Altered Expression of Retinal Molecular Markers in the Canine *RPE65* Model of Leber Congenital Amaurosis

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PURPOSE. Leber congenital amaurosis (LCA) is a group of childhood-onset retinal diseases characterized by severe visual impairment or blindness. One form is caused by mutations in the *RPE65* gene, which encodes the retinal pigment epithelium (RPE) isomerase. In this study, the retinal structure and expression of molecular markers for different retinal cell types were characterized, and differences between control and *RPE65* mutant dogs during the temporal evolution of the disease were analyzed.

METHODS. Retinas from normal and mutant dogs of different ages were examined by immunofluorescence with a panel of 16 different antibodies.

RESULTS. Cones and rods were preserved in the mutant retinas, and the number of cones was normal. However, there was altered expression of cone arrestin and delocalization of rod opsin. The ON bipolar cells showed sprouting of the dendritic arbors toward the outer nuclear layer (ONL) and retraction of their axons in the inner nuclear layer (INL). A decreased expression of GABA, and an increased expression of intermediate filament glial markers was also found in the mutant retinas. These changes were more evident in the adult than the young mutant retinas.

CONCLUSIONS. The structure of the retina is well preserved in the mutant retina, but several molecular changes take place in photoreceptors and in bipolar and amacrine cells. Some of these changes are structural, whereas others reflect a change in localization of the examined proteins. This study provides new information that can be

applied to the interpretation of outcomes of retinal gene therapy in animal models and humans. (*Invest Ophthalmol Vis Sci.* 2010;51:6793–6802) DOI:10.1167/iovs.10.5213

Leber congenital amaurosis (LCA) comprises a group of childhood-onset, autosomal recessive retinal diseases that results in severe visual impairment or blindness.¹ One form of LCA is caused by mutations in the *RPE65* gene,² which encodes the 65-kDa retinal pigment epithelium (RPE)-specific isomerase involved in visual pigment regeneration.^{3,4} The *RPE65* protein has an essential role in maintaining retinal function and photoreceptor viability, and mutations in this protein affect the essential pathways involved in the processing and metabolism of vitamin A and retinoid cycling between the RPE and photoreceptors.⁵ Mutations in *RPE65* occur naturally in dogs^{6,7} and mice⁸ and have been experimentally produced by transgenic methods.⁹

RPE65 deficiency results in the accumulation of lipid inclusions containing all-*trans*-retinyl-esters in the RPE and undetectable levels of the 11-*cis* retinaldehyde chromophore complexed to opsin, together with rod and cone photoreceptor dysfunction.^{6–11} Depending on the animal model and strain studied, photoreceptor degeneration varies. In general, dogs show late-onset photoreceptor degeneration (after 5 years of age) that progresses slowly.^{6,11} In mice, on the other hand, retinal degeneration occurs early and is progressive, affecting cones more severely than rods.¹² A comprehensive review of the differences and similarities in disease between the different animal models and humans has been recently published.¹³

Recent studies have shown the dramatic and stable restoration of retinal and central visual function in *RPE65* mutant dogs after a single subretinal injection of AAV2 viral vectors containing normal human or canine *RPE65* cDNA.^{11,13–15} In parallel, safety studies have been completed in humans^{16,17} and a suitable patient population identified¹⁸ for human clinical trials. Three phase 1 clinical trials have been initiated,^{19–22} and 1-year treatment results have been reported^{23–25} that demonstrate stability²³ and improvement in retinal function in treated areas.^{22,24}

What is unknown at this time in the animal and human studies, however, is the extent of retinal reorganization and remodeling that occurs secondary to the disease, the cell layers affected, and whether these changes are progressive. Equally important is the assessment of reversibility of the damage after gene therapy. Since these dogs are congenitally blind, it is possible that normal postnatal retinal development and organization would be altered as a consequence of the disease.

