

Tracking Dendritic Shrinkage of Retinal Ganglion Cells after Acute Elevation of Intraocular Pressure

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PURPOSE. To investigate dendritic changes of retinal ganglion cells (RGCs) and the rate of dendritic shrinkage after retinal ischemia induced by acute elevation of intraocular pressure (IOP).

METHODS. After elevating the IOP to 110 mm Hg for 30, 60, 90, and 120 minutes, a confocal scanning laser ophthalmoscope (CSLO) was used to serially image the retinas of the Thy-1 YFP transgenic mice in vivo for 1 to 3 months. Dendritic and axonal arborizations of 52 RGCs were visualized and followed longitudinally. Dendritic field, dendritic branching complexity (modified Sholl analysis), axonal diameter, and cell body area were measured. A total of 426 longitudinal measurements of dendritic field and dendritic complexity were analyzed for estimation of rate of change with linear mixed modeling.

RESULTS. There were no morphologic changes of RGCs after 30 ($n = 12$) or 60 ($n = 12$) minutes of ischemia. After 90 minutes of ischemia ($n = 19$), 78.9% of RGCs showed progressive loss of dendrites, axon, and cell body, 5.3% had only mild reduction of branching complexity and shrinkage of dendritic field whereas 15.8% showed no morphologic changes. All RGCs lost dendritic and axonal arborizations after 120 minutes of ischemia ($n = 9$). The rates of reduction of dendritic field were 11.7% per day (95% confidence interval, 5.0%–18.4% per day) after 90 minutes, and 15.1% per day (10.3%–19.9% per day) after 120 minutes of ischemia.

CONCLUSIONS. RGCs demonstrated dendritic shrinkage after 90 to 120 minutes, but not after 30 to 60 minutes of ischemia. In vivo imaging of dendritic changes could provide a sensitive approach to measure the rate of dendritic shrinkage after acute IOP elevation. (*Invest Ophthalmol Vis Sci.* 2011;52:7205–7212) DOI: 10.1167/iovs.10.6868

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Acute angle closure and retinal vascular occlusion are common forms of retinal ischemic insults encountered in clinical practice. Loss of retinal ganglion cells (RGCs) and impairment of visual function are inevitable if the duration of ischemia is prolonged. Understanding the time course of neuronal degeneration after ischemic injury is thus pertinent to identifying a potential window for therapeutic intervention. In experimental investigation, a conventional model to induce retinal ischemia is to increase the intraocular pressure (IOP) above the retinal perfusion pressure (typically above 90 mm Hg in mice and rats).^{1–3} Damage is examined by counting the RGC number in a retinal whole mount after retrograde labeling with a neuronal tracer. This approach, however, only allows cross-sectional analysis of RGC survival at a specific time point after the injury. Using a blue-light confocal scanning laser ophthalmoscope (CSLO) to serially image the Thy-1 CFP mice (a transgenic mouse model with retinal neurons intrinsically labeled with cyan fluorescent proteins under the control of the Thy-1 promoter) in vivo, we observed variable loss (14.5%–79.5%) of RGCs after elevating the IOP to 115 mm Hg for 90 minutes.⁴ Notably, there was no detectable reduction in the number of RGCs when the duration of ischemia was shortened to 45 minutes. Yet, blue-light CSLO imaging on the Thy-1 CFP mice could not examine axonal and dendritic arborizations of individual RGCs. We recently developed an imaging model that allows measurement of dendritic and axonal changes in Thy-1 YFP transgenic mice.⁵ Because of strain-to-strain variation in transgene expression,⁶ less than 1% of RGCs are labeled in the Thy-1 YFP mice. The relatively low density of yellow fluorescent protein (YFP)-positive RGCs in the retina of these mice facilitates visualization and quantitative analysis of dendritic arborization. We hypothesized that dendritic shrinkage would be an early sign of RGC dysfunction and might be evident even after a relatively short duration of ischemia. In this study, we applied a CSLO to image and follow the changes of axonal and dendritic arborizations of RGCs after 30, 60, 90, and 120 minutes of ischemia, and the rates of dendritic shrinkage were measured respectively.

METHODS

Animals

Three- to six-month-old transgenic mice in which Thy-1 promoter sequences drive expression of the enhanced YFP, designated B6.Cg-TgN (Thy1-YFP)16Jrs, were generated by Feng and associates.⁶ As only a small proportion of RGCs express fluorescent protein, detailed dendritic and axonal structures could be distinctively recognized. Breeding pairs of Thy1-YFP16Jrs mice were obtained from the Jackson laboratory (Bar Harbor, ME). The environment was kept at 21°C with a 12-hour light and 12-hour dark cycle. All mice were fed ad libitum. Animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In Vivo CSLO Imaging

An illuminating wavelength of 488 nm was used to image the Thy-1 YFP mice (HRA2; Heidelberg Engineering, GmbH, Dossenheim, Germany). A 55° wide field lens was mounted to the camera to increase the field of view of the fundus. The scan rate of the CSLO was 16 frames per second. Eye-tracking (a retinal recognition technology enabling the same retinal location being “locked on”) was activated during imaging. Fifteen images at the same retinal location at the same focal depth were captured. These images were averaged automatically by the built-in software to augment the signal-to-noise ratio and simultaneously displayed on a computer screen. Each retinal frame represents approximately 40% of total retinal area at an optical resolution 5 μm to 10 μm . The optical resolution is limited by the numerical aperture of the optical system, which is given by the beam diameter at the pupil and the focal length of the eye. The digital pixel resolution is 5 μm per pixel (high-resolution mode) and 10 μm per pixel (high-speed mode). A 10-second rest interval was provided for every 15 seconds of imaging to allow eye blinking and to keep the corneal surface moist.

