

Early-Onset Progressive Degeneration of the Area Centralis in RPE65-Deficient Dogs

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Submitted: March 24, 2017

Accepted: May 22, 2017

Citation: Mowat FM, Gervais KJ, Occelli LM, et al. Early-onset progressive degeneration of the area centralis in RPE65-deficient dogs. *Invest Ophthalmol Vis Sci.* 2017;58:3268–3277. DOI: 10.1167/iovs.17-21930

PURPOSE. Retinal epithelium-specific protein 65 kDa (RPE65)-deficient dogs are a valuable large animal model species that have been used to refine gene augmentation therapy for Leber congenital amaurosis type-2 (LCA2). Previous studies have suggested that retinal degeneration in the dog model is slower than that observed in humans. However, the area centralis of the dog retina is a cone and rod photoreceptor rich region comparable to the human macula, and the effect of RPE65 deficiency specifically on this retinal region, important for high acuity vision, has not previously been reported.

METHODS. Spectral-domain optical coherence tomography, fundus photography, and immunohistochemistry of retinal wholemounts and sagittal frozen sections were used to define the time-course and cell-types affected in degeneration of the area centralis in affected dogs.

RESULTS. Area centralis photoreceptor degeneration was evident from 6 weeks of age, and progressed to involve the inner retina. Immunohistochemistry showed that RPE65-deficient dogs developed early loss of S-cone outer segments, with slower loss of L/M-cone outer segments and rods.

CONCLUSIONS. Early-onset severe photoreceptor degeneration in the area centralis of dogs with RPE65-deficiency offers a model of the early foveal/perifoveal degeneration in some patients with LCA2. This model could be used to refine interventions aiming to improve function and halt the progression of foveal/perifoveal photoreceptor degeneration.

Keywords: canine model, LCA2, RPE65, cone photoreceptors, retinal degeneration, fovea, area centralis

Retinal epithelium-specific protein 65 kDa (RPE65) is an essential isomerase in the visual cycle that regenerates the chromophore 11-*cis*-retinal in the retinal pigment epithelium (RPE), enabling the reconstitution of light sensitive photopigments active in rod and cone photoreceptors.¹ Although there is growing evidence of an alternative chromophore regeneration cycle specific to cone photoreceptors involving Müller glia,² RPE-derived chromophore is also required for normal cone function.³

Clinical trials of gene therapy to treat human Leber congenital amaurosis type-2 (LCA2), a childhood-onset blinding photoreceptor degenerative disease associated with mutations in *RPE65*, have been supported by numerous proof-of-concept preclinical trials in RPE65-deficient dogs possessing a null mutation in *Rpe65*.^{4–8} These first human trials have reported some rescue of retinal function^{9–11}; although one trial showed

that despite therapy, photoreceptor degeneration progressed.¹² We have postulated that the first generation of gene therapy vectors provided inadequate levels of RPE65 resulting in insufficient 11-*cis* retinal to preserve photoreceptors.¹³ The need to optimize gene delivery and dose to increase effectiveness in human patients has necessitated further preclinical studies, many involving the canine model. These studies have guided our understanding of the available treatment window,^{12,14} the effect of repeated treatment,^{15,16} and dose-response effects.^{13,17}

Although historically considered a disease that affects rod photoreceptors more severely and at an earlier stage than cone photoreceptors, many young LCA2 patients suffer significant cone dysfunction¹⁸ with short wavelength light sensitive cones (S-cones) most susceptible to dysfunction.³ The central retinal fovea contains high concentrations of cone photoreceptors



TABLE. Details of RPE65 Deficient and Control Eyes Used During the Study

Animal ID Number	Group	Sex	Ages at SD-OCT, mo	Age at Euthanasia, mo	Use of Eyes
13-025	control	M	<u>10.2</u>	10.5	OD: C, OS: NA
12-102	control	F	<u>17.4</u>	17.6	OD: C, OS: C
11-040	control	F	<u>38.6</u>	38.7	OD: W - S, OS: W - LM
11-041	control	F	24.8, <u>32.5</u>	NA	NA
13-076	1	M	<u>1.2</u>	1.5	OD: C, OS: W - LM
13-078	1	M	<u>1.2</u>	1.5	OD: C, OS: W - S
12-003	1	F	1.3, 3, <u>6.3</u>	9.3	OD: W - S, OS: C
12-008	1	F	1.3, 3, <u>6.3</u>	9.3	OD: C, OS: W - LM
12-007	1	F	1.3, 3, <u>6.3</u> , 12.4, 26.56	NA	NA
09-079	2	M	NP	13.1	Semithin
09-077	2	M	NP	13.1	Semithin
12-068	2	M	<u>15.2</u>	15.2	OD: NA, OS: W - S
12-070	2	F	<u>15.2</u>	15.2	OD: NA, OS: W - LM
12-066	2	M	<u>16.9</u>	16.9	OD: NA, OS: C
12-071	2	F	<u>16.9</u>	16.9	OD: NA, OS: C
09-019	2	M	NP	19.8	Semithin
11-008	2	M	<u>18.1</u>	20.8	OD: W - LM, OS: C
11-009	2	M	<u>17.8</u>	20.8	OD: C, OS: W - S
08-038	3	M	NP	28.8	Semithin
09-076	3	F	<u>35.5</u>	55.6	OD: W - S, OS: NA
09-080	3	F	25.5, 35.5, <u>55.2</u>	55.6	OD: W - LM, OS: NA
06-050	3	M	<u>94.4</u>	94.9	OD: NA, OS: W - S
06-079	3	F	NP	64.4	OD: C, OS: C
02-152	3	M	112.4, <u>121.1</u>	122.3	OD: C, OS: NA

Underlined values are the values used in SD-OCT analysis for this manuscript. OD, right eye; OS, left eye; C, cryosections; W, wholemounts (S, S-cones; LM, L/M-cones); NP, not performed; NA, not available.

