Inner Retina Remodeling in a Mouse Model of Stargardt-like Macular Dystrophy (STGD3)

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PURPOSE. To investigate the impact of progressive age-related photoreceptor degeneration on retinal integrity in Stargardt-like macular dystrophy (STGD3).

METHODS. The structural design of the inner retina of the ELOVL4 transgenic mouse model of STGD3 was compared with that of age-matched littermate wild-type (WT) mice from 1 to 24 months of age by using immunohistochemistry and confocal microscopy and by relying on antibodies against cell-type-specific markers, synapse-associated proteins, and neurotransmitters.

RESULTS. Müller cell reactivity occurred at the earliest age studied, before photoreceptor loss. This finding is perhaps not surprising, considering the cell's ubiquitous roles in retina homeostasis. Second-order neurons displayed salient morphologic changes as a function of photoreceptor input loss. Age-related sprouting of dendritic fibers from rod bipolar and horizontal cells into the ONL did not occur. In contrast, with the loss of photoreceptor sensory input, these second-order neurons progressively bore fewer synapses. After rod loss, the few remaining cones showed abnormal opsin expression, revealing tortuous branched axons. After complete ONL loss (beyond 18 months of age), localized areas of extreme retinal disruptions were observed in the central retina. RPE cell invasion, dense networks of strongly reactive Müller cell processes,

and invagination of axons and blood vessels were distinctive features of these regions. In addition, otherwise unaffected cholinergic amacrine cells displayed severe perturbation of their cell bodies and synaptic plexi in these areas.

CONCLUSIONS. Remodeling in ELOVL4 transgenic mice follows a pattern similar to that reported after other types of hereditary retinopathies in animals and humans, pointing to a potentially common pathophysiologic mechanism. (*Invest Ophthalmol Vis Sci.* 2010;51:2248–2262) DOI:10.1167/iovs.09-4718

Stargardt-like dystrophy (STGD3, MIM 600110; Mendelian Inheritance in Man; National Center for Biotechnology Information, Bethesda, MD) is an autosomal-dominant juvenile form of atrophic macular degeneration characterized by macular flecks followed by central atrophy of the retina and retinal pigment epithelium (RPE) and by a progressive decline in visual acuity to 20/200 or worse.^{1–4} Although there is no accurate determination of the incidence of STGD3, it is less than the estimated 1:10,000 incidence of its autosomal recessive counterpart, STGD1.⁵

Disease-causing mutations have been identified in exon 6 of the *ELOVL4* gene,^{6–8} a widely conserved gene predicted to encode a protein of 314 amino acids likely to be involved in the Elongation Of Very Long-chain fatty acids.^{9–11} These mutations all result in a truncation of the wild-type ELOVL4 protein C terminus. How the mutant ELOVL4 protein initiates Stargardt-like dystrophy in humans is largely unknown. Of interest, however, deletion of the protein C terminus results in loss of the putative di-lysine motif necessary for retention of transmembrane proteins in the endoplasmic reticulum (ER). Moreover, in vitro studies further show that, when coexpressed with the wild-type ELOVL4 protein in COS-7 and HEK293 cell lines, mutant proteins form dense, cytoplasmic, aggresome-like inclusions, inhibit ER retention of the wild-type protein, sequester the wild-type protein into the aggresomes, and upregulate ER chaperones, as seen in the stress response to misfolded/unfolded proteins.^{12–15} Both approaches therefore point to a dysfunction of the wild-type protein due to cellular mislocalization as a critical step in the beginning of the disease process.¹⁶

In parallel to these molecular investigations, Karan et al.¹⁷ developed a STGD3 transgenic mouse model expressing a mutated *ELOVL4* gene (5-bp deletion corresponding to the human mutation delAACTT at position 790 to 794 of the open reading frame) in C57/BL6 mice. Since *Elovl4* is specifically expressed in photoreceptors of the adult mouse retina,^{18,19} the mutated *ELOVL4* transgene was engineered to be under the control of the human interphotoreceptor retinoid-binding protein (IRBP) promoter, a carrier glycoprotein secreted exclusively by photoreceptors.²⁰ The resulting transgenic (TG) mice were classified into three lineages according to the expression level of the *ELOVL4* transgene (TG3 > TG2 > TG1). In each line, the severity of the observed STGD3 phenotype correlated with the expression level of the *ELOVL4* transgene; pheno-

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Supported by Canadian Institutes of Health Research (CIHR) Grant 151145; National Institutes of Health Grant EY12951 (JRS); an Alberta Heritage Foundation for Medical Research (AHFMR) equipment grant; Canadian National Institute for the Blind; Olive Young Foundation and The Lena McLaughlin Foundation (Mona & Rod McLennan). FG was supported by INSERM (Institut National de la Santé et de la Recherche Médicale)/CIHR (Canadian Institutes of Health Research) cooperation program (2006) and French Foreign Office (2009). SCM was supported by the Foundation Fighting Blindness Canada. PRF was supported by an AHFMR summer studentship. YS is an AHFMR Senior Scholar and a recipient of the Barbara Tuck/MacPhee Family Vision Research Award in Macular Degeneration. The RT97 antibody developed by John Wood was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA).

Submitted for publication October 1, 2009; revised November 2, 2009; accepted November 9, 2009.

Disclosure: S. Kuny, None; F. Gaillard, None; S.C. Mema, None; P.R. Freund, None; K. Zhang, None; I.M. MacDonald, None; J.R. Sparrow, None; Y. Sauvé, None

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types included fundus defects, a central-to-peripheral pattern of photoreceptor and pigment epithelium degeneration, age-related lipofuscin accumulation, and electroretinogram (ERG) abnormalities.

Although photoreceptor degeneration has been described in this STGD3-like mouse model, it remains unknown how the primary defect may affect the structural design of the inner retina. Detailed understanding of secondary degeneration is essential to establishing the limitations of therapeutic intervention to cure or prevent STGD3. Furthermore, the elucidation of secondary retinal degeneration has relevance to other retinal dystrophies typified by regional atrophy and excessive lipofuscin accumulation, such as the recessive form of Stargardt (STGD1) and the dry form of age-related macular degeneration (AMD).

The purpose of this study was therefore to examine the impact of photoreceptor degeneration on the integrity of inner retina components (horizontal cells, bipolar cells, amacrine cells, Müller cells, retinal ganglion cell axons, and blood vessels).