Ischemic-Reperfusion Injury with Acute Intraocular Pressure Elevation

Retinal ischemia was induced by acute elevation of intraocular pressure. After anesthetizing the mice (aged 3 to 6 months) by intraperitoneal injection of ketamine (100 mg/kg, Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (9 mg/kg, TranquiVed; Vedco, Inc., St. Joseph, MO), the anterior chamber was cannulated with a fine glass pipette connected by polyethylene tubing to a reservoir containing phosphate buffered saline. The intraocular pressure was raised to 110 mm Hg by elevating the reservoir for 30, 60, 90, and 120 minutes. Leakage was constantly monitored under an operating microscope during the experiment (leaks can be easily detected as dripping and wetting). A tight seal was formed between the glass pipette and the cornea in all animals. No leakage was observed. The glass pipette was then removed. Topical antibiotic and steroid ointment (TobraDex; Alcon Laboratories, Inc., Fort Worth, TX) was applied to the conjunctival sac.

Confocal Laser Scanning Microscopy on Retinal Whole Mount

Mice were anesthetized as described and exsanguinated by perfusion with oxygenated mammalian Ringer's solution containing lidocaine hydrochloride (0.1 mg/mL, Xylocaine; Astra USA, Inc., Westborough, MA) and heparin sodium (500 U/mL, Heparin; Elkins-Sinn, Inc., Cherry Hill, NJ). Transcardial perfusion was then continued with fixative (approximately 20 mL 4.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4). Retinas were dissected after identifying the inferior area of the retina by the inferior position of the ophthalmic artery. They were placed in fixative for 3 hours, rinsed in 0.1 M phosphate buffer, and then mounted flat on a glass slide. Retinas were mounted on glass slides with coverslips (Fluoromount-G; SouthernBiotech, Birmingham, AL). The RGCs were imaged at magnification 20 \times by a confocal laser scanning microscope (Nikon C1 Confocal system, Tokyo, Japan).

Immunohistochemistry

The retina was incubated in a blocking solution containing 10% normal goat serum (NGS), 2% bovine serum albumin, and 0.5% Triton X-100 in phosphate-buffered saline (PBS; pH 7.4). The mouse anti-SMI-32 monoclonal antibody was purchased from Sternberger Monoclonals (Baltimore, MD). The primary antibody (1:100) was diluted in 5% normal goat serum, 1% BSA, 0.3% Triton X-100 in phosphate-buffered saline and applied for 4 days. Secondary antibody conjugated with rhodamine red (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA) was applied for 2 hours. The retina was mounted (GB-mount; Golden Bridge International [GBI] Life Science Inc, Mukilteo, WA).

Measurement of Axonal, Dendritic, and Cell Body Parameters

The CSLO images were exported to a computer for image analysis (MATLAB R2007a; The MathWorks, Inc., Natick, MA). The following four parameters were measured in each RGC: (1) cell body area; the area bounded by the cell body contour, (2) axon diameter; the thickness of the axon segments nearest to the cell body, (3) dendritic field; the area bounded by connected line segments joining the ends of all terminal dendritic branches, and (4) branching complexity (measured by modified Sholl Analysis).⁷ Branching complexity was measured as the mean of the function $N(r)$ representing the number of intersections of the skeletonized dendrites (N) for each of the concentric circles with radius (r). The mean of $N(r)$ was calculated by dividing the definite integral of $N(r)$ by $(r_{\text{max}} - r_{\text{min}})$, where the interval $[r_{\text{min}}, r_{\text{max}}]$ was chosen in which $N(r)$ was larger than zero within the intervals. Good measurement repeatability was found in all four parameters with intraclass correlation coefficients ranged between 0.928 and 0.998. Retinal image calibration was based on a schematic eye model for the C57BL/6J mouse derived by Remtulla and Hallett.⁸ It was estimated that 1° of field is subtended by 30 μm of retina at $\lambda = 488$ nm.

Statistical Analysis

Statistical analysis was performed with a computer software (Stata, version 10.0, StataCorp, College Station, TX). Linear mixed modeling was applied to estimate the rate of dendritic shrinkage. The model was fitted with fixed coefficients (fixed effects) on baseline dendritic field (or baseline dendritic complexity), follow-up time, and the interaction between follow-up time and baseline dendritic field (to evaluate if baseline dendritic field is related to the rate of dendritic shrinkage), with random intercepts and coefficients (random effects) at both the mouse, the eye, and the cell levels (cell nested within eye; eye nested within mouse) for the effect of time. $P < 0.05$ is considered to be statistically significant.

RESULTS

Fifty-two RGCs from 19 eyes with distinct axonal and dendritic arborization patterns visualized with the CSLO at baseline were randomly selected for longitudinal imaging. The mean number of cells analyzed in each eye was 2.6 and the range was between 1 and 9. They were imaged at baseline, day 1, day 3, week 1, 2, 3, and 4 and then every 1 to 2 weeks for 2 months after acute elevation of intraocular pressure. Only images with sufficient quality were selected for quantitative analysis. Figure 1 shows the baseline morphologic measurements. The mean dendritic field, dendritic branching complexity, axonal diameter, and cell body area were 49,451 μm^2 , 8.9 (modified Sholl analysis⁷), 8 μm and 1398 μm^2 , and the ranges were 16,526–142,715 μm^2 , 5.4–14.5, 4–15 μm , and 704–2616 μm^2 , respectively. Sholl analysis of the mean (\pm SEM) number of dendritic intersections of the 52 RGCs is shown in Figure 1E.