To address the first question, we used a panel of antibodies that characterize the expression and localization of molecular markers in wild-type (wt) and mutant retinas. The purpose was to investigate the effect of the functional deletion of the *RPE65* gene product on the expression of different molecular markers in retinal cells in disease. We found evidence of altered expression of some molecular markers, but a remarkable preservation

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TABLE 1. Dogs Used in the Study

Status	Dog ID	Eye	Terminal Age
Normal	EM170	OD	4 months
Normal	EM171	OS	4 months
Normal	7560	OS	18 months
Normal	7561	OS	24 months
Normal	7559	OS	27 months
<i>RPE65</i> ^{-/-}	Br129	OS	4 months
<i>RPE65</i> ^{-/-}	Br113	OS	10.5 months
<i>RPE65</i> ^{-/-}	Br118	OS	15 months
<i>RPE65</i> ^{-/-}	Br89	OS	17.1 months

Identification of the dogs used in the present study.

of the retinal structure, despite the severe accompanying functional deficits that were present.

METHODS

Animals and Tissue Fixation

Five wt and four *RPE65*-mutant dogs were studied. The dogs (control and mutant) were maintained at the Retinal Disease Studies (RDS) facility (Kennett Square, PA) or were part of a separate research colony (control) located at the Baker Institute for Animal Health (Cornell University, Ithaca, NY). The dogs were housed in a cyclic light environment (7 AM lights on/7 PM lights off), and the eyes were collected during the first 4 to 5 hours of the light cycle. All procedures were done in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The wt dogs were homozygous normal (+/+) at the *RPE65* locus; the dogs were 4 ($n = 2$), 18 ($n = 1$), and 24 to 27 ($n = 2$) months of age. Of the four homozygous mutant dogs, one was 10.5 months of age (referred to as young adult mutant; dog Br 113), and two were 15 and 17.1 months of age (referred to as adult mutant; dogs Br118 and Br89, respectively; Table 1). In the 4- to 17.1-month time span, the retina of this strain of *RPE65* mutant dogs shows some structural changes, but not overt degeneration.¹¹ This differs from the findings in the initial description of the disease where photoreceptor degeneration was reported to occur as early as at 7 months.^{26,27}

The mutant dogs are part of a research strain of mixed-breed dogs maintained at the RDS facility. The disease in this strain derives

from a single mutant Briard dog and is caused by a 4-bp deletion in the canine *RPE65* gene.⁶ The dog identification prefix used in Table 1 is based on the breed of origin (Br, Briard) of the disease. Molecular diagnostic testing has determined that this strain is homozygous normal for other genes/loci that are responsible for inherited retinal degeneration in dogs (*prcd*, *erd*, *CNGB3*, *PDE6B*, *RHO*, and *RPGR*).²⁸

The dogs were anesthetized with intravenous pentobarbital. The eyes were enucleated, and the dogs were euthanized with a barbiturate overdose. The retinas were processed using two different methods that gave the same results for the antibodies tested. In the first method, the posterior segments of the eye cup were isolated and fixed for 3 hours in 4% paraformaldehyde prepared in 0.1 M phosphate-buffered saline (PBS) at 4°C. The posterior segments were then trimmed into superior and inferior quadrants that extended from the optic nerve to the ora serrata, cryoprotected 24 hours in 0.1 M PBS containing 30% sucrose at 4°C, and embedded in optimal cutting temperature (OCT) medium. In the second method, the entire globe was fixed for 3 hours in 4% paraformaldehyde prepared in 0.1 M PBS at 4°C, the posterior segment then was isolated and the eye cup fixed for an additional 42 hours at 4°C in 2% paraformaldehyde prepared in 0.1 M PBS. The tissue was trimmed in the same manner as in the first method, sequentially cryoprotected during 24 hours in 15% and 30% sucrose in PBS at 4°C, and embedded in OCT. Cryosections were cut at 10- to 12-μm thickness and stored at -80°C until used.

Immunocytochemistry

Different antibodies were used to identify molecular markers that define different cellular compartments or layers of the differentiated retina. These included the RPE, rod and cone photoreceptors, outer (ONL) and inner (INL) nuclear layers, and ganglion cell layer (GCL). In addition, the expression of glial molecular markers was evaluated, and an *RPE65* antibody was used to confirm the presence or absence of immunolabeling in the normal and mutant retinas, respectively. Double immunolabeling with different combination of antibodies was used to visualize various cellular classes when the primary antibodies were raised in different species. Mounted sections were washed two times for 10 minutes each in 1 M PBS containing 0.25% Triton X-100 at pH 7.4 and then incubated overnight at room temperature with the primary antibodies. The antibodies used and their concentrations are listed in Table 2. All immunocytochemistry experiments were per-