MATERIAL AND METHODS

Animals

Breeding and Genotyping. Heterozygous females from the ELOVL4/TG2 mouse model of STGD3 (imported from the colony described by Karan et al.¹⁷) were bred with C57BL/6N male mice (Charles River Laboratories, Wilmington, MA) in a colony maintained at the University of Alberta. Line TG2 (with intermediate levels of transgene expression) was preferred over line TG3, for its relatively slow photoreceptor degeneration, which allows thorough investigation of both anatomic and functional retinal alterations as well as adequate time for intervention with potential therapeutic treatments. Litters were genotyped by PCR using *Elovl4* primers 5'-TGTAGCAGACTGGC-CGCTGAT-3' (forward) and 5'-CTCTGAAGATGAAAAGGTTAAGCA-3' (reverse) and *ELOVL4* primers 5'-GCAGTCTCCTTGGCCTACAC-3' (forward) and 5'-GAATTCAACTGGGCTCCAAA-3' (reverse). All animals were maintained on a 12:12 light-dark cycle (to ensure a normal production of the IRBP mRNA²¹), a temperature of 21°C, relative humidity of ~50%, and water and a rodent diet (Laboratory Rodent Diet 5001 from LabDiet; Nutrition International, Richmond, IN) supplied ad libitum. The experiments were performed in accordance with the Institutional Animal Care and Use Committee (University of Alberta), the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research, and the guidelines laid down by the National Institutes of Health regarding the care and use of animals for experimental procedures.

Transgene Expression Levels. To verify the phenotype of the transgenic mouse model bred in our laboratory, we used semiquantitative RT-PCR to examine the expression level of the mutated human *ELOVL4* transgene in nine transgenic mice at 9 months of age from four different litters. Total RNA was isolated from the retina (TRIzol; cat. no. 15596-026; Invitrogen, Carlsbad, CA). Total RNA (0.5 µg) was treated with DNase I (Amplification Grade; cat. no. 18068-015; Invitrogen) and cDNA synthesis and PCR were performed (SuperScript One-Step RT-PCR with Platinum *Taq*; cat. no. 10928-034; Invitrogen). The primers used for RT-PCR were 5'-GTGTGGCTGGGTCCAAA-3' (forward) and 5'-TTGGGAAGGGGCAGTC-3' (reverse), which bind within exons 2 and 6, respectively, in humans and mice. The cDNA was digested with *EcoRI* and separated on a 2% TAE gel. The human *ELOVL4* cDNA contains an *EcoRI* site that is absent in the endogenous mouse *Elovl4* cDNA, allowing us to distinguish human transgene products (384 and 173 bp) from the endogenous mouse product (557 bp). The net intensity of the bands was quantified (Image Station 440 with Imaging software ver. 4.0.3; Eastman Kodak, Rochester, NY). Liver tissue of one ELOVL4 transgenic mouse served as a negative control tissue.

Genotyping for Met450Leu Mutation in the RPE65 Gene. Since the Rpe65-Leu450Met variant (found in inbred strains of mice) has been linked with a phenotype involving reduced A2E level accumulation,²² heterozygous ELOVL4 transgenic mice were genotyped further for this variant by PCR amplification of DNA derived from mouse tails with the primers 5'-ACCAGAAATTTGGAGGGAAAC-3' (forward) and 5'-CCCTTCCATTTCAGAGCTTCA-3' (reverse) and digestion of the 545-bp product with the *MwoI* restriction enzyme (New England Biolabs, Beverly, MA).

A2E Measurement

The major component of lipofuscin is the fluorophore A2E (2-[2,6-dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E-hexatrienyl]-pyridinium). The levels of A2E and the related molecule *iso*-A2E were measured as previously described.^{22,23} In brief, mouse posterior eye cups ($n = 6$ per time point) were homogenized, extracted with chloroform/methanol (2:1), analyzed by HPLC (Alliance system, with an Atlantis dC18 3-µm, 4.6 × 150-mm column; Waters Corp., Milford, MA), and monitored by photodiode array. For the mobile phase, gradients of water and acetonitrile with 0.1% TFA were used with a flow rate of 0.8 mL/min. The bis-retinoid lipofuscin compounds A2E and *iso*-A2E were identified on the basis of UV-visible absorbance spectra and elution times that correspond to authentic synthetic compounds.^{22,24,25} Molar quantity per mouse eye was determined by using standard curves constructed from known concentrations of purified external standards.

Expression of Specific Retinal Cell Markers

Primary Antibodies. The primary antibody sources, hosts, and dilutions used for immunofluorescence are provided in Table 1. Their specificity can be assessed as follows. The anti-bassoon monoclonal antibody recognizes a major band of ~400 kDa (the apparent molecular mass of the protein) on immunoblots from rodent brain. At the retinal level, this antibody displays punctate distributions in both the outer (OPL) and the inner (IPL) plexiform layers,²⁶ corresponding respectively to active zones of rod and cone ribbon synapses and to conventional presynaptic structures of GABAergic amacrine cells.²⁷ The anti-calbindin D-28k antibody recognizes a major product at ~28 kDa in Western blot analysis of rodent brain (manufacturer's data sheet). In the mouse retina, it stains horizontal cells and some amacrine cells.²⁸ Immunostaining is absent in calbindin D-28k-knockout mice.²⁹ The affinity-purified anti-choline acetyltransferase (ChAT) antibody detects a ~70-kDa product in Western blot analysis of rat brain extracts (manufacturer's data sheet). In cryosections, the labeling pattern matches that obtained in rodents with other ChAT antisera.^{30,31} The monoclonal anti-glial fibrillary acidic protein (GFAP) antibody cocktail labels a major band at ~50 kDa on Western blot analysis of mouse retina (Supplementary Fig. S1, <http://www.iovs.org/cgi/content/full/51/4/2248/DCD>). This antibody stains retinal astrocytes and Müller cells.^{26,32} The affinity-purified anti-glutamine synthetase (GS) antibody recognizes a ~45-kDa protein from rat brain extract (manufacturer's data sheet). It stains murine Müller cells to their full extent specifically.³³ The affinity-purified polyclonal anti-metabotropic glutamate receptor 6 (mGluR6) antibody provides a punctiform staining at the dendritic tips of rod bipolar cells and ON-type cone bipolar cells in the OPL (see Fig. 3).^{34,35} The specificity of this widely used antibody has been described elsewhere.³⁶ The monoclonal anti-neurofilament antibody (NF-200 kDa, also referred to as RT97) recognizes the phosphorylated 200-kDa isoform of the neurofilament triplet from rat brain extract.³⁷ The staining pattern was consistent with that shown in previous studies.^{38,39} Cone photoreceptors were labeled using anti-M/L- and anti-S-opsin polyclonal antibodies, which stain outer segments and cell membranes of specific types of cones in mouse retina.^{40,41} The polyclonal and monoclonal anti-protein kinase C, α subunit (PKC α) antibodies detect a major band (~80 kDa) as well as minor bands corresponding respectively to the β I and β II isoforms