Morphologic Changes of RGCs after Ischemic Injury

There were no detectable changes in axonal or dendritic structures after 30 ($n = 12$) (Fig. 2A) or 60 minutes ($n = 12$) of ischemia. After 90 minutes of ischemia ($n = 19$), 78.9% ($n = 15$) of RGCs showed progressive loss of the dendrites, the axon, and then the cell body at 1 to 2 weeks after the injury (Fig. 2B). A complete loss of dendritic tree was evident as early as day 3 after the injury. No detectable changes were seen in 15.8% ($n = 3$) at 2 months after the injury. In one RGC (5.3%), there was only mild reduction of branching complexity and shrinkage of dendritic field (Fig. 3). All RGCs showed rapid loss of dendrites, axon, and cell body within 1 week after 120 minutes of ischemia ($n = 9$). There were no significant differ-

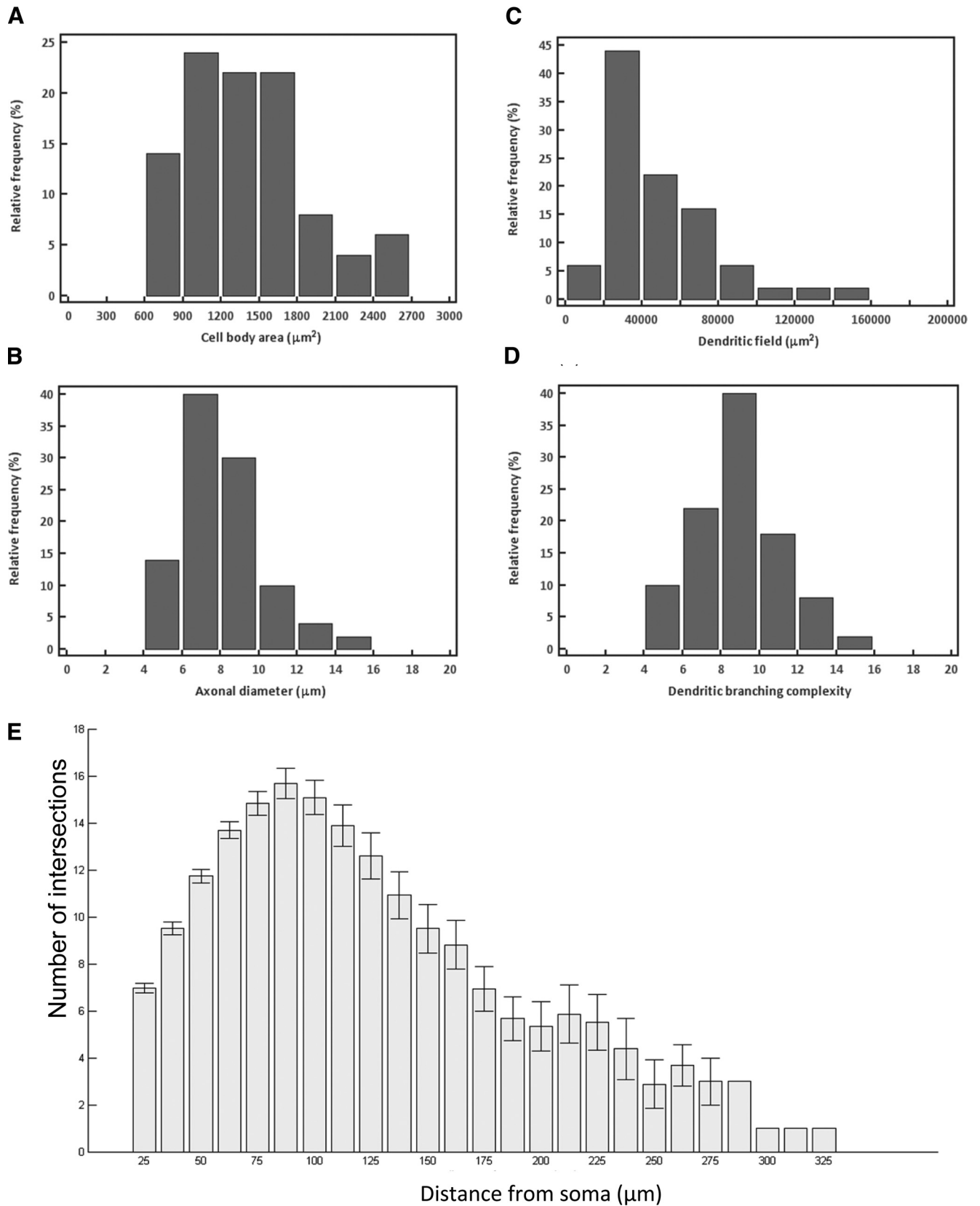


FIGURE 1. Histograms of baseline measurement of (A) cell body area, (B) axonal diameter, (C) dendritic branching complexity (modified Sholl analysis), and (D) dendritic branching complexity of 52 retinal ganglion cells imaged *in vivo* in the study. (E) Sholl analysis of the *in vivo* images showing the mean (\pm SEM) number of dendritic intersections.