mediating central high-quality vision, and foveal and perifoveal cone degeneration occurs early in life in some LCA2 patients.^{3,19,20} The mechanism leading to cone degeneration is still unclear, although competition for small amounts of residual chromophore in diseased eyes^{21,22} and toxicity from mislocalized cone opsin²³ are theorized to contribute. LCA2 patients typically suffer early rod photoreceptor loss from childhood (and perhaps also prenatally) making the disease more aggressive than in mouse models and previous reports of the RPE65-deficient dog.^{10,19,24}

Previous studies in the RPE65-deficient dog have compared cone loss between the peripheral and central retina. No significant cone loss was identified in one study between 4 and 17 months of age.²⁵ Another study showed more severe loss of short wavelength sensitive opsin positive cones (S-cones) in the central but not peripheral retina of dogs between 18 and 30 months of age.²⁶ Neither study found regional differences in long/medium wavelength sensitive opsin positive cones (L/M-cones). We previously reported a more severe loss of S-cones than L/M-cones in a study that concentrated on the central portion of the retina and included a wider range of ages of study dogs.¹⁴ Gene augmentation therapy in RPE65-deficient dogs results in preservation of S-cones, but only in the treated region of retina.¹⁴ Although dogs do not have a fovea, the area centralis in the temporal cone-rich retinal visual streak contains the highest density of cones in the canine retina, approaching the cone density of the human macula/fovea.^{27,28} The effect of RPE65-deficiency on this region remains to be established.

In this study, we report the clinical and histologic features of an early-onset progressive photoreceptor degeneration of the area centralis in a colony of RPE65-deficient dogs. We show early thinning of the area centralis is associated with a loss of S-cone opsin immunoreactivity within a few months of birth, starting in the center of the area centralis. The extent of S-cone loss expands significantly with age to involve the cone-rich visual streak and also the adjacent superior retina. In contrast,

L/M-cones and rods degenerate more slowly, beginning at approximately 10 months of age, with the loss initially localized to the area centralis and inferior retina, respectively. The rapid photoreceptor degeneration involving the area centralis described here may reflect the situation in the retina of LCA2 patients and may provide an opportunity to assess the effectiveness of therapy to slow cone photoreceptor loss.

MATERIALS AND METHODS

Animals

RPE65-deficient dogs homozygous for a null mutation in *Rpe65* were derived from a colony originally established and maintained at Michigan State University. Animal care and use complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the Michigan State University's Institutional Animal Care and Use Committee. Animals were housed under a 12-hour light to dark cycle, and tissues were harvested in the light. Eyes from 24 dogs were examined (13 males and 11 females; 20 RPE65-deficient dogs and four wild-type dogs). Not all eyes were available for both clinical and histologic evaluation; 36 eyes were available for clinical evaluation, and 32 eyes were available for postmortem evaluation. The Table outlines the details of use of each animal. Of 11 affected retinas and 2 unaffected retinas utilized for wholemount analysis, 7 were labeled with S-cone opsin, and 6 were labeled with L/M-cone opsin. Four eyes from four dogs were used for retinal semithin evaluation.

Retinal Imaging

Wide-angle color digital fundus imaging (RetCam II, Clarity Medical Systems, Pleasanton, CA, USA) was performed on 20/24 animals. Confocal scanning laser ophthalmoscopy (cSLO)

