Structural Correlation Between the Nerve Fiber Layer and Retinal Ganglion Cell Loss in Mice with Targeted Disruption of the *Brn3b* Gene

Andrew S. Camp,¹ Marco Ruggeri,¹ Gustavo C. Munguba,¹ Mary L. Tapia,¹ Simon W.M. John,² Sanjoy K. Bhattacharya,¹ and Richard K. Lee¹

PURPOSE. Mice with a targeted disruption of *Brn3b* (knockout *Brn3b*^{-/-}) undergo the loss of a majority of retinal ganglion cells (RGCs) before birth. Spectral domain optical coherence tomography (SD-OCT) allows for the noninvasive examination of *Brn3b*^{-/-} cellular loss in vivo.

METHODS. The central retinas of *Brn3b*^{-/-} and phenotypically wild-type (*Brn3b*^{+/+} and *Brn3b*^{+/-}) mice were imaged by SD-OCT. The combined nerve fiber layer (NFL) and inner plexiform layer (IPL) were manually segmented and thickness maps were generated. The results were confirmed by histologic and immunofluorescence cell counts of the RGC layer (RGCL) of the same retinas.

RESULTS. The combined NFL and IPL of the *Brn3b*^{-/-} retinas were significantly thinner, and the histologic cell counts significantly lower, than those of the phenotypically wild-type retinas (paired *t*-test; *P* < 0.01 and *P* < 0.01, respectively). The combined NFL and IPL thickness and the histologic cell count correlated highly (*R*² = 0.9612). Immunofluorescence staining revealed significant RGC-specific loss in *Brn3b*^{-/-} retinas (paired *t*-test; *P* < 0.01). The distribution of combined central NFL and IPL loss was not localized or sectorial.

CONCLUSIONS. The strong correlation between the combined layer thickness and histologic cell counts validates manual OCT segmentation as a method of monitoring cell loss in the RGCL. A retinal thickness map assessed if combined NFL and IPL thickness loss in *Brn3b*^{-/-} eyes was topographically specific. Generalized RGC and combined NFL and IPL loss was observed in the *Brn3b*^{-/-} retinas, in contrast to topographically specific RGC loss observed in glaucomatous DBA/2J eyes. (*Invest Ophthalmol Vis Sci.* 2011;52:5226–5232) DOI:10.1167/iovs.10-6307

From the ¹Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida, and the ²Howard Hughes Medical Institute, The Jackson Laboratory, Bar Harbor, Maryland.

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Corresponding author: Richard K. Lee, Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, 900 NW 17th Street, Miami, FL 33136; rlee@med.miami.edu.

Animal models of glaucoma have significantly advanced research for this clinically important optic neuropathy. Glaucoma is a sight threatening optic neuropathy that is characterized by chronic progressive retinal ganglion cell (RGC) death. Animal models have allowed an understanding of the complex genetic and biochemical pathways involved in the death of RGCs.¹ Targeted disruption of the gene encoding BRN3B, a POU domain transcription factor, results in the loss of approximately 70% of RGCs prenatally in mice.^{2–5} The RGC layer (RGCL) loss seen in *Brn3b* knockout mice (*Brn3b*^{-/-}) is specific to RGCs and affects all RGC subtypes and dendritic projections to the inner plexiform layer (IPL).^{6,7} BRN3B is crucial for early RGC differentiation, dendritic pathfinding, and central axonal projections. *Brn3b* knockout mice exhibit severe visual sensory defects secondary to RGC loss.^{7–11}

Study of the role of BRN3B in RGC development and maintenance relies on the histologic examination of enucleated eyes. Spectral domain optical coherence tomography (SD-OCT) allows for the noninvasive visualization of retinal morphology and quantification of the nerve fiber layer (NFL) in vivo by measuring the intensity of backscattered light through retinal sections.^{12–15} SD-OCT is currently used in the clinical assessment of glaucoma. Advances in SD-OCT technology have enabled researchers to study small-scale mouse eyes with progressively improved resolution.^{16–19} Recent studies using manual segmentation of retinal layers illustrate the capability of SD-OCT to measure the thickness of retinal structures in mice but did not focus on topographical NFL degeneration across the entire retina or provide histologic correlation to actual cell counts.^{20–22} Development of SD-OCT systems specifically designed for small animal imaging is expected to facilitate the use of OCT imaging for studies of retinal morphology and quantification through the assessment of retinal layer thickness in living mice. This will aid in noninvasive clinical follow-up for various experiments pertaining to retinal cell biology and retinal cell degeneration studies.