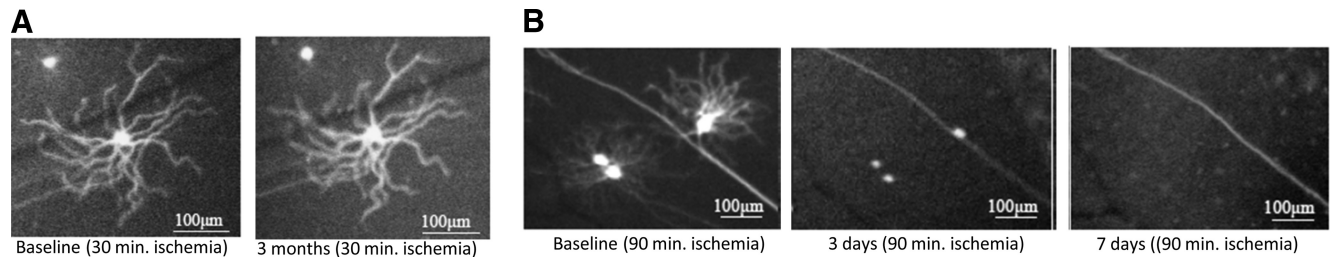


FIGURE 2. Morphologic changes of RGCs visualized in vivo with a confocal scanning laser ophthalmoscope before and after retinal ischemia induced by elevating the intraocular pressure to 110 mm Hg. **(A)** No morphologic changes were observed after 30 minutes of retinal ischemia. **(B)** After 90 minutes of ischemia, three RGCs showed complete loss of dendritic trees and axons at day 3, and then loss of the cell body at day 7.

ences in the baseline dendritic field, dendritic branching complexity, axonal diameter, and cell body area among the 4 groups of RGCs with different durations of retinal ischemia ($P \geq 0.724$).

Histologic Validation

Figures 3A and 3B illustrate the shrinkage of dendritic tree and reduction in branching complexity of an RGC at 107 days after 90 minutes of ischemia. The colocalization between YFP and anti-SMI32 (Figs. 3C–E) confirmed the morphologic changes observed with in vivo imaging were not secondary to redistribution of cytoplasmic YFP. Figure 4 shows Sholl analysis of RGCs from normal Thy-1 YFP mice. There was close corre-

spondence in Sholl analysis between anti-SMI32 staining and YFP fluorescence in the retinal flat mount (Fig. 4A), and between in vivo and in vitro YFP fluorescent images (Fig. 4B).

Rate of Change of Dendritic Field and Branching Complexity

A total of 426 longitudinal measurements consisting of 213 dendritic field and 213 branching complexity measurements were included for estimation of rate of change with linear mixed modeling. There was no significant change in dendritic field and branching complexity after 30 minutes or 60 minutes of ischemia ($P \geq 0.069$) (Figs. 5A and 5B and Figs. 6A and 6B).