TABLE 2. Primary Antibodies

Antigen	Host	Source, Catalog No.*	Working Concentration	Normal Retinal Localization
RPE65	Rabbit polyclonal	T. Michael Redmond	1:10,000	RPE
RPE65	Mouse monoclonal	Novus, NB 100-355	1:500	RPE
hCAR	Rabbit polyclonal	Cheryl Craft, LUMIF	1:10,000	Cone photoreceptors
M/L opsin	Rabbit polyclonal	Chemicon, AB5405	1:10,000	OS of M/L cones
S opsin	Rabbit polyclonal	Chemicon, AB5407	1:5,000	OS of S cones
Rod opsin	Mouse monoclonal	Paul A. Hargrave, R2-12N	1:300	OS of rods
PKCα	Mouse monoclonal	Santa Cruz, SC-8393	1:2,000	Rod bipolar cells
Goα	Mouse monoclonal	Chemicon, MAB3073	1:5,000	ON cone bipolar cells
Synaptophysin	Mouse monoclonal	DAKO, MO776	1:100	Synapses photoreceptor-bipolar cells, IPL
TH	Rabbit polyclonal	Chemicon, AB152	1:500	Dopaminergic amacrine cells
GABA	Rabbit polyclonal	Chemicon, AB5016	1:50	GABAergic amacrine cells
Calretinin	Rabbit polyclonal	Sigma-Aldrich, C7479	1:1,000	Amacrine cells and any RGCs
BDNF	Rabbit polyclonal	Santa Cruz, SC-546	1:500	Amacrine cells and any RGCs
p75	Mouse monoclonal	Santa Cruz, SC-13577	1:1,000	IPL and Müller cells
CRALBP	Rabbit polyclonal	John Saari	1:1,500	Müller cells, RPE
Vimentin	Mouse monoclonal	Dako, M0725	1:2,000	Müller cells and astrocytes
GFAP	Rabbit polyclonal	Dako, Z0334	1:1,000	Astrocytes

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formed in the control and mutant retinal sections simultaneously to ensure consistency of all procedures.

The antibodies were diluted with PBS containing 0.25% Triton X-100. After incubation, sections were rinsed two times for 10 minutes each time in PBS. Afterward, the sections were incubated for 1 hour in darkness with secondary antibodies diluted in PBS containing 0.25% bovine serum albumin (BSA). Secondary antibodies included goat anti-rabbit IgG conjugated to either Bodipy FL (505–513) or AlexaFluor (488) for green fluorescence, and goat anti-mouse IgG conjugated to Texas red (595–615) or Alexa Fluor (568) for the red fluorescence. All secondary antibodies were from Invitrogen-Molecular Probes (Eugene, OR). DAPI (4',6-diamidino-2-phenylindole) was used for nuclear labeling. The sections were washed two times for 10 minutes each time in PBS, mounted in 50% PBS-glycerol and coverslipped. The tissue was examined with an epifluorescence microscope (Axioplan; Carl Zeiss Meditec, Oberkochen, Germany), and the images were digitally captured (Spot 3.3 camera; Diagnostic Instruments, Inc., Sterling Heights, MI) and imported into a graphics program (Photoshop; Adobe, Mountain View, CA) for display and analysis. To compare the immunofluorescence observations between the control and mutant retinas, we captured all the retinal images and processed them using the same exposure times and settings.

Confocal microscopy was performed (model FV500; Olympus, Hamburg, Germany) to verify the presence of fluorescent label in processes and dendrites of bipolar cells. Confocal images obtained as stacks of images (1 μ m thickness) were analyzed with the confocal software (Fv10-ASW1.6; Olympus).

Quantification of hCAR-Labeled Cones

We quantified the number of cone photoreceptors labeled with the human cone arrestin (hCAR) antibody. For each experimental group (wt: two retinas at 4 months [young], and two retinas at 24 and 27 months [adult]; mutant: one retina each at 4 and 10.5 months [young] and 15 and 17 months [adult]), at least two sections from each wt and mutant dog were counted. Mosaics of the retinal section at 20 $\times$ were used to count immunolabeled cells along the retinal section with a motorized fluorescence microscope (Imager M1 with Axiovision Rel. 4.7 software; Carl Zeiss Meditec), and the linear distance of each mosaic was measured. The number of hCAR-positive cells was expressed as the mean/100 μ m of retinal length. For statistical analysis, Student's *t*-test was applied.