TABLE 1. List and Details of Antibodies Used in This Study

Antibody	Antigen	Host	Source (Cat. No.)	Dilution
Bassoon	Amino acids 738-1035 of recombinant rat protein	Mouse	Stressgen (VAM-PS003E)	1:500
Calbindin D-28k	Full-length recombinant rat protein	Rabbit	Swant (CB38a)	1:1000
ChAT	Full length human placental enzyme	Goat	Chemicon (AB144P)	1:200
GFAP	GFAP derived from Bigner-Eng clones MAb1B4, MAb2E1 and MAb4A11	Mouse	Covance (clone SMI-22R)	1:1000
Glutamine synthetase	Full sequence of the sheep protein	Mouse	BD Transduction Labs (610517; clone 6)	1:1000
mGluR6	Synthetic peptide containing C-terminal sequence 853-871	Rabbit	Nakanishi, Kyoto University, Japan	1:1000
NF-200 kDa	Semipurified rat neurofilaments	Mouse	DSHB, Univ. Iowa (RT97)	1:50
M/L-opsin	Last 42 amino acids of recombinant human red/green opsin	Rabbit	Chemicon (AB5405)	1:500
S-opsin	Last 38 amino acids of recombinant human blue-sensitive opsin	Rabbit	Chemicon (AB5407)	1:500
S-opsin, for double labeling with M/L opsin	20 amino acids within 1-50 of human blue-sensitive opsin (EFYLFKNISSVGPWDGPQYH)	Goat	Santa Cruz Biotech (sc-14363)	1:200
PKC α (C-20)	Amino acids 650-690 at the C-terminus of human PKC α	Rabbit	Santa Cruz Biotech (sc-208)	1:200
PKC α (H-7)	Amino acids 640-690 at the C-terminus of human PKC α	Mouse	Santa Cruz Biotech (sc-8393)	1:200
Vimentin (C-20)	Epitope mapping at the C-terminus of human vimentin; localized within amino acids 411-461, approximately 12-24 amino acids in length (commercially sensitive)	Goat	Santa Cruz Biotech (sc-7557)	1:100

Stressgen Bioreagents, Victoria, BC, Canada; Swant, Bellinzona, Switzerland; Chemicon, Temecula, CA; Covance, ; BD Transduction Laboratories, Lexington, KY; Santa Cruz Biotechnology, Santa Cruz, CA.

(manufacturer's datasheets). These antibodies label both rod bipolar cells and a subset of amacrine cells as efficiently as other anti-PKC α antibodies.^{28,31} The polyclonal anti-vimentin antibody labels retinal Müller cells and astrocytes in the mouse retina.⁴² TRITC-conjugated lectin from *Bandeiraea simplicifolia* (BS-I; L5264; Sigma-Aldrich, St. Louis, MO), a pan-endothelial binding agent, was used to detect blood vessels.

Immunohistochemistry. Transgenic ELOVL4 and WT control mice were studied immunohistochemically from 1 to 24 months of age (WT: 1 month, $n = 1$; 3 months, $n = 6$; 6 months, $n = 3$; 9 months, $n = 5$; 12 months, $n = 6$; 18 months, $n = 4$; and 24 months, $n = 6$. Transgenic ELOVL4: 1 month, $n = 1$; 3 months, $n = 6$; 6 months, $n = 3$; 9 months, $n = 16$; 12 months, $n = 6$; 18 months, $n = 3$; and 24 months, $n = 5$). All animals (with the exception of those 1 month old) were used for ONL row counts; a subset was used for bipolar cell counts and for cholinergic amacrine cell counts. After anesthesia (Euthanyl; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada), the eyes were enucleated between 0900 and 1100 hours. The corneas were punctured, and the eyes were immersed for 30 minutes at room temperature in 4% paraformaldehyde. The lenses were removed, and the eyes were fixed for an additional 30 minutes in 4% paraformaldehyde and cryoprotected in graded sucrose concentrations at 4°C. On the following day, the eyes were embedded (Shandon Cryomatrix; Anatomic Pathology, Pittsburgh, PA), flash frozen in liquid nitrogen, and stored at -80°C until sectioned. Cross-sections at 20 μ m were cut parallel to the temporonasal axis through the optic nerve head and mounted (Superfrost/Plus glass slides; Fisher Scientific, Pittsburgh, PA). After hydration in PBS (pH ~7.3), the sections were blocked for 1 hour in PBS+0.3% Triton X-100+10% goat (or horse) serum (same species as the secondary antibody), and reacted overnight in a humid container with the primary antibodies, either alone or in combination, diluted in a 1:10 solution of the previous blocking medium. The following day, the sections were washed extensively in PBS, reacted for 1 hour with species-appropriate secondary antibodies conjugated to Alexa fluorescent dyes (Molecular Probes, Eugene, OR) diluted 1:1000 in a 1:10 solution of the blocking medium, and washed exten-

sively in PBS. Finally, slides were coated with antifade reagent with DAPI (Prolong Gold; Molecular Probes, cat. no. P36931, Eugene, OR) and coverslipped. All reactions were performed at room temperature. Images were captured on a confocal microscope (model LSM510; Carl Zeiss Meditec, Dublin, CA, with a Plan-Neofluar 40 $\times$ /1.3 oil objective). Two regions were considered: the center ($400 \pm 200 \mu$ m from the optic disc) and the periphery ($500 \pm 200 \mu$ m from the ora serrata). Images ($230.3 \times 230.3 \mu$ m at zoom setting 1) were projections of z -stacks of 6 to 10 slices of 1 μ m. Brightness and contrast levels were adjusted if necessary with image-management software (Photoshop CS2, ver. 9.0.2; Adobe, San Jose, CA).