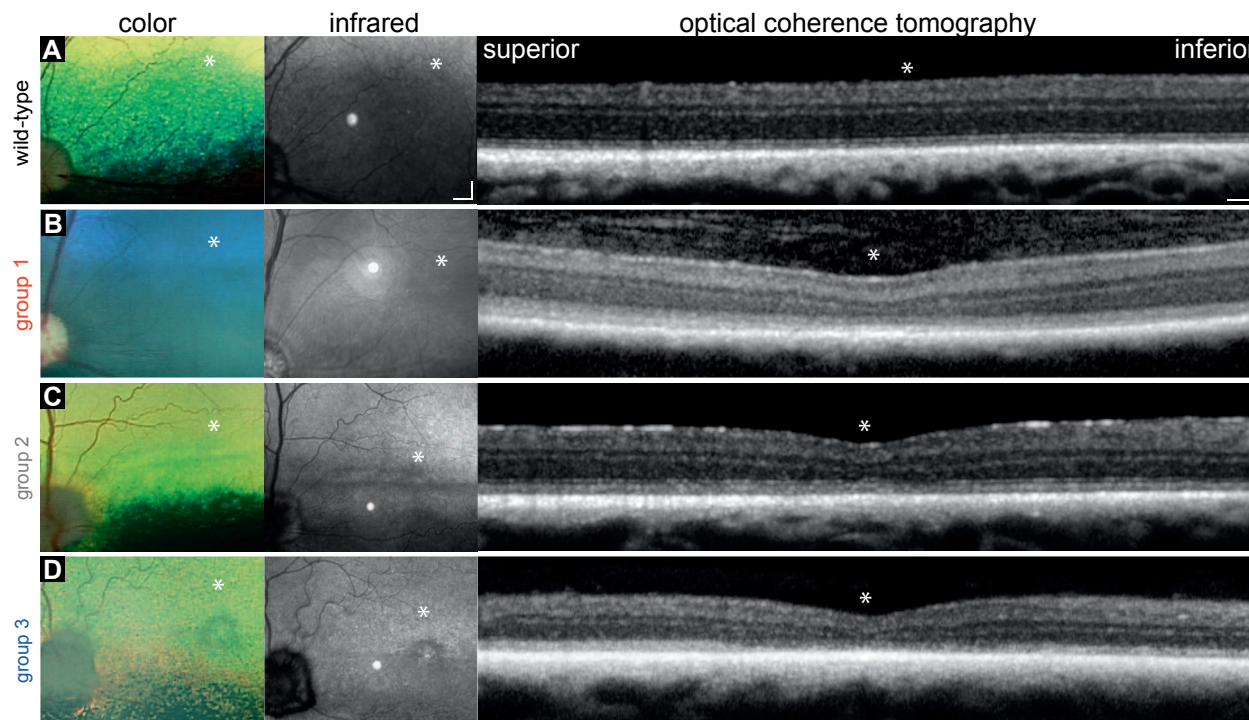


FIGURE 1. Early and progressive loss of the photoreceptor layers in the area centralis of young RPE65-deficient dogs. Images A–D show the area centralis region using color fundus photography (*left image*), infrared cSLO (*middle image*), and SD-OCT sagittal section (*right image*). Asterisks in each image have been placed just superior (*left and middle images*) or vitread (*right image*) to the area centralis. There is a focusing beam artifact (*white spot*) present in the infrared cSLO images. In the wild-type control dog, the area centralis is similar in appearance to the surrounding retina (38.6 months wild-type shown in A). In young group 1 animals, particularly those imaged prior to maturation of the tapetum lucidum, the thinning of the area centralis was not obvious on color and infrared images, but its presence was clearly detectable on SD-OCT imaging (1.2 months RPE65-deficient shown in B). The lesion was identified on fundus imaging (color and infrared cSLO) in group 2 animals (18.1-month-old in C), and in group 3 animals the lesion occupied a larger area and had progressed so that the outer nuclear layer was difficult to identify using SD-OCT (121.1-month-old in D). Scale bar (x and y scales shown) in infrared image in A represents 1 mm (applies to all infrared scans), and in SD-OCT cross-section represents 100 μm (applies to all SD-OCT images).

and spectral-domain optical coherence tomography (SD-OCT) imaging (Spectralis HRA+OCT, Heidelberg Engineering, Carlbad, CA, USA) was performed under general anesthesia on almost all animals at least once (Table). Pupils were pharmacologically dilated (Tropicamide Ophthalmic Solution 1%; Falcon Pharmaceuticals, Fort Worth, TX, USA), and eyes were maintained in primary gaze for imaging. Wide-field fundus images were obtained using cSLO and cross-section images by SD-OCT with an 820-nm wavelength laser and 55 and 30° lenses. Vertical and horizontal sections of the area centralis at 180- μm intervals were quantified. Thicknesses of the outer and inner nuclear layers and total retinal thickness were measured.

Retinal Wholemount, Frozen, and Semithin Sections and Immunohistochemistry

Following humane euthanasia by barbiturate overdose (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA), eyes were removed and processed for immunohistochemistry (IHC) as previously described as either retinal wholemounts²⁷ or frozen sections.²⁹ In vertical sagittal frozen sections, the location of the area centralis was estimated using retinal photographs and by calculating the horizontal distance of the area centralis from the optic nerve head (expressed as a ratio of the horizontal diameter of the optic nerve head). The width of the optic nerve head was measured during sectioning and used to calculate the approximate horizontal distance to the area centralis. IHC was performed as previously described using

antibodies for cone arrestin (human cone arrestin; CAR; labels all cones; kind gift of Cheryl Craft, University of Southern California),^{14,30} L/M- and S-cone opsin counterstained with peanut agglutinin (cone subtypes),¹⁴ rhodopsin (rods),¹⁴ protein kinase C- α (rod bipolar cells),²⁹ and glial fibrillary acidic protein (1:500 dilution, activated glial cells; Dako North America, Inc., Carpinteria, CA, USA). 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Corp., St Louis, MO, USA) nuclear counterstain was used.

To obtain semithin sections, globes were fixed in 2.5% glutaraldehyde and 2.5% paraformaldehyde. After fixation, a 0.5-cm by 0.5-cm square of retina containing the area centralis was collected. This was postfixed in 2% osmium tetroxide, dehydrated in acetone series, and infiltrated and embedded in spur resin.³¹ Serial sections at 2- μm intervals were stained with Toluidine blue.

Imaging and Analysis

To quantify cone subtypes in retinal wholemounts, fluorescent microscopy images were taken at 40 \times magnification using a Nikon Eclipse 80i microscope (Nikon Instruments, Inc., Melville, NY, USA) equipped with a CoolSnap ESv camera (Photometrics, Tucson, AZ, USA). The camera position on the microscope was adjusted to orient the visual streak horizontally on the image, or to orient the dorsal retinal vein vertically when the visual streak was not clearly visible. Images were taken at 1-mm intervals in horizontal and vertical planes through the area centralis. For fine mapping of the region of

the area centralis, contiguous images were obtained vertically and horizontally surrounding the area centralis spanning $168 \times 250 \mu\text{m}$. Images were randomized and masked to the location and identity of the animal, loaded into an image analysis program (ImageJ, National Institutes of Health, Bethesda, MD, USA), and all cone matrix sheaths (labeled with peanut agglutinin) and cone opsin subtype outer segments (labeled with S- or L/M-opsin) were manually counted.

To quantify rod and cone numbers in retinal sections, images were taken of vertical sections of the area centralis region stained with CAR to identify cones and DAPI to identify nuclei. In images taken $340\text{-}\mu\text{m}$ apart (images spanned a $112.5\text{-}\mu\text{m}$ length of retina), the total number of nuclei in the outer nuclear layer and cone (CAR immunolabeled) nuclei were counted, and cone density per $500\text{-}\mu\text{m}$ length of retina was calculated. The number of rod nuclei were calculated as total outer nuclear layer nuclei minus cone nuclei as previously described.¹⁴

Statistical Analysis

RPE65-deficient animals were placed into three age groups for analysis: group 1 = 1.2 months to 10 months; group 2 = 10 months to 24 months; group 3 = 24 months to 123 months. A 2-way ANOVA was used to compare the three age groups to each other, and to wild-type controls. Variables for 2-way ANOVA included age (grouped) and location (distance from the center of the area centralis). A Bonferroni posttest correction was used to compare all RPE65-deficient dog groups with the control group.