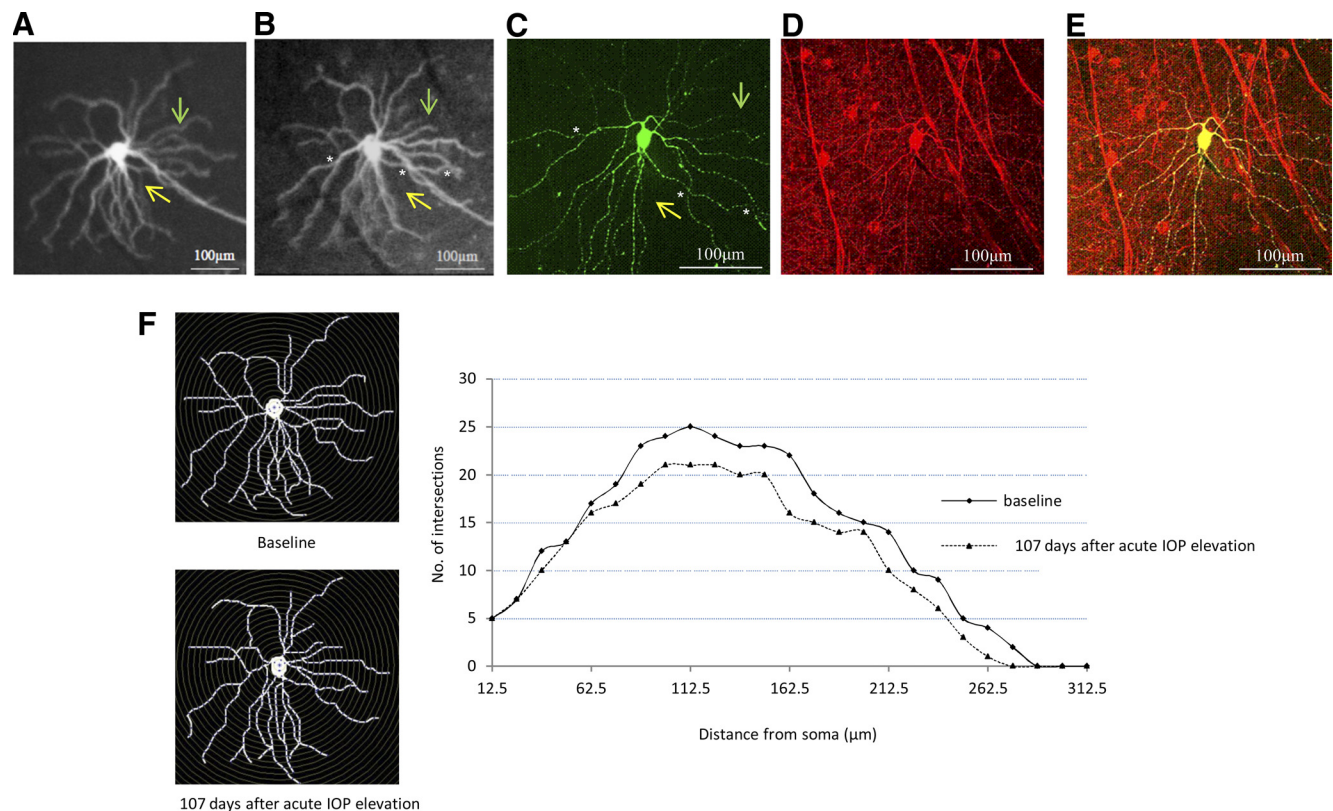


FIGURE 3. A retinal ganglion cell showing loss (yellow arrows) and shortening of dendrites (green arrows) after 90 minutes of retinal ischemia visualized in vivo with a confocal scanning laser ophthalmoscope imaged at **(A)** baseline, and **(B)** 107 days after the injury. The retina was dissected immediately after the last in vivo image capture and stained with anti-SMI32, an anti-neurofilament antibody. Images **(C)–(E)** were derived from a compressed *Z* stack captured under a confocal scanning laser microscope. **(A)** At baseline, the dendritic field and dendritic branching complexity (modified Sholl analysis) were $114,800 \mu\text{m}^2$ and 11.7, respectively. **(B)** Three months after retinal ischemia, they were $104,888 \mu\text{m}^2$ and 11.3, respectively. The colocalization **(E)** between **(C)** YFP and **(D)** anti-SMI32 suggests that the dendritic changes observed in vivo were not secondary to a redistribution of cytoplasmic YFP. Asterisks in **(B)** and **(C)** show the corresponding locations. Sholl analysis of the in vivo images **(A, B)** before and 107 days after the ischemic injury measured the number of dendrites intersecting with a series of concentric circles, spaced at 12.5- μm intervals centered on the soma **(F)**.

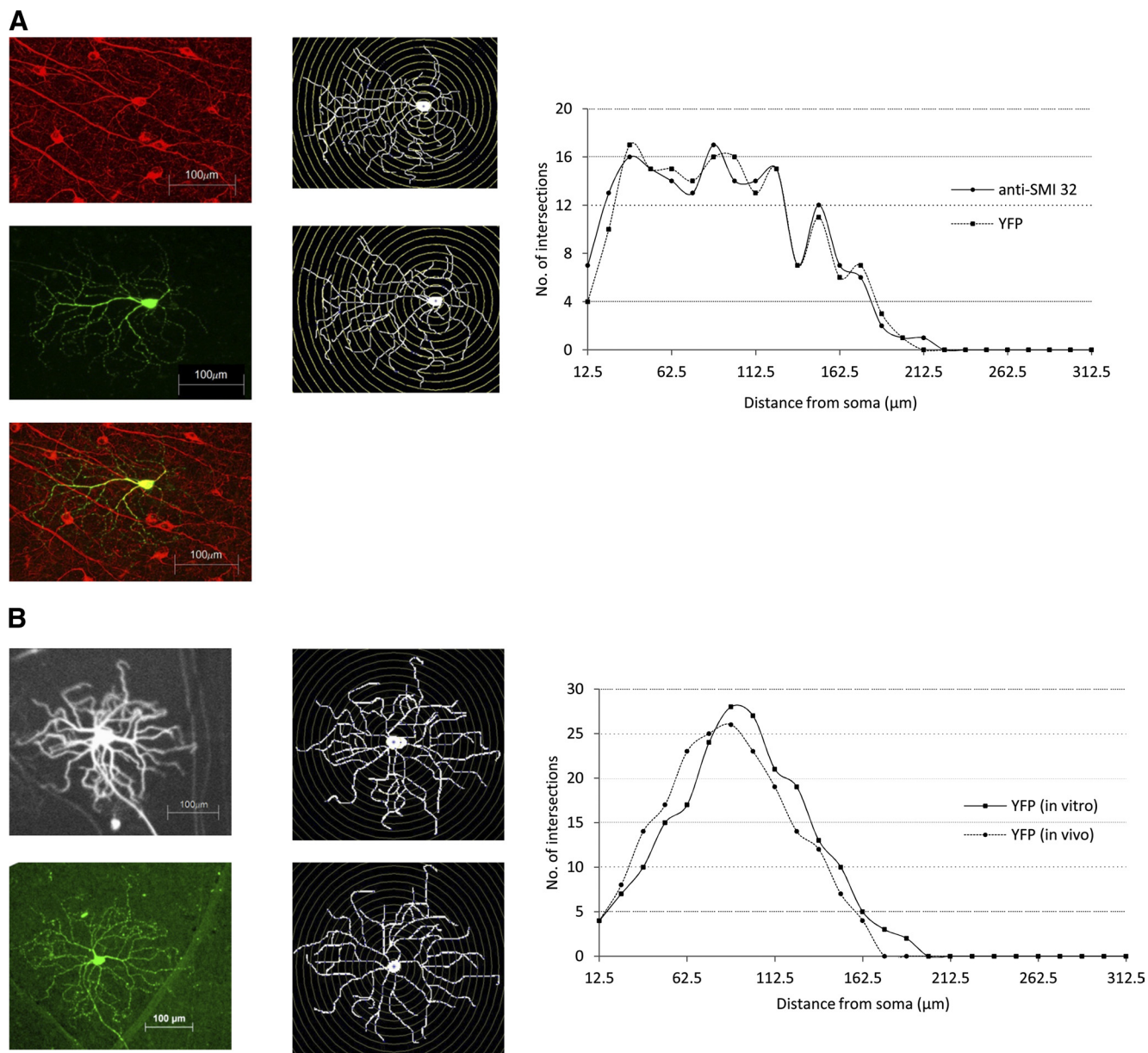


FIGURE 4. Sholl analysis of RGCs from normal Thy-1 YFP mice comparing (A) anti-SMI32 staining and YFP fluorescence in a retinal flat mount, and (B) between in vivo and in vitro YFP fluorescent images. Sholl analysis measured the number of dendrites intersecting with a series of concentric circles, spaced at 12.5-μm intervals centered on the soma. Adapted from Leung CK, Weinreb RN, Liu S, et al. Long term in vivo imaging and measurement of dendritic shrinkage of retinal ganglion cells (RGCs). *Invest Ophthalmol Vis Sci.* 2011;52:1539-1547. © ARVO.