ERG Recordings

To determine the rate and extent of retinal functional loss with age, dark- and light-adapted ERG responses were respectively recorded in transgenic ELOVL4 and age-matched WT mice (WT: 1 month, $n = 8$; 3 months, $n = 7$; 6 months, $n = 7$; 9 months, $n = 8$; 12 months, $n = 8$; 18 months, $n = 18$; and 24 months, $n = 5$. transgenic ELOVL4: 1 month, $n = 8$; 3 months, $n = 7$; 6 months, $n = 8$; 9 months, $n = 7$; 12 months, $n = 7$; 18 months, $n = 6$; 24 months, $n = 8$), as previously described (Sauvé Y, et al. *IOVS* 2007;43:ARVO E-Abstract 15).²⁶ In brief, the mice were dark-adapted for 1 hour before anesthesia with a mixture of ketamine (62.5 mg/kg IP) and xylazine (12.5 mg/kg IP) and the pupils dilated with 1% tropicamide. Body temperature was monitored with a rectal thermometer and maintained at 38°C with a homeothermic electric blanket. Simultaneous bilateral recording was achieved with active gold loop electrodes (placed on each cornea) and a subdermal platinum reference electrode (placed behind each eye); a subdermal ground platinum electrode was placed on the mouse's scruff. Light stimulation (10- μ s flashes), signal amplification (0.3-300-Hz bandpass), and data acquisition were provided by an electrophysiology system (Espion E²; Diagnosys LLC, Littleton, MA). For each animal, only one eye was considered for statistical comparisons. It corresponded to the eye associated with the dark-adapted b-wave of maximum amplitude.

FIGURE 1. Confirmation of the ELOVL4/TG1-2 mouse phenotype. (A) RT-PCR results from 9-month-old WT (W) and ELOVL4/TG1-2 mice 1 to 9; L, ladder; C, control (liver, devoid of *ELOVL4* expression). (B) Expression levels of the *ELOVL4* transgene. (C) Examples of retinal architecture in ELOVL4/TG1-2 mice 4 and 8; the respective number of ONL rows is 9 to 10 and 5 to 6. Green: mGluR6; red: PKC α ; blue: DAPI. (D) Total A2E levels (picomole per eye) as a function of age, in ELOVL4/TG1-2 and WT mice. Values (from six pooled eye cups per each group and per time point) were 12.41 and 55.80 picomoles/retina in 3- and 10-month-old ELOVL4 mice, respectively, and 5.6 and 15.37 picomoles/retina in the age-matched WT. Data are based on single measures of six eye cups per homogenate; hence, the absence of error bars in (D). To confirm the reliability of the results, we compared the 10-month WT values with values obtained from an independent study in which WT mice (also Rpe65-Leu450Met) were fed a homemade diet from 6 to 12 months of age. Analysis of three distinct homogenates made from six eye cups each (at 12 months of age) gave similar values (total A2E: 6.26, 10.51, and 10.85 picomoles/eye) to those reported here at 10 months of age (15.37 picomoles/eye). Scale bar, 50 μ m.

Statistics

Cell counts were performed on previously stored confocal images and tested for significance with nonparametric statistics. Single-point comparisons were performed with the Mann-Whitney U test, and multiple point comparisons were performed with the Kruskal-Wallis test. Data in graphs represent the average $\pm$ SD.

RESULTS

Assessing the STGD3 Phenotype in a Line of Transgenic ELOVL4 Mice

Transgene Expression Levels. Three bands were obtained after *Eco*RI digestion of *ELOVL4/Elovl4* RT-PCR products (Fig. 1A): an upper band (557 bp), corresponding to the endogenous mouse *Elovl4* product, and two smaller products (384 and 173 bp, respectively), corresponding to the human *ELOVL4* transgene.¹⁷ The relative transgene expression level was determined as being the ratio of the net intensity of the 384-bp band + the 173-bp band (human *ELOVL4* transgene) over the 557-bp band (mouse endogenous *Elovl4*). This ratio

was 1.34 ± 0.21 (range, 1.10–1.70; Fig. 1B) for the nine ELOVL4 mice studied, supporting a stable transgene expression in experimental animals.

Such a value, however, is nearly between those reported¹⁷ for line TG1 and line TG2 (0.6 and 3.3, respectively; Fig. 1B). If correct, transgenic ELOVL4 mice in our colony would exhibit a photoreceptor degeneration much slower than that reported for the original line TG2. Figure 1C shows examples of the structure of the outer retinas (contralateral to those used for transgene expression) from two of the transgenic ELOVL4 mice dedicated to this study. Despite similar transgene expression (ratios of 1.6 and 1.2 in animals 4 and 8, respectively), there was some variability in the number of ONL rows, with 9 to 10 and 5 to 6 rows remaining in animals 4 and 8, respectively, at 9 months of age. Indeed, in 16 retinas collected from distinct transgenic ELOVL4 mice at that age, the range of variability (including all eccentricities) was four to nine rows, which is significantly higher than the average of two reported for the ELOVL4 line TG2 at the same age.¹⁷ On the basis of these findings, the ELOVL4 mouse line that we studied was intermediate between the original lines TG1 and TG2 and therefore will be referred to in the remaining text as ELOVL4/TG1-2. Anatomic and functional phenotypes reported in the following sections support this view.

Genotyping for the Rpe65-Leu450Met Variant. Heterozygous ELOVL4/TG1-2 mice exhibited all three bands corresponding to Leu-450: the 180- and 365-bp digested bands and the 545-bp undigested band (data not shown). These results confirm that these ELOVL4/TG1-2 mice possess the Rpe65-Leu450Met variant classically associated with reduced levels of A2E accumulation.

A2E Levels. Measurements of all-*trans* A2E and *iso*-A2E at 1, 3, and 10 months of age demonstrated increased levels with aging in both the WT and ELOVL4/TG1-2 mice (Fig. 1D). In support of the genetic analysis, both mouse types exhibited low levels of these pigments at 1 month of age (2.0 vs. 1.0 picomole per eye in the WT and ELOVL4/TG1-2 mice, respectively), on average three to four times lower than levels in mice with the normal Leu variant at position 450.²² The age-related increase in total A2E levels was steeper in the ELOVL4/TG1-2 mice, and these levels were consistently higher at 3 and 10 months of age (2.2- and 3.6-fold, respectively) than those in the WT mice.

