker⁴³ reported significant loss of RGCs in older eyes only near the fovea, which they estimate would result in less than 7% loss for the total RGC count over a span of 40 years and correspond to predominantly thinner axons of the papillomacular bundle.

We did not find evidence of any age-related loss of RNFLT using the longitudinal data. This might be due to the limited follow-up duration, the longest of which was 72 measurements spanning 3 years. To our knowledge, there are only two other longitudinal studies of RNFLT in healthy eyes published to date. O'Leary et al.⁴⁴ followed 33 eyes of 33 healthy volunteers for a median of 3 years and six exams, measuring RNFLT every 6 months by time domain OCT. They reported a longitudinal rate of change for RNFLT that was actually positive ($0.6 \mu\text{m}/\text{y}$), though not significantly different from zero.⁴⁴ This rate was derived from a linear mixed-effects model that included baseline age and RNFLT, thus the rate observed during follow-up is adjusted for effects of these baseline variables. O'Leary et al.⁴⁴ did not report the observed change (unadjusted) for RNFLT over time.

In a similar study, Leung et al.⁴⁵ reported a cross-sectional rate of $0.33 \mu\text{m}/\text{y}$ based on 200 eyes of 100 healthy control subjects. In their study, Leung et al.⁴⁵ also randomly selected 35 subjects for longitudinal follow-up, measuring RNFLT by SDOCT every 4 months for an average of 30 months. Though Leung et al.⁴⁵ found that this group of 70 eyes lost an average of only $1.08 \mu\text{m}$ from baseline to final follow-up (consistent with a longitudinal rate of $0.43 \mu\text{m}/\text{y}$), they also used a linear mixed-model to adjust for other variables, of which they found baseline RNFLT to be most important. After adjusting for baseline RNFLT, they reported an average longitudinal rate of $0.52 \mu\text{m}/\text{y}$ and concluded that “a greater baseline RNFL thickness was associated with a faster rate of change.”⁴⁵ However, it was not stated whether the baseline exam used in the model was also included in the longitudinal series, which would have been an error in that a thick initial RNFLT would bias toward a more negative apparent slope. A straight-forward simulation of injected noise in to a series of otherwise

constant measurements will demonstrate that the apparent rate is inversely related to the apparent baseline (real signal plus noise) when the latter is included in the calculation of the former, but unrelated when the first measurement is not included in the calculation of slope. Therefore, if Leung et al.⁴⁵ included the same initial observation as both the “baseline” and the first measurement within the longitudinal series, then their conclusion about its significance as a strong predictor of subsequent rate would be erroneous. In any case, it is likely that the estimates of longitudinal rate derived from their model are exaggerating the true rate of change. Indeed, the rate predicted by the Leung et al.⁴⁵ model for an average healthy eye (i.e., an eye beginning with an RNFLT of 100 μm would lose $-0.62\%/y$), suggests that the entire dynamic range of RNFLT would be lost within a lifetime even without pathology.

The results of the present study further indicate that declining optical quality of the aging eye is likely to affect OCT scan quality in a manner that alters the representation of true RNFL tissue thickness within B-scan images, influencing the segmentation process to produce thinner measurements of RNFLT, as Rao and colleagues²⁶ hypothesized. We found that the scan quality artifact is responsible for a small portion ($\sim 10\%$ – 25%) of the age-related decline in RNFLT. This result is also consistent with the findings of other investigators who used attenuating filters (without a scattering component)³² or refractive error to corrupt scan quality,^{27,30} though the latter approach could also include an influence on RNFLT due to changes in lateral magnification.^{39,46} These results are also consistent with changes in scan quality and RNFLT following cataract extraction.^{31,32} Other factors potentially contributing to the discrepancy between rates of axon loss and RNFL thinning with age might include the possibility that RNFL tissue reflectance declines with age (see [Supplemental Figure 3](#)) and that the caliber of major retinal blood vessels declines with age,⁴⁷ which might also influence segmentation algorithms and apparent thickness.^{48,c.f.38} Recently developed techniques might prove useful for addressing these questions.^{39,49,50} In fact, one recent study by Patel and colleagues found that thinning of the major retinal blood vessels with increasing age accounts for approximately 12% of the apparent aging effect for RNFLT (Patel, Nimesh and Harwerth, Ron, personal e-mail communication, January 2014). Our estimate (11.6%) was very close to their value, despite the fact that our automated vessel segmentation is based on detecting vessel shadows and thus is likely a

measurement of lumen diameter, whereas Patel et al.³⁹ measure the entire (outer) vessel diameter. We also limited our analysis to only vessels with a diameter greater than 50 μm . Another study has reported thickening of vessel walls with age,³⁸ which could offset some of the effect observed in our study.

In summary, this study found that the loss of optic nerve axons with age does not provide a geometrically sufficient explanation for the age-related rate of RNFLT decline. In fact, the prediction based on optic nerve axon loss was approximately one-third the rate observed in cross-sectional RNFLT data. A portion ($\sim 15\%$) of this difference is likely due to an artifact produced by optical degradation as eyes age ($\sim 15\%$ of the apparent aging effect, or $\sim 23\%$ of the discrepancy). Another considerable portion ($\sim 12\%$) of the apparent aging effect ($\sim 18\%$ of the discrepancy) is due to thinning of the retinal vessels with age, which leaves approximately one-third the overall effect unexplained. Nevertheless, cross-sectional estimates of the decline in RNFLT with age derived from SDOCT scans appear to over-estimate both axon loss and the actual rate of decline in RNFLT. The results further indicate that current models used to predict retinal ganglion cell losses should be reconsidered.^{51–56}

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