After 90 minutes of ischemia, the mean rate of reduction of dendritic field was 11.7% per day (95% confidence interval: 5.0%-18.4%) ($P = 0.001$) (Fig. 5C), and that of branching complexity was 11.1% per day (5.0%-17.2%) ($P < 0.001$) (Fig. 6C). After 120 minutes of ischemia, they were 15.1% per day (10.3%-19.9%) ($P < 0.001$) (Fig. 5D), and 15.9% per day (11.3%-20.5%) ($P < 0.001$) (Fig. 6D), respectively. The baseline dendritic field was significantly associated with the rate of dendritic shrinkage ($P = 0.002$). The rate of dendritic shrinkage was reduced by 1.8% (0.7%-2.9%) for every 10,000-μm² increase in dendritic field.

DISCUSSION

With long-term in vivo imaging of dendritic and axonal structures of RGCs, we showed that the RGCs have relatively high

tolerance of retinal ischemia. Even when the IOP was elevated to 110 mm Hg for 30 to 60 minutes, there were no detectable changes in dendritic and axonal arborizations. After 90 and 120 minutes of high IOP elevation, a significant proportion of RGCs showed progressive shrinkage of dendritic trees followed by loss of the axon and then the cell body. With longitudinal measurement of dendritic field, the rate of dendritic shrinkage was estimated at 11.7% per day and 15.1% per day after 90 and 120 minutes of ischemia, respectively, which was faster than that after optic nerve crush.⁵ Tracking dendritic changes with in vivo imaging would be a sensitive approach to evaluate the health of RGCs and could serve as an important biomarker for different types of optic nerve injury.

There is a paucity of published data reporting the morphologies of RGCs after elevation of intraocular pressure. Prior studies have been derived from histologic examination of ret-