Number of Photoreceptor Rows. The number of photoreceptor rows in the ONL was estimated based on the number of rows of DAPI-stained nuclei in this layer; only one retina per animal (right eye) was considered (Fig. 2A). The rate of the degenerative process was approximately two times slower than that reported for ELOVL4/TG2.¹⁷ We observed an ONL depleted to one photoreceptor row after 18 months of age (Fig. 2A), whereas TG2 displayed a similar loss by 10 months of age.¹⁷ Statistically significant differences in the number of photoreceptor rows between WT and ELOVL4/TG1-2 mice occurred as early as 3 months of age in the central retina and from 9 months of age in the periphery ($P < 0.05$, multiple ANOVA with post hoc group comparisons). The ratio of number of rows in the center over that in the periphery (Fig. 2B) was lower in the ELOVL4/TG1-2 than in the WT ($P < 0.05$, multiple ANOVA with post hoc group comparisons, from 9 months of age onward). By 24 months of age, there was no discernable ONL in the ELOVL4/TG1-2 mice.

There was a tendency for greater anatomic variability in the ELOVL4/TG1-2 compared with the WT mice (Figs. 2C, 2D); hence, the analysis of data pooled from many animals cannot optimally show that the ELOVL4 mutation does indeed result in greater photoreceptor loss in the center than in the periphery of the retina. This center-versus-periphery gradient is better evidenced when individual animals are analyzed separately

FIGURE 2. Age-related loss of photoreceptors and retina function. (A) Age-related loss of photoreceptors in ELOVL4/TG1-2 compared with WT mice. At all ages in the WT, there were more photoreceptors in the center than in the periphery of the retina. This finding is demonstrated by representing the ratio of photoreceptor number in the center versus the periphery as a function of age (B). In ELOVL4/TG1-2 mice, this ratio was inverse, reflecting more pronounced photoreceptor loss in the center than the periphery of the retina. (C, D) Examples of variability in photoreceptor rows in ELOVL4/TG1-2 compared with age-matched WT mice at 3 and 18 months of age (blue, DAPI-stained cell bodies). Specimens with the highest and lowest number of rows are shown on the left and right of each panel, respectively. Each image is from a different mouse. (E–G) ERG parameters as a function of age in WT and ELOVL4/TG1-2 mice, showing dark-adapted (E) a- and (F) b-wave amplitudes and light-adapted (G) b-wave amplitude and (H) flicker fusion. Scale bar, 20 μ m.

(Fig. 2B). Therefore, to overcome this interindividual variability, morphologic and structural changes in the inner retina will be expressed as a function of photoreceptor rows remaining in the ONL at specific eccentricities (center versus periphery) in addition to age.

Retina Function as Assessed with the ERG. Compared with WT, in which there was no significant change with age in any of the ERG components studied, the ELOVL4/TG1-2 mice showed a progressive deterioration in ERG responsiveness with age (Figs. 2E–H). Decline in dark-adapted a- and b-wave amplitudes in ELOVL4/TG1-2 versus WT mice began by 6 and 12 months of age, respectively. Light-adapted b-wave amplitudes and flicker fusion frequencies were lower than in age-matched WT mice by 18 months of age. Decline in ERG responsiveness was between that previously reported¹⁷ for lines TG1 and TG2.

Loss of Synapses in the OPL

Before any significant photoreceptor loss, there was no noticeable reduction in the thickness of the OPL, as indicated by the number of rows of labeled synaptic sites. Figure 3 shows an example of central retinas at 1 month of age (number of ONL rows, 11–13 in both WT and ELOVL4/TG1-2) reacted for mGluR6, a marker of postsynaptic sites localized on the dendrites of ON-type bipolar cells. The number and distribution of mGluR6-positive labels were similar between the WT and ELOVL4 groups at 1 month. The progressive decline in the number of photoreceptor rows was associated with presynaptic loss, which also was related to reduction in the thickness of the OPL. At 9 months of age, OPL thickness and the number of bassoon-labeled presynaptic sites were dramatically reduced.

FIGURE 3. Loss of synapses in the OPL of ELOVL4/TG1-2 mice compared with age-matched WT mice in central retinas (blue, DAPI-stained cell bodies). *Insets* in the first and second columns (indicated by *boxes*) are shown in third and fourth columns, respectively. Scale bars: (first and second columns) 50 μ m; (third and fourth columns) 10 μ m.

The loss of ONL rows was also concomitant with a reduction in the number of postsynaptic sites: 9-month-old ELOVL4/TG1-2 retinas with a reduced number of photoreceptor rows (five to six) had significantly fewer postsynaptic sites (labeled with mGluR6) in the OPL than did the age-matched WT mice (10–11 photoreceptor rows; Fig. 3). Further loss of synaptic sites (as revealed by bassoon labeling) was found in older ELOVL4/TG1-2 retinas (18 months of age) having only two to three ONL rows (Fig. 3). At 24 months of age, mGluR6 labeling did not reveal any distinct OPL. Instead, mGluR6 labeling was very sparse, with punctate staining limited to cell bodies that were localized at the outer limit of the inner nuclear layer (INL). Finally, the bottom row of Figure 3 shows an example of fewer photoreceptor synapses (stained for bassoon) on horizontal cells (stained for calbindin) in the ELOVL4/TG1-2 retina (5–6 photoreceptor rows) compared with the WT retina ($\approx$ 12 photoreceptor rows) at 9 months of age. In conclusion, the reduction of synaptic burden (from photoreceptor axon terminals) onto secondary neurons (bipolar and horizontal cells) is directly related (in a temporal fashion) to the extent of photoreceptor loss.