RESULTS

Photoreceptor Loss Within the Area Centralis Begins Early and Progresses to Involve the Inner Retina

Using fundus photography (color and infrared via cSLO) and SD-OCT to examine eyes of affected animals of different ages ($n = 4$ control dogs, 5 group 1, 6 group 2, 4 group 3), we determined that area centralis outer retinal thinning in RPE65-deficient dogs started at an early age and was progressive (Fig. 1). The precise location of the center of the area centralis in normal dogs can be difficult to identify on these images although the retinal location is well described.²⁷ On SD-OCT imaging, the area centralis can be identified by a subtle thickening of the ganglion cell layer and a slight thickening of the inner/outer segment layers (Fig. 1A). At the earliest time point assessed (5–6 weeks of age), the lesion in the area centralis in RPE65-deficient dogs was difficult to identify on color fundus photographs because at this age the tapetum lucidum had not developed fully and the image lacked contrast. However, the lesion was identifiable on infrared cSLO images as a streak of slightly altered reflectivity and easily identified on SD-OCT cross-sectional scans because of retinal thinning (Fig. 1B). With increasing age and maturation of the tapetum lucidum, the lesion was also identifiable on color fundus photography (as a linear or circular region of tapetal hyper-reflectivity). As monitored by SD-OCT cross-sectional imaging, the retina became progressively thinner in the area centralis. There was also a loss of definition of the external limiting membrane and ellipsoid zone (Figs. 1C–D). Measurement of SD-OCT images revealed a focal decrease in retinal thickness (Figs. 2A, 2B) and outer nuclear layer thickness (Figs. 2C, 2D) in the center of the area centralis in eyes of group 1 and 2 dogs (up to 24 months of age) in both the horizontal and vertical meridians. In group 3 dogs, retinal thinning had extended to

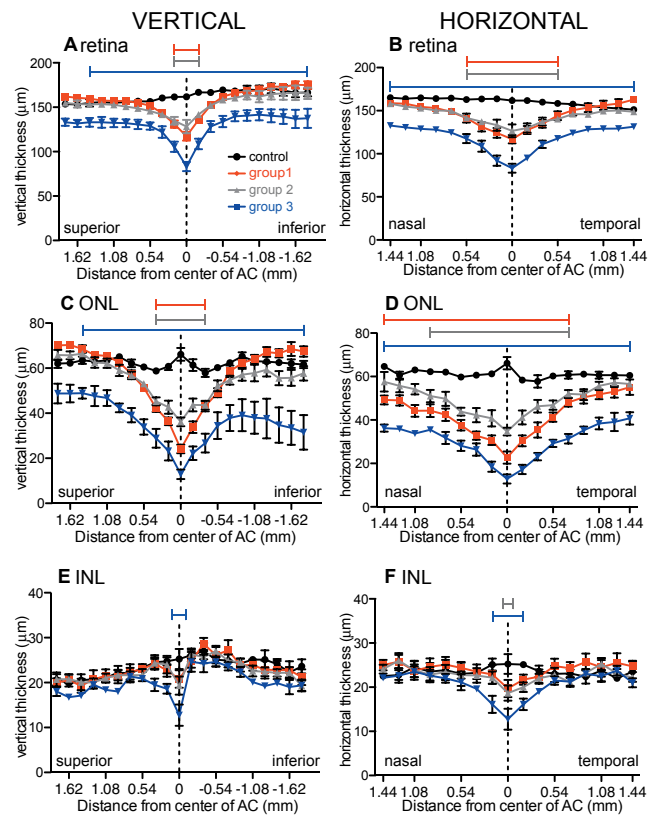


FIGURE 2. Quantification of the loss of retinal layers vertically through the area centralis and horizontally along the visual streak. SD-OCT scans were analyzed to measure thickness of retinal layers in the vertical and horizontal meridian. Colored bars above the graphs indicate where differences between the corresponding group and controls were significant ($P < 0.05$) using a 2-way ANOVA (age and location were the variables assessed). Total retinal thickness was smaller in the area centralis with expansion of the thinning in the vertical direction in group 3 dogs (A); a wider area was affected in the younger animals in the horizontal meridian (B). Outer nuclear layer thickness reflected this change, in both the vertical (C) and horizontal (D) meridians. Inner nuclear layer thickness was reduced in group 3 animals in the center of the area centralis in both the vertical (E) and horizontal (F) meridians and also in the horizontal meridian in group 2 animals. *N*, numbers: 4 control dogs, 5 group 1 dogs, 6 group 3 dogs, and 4 group 3 dogs.

involve a wider area of the area centralis both vertically and horizontally. The thickness of the inner nuclear layer was comparable to wild-type controls in younger animals, but became focally thinned in the center of the area centralis of the eyes of older group 3 dogs (Figs. 2E, 2F), and also in group 2 dogs in the horizontal meridian. Even in younger individuals, the length of retina affected along the horizontal meridian of the visual streak was greater than that seen in the vertical scans, indicating that the visual streak in the region of the area centralis, as well as the area centralis itself were affected at an early stage.