We evaluated the capability of manual segmentation of SD-OCT images to identify differences in retinal layer thickness resulting from the loss of cells in the RGCL in living mice. We used three-dimensional (3D) manual segmentation of SD-OCT images to quantify and compare the combined NFL and IPL thicknesses in living *Brn3b*^{-/-} and phenotypically wild-type (*Brn3b*^{+/+} and *Brn3b*^{+/-}) mice noninvasively. We show that SD-OCT measurements of the combined NFL and IPL correlate well with histologic loss of cells in the RGCL. Immunofluorescence staining further detailed a significant loss specific to RGCs in *Brn3b*^{-/-} retinas. In addition, we determined that RGC and combined NFL and IPL loss is diffuse in the *Brn3b*^{-/-} central retina, unlike the focal loss of RGCs in the DBA/2J mouse glaucoma model. These preliminary findings set the stage for future studies using SD-OCT segmentation to track changes in retinal architecture secondary to cell loss.

METHODS

Animals

Experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and within the guidelines of the University of Miami Institutional Animal Care and Use Committee. The mice used in this study were 2-month-old *Brn3b*^{+/+}, *Brn3b*^{+/-}, and *Brn3b*^{-/-} mice (originally from Lin Gan, University of Rochester, NY) bred into a DBA/2J genetic background. Although DBA/2J develop increased intraocular pressure with age, no histologic changes have been reported in the first 3 to 5 months.²³ *Brn3b*^{+/+} and *Brn3b*^{+/-} retinas are phenotypically wild-type and were used as controls. The genetic background of the mice used in this study was confirmed by PCR genotyping. In addition, the optic nerves from *Brn3b*^{-/-} eyes were significantly smaller in caliber than *Brn3b*^{+/-} and *Brn3b*^{+/+} nerves. Mice were kept on a 12-hour light/dark cycle and were fed standard rodent chow available ad libitum.

SD-OCT Mouse Imaging

An SD-OCT system designed for small animal imaging (BiopTigen, Research Triangle Park, NC) was used for in vivo imaging of mouse retina. The axial resolution of the system is approximately 5 μ m in retinal tissue. Mice were anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture then placed on a positioning stage. The head was fixated on a stabilizing bar and a heating blanket was placed under the anesthetized mouse. The eyes were dilated with topical tropicamide and the corneas were kept moist during imaging with the regular application of artificial tears (Systane, Alcon, TX). Raster scans centered on the optic disc consisted of 1000 $\times$ 100 (horizontal $\times$ vertical) depth scans covering an area of 1 $\times$ 1 mm² of the mouse retina. Alignment of the mouse took less than 1 minute, and image acquisition took approximately 2 seconds per eye.

SD-OCT Retinal Layer Segmentation

Monitoring the thickness of the RGC containing NFL is of particular interest in studying mouse models of glaucoma. The IPL is also expected to be thinner in *Brn3b*^{-/-} mice because of the loss of dendritic projections from the reduced numbers of RGCs in the RGCL and NFL. The NFL and IPL have similar scattering properties, and both appear as bright layers in the OCT images. For this reason, and because the NFL is on average very thin compared to the IPL, visual separation of the two layers is difficult, and therefore manual segmentation of the combined NFL and IPL was obtained for the OCT images of the *Brn3b*^{-/-}, *Brn3b*^{+/-}, and *Brn3b*^{+/+} mouse retinas. Thickness maps resulting from segmentation of the NFL and IPL boundaries were calculated together with the average combined layer thickness and SD for each eye. The retinal thicknesses of the *Brn3b*^{-/-} and phenotypically wild-type mice were compared using a paired *t*-test. *P* < 0.05 was considered statistically significant. The genotype of two eyes was masked and segmentation was repeated with similar results to validate the reliability of this manual segmentation method.