Remodeling of Rod Bipolar Cells

Rod bipolar cells are second-order retinal neurons involved in the transmission of low-energy light signals from rod photoreceptors to retinal ganglion cells. In the rod-dominant mouse retina, these cells are among the most numerous bipolar cells

(40%–50% of the total population), and can be labeled specifically for PKC- α , which offers an indisputable advantage over any other types of bipolar cells in immunohistological approaches. In WT mice, the only visible age-related change in rod bipolar cells was the progressive increase in the number and length of their dendritic processes projecting into the ONL (Fig. 4A; 18 months of age); some of these processes even spanned the entire extent of the ONL. Despite their aberrant projection in the ONL, these dendrites maintained, at their extremity, an association with the presynaptic marker bassoon (Fig. 3, third row, third column: WT at 18 months of age). This type of age-related dendritic remodeling was observed in all the WT retinas. Its frequency increased from none at 1 month to several very long dendritic processes by 18 months of age and tended to be exacerbated in the periphery compared with that in the center of the retina.⁴³ This phenomenon occurred rarely in ELOVL4/TG1-2 retinas and mostly in the periphery, where at least four photoreceptor rows were retained (not illustrated). The most common observation in ELOVL4/TG1-2 mice was not an extension but rather a progressive loss of rod bipolar cell dendrites, which was proportional to the loss of photoreceptors, to the point of no observable dendrites in areas devoid of photoreceptors (Fig. 4B, 18 months of age). Dendritic pruning in ELOVL4/TG1-2 retinas was first detectable in the central retina (as early as 3 months of age), and then encompassed the whole retina (from 6 months onward) as the photoreceptor population declined in the periphery of the

FIGURE 4. Age-related changes in rod bipolar cells (PKC α -labeled; A–C) and horizontal cells (calbindin-labeled; E–H) in WT and ELOVL4/TG1-2 mice. Examples taken at 18 (A, B, E, F) and at 24 (C, G, H) months of age. (A, E, arrows) Dendrites extending into the ONL. (C, arrow) Horizontally oriented bipolar cell dendrites; (*) tortuous and beaded axons (two on the left and one on the right of the symbol). (D) Average number ($\pm$ SD) of rod bipolar cells (per retinal cross section window of $230.3 \times 230.3 \mu\text{m}$) as a function of age; filled and open symbols correspond to center and periphery, respectively. (G, arrows) Two cell bodies, abnormally oriented. (H, arrows) Processes extending aberrantly into the INL. Scale bar: (A, B, E, F) $50 \mu\text{m}$; (C, G, H) $15 \mu\text{m}$.

retina. In the oldest ELOVL4/TG1-2 retinas studied (24 months of age), changes in rod bipolar cell body orientation occurred, especially in those areas where the INL was severely distorted (see Severe Remodeling section, later). As exemplified in Figure 4C, horizontally oriented somata of rod bipolar cells, projected truncated dendrites sideways along the INL rather than

into the ONL. Loss of rod bipolar cells became statistically significant only at the latest time point examined, 24 months of age (Fig. 4D).

Horizontal Cell Remodeling

B-type horizontal cells (the only horizontal cell type found in the mouse, also referred to as H2 type) underwent analogous age-related remodeling similar to that of rod bipolar cells. Changes observed in the WT retinas consisted of lengthening, not retraction, of horizontal cell dendritic processes (Fig. 4E). Such elongations again occurred in an age- and eccentricity-dependent fashion, with the peripheral retina being more affected than the central retina. In contrast to the WT mice, horizontal cell dendritic processes in ELOVL4/TG1-2 retinas became sparser (Fig. 4F) as photoreceptor loss progressed from the central to the peripheral retina. At 24 months of age, only a very few horizontal cells were detected in the center of ELOVL4/TG1-2 retinas (not illustrated). The remaining cells had their somata displaced toward the outer retina (Fig. 4G) and their processes oriented upside down (toward the inner rather than outer retina; Fig. 7H). In addition, the ingress of numerous horizontal cell processes into the inner retina occurred at 24 months (Fig. 4H). The loss of horizontal cells followed the same regional pattern as described for rod bipolar cells, being more pronounced in the central than in the peripheral retina.

Remodeling of Cholinergic Amacrine Cells

Figure 5 shows examples of ChAT-expressing amacrine cells in a WT and an ELOVL4/TG1-2 retina at 18 months of age. Up to this age, cholinergic amacrine cells in both groups formed two distinct rows of cells: one located in the proximal aspect of the INL and the other in the ganglion cell layer (GCL). Occasionally, ChAT-labeled cells were seen in the outermost aspect of the INL; a finding previously reported in the C57BL/6 mouse strain.⁴⁴ ChAT-positive cells formed two dendritic strata in the IPL, one between sublaminae 1 and 2 and the other between sublaminae 3 and 4. These two strata remained distinct from each other and were evenly labeled without apparent alteration in their thickness. In both experimental groups, the density of ChAT amacrine cells remained stable with age (Figs. 5E, 5F). Other types of amacrine cells, such as PKC- or calbindin-positive cells, did not undergo any noticeable morphologic changes either. Changes in morphology, distribution pattern, and dendritic layering of the ChAT-positive cholinergic amacrine cell population in ELOVL4/TG1-2 mice occurred only at the latest time point studied (24 months of age), in areas of severe retinal distortion (see Severe Remodeling, including Figs. 7I, 7K).

Müller Cell Reactivity

Müller cell reactivity (estimated from GFAP expression) in both ELOVL4/TG1-2 and WT mice was more pronounced in the peripheral than in the central retina and was stronger in ELOVL4/TG1-2 than in age-matched WT retinas. In ELOVL4/TG1-2 retinas, this reactivity appeared at the earliest time point analyzed (3 months of age; not illustrated) and increased with age (Fig. 6A, 18 months of age). Western blot results indicated that the levels of GFAP expression in 9-month-old ELOVL4/TG1-2 mice (i.e., at the beginning of photoreceptor loss; Fig. 2A) were eight times those of age-matched WT retinas (see Supplementary Fig. S1, <http://www.iovs.org/cgi/content/full/51/4/2248/DC1>). A similar age-related Müller cell reactivity was observed by using another glial cell marker, vimentin (Fig. 6A), but without any clear center-to-periphery gradient in labeling. Vimentin was strongly expressed throughout the ELOVL4/TG1-2 retina, including the OPL. Finally, labeling of all

FIGURE 5. Lack of morphologic changes in cholinergic, ChAT-expressing amacrine cells up to 18 months of age in the center and periphery of WT and ELOVL4/TG1-2 retinas. (A, B) WT mice, center and periphery respectively. (C, D) ELOVL4/TG1-2 mice, center and periphery, respectively. The number of ONL rows was 11 for the center and periphery in WT and 0 for the center and 4 for the periphery in ELOVL4/TG1-2. The pattern of distribution of cell bodies and their synaptic terminals in the IPL were similar in both WT and ELOVL4/TG1-2 mice. (E–F) The number (average $\pm$ SD) of cholinergic amacrine cells bodies (per retinal cross section window 600 μ m in width) did not vary as a function of age in WT or ELOVL4/TG1-2 mice; counts are presented separately for cells distributed along the outer (E) and inner (F) layers. Scale bar, 50 μ m.