RESULTS

Morphologic and immunocytochemical assessment was always performed in the same region of the normal and mutant retina. When applicable, these are identified in the figures as *central* (central 1/3), *midpoint* (middle 1/3), and *peripheral* (peripheral 1/3) in sections that extended from the optic disc to the ora serrata. The RPE65 mutant retina shows normal structure, and no evidence of photoreceptor degeneration was noted at the time points examined. In general, the same changes in the expression of the molecular markers were found when comparing the young (4 months), young adult (10.5 months), and adult (15 and 17.1 months) animals; however, more marked changes often were observed in the adults. With the exception of the lack of RPE65 protein expression in the mutants, in no case was there loss of cells or complete absence of immunolabeling. In contrast, there were no differences in the expression of any of the markers examined in the normal control dogs, regardless of age. A summary of the changes in labeling observed in the normal and mutant retinas is presented in Table 3.

Retinal Pigment Epithelium

The RPE of wt retina was uniformly labeled with the RPE65 antibodies, but the mutant retinas showed complete absence of labeling (Figs. 1B, 1C, 1E, 1F, 1H, 1I, 1K, 1L). In contrast, CRALBP equally labeled both the normal and mutant RPE. Other than the presence of lipid inclusions that represented the accumulation of all-*trans*-retinyl-esters, no other changes were observed in the RPE of the mutant dogs.

Photoreceptor Cells

Three antibodies were used to label cone photoreceptors: human cone arrestin (hCAR) and opsins (M/L and S). The normal and mutant M/L- and S-cones were labeled with hCAR antibody throughout the retina. However, whereas normal cones showed intense hCAR labeling throughout the cell (Figs. 1D, 1G, 1J), label in the mutants was located primarily in the outer segments (OS) and pedicles, and there was decreased immunostaining in the inner segments (IS), cell bodies, and axons (Figs. 1E, 1F, 1H, 1I, 1K, 1L). This finding was uniformly present in the central (Figs. 1D–F), midpoint (Figs. 1G–I), and peripheral (Figs. 1J–L) regions. On the other hand, antibodies directed against M/L-

TABLE 3. Summary of the Labeling Results with the Different Molecular Markers in Mutant Dog Retinas

Molecular Marker	Summary of Alterations in RPE65 Mutant Dog Retinas
RPE65	Absence of RPE65 labeling
hCAR	Decreased labeling in IS and axons of cones
M/L opsin	Normal
S opsin	Normal
Rod opsin	Normal rod OS labeling; delocalization into the perinuclear region and axons
PKC α	Retraction of synaptic terminals and increase in terminal size in all rod bipolar cells. Sprouting of dendritic arbors.
Go α	Sprouting of the dendritic arbors of ON cone and rod bipolar cells
Synaptophysin	Normal
TH	Normal
Calretinin	Normal
GABA	Decreased expression in horizontal and GABAergic amacrine cells
BDNF	Normal
p75	Increased Müller cell processes and enveloping of RGCs
Vimentin	Increase in Müller cell fiber labeling in outer layers, more prominent in young mutant retina
CRALBP	RPE normal; decreased expression in adult Müller cells
GFAP	Increase in astrocyte and Müller cell labeling in young mutant retina

FIGURE 1. Immunohistochemical localization of hCAR and RPE65 in normal (wt) and young and adult affected retinas. The proteins were labeled in green (RPE65) and red (hCAR) and the cell nuclei were stained with DAPI (blue). The images were taken from the central (D–F), mid-point (G–I), and peripheral (J–L) retinal regions. RPE65 was present in the wt retinas (A, D, G, J), but absent in the affected retinas, regardless of age. The hCAR antibody labeled the entire cone in the wt retinas, whereas in the affected retinas, the distribution was polarized with labeling primarily in the OS and pedicles. Note that in both the wt and mutant retinas, the peripheral cones were short and broad. Representative images from different animals have been used to illustrate the salient findings: normal (wt) (dog 7560, 18 months of age, A; dog 7559, 27 months of age, D, G, J), young affected *RPE65*^{−/−} retinas (dog Br129, 4 months of age, B, E, H, K), and adult affected *RPE65*^{−/−} retinas (dog Br118, 15 months of age, C; dog Br113, 10.5 months of age, F, I, L). Scale bar, 20 μ m.