Histology and Cell Counts

Immediately after SD-OCT imaging, the eyes were harvested and fixed in formalin and were then processed and embedded in paraffin blocks. Five-micrometer hematoxylin and eosin (H&E)-stained sections across the entire retina of the globe were prepared. The retinal sections were examined by light microscopy using 400 $\times$ total magnification. The total number of cells in the RGCL was manually counted across the entire retinal section in 80- μ m increments. Cell counts of retinal sections taken from the peripheral retina were variable because of the preparation methods. The average was taken of the central 75% of the cell counts for each eye to minimize bias introduced by variable far peripheral retina cell counts. The central 75% of the cell counts of the *Brn3b*^{-/-} and phenotypically wild-type mice were compared using a paired *t*-test. *P* < 0.05 was considered statistically significant.

Immunofluorescence Staining

Five-micrometer sections of all globes were also prepared for immunofluorescence staining by fixation in formalin and 1X phosphate buffered saline (PBS; 127 mM NaCl, 2.7 mM KCl, and 10 mM phosphate) at 25°C. After fixation, globes were embedded in paraffin, sectioned, and mounted on glass slides. Sections were examined 160 μ m to the left and right of the peak cell count as previously determined through histologic cell counts. Two sections per eye of the *Brn3b*^{-/-}, *Brn3b*^{+/-}, and *Brn3b*^{+/+} mice were stained. Primary antibodies were: rabbit anti-calretinin (1:100 dilution, #232A-74; Cell Marque, Rocklin, CA) and mouse anti-Brn3a (1:100 dilution, #SC-8429; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were: goat anti-rabbit Alexa 488 (1:100 dilution, #A11034; Molecular Probes, Eugene, OR) and goat anti-mouse Alexa 546 (1:100 dilution, #A11030; Molecular Probes). Sections were deparaffinized in xylene and sequential alcohol rinses, then treated with 95°C Trilogy (CMX833-C; Cell Marque) antigen retrieval reagent. Tissue sections were subsequently blocked with Rodent Block M (RBM96; BioCare Medical, Concord, CA) for 30 minutes to reduce nonspecific binding. Slides were incubated overnight at 4°C in PBS containing primary antibodies, washed with PBS, incubated in antibody buffer containing secondary antibodies for 1 hour at room temperature, washed with PBS, and mounted onto glass coverslips using DAPI vectashield (Vector, Burlingame, CA). Images were collected on an inverted microscope (Axiovert 200M; Carl Zeiss Meditec, Oberkochen, Germany) running the Zeiss AxioVision 4.7.2 on PC (Zeiss Inc., Thornwood, NY). Confocal microscopy images were obtained in a Leica TCP SP5 spectral confocal microscope (Leica, Exton, PA; 63 $\times$, water-immersion, 1.2 NA objective). Cell counts were taken across the extent of each retinal section examined. The total anti-Brn3a cell counts and anti-calretinin cell counts of the *Brn3b*^{-/-} and phenotypically wild-type mice were compared using a paired *t*-test. *P* < 0.05 was considered statistically significant.

RESULTS

SD-OCT Imaging of Retinas of Mice with Targeted *Brn3b* Gene Disruption and Controls