S-Cone Outer Segment Loss Is Widespread and Precedes More Focal Loss of L/M-Cone Outer Segments

The area centralis contains a high density of both rod and cone photoreceptors and the density of cones peaks in the central portion.²⁷ Retinal wholemounts from RPE65-deficient dogs of various ages ($n = 1$ control retina, 2 for each opsin

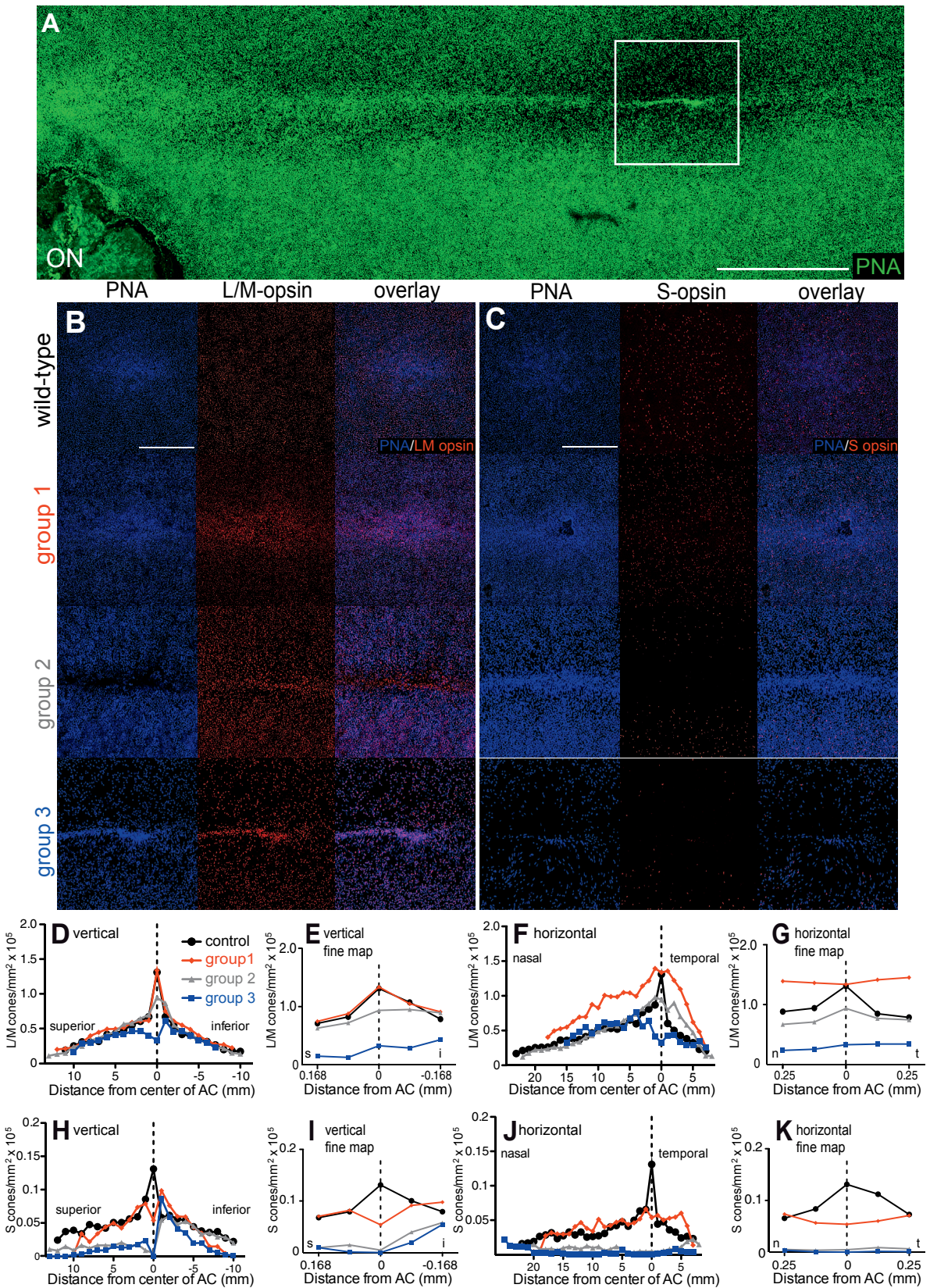


FIGURE 3. Cone subclass distribution in retinal wholemounts of the area centralis and visual streak at different ages in RPE65-deficient dogs. The area centralis region (delineated by a *white box* in **A**) was markedly depleted of cone photoreceptor outer segments (peanut agglutinin labeling of the cone matrices in *green*) in older RPE65-deficient dogs as shown in the low power overview (**A**; 55.6-month-old, group 3 dog). In many dogs a linear horizontal streak of residual peanut agglutinin labeling remained across the area centralis. Progressive loss of L/M-opsin cone outer segments occurred (*red*, all cone matrix sheaths labeled with PNA in *blue*) in the area centralis region (**B**), with progression most obvious between groups 2 and 3. This left a residual narrow central horizontal streak of L/M-opsin labeling cells, whereas the loss of S-cone labeled outer segments (labeled in

red, all cone matrix sheaths labeled with PNA in *blue*) occurred at an earlier stage (C). Quantification of L/M-cone outer segment density is shown in graphs D–G. In the vertical meridian, depletion of L/M-cone outer segments was noted in the area centralis of group 2 eyes, progressing in extent but still restricted to the area centralis region in group 3 eyes (D, fine mapping shown in E). In the horizontal meridian, depletion of L/M-cone outer segments was noted in the area centralis of group 2 eyes, progressing in extent but still restricted to the area centralis in group 3 eyes (F, fine mapping shown in G). Quantification of S-cone outer segment density is shown in graphs H–K. In the vertical meridian, depletion of S-cone positive outer segments was noted in the center of the area centralis of group 1 eyes, progressing in extent to involve most of the quantified superior retina in group 2 and 3 eyes (H, fine mapping shown in I). In the horizontal meridian, S-cone outer segments were depleted in the area centralis region in group 1 eyes, this extended to involve the whole horizontal length of the visual streak in group 2 and 3 eyes (J, fine mapping shown in K). Note the difference in scale of S- and L/M-cone *y* axes. Representative images shown in B and C were images from eyes at the following ages: control: 38.6 months; group 1: 1.5 months; group 2: 20.8 months; group 3: 55.6 months. In A: ON, optic nerve; PNA, peanut agglutinin; rg opsin, L/M-opsin; b opsin, S-opsin; n, nasal; t, temporal; s, superior; i, inferior. Scale bar in A = 1 mm; scale bar in B and C = 250 μ m. *N* = 1 independent eye control for each of LM and S opsin, 2 for each group).