Müller cells with the constitutive marker, glutamine synthetase, showed that Müller cells were highly disorganized and abnormally clustered at 24 months of age; a few processes could even be seen to invade the subretinal space in circumscribed areas (Fig. 6B).

Severe Remodeling

At the latest time point studied (24 months of age), when the ONL had disappeared, the coarse structure of the inner retinal layers sustained severe damage (Fig. 7). Cells from the RPE migrated into the inner retina of the ELOVL4/TG1-2 mice (Fig. 7A). RPE cell migration occurred in an eccentricity-dependent fashion, with the central retina being more affected than the peripheral retina. Also respecting this severity pattern, were large inner retinal blood vessels extending obliquely into the outer retina. These abnormal vessels most likely supplied the intermediate vascular plexus (Fig. 7B) and tended to cluster with the migrated RPE cells. Figure 7B shows an example where pigment cells were found along the pathologic course of a large vessel in the INL and IPL, approaching the ganglion cell layer. Of note, vascular changes in the present model did not occur during the acute phase of photoreceptor degeneration and did not affect the choroidal plexus. Several other pathologic features were observed in ELOVL4/TG1-2 retinas at 24 months of age, including large bundles of axons (labeled with RT97) extending upward from the retinal ganglion cell layer into the IPL, with other axon bundles (presumably from B-type horizontal cells) extending downward, from the INL into the IPL (Fig. 7C, arrow). Strongly reactive glial processes from Müller cells and astrocytes (Figs. 7D–F) colocalized with migrated RPE cells into the INL, and in some cases formed a glial continua, mainly in the nerve fiber layer (NFL). Rod bipolar cell somata were displaced to the innermost part of the INL, and the ones appropriately localized in the outermost part of the INL often had abnormal orientations (Fig. 7G). Horizontal cell somata also had abnormal cell body orientation, and their outermost synaptic plexi extended into the INL (Fig. 7H), and the bistratified synaptic plexi of ChAT amacrine cells was completely interrupted in some areas (Fig. 7I). Some calbindin-labeled cell bodies (retinal ganglion cell or amacrine cell) were localized in the outer side of the nerve fiber layer (Fig. 7J), and cholinergic amacrine cells appeared to send horizontal processes between each other (Fig. 7K).

Age-Related Changes in Opsin Expression

In the WT mice of all ages, M/L- and S-opsin staining was restricted to the outer segments of the cone photoreceptors (Fig. 8A). In some instances, the cone cells were labeled in their entirety and showed the stereotypical radial array of their axons and inner and outer segments (not illustrated). In contrast, the ELOVL4/TG1-2 mice exhibited a gradual loss of photoreceptors until approximately 18 months of age, when very few cones remained (Mema SC, et al. *IOVS* 2007;48:ARVO E-Abstract 23). Early changes were detected by 6 months of age. In addition to labeling-truncated outer segments, double staining for S- and M/L-opsins revealed double-labeled subretinal spherical deposits (Fig. 8B) not found in WT retinas at any ages. Remodeling was more severe at later ages. By 18 to 24 months, staining for opsins on retinal flatmounts revealed intensely labeled somalike structures associated with aberrant sprouts and large swellings (Figs. 8C, 8G–J), which were preferentially localized at the periphery of the retina. On similarly processed, age-matched retinal cross sections, opsin labeling was confined to the outermost part of the retina (Figs. 8E, 8F). Furthermore, as opposed to the WT and younger ELOVL4/TG1-2 mice, M/L- and S-opsin expression never co-localized in the same cone in the 18- to 24-month-old ELOVL4/TG1-2 mice.

FIGURE 6. Increased Müller cell reactivity in WT and ELOVL4/TG1-2 mice. (A) Examples of Müller cell reactivity using the markers GFAP (age: 18 months) and vimentin (age: 12 months), respectively. (B) Examples of labeling of the entire population of Müller cells using the constitutive marker GS, in WT and ELOVL4/TG1-2 mice at 24 months of age; arrow: Müller cell processes invading the subretinal space. Scale bar: (A) 50 μ m; (B) 20 μ m.

DISCUSSION

The present work describes for the first time the morphologic alterations occurring over a 2-year period in the retinas of transgenic mice (ELOVL4/TG1-2 line) bearing a genetic mutation leading, in humans, to an autosomal-dominant juvenile form of atrophic macular degeneration, Stargardt-like dystrophy (STGD3). Such a prolonged investigation period was dictated by the slow degenerative process observed in this transgenic mouse line (Fig. 2A), but it also permitted observation, in parallel, of age-related changes in retinal circuitry in the normal mouse (C57BL/6N).

The ELOVL4 Transgenic Mouse Model

To assess the ELOVL4/TG1-2 phenotype, we measured transgene expression and lipofuscin levels. Transgene expression between the ELOVL4/TG1-2 mice was found to be comparable, which confirmed the validity of comparing observations from different animals at various time points. The ratio of ≈ 1.3 (mutated *ELOVL4* over endogenous *Elovl4* expression level) is in agreement with both our anatomic (number of photoreceptor rows) and functional findings (ERG) and therefore points to a degeneration rate between the original lines TG1 and TG2 described by Karan et al.¹⁷

Lipofuscin levels (expressed as A2E levels) have been shown to increase with age in the RPE.²³ As expected of a

model of STGD3 dystrophy, this accumulation is exacerbated (both in terms of levels and rate of buildup) in ELOVL4 transgenic mice compared with WT.¹⁷ The A2E level in the 3-month-old ELOVL4/TG1-2 mice was approximately 10% of that in age-matched TG2 mice (119 picomoles/retina; Fig. 4 in Karan et al.,¹⁷); the level in the 10-month-old ELOVL4/TG1-2 mice was still half the level in the 3-month-old TG2 mice. These values further support that the ELOVL4 transgenic mouse phenotype is intermediate between the TG2 and TG1 lines.