(Figs. 2A–C) and S- (Figs. 2D–F) cone opsins showed the same labeling pattern in the wt and mutant retinas. Labeling was limited to the cone OS, and no delocalization of cone opsins was detected in diseased retinas.

Cones were structurally normal in the mutants, and, qualitatively, there was no cone loss at the ages examined. To ascertain that mutant cones were preserved during the course of the disease, we quantified the number of hCAR-labeled cones in retinal sections. There were no differences in the number of cones between the wt and mutant animals in the two age groups examined, and both showed a comparable decrease in cones associated with aging (Fig. 3).

Rod opsin antibodies were used to assess rod photoreceptors. The expression of rod opsin was limited to the rod OS of the wt retinas (Fig. 2G). In the mutants, opsin labeling was also present in the rod OS, but delocalization of opsin into the perinuclear region and axons was observed as well. These findings were uniformly present from the central to the periph-

eral regions in both the young and the older mutant animals (Figs. 2H, 2I).

Inner Retina

The inner retinal neurons showed more severe disease-associated abnormalities. Bipolar cells were examined with antibodies that labeled PKC α and Go α . Both antibodies labeled the entire cell; PKC α labeled all rod bipolar cells, whereas Go α labeled ON-cone and rod bipolar cells. In addition, PKC α -positive rod bipolar terminals and Go α -positive dendritic arborizations were examined with a confocal microscope. Changes in bipolar cells were more severe in the young adult retinas than in the young mutant ones (Fig. 4).

In all the mutant dogs, we observed a retraction of the axonal terminals in PKC α -labeled rod bipolar cells. The retraction was accompanied by an apparent increase in terminal size best visualized by confocal imaging (Figs. 4A–F, 5). Double labeling with PKC α and synaptophysin confirmed the PKC α

FIGURE 2. Immunohistochemical localization of RPE65, M/L- and S-cone opsins, and rod opsin in normal (wt) and affected retinas. The proteins were labeled in *green* (RPE65) and *red* (different opsins), and the cell nuclei were stained with DAPI (*blue*). RPE65 was present in the wt retinas (A, D, G) but absent in the affected retinas regardless of age. Cone opsin antibodies labeled cone OS in the control as well as in the mutant retinas, and the number of cones labeled were comparable in both groups. Rod opsin antibodies labeled rod OS in the wt retinas, but in the mutant retinas opsin was also delocalized into the perinuclear and axonal regions of the cells. These changes were similar in the young and in adult affected retinas. Representative images from different animals were used to illustrate the salient findings: normal retinas (wt) (dog EM171, 4 months of age, A, D, G); dog 7561, 24 months of age, G); young affected retinas (dog Br129, 4 months of age, B, E, H), and adult affected retinas (dog Br113, 10.5 months of age, C, I; dog Br118, 15 months of age, F). Scale bar, 20 μ m.

changes, but showed no changes in the expression of synaptophysin in the outer (OPL) and inner (IPL) plexiform layers (Fig. 5). The retraction of the PKC α axons is associated with a more compact IPL that is more evident with the synaptophysin labeling (Fig. 5B) than with Go α (Figs. 4G-I). Go α labeling showed distinct sprouting of the dendritic arbors toward the photoreceptors in the mutant retina (Figs. 4G-L). The changes observed consisted in elongation of dendritic processes that extended into the ONL and were more evident in the adult than in the young mutant retinas. In some cases, these extensions projected into the middle of the ONL (data not shown).

With other antibodies, we found variable expression changes in the mutant retina that, in some cases, were age-dependent. Tyrosine hydroxylase (TH), which labels a subclass of dopaminergic amacrine cells, showed no appreciable varia-

tion in expression level and label distribution (Figs. 6A-C). Similarly, calretinin labeling of horizontal and AII amacrine cells was the same in the control and mutant retinas, regardless of age (Figs. 6D-F). In contrast, GABA expression was decreased in both amacrine and horizontal cell bodies, the dendritic arborizations of the cells that formed laminae in the OPL and IPL were faintly labeled, and the layered organization of these was not readily visible (Figs. 6G-I). The changes observed were more evident in the adult animals.