SD-OCT images were obtained for eyes of 2-month-old mice: *Brn3b*^{-/-}, *Brn3b*^{+/-}, and *Brn3b*^{+/+}. *Brn3b*^{+/-} and *Brn3b*^{+/+} eyes are phenotypically wild-type.⁴ All images were of sufficient resolution for use in this study despite moderate anterior chamber opacity related to transient cataract formation in some eyes. SD-OCT images were obtained near the center of the posterior pole from *Brn3b*^{+/+}, *Brn3b*^{+/-}, and *Brn3b*^{-/-} retinas (Figs. 1A, 1C, 1E). The SD-OCT images were compared to histologic H&E-stained samples of the same eye (Figs. 1B, 1D, 1F). The anatomic layers of the retina can be seen in both the SD-OCT image and the H&E stain and are labeled accordingly (Fig. 1). A qualitative difference in both NFL density and cell count was observed between *Brn3b*^{-/-} and phenotypically wild-type eyes. Phenotypically wild-type eyes have visually thicker RGCL cell counts than those of *Brn3b* deficient retinas. Histologic sections and SD-OCT images at various depths were compared and found to be in agreement with each other, demonstrating the anatomic correlation between histology and imaging (Fig. 1).

Quantitative Analysis of Retinal Layer Thickness

OCT images were manually segmented for all eyes and thickness maps of the combined NFL and IPL were generated (Figs. 2A, 2B, 2C). The thickness map is displayed over a circular area with an equivalent diameter of 0.7 mm. Segmentation of the combined NFL and IPL was not accurate in the optic disc region because of the progressive taper of the retinal layers toward the optic nerve head. For this reason, thickness maps were calculated after removing data from a circular area

FIGURE 1. Representative comparison of SD-OCT B-scan retinal cross-sections (A, C, E) to hematoxylin and eosin (H&E)-stained retinal histologic sections (B, D, F) from the same mice. All retinal layers can be distinguished in both the SD-OCT images and H&E sections. Retinal layers are labeled as follows: neurofiber layer/retinal ganglion cell layer (NFL/RGCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor inner segment/outer segment (IS/OS), and RPE. The NFL of the *Brn3b*^{-/-} retina (A and B) is qualitatively thinner than that of the *Brn3b*^{+/-} (E and F) and *Brn3b*^{+/+} (C and D) retinas.

with a 245- μ m diameter centered on the optic disc. Color bars ranging from 0 to 100 μ m are shown together with the thickness maps.

The retinal blood vessel pattern can be distinguished on the thickness map as a thickening of the segmented complex layer.

The locations of the retinal arteries correlate well with the arteries visualized in the raw SD-OCT Z-stack en face fundus images displayed in grayscale in Figure 2 (D-F). This validates the ability of the segmentation program to accurately track the contours of the retinal layers. The retinal thickness maps of the

FIGURE 2. The representative three-dimensional segmentation of the NFL and IPL of *Brn3b*^{-/-} (A), *Brn3b*^{+/-} (B), and *Brn3b*^{+/+} (C) retinas yields a retinal thickness map. Thickness loss is uniform across the *Brn3b*^{-/-} retina. Retinal artery locations in the OCT generated fundus images of the *Brn3b*^{-/-} (D), *Brn3b*^{+/-} (E), and *Brn3b*^{+/+} (F) mice match well with the retinal arteries seen in the thickness map.

phenotypically wild-type retinas (Figs. 2B, 2C) are yellow-shifted when compared to those of the *Brn3b* knockout retina (Fig. 2A). This indicates that the phenotypically wild-type retinas have a greater combined NFL and IPL thickness than the knockout retina. The uniform color of the thickness map of the *Brn3b* knockout retina indicates a relatively even loss of combined layer thickness across the central retina.

The average thickness and SD of the combined NFL and IPL was calculated for each eye using a previously described segmentation algorithm modified for use with images obtained from the BiopTigen system.²⁴ *Brn3b*^{-/-} eyes have thinner inner retinal layers with no overlap of SDs compared to thicker, phenotypically wild-type mouse retinal layers (Fig. 3). The average thickness of the combined NFL and IPL of *Brn3b*^{-/-} retinas is significantly less than that of phenotypically wild-type mice (paired *t*-test; $P < 0.01$). Phenotypically wild-type mice, *Brn3b*^{+/-} and *Brn3b*^{+/+}, have retinal layers of similar thickness, suggesting no *Brn3b* gene expression dosage effect on the combined NFL and IPL thickness (Fig. 3).