subtype and each group) were analyzed to determine two parameters: firstly, whether cones were lost in the area centralis and visual streak as the outer nuclear layer thinned, and secondly, whether the two cone subtypes were affected to different extents. Loss of cone outer segments (demonstrated by loss of peanut agglutinin staining) was apparent at low magnification in the area centralis and surrounding region of eyes from group 2 and 3 dogs (Fig. 3A), and progressive depletion of both L/M- and S-opsin expressing cone outer segments was evident in affected eyes (Figs. 3B–C). However, cone outer segments were preserved in a horizontal streak in the center of the area centralis, extending along the visual streak (Figs. 3A, 3B). These residual cones contained L/M-opsin (Fig. 3B, also shown in the L/M opsin panel in Fig. 3G). Interestingly, L/M cone numbers appeared higher in the visual streak of group 1 animals compared with control. L/M-cone outer segment loss occurred at a slower rate than that of S-cones with depletion only apparent in eyes from groups 2 and 3 (Fig. 3B). In contrast, S-cone outer segment loss occurred in groups 1 to 3 (Fig. 3C). Regional quantification of cone subtypes in the area centralis confirmed these findings (Figs. 3D–K): in the vertical (Figs. 3D, 3E) and horizontal (Figs. 3F, 3G) meridians, group 2 eyes had mild L/M-cone outer segment loss, whereas group 3 eyes showed a more profound L/M-cone outer segment loss. L/M-cone outer segment loss expanded in vertical and horizontal extent between group 2 and group 3 eyes. S-cone outer segment depletion was identified in group 1 eyes (Figs. 3H, 3D), expanding to also involve a large proportion of the region superior to the area centralis in group 2 and 3 eyes. In the horizontal meridian along the visual streak, group 1 eyes demonstrated focal area centralis S-cone outer segment loss, progressing in group 2 and 3 eyes to result in almost complete S-cone outer segment absence along the entirety of the visual streak (Figs. 3I, 3J).

The Area Centralis Undergoes Rod Loss and Inner Retinal Alterations

Sagittal retinal cross-sections and IHC were used to examine the area centralis in eyes of different ages of affected dogs. By 64 months of age, there was almost complete cone nuclei and inner/outer segment loss in the area centralis (Fig. 4A). Semithin sections confirmed the SD-OCT findings of total retinal and outer nuclear layer thinning in the center of the area centralis (Figs. 4B–C). The remaining photoreceptors had nuclear morphologic features identifying them as rods and cones, and cone inner segments could also be discerned (Fig. 4C). At the center of the affected area, there was also displacement of the inner nuclear layer cells into the outer plexiform layer. There was expected accumulation of vacuoles in the RPE apparent in semithin sections.

Imaging and analysis of vertical sections of the area centralis (Figs. 4D–F) revealed that there were fewer rods in

the area centralis than surrounding areas (wild-type control eyes also had lower numbers of rods in the center of the area centralis where the number of cones peaked). There was a significant depletion of rods within the area centralis and inferior to it in group 3 eyes (Fig. 4D). The total number of cones in the area centralis in group 3 eyes was significantly reduced (Fig. 4E, 4F). Insufficient sections were available to compare cone subtypes statistically; however, histologic sections followed the same trend as retinal wholemounts; S-cone outer segments were depleted in eyes from groups 1 to 3, compared with L/M-cone outer segments, which only appeared depleted in group 3 eyes (Fig. 4G). Glial cell activation was present even in group 1 eyes, and progressively increased in eyes from groups 2 and 3, with glial fibrillary acidic protein immunolabeling extending into the outer nuclear layer and inner/outer segment regions (Fig. 4H). Rod bipolar cell nuclei displacement into the outer plexiform layer was also identified in group 1 eyes similar to that seen in semithin sections, and thinning and loss of organization of the outer plexiform layer axons (protein kinase $c\alpha$ labeled, rod bipolar cells) was evident in group 2 and 3 eyes (Fig. 4I). All changes were more severe in the area centralis than surrounding retinal regions.

DISCUSSION

In this manuscript, we show that photoreceptors in the area centralis of RPE65-deficient dogs degenerate at an early age, which is in contrast to other retinal regions where retinal thinning is not established until dogs are several years of age. This previously unreported early regional photoreceptor loss may more closely reflect the timing of photoreceptor loss observed in many LCA2 patients. In vivo SD-OCT retinal imaging showed an initial loss of definition of the ellipsoid zone in the affected area as early as 6 weeks of age, during the final stages of retinal maturation in the dog.³² This loss of ellipsoid zone definition is a common finding during photoreceptor degeneration and may represent abnormalities in the photoreceptor inner and outer segment organization; in humans this finding correlates with loss of visual acuity.³³ The changes in the center of the area centralis were progressive, leading to a dramatic thinning of the outer nuclear layer and associated loss of photoreceptors. In addition to area centralis photoreceptor loss, we identified glial cell activation and loss of rod bipolar cell axons. Alterations in second order neurons and other retinal cell types accompanying photoreceptor loss is well documented³⁴ and has previously been reported in RPE65-deficient dogs,²⁶ although here we show that the cells in the area centralis suffer earlier and more profound changes.