Ganglion Cells and Retinal Glia

BDNF was expressed in RGCs and a subpopulation of amacrine cells in the normal and mutant retinas. Although quantification of ganglion cells and classes was not performed, there appeared to be no appreciable labeling differences between the control and mutant retinas in regard to BDNF expression. The expression of p75 was increased in the inner Müller cell processes early in the disease. Moreover, these cells enveloped the ganglion cell somas (Figs. 7A-C).

We used glial markers to examine the expression changes in Müller cells and astrocytes resulting from disease. Vimentin labeling was predominantly located in the Müller cell processes of the control inner retina (Fig. 7D). In the mutants, there was increased vimentin expression in the outer layers, and fine radial fibers extended through the OPL and into the ONL. These were more prominent in the young versus the adult mutant animals (Figs. 7E, 7F). The mutant retinas of all ages also showed disorganization of the Müller cell processes in the IPL that was evident with CRALBP immunostaining, but comparable labeling of the RPE with this antibody was observed (Figs. 7G-I). In Müller cells and processes, labeling was comparable with wild-type. Immunolabeling of the control retina with GFAP showed labeling of the processes and cell

FIGURE 3. The number of hCAR-labeled cones in wt and mutant retinas at different ages. There were no differences in the number of cones between the wt and mutant, but the number of cones was comparably decreased between the young and adult retinas of both genotypes (** $P \leq 0.01$).

FIGURE 4. Immunohistochemical localization of PKC α and Go α in bipolar cells from normal (wt) and affected retinas. The proteins were labeled in green (A, G, RPE65) and red (A– PKC α ; G–L, Go α), and the cell nuclei were stained with DAPI (A, G, blue). Retraction of the axonal terminals of the rod bipolar cells was seen in the affected retinas labeled with PKC α (B, C). (D–F) Higher resolution of the bipolar terminals by confocal microscopy. In the affected retinas, there was sprouting of dendritic arbors of Go α ON-bipolar cells that extended toward the ONL in the affected retinas (compare H, I, K, L with G, J, and in confocal microscope detail in J–L). Representative images from different animals are used to illustrate the salient findings: normal retinas (wt) (dog 7560, 18 months of age, A, D, G, J), young affected retinas (dog Br129, 4 months of age, B, E, H, K), and adult affected retinas (dog Br118, 15 months of age, C, F; dog Br89, 17 months of age, I, L). Scale bar, 20 μ m.

bodies of the inner retinal astrocytes in the GCL. In the young mutant retinas, however, there was an increase in GFAP expression in the nerve fiber layer region, and inner radial processes of the Müller cells. This change appeared less distinct in the adult retina (Figs. 7J–L).

DISCUSSION

Mutations in the *RPE65* gene cause early-onset, profound visual dysfunction or blindness in children^{2,18} and experimental animals (dogs^{6,7} and mice⁸); a similar phenotype occurs in transgenic mice.⁹ Because of such early and severe visual dysfunction, the question arises as to whether changes occur in the retinas of the mutant animals that affect the structure,

molecular expression, and/or neural networks of the photoreceptors and other retinal neurons.

Gene therapy in dogs and mice has demonstrated restoration of retinal function after transfer of the normal cDNA to the RPE.^{11,14,29} In both species, rescue of retinal function has been accompanied by restoration of some aspects of visual cortical activity measured noninvasively.^{15,29} This recovery implies that the neural networks in both the retina and retinocortical pathways are to some extent preserved and that a degree of plasticity may be present that allows for the restoration of functional connections after such prolonged periods of blindness.