Cell Counts and Retinal Layer Thickness Correlations

A comparison of RGCL cell counts across the entire retinas of H&E-stained *Brn3b*^{+/+}, *Brn3b*^{+/-}, and *Brn3b*^{-/-} eyes shows a clear reduction in cell numbers across the entire retina of *Brn3b*^{-/-} eyes. The cell counts of the *Brn3b*^{-/-} retinas are consistently lower than the phenotypically wild-type *Brn3b*^{+/-} and wild-type *Brn3b*^{+/+} retinas across the breadth of the eye (Fig. 4A). The averages of the top 75% of the H&E-stained RGCL cell counts for all eyes, with error bars representing the single maximal cell count per section of each eye, are shown in Figure 4B. The *Brn3b*^{-/-} eyes have significantly lower RGCL cell counts than the phenotypically wild-type eyes (paired *t*-test, $P < 0.01$).

The reduced cell count in the RGCL of *Brn3b*^{-/-} retinas was confirmed and further explored through immunofluorescence staining. The cell counts in the RGCL of *Brn3b*^{-/-} retinas are reduced in both the Brn3a-stained (Fig. 5B) and calretinin-stained (Fig. 5C) images when compared to the equivalently stained *Brn3b*^{+/-} retinas (Figs. 5E, 5F, respectively) and *Brn3b*^{+/+} retinas (Figs. 5H, 5I, respectively). A significant reduction of both Brn3a- and calretinin-stained cells in the RGCL of *Brn3b*^{-/-} retinas was observed when compared to phenotypically wild-type *Brn3b*^{+/-} and *Brn3b*^{+/+} retinas (paired *t*-tests; $P < 0.01$). However, when the Brn3a-stained cell count was subtracted from the calretinin-stained cell count, no significant difference was found between *Brn3b*^{-/-}

FIGURE 3. Retinal thicknesses and SDs (error bars) were calculated using data from the retinal thickness map. *Brn3b*^{-/-} mice have thinner retinas and nonoverlapping SDs with phenotypically wild-type (*Brn3b*^{+/+} and *Brn3b*^{+/-}) mice.

FIGURE 4. (A) Cell counts in the NFL of a *Brn3b*^{-/-}, *Brn3b*^{+/-}, and a *Brn3b*^{+/+} mouse shows a reduction in cell number in the *Brn3b*^{-/-} NFL. (B) Averaging the top 75% of the cell counts for all mice shows a reduction in cell numbers in *Brn3b*^{-/-} versus phenotypically wild-type retinas. Error bars represent the maximum cell count for each retina.

retinas and phenotypically wild-type retinas (paired *t*-test; $P = 0.34$). The calculated average top 75% H&E-stained cell counts were plotted as an independent variable to the dependent SD-OCT calculated NFL and IPL thickness (Fig. 6). A correlation between the average cell count in the RGCL and the calculated thickness of the NFL and IPL was observed ($R^2 = 0.9612$; Fig. 6).

DISCUSSION

Study of the RGCL and the NFL has typically been dependent on the histopathologic analysis of retinas from enucleated eyes or retinal cell cultures. These techniques allow only a static picture of in vivo retinal changes. Most *Brn3b*-dependent RGCs undergo apoptosis perinatally or in early postnatal development, so *Brn3b*^{-/-} retinas likely undergo a majority of their alterations before adulthood.⁵ However, RGCs continue to express *Brn3b* throughout the lifetime of the cells, suggesting an important role for BRN3B in the maintenance of RGC survival.²⁵ The development of a *Brn3b*-dependent Cre/lox system for genetic ablation of adult *Brn3b*-expressing RGCs lays a framework for study of *Brn3b* expression and the role of *Brn3b*-expressing RGCs in adult mice.²⁶ As such, the development of a noninvasive retinal monitoring system capable of following individual mice across multiple time points may play an important role in the in vivo study of *Brn3b* in adult mouse RGCs. More importantly and generally, noninvasive imaging of the retina will allow for sequential in vivo study of the natural history of the changes the retina undergoes under certain model disease conditions, such as glaucoma and retinal degeneration, as is observed in human eyes.