Photoreceptor loss in the area centralis and the immediately surrounding retina was characterized by immunohistology of retinal wholemounts and sagittal sections, and

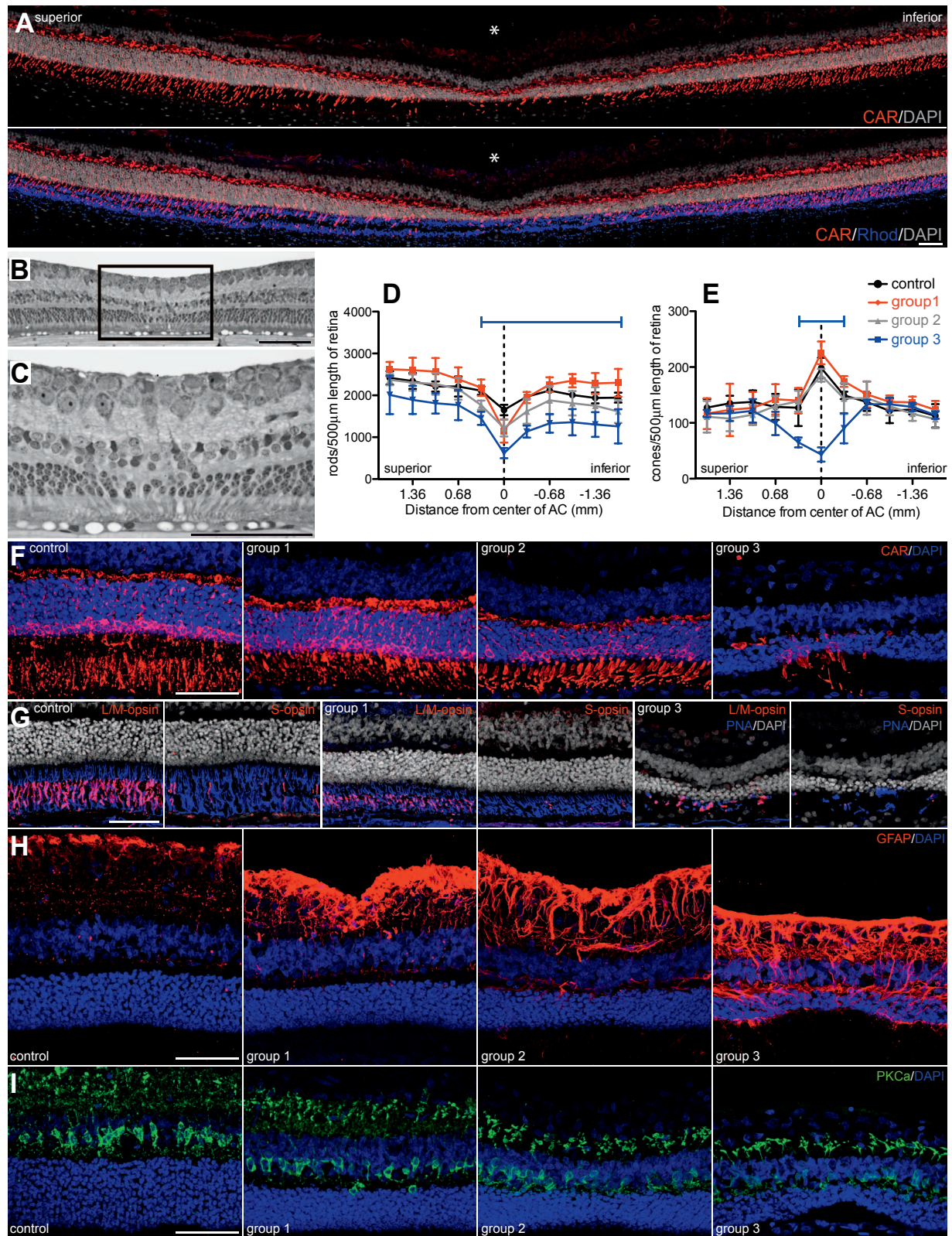


FIGURE 4. Histology and IHC of vertical cross-sections of the area centralis region. Loss of photoreceptors in the area centralis region was identifiable in sagittal retinal sections (A shows a 64.4-month-old, group 3 RPE65-deficient retina, vitread to area centralis marked with an *asterisk*), the outer nuclear layer was thinned (DAPI in *gray*), and there was significant depletion of cones (cone arrestin in *red*) and rods (rhodopsin in *blue*), although in the very center of the region a small number of cones remained and across the region some rods were still present. Images of semithin sections in B and further magnified in C illustrate the profound loss of photoreceptor nuclei in the area centralis of a group 2 (13.1-month-old) RPE65-deficient dog. In RPE65-deficient dogs, rod photoreceptor nuclei were progressively depleted, particularly from the area centralis and the retinal region inferior to it (D), the difference was significantly different from control retinas in group 3 (*blue line* above the graph outlines the region of statistically significant difference; $P < 0.05$, between group 3 and controls). Cone depletion was significant in the area centralis region in

group 3 animals (E, blue line delineates the region of significant difference between group 3 and controls, $P < 0.05$). F-I show representative sections of the area centralis regions of eyes from controls and groups 1 to 3. In the area centralis of controls and group 1 RPE65-deficient dogs, there was a dense cluster of cone photoreceptors (F, CAR in red); relative cone numbers declined with age, with very few remaining in the area centralis of group 3 animals, with the exception of some surviving cones immediately in the center of the area centralis. The effect on cone subclasses mirrored that seen in retinal wholemount analysis, with a relatively slower loss of L/M-cone outer segments compared with an early reduction in S-cone outer segments (G, L/M- or S-opsin in red, PNA labeling cone matrix sheaths in blue). The inner retina was also altered from an early age in RPE65-deficient eyes. GFAP (H, labeled in red) labeling showed a progressive glial reaction in group 1 to 3 eyes. GFAP positive processes extended through the region of the remaining photoreceptor nuclei and through the region of the outer limiting membrane in group 3 eyes. Rod bipolar cells (I, PKCa labeled in green) were also affected at an early stage in RPE65-deficient eyes, with displacement of cell bodies into the outer plexiform layer (this can also be seen in the semithin section C) and shortening of both axons and dendrites noted in group 1 to 3 eyes. DAPI, nuclear counterstain; CAR, cone arrestin; B opsin, S-cone opsin; PNA, peanut agglutinin; RG opsin, L/M-cone opsin; GFAP, glial fibrillary acidic protein; PKCa, protein kinase C α . Scale bars in A, F-I = 50 μ m; B, C = 25 μ m.

although the numbers of retinas utilized for each group was small, the differences were striking, particularly in groups 2 to 3. S-cone opsin labeling in retinal wholemounts was decreased prior to loss of L/M-cone opsin immunolabeling. Sagittal sections highlighted the outer nuclear layer thinning at the center of the area centralis, resulting from loss of both rods and cones.