Together with a good safety profile of AAV-mediated retinal gene therapy, promising studies in animals have provided the impetus toward human gene therapy for this devastating class

FIGURE 5. Confocal fluorescent immunohistochemical localization of PKC α and synaptophysin in normal (wt), young, and adult affected retinas. The proteins were labeled in green (synaptophysin) and red (PKC α), and the cell nuclei were stained with DAPI (blue). Note the retraction of the axonal terminals of the bipolar cells double labeled with PKC α and synaptophysin, in yellow, in the affected retina. Representative images are used to illustrate the salient findings: normal retina (wt) (dog 7559; 27 months of age, A) and adult affected retina (dog Br118; 15 months of age, B). Scale bar, 20 μ m.

of diseases. Reports of three phase 1 clinical trials have been published, and all show safety of the therapy and positive measures of functional recovery in a subset of patients.^{19–24,28} What is now necessary is to characterize the structural and synaptic changes that occur in the mutant retina and then to determine whether these are reversed or modified after successful gene therapy. As this analysis is not possible in patients, studies in animal models will provide insights into these fundamental issues.

To address the first question, we used a panel of antibodies that characterize the expression and localization of molecular markers in the differentiated normal retina and examined the

expression changes that occur in the mutants. We selected the canine *RPE65* model because the disease and the anatomic and functional features of the eye are comparable between humans and dogs. In both, eye size, and surgical approaches for treatment are similar,³⁰ although the human disease appears somewhat more severe.^{11,13,14,18}

In the present study, we show evidence that outer and inner retinal neurons in a dog *RPE65*-LCA model undergo a series of changes in the expression of retinal cell-specific markers. These occurred at a time when retinal structure is preserved and there is no evidence of photoreceptor and/or inner retinal degeneration. As such, they reflect the effect of

FIGURE 6. Immunohistochemical localization of RPE65, TH, calretinin, and GABA in normal (wt) and affected retinas. The proteins were labeled in green (A, D, G, RPE65) and red (TH, A–C; calretinin, D–F; GABA, G–I), and the cell nuclei were stained with DAPI (A, D, G, blue). The GABA label was lower than the control in the young affected retina (H), and immunolabeling was further decreased in the adult affected retinas (I). There were no differences between normal and affected retinas with TH and calretinin. Representative images from different animals are used to illustrate the salient findings: normal retinas (wt) (dog 7561, 24 months of age, A, D, G), young affected retinas (dog Br129, 4 months of age, B, E, H), and adult affected retinas (dog Br89, 17 months of age, C, F, I). Scale bar, 20 μ m.

FIGURE 7. Immunohistochemical localization of BDNF, p75, vimentin, CRALBP, RPE65, and GFAP in normal (wt) and affected retinas. (A–C) RGCs labeled with BDNF (green) and Müller cells with p75 (red). (B, C) Retinal ganglion cells were enveloped by p75-labeled processes in the affected dogs. Vimentin expressed in Müller cells labeled in red (D–F) was increased in the affected dogs (E, F). CRALBP expressed in Müller cells and RPE labeled in green (G–I) was disorganized in the affected dogs (H, I). Red label shows an increase in GFAP expression in affected dogs (J–L), and green shows RPE65 expression (J–L). Representative images from different animals were used to illustrate the salient findings: normal (wt) (dog 7560, 18 months of age, A, D, G, J), young affected retinas (dog Br129, 4 months of age, B, E, H, K), and adult affected retinas (dog Br118, 15 months of age, C, F, I, L). Scale bar, 20 μ m.

loss of function on the expression of cell-specific molecular markers and not events associated with a degenerative process. Furthermore, although differences were noted between the young dogs and those classified as young adult or adult, in general, these differences were modest and qualitative, indicating that they occurred early in the disease process and were relatively stable.

Antibodies against different opsin classes (rod opsin and M/L and S opsin) and hCAR were used to evaluate the rod and cone photoreceptors. Although cones showed a change in the distribution of hCAR labeling, they were normal in structure and in localization of cone opsins. Furthermore, there was no evidence of cone degeneration or loss, and the number of cones was the same as in the control. These results are strikingly different from those reported in *RPE65* mutant mice in which shortening of the cone OS occurs early, and M/L and S opsin proteins fail to traffic properly. Cones degenerate subsequently and appear more vulnerable than rods to the *RPE65* defect.^{31,32} That the cone disease in mice is secondary to the biochemical defect is demonstrated by the rescue of cones