The SD-OCT imaging system (BiopTigen) used in this study provides high-resolution images of the retinal layers in living mice. OCT studies in human eyes have long shown the relationship between NFL thickness and optic nerve function in glaucomatous patients.^{27,28} The small pupil size and optics of the mouse eye has slowed the development of an OCT system

FIGURE 5. Representative comparison of flattened Z-stack images of Dapi- (A, B, C), Brn3a- (D, E, F), and calretinin-stained (G, H, I) retinal cross sections from *Brn3b*^{-/-}, *Brn3b*^{+/-}, and *Brn3b*^{+/+} mice. Retinal layers are labeled as previously indicated. The RGCL of *Brn3b*^{-/-} retinas (A, D, G) has significantly fewer Brn3a- and calretinin-stained cells than the RGCL of *Brn3b*^{+/-} (B, E, H) and *Brn3b*^{+/+} (C, F, I) retinas.

with similar resolution for mouse eyes. Now that such an animal SD-OCT imaging system is commercially available, research using mouse models can be expected to accelerate on a much greater scale. The results of our preliminary work show the utility of manual segmentation of SD-OCT images in determining retinal layer thickness as correlated to histologic cell counts. Ideally, automated segmentation will be forthcoming for this technology. We found a strong correlation between the histologic number of cells in the RGCL and the combined thicknesses of the NFL and IPL measured by SD-OCT ($R^2 =$

FIGURE 6. Scatter plot using NFL cell counts as the independent variable and calculated retinal thickness as the dependent variable. A strong correlation between cell counts and calculated retinal thickness is observed ($R^2 = 0.9612$).

0.9612; Fig. 6). This finding supports observing changes in NFL and IPL thickness by SD-OCT longitudinally in vivo in individual mice as measures of RGCL loss associated with mouse models of glaucoma. This methodology could be used in other progressive mouse models of glaucoma to characterize the temporal relationship between thinning of the NFL and cellular loss in the RGCL by examining multiple timepoints in the progression of the disease.

Our RGCL cell counts confirm previous histologic work showing RGC loss in *Brn3b*^{-/-} mice. We show a significant reduction in the total H&E-stained RGCL cell count of *Brn3b*^{-/-} retinas compared with phenotypically wild-type retinas (paired *t*-test; $P < 0.01$). The total cell loss in the RGCL is on a similar scale as the 30% previously reported.⁴ Immunofluorescence staining and cell counts further characterize the cell types found in the RGCL of the mice used in this study (Fig. 5). Displaced amacrine cells are present in the RGCL of *Brn3b*^{-/-} retinas in addition to RGCs, so it is necessary to determine that the total cell loss observed is not related to the loss of displaced amacrine cells alone.²⁹ Brn3a is a well characterized marker of RGCs, and calretinin stains both displaced amacrine cells and a majority of RGCs.^{30–32} We show a significant loss of Brn3a-labeled RGCs in the *Brn3b*^{-/-} retinas similar to the 70% loss of RGCs previously reported in *Brn3b*^{-/-} mice^{2,4} (paired *t*-test; $P < 0.01$).

A significant loss in calretinin-labeled cells in the RGCL of *Brn3b*^{-/-} retinas further confirms the total cell loss observed in the H&E-stained sections (paired *t*-test; $P < 0.01$). The lack of

statistically significant cell loss after the subtraction of Brn3a-stained RGCs from the total calretinin-stained cell count suggests that the loss of RGCs is largely responsible for the total cell loss observed in *Brn3b*^{-/-} retinas (paired *t*-test; *P* < 0.34). However, the lack of significance in our work could be related to sample size or the limitations of immunofluorescence staining. Future work could more accurately characterize cell types through retrograde labeling.³³ Displaced amacrine cells could also be tracked more accurately and specifically as better markers are developed. Additional work will be necessary to characterize the specific cell types lost in other glaucoma models. Despite these limitations, we believe that this work supports the use of SD-OCT measurements of RGCL thickness as a general marker of total cell loss in the RGCL and RGC-specific cell loss in the *Brn3b*^{-/-} mouse model.