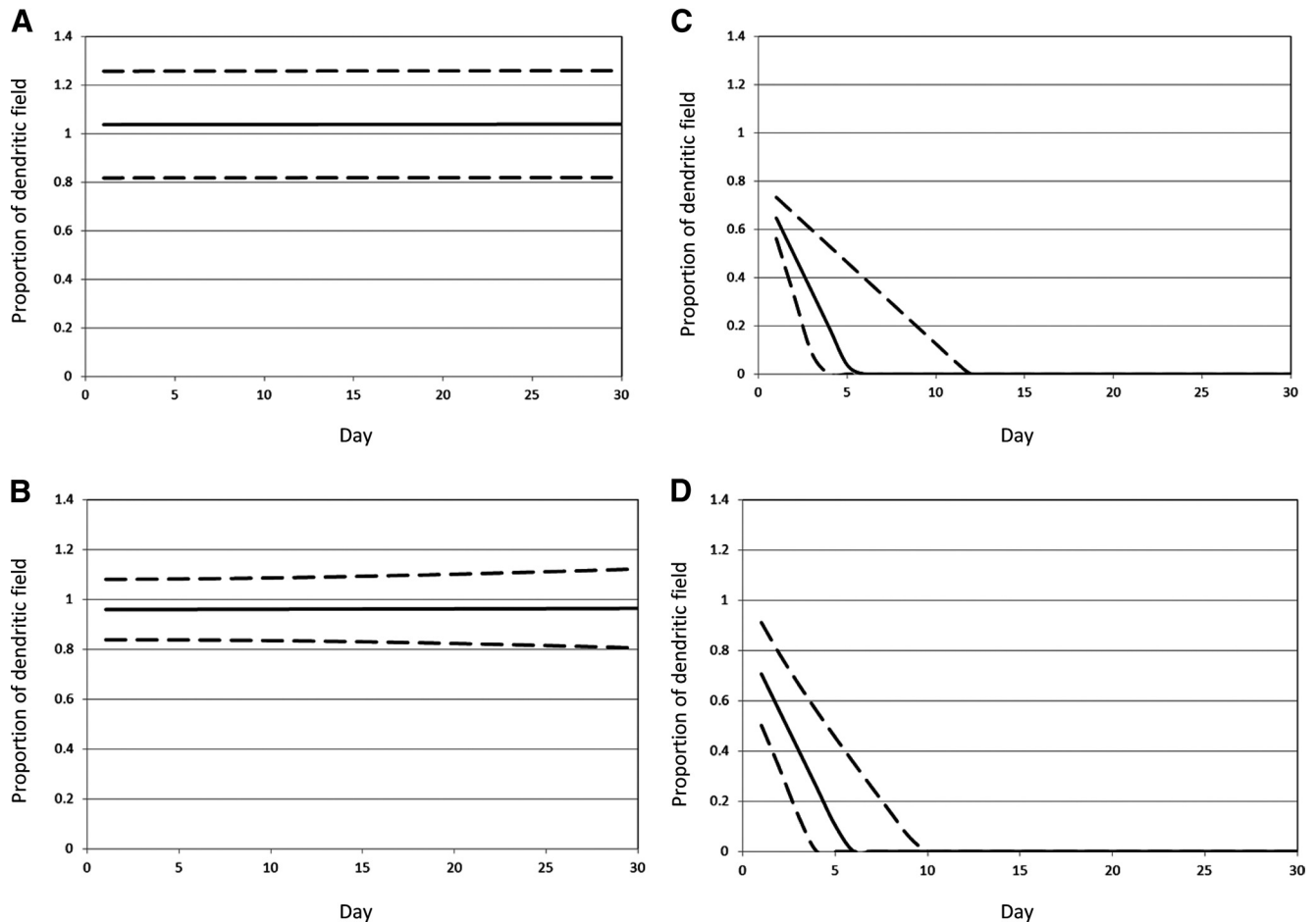


FIGURE 5. Longitudinal profiles of changes of dendritic field with 95% confidence intervals (*dotted lines*) estimated by linear mixed modeling after (A) 30, (B) 60, (C) 90, and (D) 120 minutes of retinal ischemia. Four RGCs with no detectable changes of dendritic morphology within 30 days after 90 minutes of ischemia were not included in the analysis.

inas dissected after injury. In the study by Weber et al. using a primate model of experimental glaucoma and intracellular staining of RGCs with Lucifer yellow, the earliest sign of RGC degeneration detected was a change in the dendritic arborization.⁹ Similar observation of dendritic pruning in human glaucoma was also reported by Pavlidis et al. with intraretinal labeling in enucleated eyes.¹⁰ It has been suggested that RGCs with larger soma and larger dendritic fields were more susceptible to damage after chronic intraocular pressure elevation.^{11–13} In contrast, they were more tolerant to dendritic damage and cell loss after acute intraocular pressure elevation.¹⁴ Observation from histologic studies, however, is always limited at a single time point. It is difficult to determine whether there were changes in the dendritic arborization without visualizing the morphologies at baseline. In addition, changes in dendritic and axonal structures could be related to severing of the axons during dissection of the retina. A group of normal controls is therefore always needed for comparison in histologic studies. With high-resolution *in vivo* imaging, we demonstrated that degeneration of RGCs begins with dendritic shrinkage and that the rate of dendritic shrinkage was associated with the size of dendritic field measured at baseline. RGCs with a larger dendritic field had a slower rate of dendritic shrinkage. Remarkably, there were no morphologic changes in the dendritic and axonal arborization after 30 and 60 minutes of ischemia. Even after 90 minutes of ischemia, a small proportion of RGCs survived with intact dendritic and axonal structures. These results concur with our previous investigation

using the blue-light CSLO to measure the number of RGC after acute intraocular pressure elevation.⁴ A reduction in RGC number (14.5%–79.5% reduction relative to baseline measurement) was observed only after 90 minutes, but not after 45 minutes of ischemia. The finding of intact dendritic and axonal morphologies further support the notion that structural damage would not be evident in a relatively short period (30–60 minutes) of ischemia. The high tolerance of RGCs to ischemic injury has been attributed to the considerable amounts of glucose in the vitreous and the capacity to extract adenosine triphosphatase from glycolysis.^{15,16} Functional impairment, however, might be detectable. Complete, reversible loss of scotopic ERG b-wave has been shown even after only 4 minutes of ischemia.¹⁶ In such a model system, functional changes of RGCs likely precede structural damage in retinal ischemia.

Of note, the conventional approach of counting the cell body may not be as sensitive as measuring the rate of dendritic shrinkage to evaluate RGC damage. As shown in Figure 2B, the presence of the cell body does not represent an intact RGC. The mean rate of dendritic shrinkage was 11.7% per day after 90 minutes, and 15.1% per day after 120 minutes of ischemia with complete loss of dendritic trees in 3 to 14 days. Treatment may be effective to preserve RGC function if initiated at a stage before complete loss of dendrites. The ability to monitor dendritic and axonal arborizations thereby provides a sensitive approach to identify a potential therapeutic window for neuroprotective treatment.