after either exogenous 11-*cis* retinal administration³¹ or gene therapy.²⁹

On the other hand, antibodies against rod opsin demonstrated extensive delocalization of the protein from the OS into the ONL in the young and adult mutant retinas. Similar findings have been reported in human retinitis pigmentosa (RP)³³ and in other animal models of retinal degeneration (transgenic pigs,³⁴ cat,³⁵ dog,³⁶ and mouse³⁷). Surprisingly, the retinas of *RPE65* mutant mice that show such prominent cone opsin mislocalization do not show a comparable change in rod photoreceptors.³⁸ To examine downstream events that result from photoreceptor dysfunction, we used antibodies that label the synaptic terminals in the OPL and IPL (synaptophysin) or different classes of bipolar cells. In the mutant retinas, the terminals of rod bipolar-PKC α cells retracted and enlarged. Such retraction has been reported in the RCS rat model and before and during the photoreceptor degenerative process induced by experimental retinal detachment.^{39,40} This phenomenon was more evident in the adult mutant retinas and may have resulted from a translocation of PKC α from the

perikarya to the terminals, which had become enlarged. Proximal changes in bipolar cells were also observed in the mutant retinas. Both PKC α - and GABA-positive cells showed increased dendritic arborizations that extended into the ONL. It is not known whether this extension made ectopic synaptic contacts in the ONL or whether they represented dendrites without any presynaptic input.

The signals of the rod and cone pathways converge in the OPL through horizontal cells and in the IPL through the AII amacrine cells. In mammals, rod signals pass into the cone pathways by means of gap junctions between AII amacrine cells that contact ON cone bipolar cells.⁴¹ Labeling of both cell classes with the calretinin antibody was normal in the mutant retina, as was labeling of dopaminergic amacrine cells with TH antibody.⁴² However, the GABAergic amacrine cells labeled with the GABA antibody⁴³ showed a decrease in labeling intensity of both the horizontal and amacrine cells that was more evident in the older animals. As GABA is an inhibitory neurotransmitter that mediates lateral surround inhibition,⁴⁴ loss of photoreceptor activity due to RPE65 mutation would be compatible with a decreased requirement for lateral inhibition and consequent decrease in GABA expression in these cells.

To assess the effect of outer retinal disease on the inner retina, we used antibodies that label different proteins in glia and RGC. In mammals, BDNF is present in RGCs and amacrine cells,⁴⁵ and no changes were detected in the distribution of this neurotrophin in the mutants. Müller cells also responded to the ongoing disease process, but this response was not uniform against all proteins examined. The low-affinity receptor p75 was expressed in Müller cells and increased in the mutant retina with expression in the radial Müller fibers and surrounding RGC. Both vimentin and GFAP showed increased expression, but the changes were more prominent in the younger animals and less distinct in the adults. In contrast, CRALBP did not show an initial increase and was reduced below control levels in the adult mutants. For the proteins examined, it is clear that Müller cells responded to outer retinal disease. This response was slight, transient, and more distinct in the younger than in the older mutant retinas, most likely indicating a lack of progressive retinal degenerative changes during the time analyzed. However, analysis of Müller cells included only a limited number of antibodies, two of which were directed at intermediate filaments. It is possible that other proteins (e.g., glutamine synthetase and carbonic anhydrase C), would respond differently as they are known to decrease in experimental retinal detachment in adult cats.⁴⁶

In conclusion, in the present study, in a dog model of RPE65-LCA, the structure of the retina was well preserved, but several molecular changes took place, not only in photoreceptors but in bipolar and amacrine cells. Some of these changes were structural, whereas others reflected a change in expression of localization of the examined proteins. Regarding the synaptic connectivity of the retina, we evaluated the vertical (photoreceptor, bipolar, and ganglion cells) and horizontal (horizontal and amacrine cells) pathways. In the absence of degeneration, lack of photoreceptor function resulted in structural changes in the bipolar cells (i.e., retraction and dilatation of PKC α terminals and increased dendritic arborizations of rod and ON cone bipolar cells). In terms of the lateral pathways, there appeared to be excellent preservation of markers that characterize both the horizontal and amacrine cells, with the exception of GABA, which was reduced in both cell types, especially in adults. These results provide the basis for a subsequent analysis of retinas treated by gene therapy, to determine the reversibility of the molecular and structural changes. These results will inform parallel gene therapy studies that are now taking place in patients with RPE65-LCA.

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