Brn3b^{-/-} mice have retinas that are 20% thinner than phenotypically wild-type mice.⁴ Although all subtypes of RGCs are equally affected in *Brn3b*^{-/-} mice, some subtypes are lost in an asymmetric topographic pattern.⁶ More extensive topographic analysis of cell loss has been performed in other mouse models of glaucoma, such as the DBA2/J mouse. DBA2/J mice develop pigmentary glaucoma because of a mutation in genes encoding melanosomal proteins.^{23,34,35} Analysis of total cell loss in retinas of aged DBA2/J mice shows damage to RGC axons beginning around the lamina, followed by clustered and fan-shaped areas of cell death across the retina with differential gene expression in areas of RGC loss.^{36–40} A similar pattern of sectorial RGC loss has also been shown in a rat ocular hypertension model.³³ Our 3D thickness model of the NFL and IPL of *Brn3b*^{-/-} eyes shows a symmetrical and nonlocalized loss of retinal thickness in the central retina (Fig. 2). These images suggest that, as a whole, cell loss in the NFL and IPL of *Brn3b*^{-/-} mice follows a generalized pattern of RGC loss and is not regionally specific—a pattern of cell loss similar to that seen in the C57BL/6 mouse.⁴¹

Only one of the two RGC subtypes described by Lin et al.⁴² showed any asymmetry in the *Brn3b*^{-/-} model, and more than 10 subtypes exist, so it is possible that the asymmetry described is subtype-specific and overshadowed by losses among the rest of the subtypes. One shortcoming of this method of retinal segmentation is the inability of SD-OCT to distinguish between RGC subtypes, so localized changes in subtypes such as that described in aged DBA/2N mice will be missed.⁴³ The restriction of the OCT field to a 1-mm² field surrounding the optic nerve head also may have missed topographical loss specific to the peripheral retina. Although analyses of topographical loss in other mouse models have shown a pattern of loss extending to the optic nerve head, a wider field will be necessary to show true homogenous loss.^{36,37,40}

Other modalities, such as scanning laser polarimetry (SLP) and confocal scanning laser ophthalmoscopy (cSLO), can be used to image the NFL in vivo. However, like SD-OCT, these imaging modalities are unable to differentiate between RGC subtypes and are further hampered by their reliance on indirect measurements of NFL thickness.^{15,44,45} The use of gene-based reporters allows for the examination of specific subtypes of RGCs to overcome the limitations in cellular subtype differentiation. GFP-labeling of RGCs in transgenic mice has been a particularly active field and provides a new method for researchers to distinguish RGC subtypes.^{46,47} However, imaging of GFP-labeled retinal cells in vivo is limited by shallow image penetration and low image resolution in vivo.⁴⁸ Ongoing research in the field of molecular contrast OCT (MCOCT) works to combine the molecular specificity of fluorescent labeling with the greater penetration and higher resolution of SD-OCT images.^{49,50} As this nascent field advances, there is great potential for contribution to the study of retinal changes in models of ocular disease.

Our work illustrates the use of SD-OCT in the study of RGC loss and supports additional work to evaluate RGC loss in mouse models of glaucoma. The correlation between total RGCL cell counts measured histologically and SD-OCT measured combined NFL and IPL thicknesses validates segmentation of SD-OCT retinal layers as a marker for RGCL cell loss in vivo. Immunofluorescence work confirms that the difference in cell count is largely related to changes in total RGC counts in the *Brn3b*^{-/-} retina. The 3D thickness map generated can be used to determine whether any observed cell loss occurs in topographically localized areas. These in vivo imaging methods will become more important as SD-OCT becomes widely available for the study of animal models for ocular disease, such as glaucoma, retinal degenerations, and other eye diseases with chronic progressive changes.

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