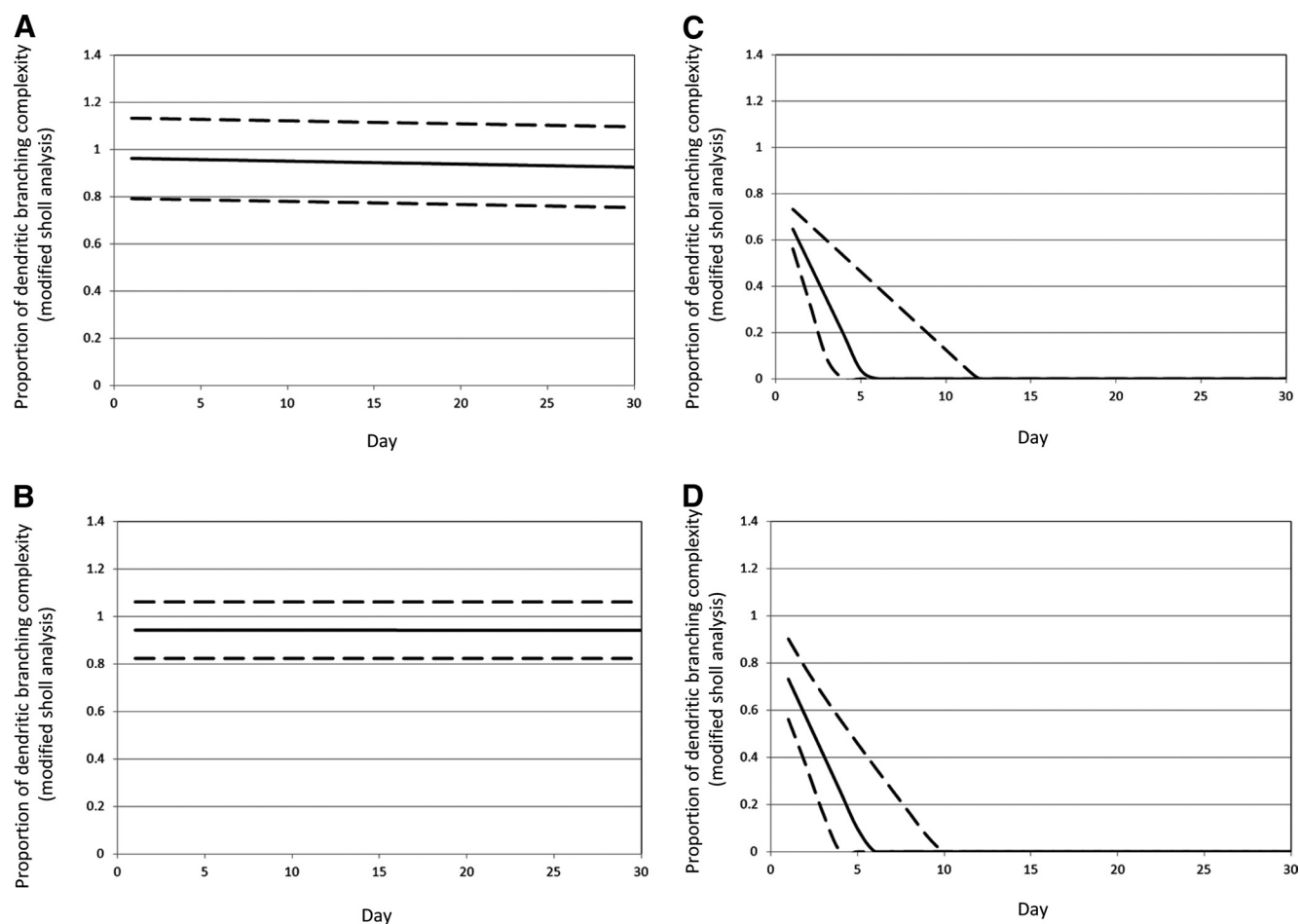


FIGURE 6. Longitudinal profiles of changes of dendritic branching complexity (modified Sholl analysis) with 95% confidence intervals (*dotted lines*) estimated by linear mixed modeling after (A) 30, (B) 60, (C) 90, and (D) 120 minutes of retinal ischemia. Four RGCs with no detectable changes of dendritic morphology within 30 days after 90 minutes of ischemia were not included in the analysis.

In vivo imaging of the Thy-1 YFP mice has been reported using a confocal scanning laser microscope.¹⁷ The advantages of using a CSLO include improvement in resolution of dendritic and axonal structures for quantitative analysis. Furthermore, imaging can be performed while the animal is awake. Different from the Thy-1 cyan fluorescent protein (CFP) strain in which over 95% of RGCs express CFP,¹⁸ only a small proportion of RGCs in the Thy-1 YFP mice express fluorescent protein. For this reason, the dendritic trees and axons of individual neurons can be discriminated and visualized. The disparity in the expression pattern has been proposed to be related to position effect variegation and repeat-induced gene silencing.⁶ Of note, the YFP-expressing RGCs likely represent a random sample of the retina as it has been shown that the expression has no predilection for specific subtypes of RGCs.¹⁹

There are a number of potential limitations that may confound the interpretation of RGC measurements in this study. Development of lens opacity is common after general anesthesia in mice.²⁰ In this study, imaging was performed without using any local or systemic anesthetic agent. The natural blinking reflex would prevent corneal drying and worsening of image quality. Imaging could thus be conducted repetitively and longitudinally for months without compromising the optical media or survival of the animal. The colocalization of anti-SMI32 antibodies and YFP in degenerating dendritic trees indicated that loss of fluorescent signals detected in vivo likely represents loss of dendrites but not redistribution of cytoplasmic YFP. Although laser exposure during repeated imaging

may theoretically bleach and reduce the intensity of fluorescent signal of RGCs and create a false impression of dendritic degeneration, there was no change in fluorescent signal for mice undergoing 30 and 60 minutes of ischemia. This observation suggested that there was no bleaching effect (Fig. 2A). It is notable that axon diameter measured in this study was generally greater than that reported in histologic studies. Unlike confocal scanning laser microscopy, it is not feasible to perform a compressed Z stack with confocal scanning laser ophthalmoscopy. As axonal and dendritic structures may reside in different focal planes, in vivo measurement could be overestimated if the measured segment is not in focus.

It has been shown that structural and functional damage to the RGCs were evident after elevating the IOP to 110 mm Hg for 60 minutes in rats.²¹ The lack of damage in our mouse model may be a result of subtotal ischemia if the blood pressure was consistently higher than 110 mm Hg. Nevertheless, an important implication is that structural damage of RGCs would only become evident when the duration of ischemia is longer than a certain threshold. It can be inferred that in acute angle closure, a clinical emergency with acute elevation of IOP, RGCs may not be damaged if the duration of IOP elevation is short. However, irreversible RGC loss could be inevitable when threshold duration is reached. Early treatment to lower the IOP is important to managing patients with acute angle closure.

In summary, RGCs appear to be relatively resistant to acute elevation of intraocular pressure with dendritic shrinkage evi-

dent only after 90 to 120 minutes, but not 30 to 60 minutes of ischemia. In vivo imaging of dendritic changes would provide a sensitive approach to track RGC damage and identify therapeutic windows for neuroprotective treatment.

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