In addition to temporal differences in photoreceptor degeneration, we identified spatial differences; with S-cone opsin immunolabeling being lost first in the area centralis and then later in the visual streak and superior retina. The apparently higher L/M cone density along the visual streak in young RPE65-deficient dogs noted in our wholemount analysis warrants further study, and may represent an aberration of quantification, or could reflect early pruning of cone photoreceptors as has been suggested to occur in the primate retina.³⁵ L/M-cone opsin labeling was lost in the area centralis and immediate surrounding retina, and rod loss occurred in the area centralis and inferior to it. In the very center of the area centralis and along the visual streak, a small number of L/M-opsin expressing cones remained, contrasting with the adjacent area centralis and visual streak, which became almost totally devoid of opsin-expressing cones. Histologic cross-sections confirmed a very reduced area centralis outer nuclear layer with a small number of remaining L/M-cones. This may represent survival of some of the small fovea-like collection of cones recently described in the center of the canine area centralis.²⁸

The early photoreceptor loss in the area centralis we report here is striking, and it is unclear why this has not previously been reported. Two other immunohistochemical studies of RPE65-deficient dogs from different research colonies investigated tempo-spatial photoreceptor loss, with one study reporting a loss of S-opsin expressing cones.^{25,26} Neither of these studies reported specifically on the area centralis, so it is not possible to compare our findings with those studies. It is conceivable that this change is unique to our colony resulting from an effect of background genetics or possibly even an environmental effect. The same four base-pair frameshift mutation causing deletion in *Rpe65* is present in all colonies of affected dogs.^{36,37} However, the background genetics of individual colonies are likely different. Our colony was founded from a single pure-bred homozygous RPE65-deficient male Briard dog crossed onto a laboratory beagle background, a breed known to have a high cone density in the area centralis and visual streak,²⁷ although how this compares to the cone density of other breeds of dog has not been reported. We hypothesize that a higher density of central photoreceptors in our beagle background RPE65-deficient dog model may put the central photoreceptors at greater risk of retinoid deficiency and subsequent area centralis and visual streak degeneration. It has been reported that in regions of high photoreceptor concentration in the retina with RPE65 deficiency, there is competition for alternative retinoids (such as 9-*cis*-retinal)³⁸

that are present at low levels. Such competition has been proposed to contribute to cone loss in a *Rpe65* hypomorph mouse model,²² and perhaps similar competition for the alternative retinoids also exists in the canine model and is more severe where photoreceptors are more densely packed in the area centralis and visual streak.

Cone photoreceptor loss has been studied in mouse models of LCA2. Since mice do not have a retinal region of higher cone density to model the human macula, regional differences would not reflect the situation in human patients. However, similar to the findings we report in dogs, earlier loss of S-cones than L/M-cones is a feature of the following three mouse models: the *Rpe65* knockout mouse,³⁹ the *Rpe65^{rd12}* spontaneously occurring mouse,⁴⁰ and the *Rpe65^{R91W}* knock-in model, which retains low levels of RPE65 activity.²² Psychophysical studies to investigate cone function in human LCA2 patients indicate that L/M-cone function is present while S-cone function is not detectable, suggesting that S-cones might be lost more rapidly.³ Advances in in vivo imaging have allowed more detailed characterization of the structural changes that occur in the retina of LCA2 patients.^{19,20,41} Imaging of the macula and fovea has shown that although patients had a loss of central cones from early childhood, some foveal cones survive for several decades.³ The small streak of surviving L/M-cones in the degenerate area centralis in the RPE65-deficient dogs we describe may model the remaining foveal cones in LCA2 patients. The loss of photoreceptors in retinal regions distant to the area centralis region in RPE65-deficient dogs does not occur until middle-age (approximately 6 to 7 years old), in contrast to human LCA2, where marked outer nuclear layer thinning in the more peripheral regions is an early change.^{3,19,41} Photoreceptor loss was even shown to be present in a 33-week preterm fetus with LCA2,⁴² and LCA2 affected children have clinically detectable thinning of the inferior retina.¹⁹ A study by Cideciyan et al.¹² suggested that the onset of degeneration in the canine RPE65-deficient retina started at 5.3, 4.9, and over 7 years of age in the superior, inferior, and nasal visual streak regions, respectively. In their study they did not specifically examine the area centralis. The relatively slow retinal degeneration in these retinal regions of RPE65-deficient dog retina makes the testing of the structural preservation in these areas following therapies costly, time consuming, and does not accurately model disease seen in human LCA2. The presence of an early and progressive photoreceptor degeneration in the area centralis of our colony of RPE65-deficient dogs may, however, facilitate such assessments.

Acknowledgments

The authors thank András Komáromy for providing additional eyes for analysis from RPE65-deficient dogs, and Annie Oh, Kristin Koehl, and Christine Harmon for animal assistance.

Supported by Donald R. Myers and William E. Dunlap Endowment, The Hal and Jean Glassen Memorial Foundation, RP Fighting Blindness (UK), and UK Medical Research Council.

Disclosure: **F.M. Mowat**, None; **K.J. Gervais**, None; **L.M. Occelli**, None; **M.J. Annear**, None; **J. Querubin**, None; **J.W. Bainbridge**, None; **A.J. Smith**, None; **R.R. Ali**, None; **S.M. Petersen-Jones**, None

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