Age-Related Changes in WT Mice

There were no gross morphologic changes in WT retinas between the ages of 1 and 24 months. In agreement with previous studies in C57BL/6N mice,^{45–47} the number of photoreceptor rows and inner retina neurons, the expression of bassoon and mGluR6, and the reactivity of glial cells did not vary noticeably with age. A slightly more intense GFAP and vimentin immunolabeling could be observed only beyond 12 months of age in the periphery of the retina as reported in aged rats⁴⁸ and humans.⁴⁹

A distinctive age-related alteration in WT mice was the sprouting of dendritic fibers from rod bipolar and horizontal cells into the ONL. Such phenomenon have been reported in normal aged mice and humans.^{28,43,50} Although its actual mechanism is currently unknown, dysregulation of calcium

FIGURE 7. Severe remodeling in 24-month-old ELOVL4/TG1-2 retinas in areas where the ONL was lacking. (A) RPE migration into the INL (*black arrow*). (B) Abnormal inner retina blood vessels (labeled with BS-I) invaginating into the outer retina (*arrows*) and merging with migrated RPE. (C) Axons (labeled for NF-200 kDa) from the innermost retina (probably ganglion cells or displaced amacrine cells ***) and from the INL (most likely horizontal cells; *arrow*), appearing to converge at the same location. (D–F) Examples of GFAP-labeled aberrant projections from the inner and the outer retina. *Arrows*: GFAP-labeled processes overlapping with migrated RPE; (***) GFAP-labeled processes extending from the GCL toward the outer retina. (A–F) Superimposition of bright-field (to show the RPE, in *black*) and fluorescence (to show specific immunofluorescent markers [*red*] and DAPI-labeled cell bodies [*blue*]). (G) Rod bipolar cell bodies (labeled for PKC α) atypically displaced toward the inner retina (*arrow*) and abnormally orientated (***). (H) Horizontal cells (labeled for calbindin) also having abnormal cell body orientation (***); note the strong invagination of the outermost calbindin-positive synaptic plexus into the presumed INL (*arrow*). (I) Cholinergic amacrine cells (labeled for ChAT) showing a complete interruption of their bistratified synaptic plexi (*arrow*). (J) Calbindin-labeled cell body (retinal ganglion cell or amacrine cell ***) abnormally localized in the outer side of the nerve fiber layer (*arrows*); +, a nearby correctly positioned calbindin-labeled cell body. (K) ChAT immunoreactivity showing, possibly, a cell on the *right* (***) sending a process (*arrow*) to a second cell on the *left* (***). Scale bar: (A–I) 50 μ m; (J) 20 μ m; (K) 10 μ m.

exchanges at the photoreceptor level (which is associated with the retraction of axon terminals, microtubule dysfunction, and abnormal synaptic transmission between photoreceptors and bipolar cells) may be a key player.⁵¹ Of relevance, the preclusion of synaptic input to bipolar cells during development induces a compensatory dendritic overgrowth in postsynaptic neurons.⁵² It is therefore conceivable that, with age, subtle changes in the local microenvironment at the RPE level alter the normal physiological status of some photoreceptors, causing axon retraction and postsynaptic dendrite towing. On the other hand, dendritic sprouting from second-order retinal neu-

rons in normal aged subjects may be a neuronal response to local upregulation of survival factors after stress (for instance, a side effect of the so-called rod-derived cone viability factor^{53,54}). Investigation of the mechanisms underlying second-order neuron dendritic growth was beyond the scope of the present study.

Photoreceptor Loss in ELOVL4 Transgenic Mice

A very different picture was observed in the ELOVL4/TG1-2 mice. As a first discrepancy, the young adult ELOVL4/TG1-2

FIGURE 8. Age-related changes in anti-opsin immunofluorescence. Double staining for S-opsin (green) and M/L-opsin (red) in a cross section from the central retina at 6 months of age, in WT (A) and ELOVL4/TG1-2 (B), shows coexpression in outer segments (yellow); note a double-labeled subretinal deposit (*) in the ELOVL4/TG1-2 but not WT retina. (C, G–J) M/L-opsin-stained flatmounts from 24-month-old ELOVL4/TG1-2 mice. (D) A z-stack reconstruction of the confocal photomicrograph shown in (C) illustrates that the presumed soma (arrows), the aberrant sprouts, and the large swellings (*) were confined in the outermost retina. The corresponding locations of the structures identified by symbols in (C) are shown in (D), and the plane of view of the z-stack shown in (D) is aligned along the dotted line in (C). (E, F) Additional examples of horizontally arrayed opsin-labeled processes, where cross sections were double stained for S-opsin (green) and M/L-opsin (red); note the absence of any co-localization (yellow) signal. Scale bar, 20 μ m.

mice displayed significantly fewer photoreceptor rows than did the age-matched WT at the center of the retina (Fig. 2A; see also Karan et al.¹⁷ for 1-month-old transgenic mice). This observation confirms a primary effect of the mutated *ELOVL4* transgene expression on the central retina, but raises questions about the nature of this effect during development. Of note, promoter-driven temporal expression profiles of both the *Irbp* and *Elovl4* genes are identical during the development of the

mouse retina.^{19,55} Significant transcript and protein levels have been reported at embryonic day (E)10 and E11, when cones begin to differentiate, but long before the expression of a critical element in rod commitment, the neural leucine zipper *Nrl* (E12.5). Expression levels of both transcripts increase postnatally and are maximal between P4 and P15, when photoreceptors are maturing. It may be of interest to examine whether the *ELOVL4* transgene impairs photoreceptor (rod) differentiation and/or strengthens normally occurring cell death in the postnatal period.

With age, there was a gradual loss of photoreceptors until approximately 18 months when very few photoreceptors remained. Rod loss preceded cone loss (Mema SC, et al. *IOVS* 2007;48:ARVO E-Abstract 23). The corresponding loss of visual function could be observed with the ERG (Sauvé Y, et al. *IOVS* 2007;48:ARVO E-Abstract 15).¹⁷ At each time point studied, there was variability in the degree of photoreceptor loss that precluded a population estimate of central-to-peripheral progression, which is otherwise present in individual animals (Table 2). This variable extent of photoreceptor loss between the age-matched ELOVL4/TG1-2 mice did not correlate with the respective transgene expression levels that appear roughly constant between the mice of the same age (Fig. 1B). Of note, the same mutation in *ELOVL4* has been shown to lead to highly variable phenotypes in humans.⁵⁶ In addition to age, intrinsic individual variability (independent of the genotype) must therefore be taken into account when determining the extent of any degenerative process.

Degenerative Process in the Inner Retina

With the loss of input from the photoreceptors, the second-order neurons in the ELOVL4 transgenic mice underwent salient morphologic changes, but rarely were dendrites elongated across the ONL. This observation contrasts with the multiple reports of dendritic sprouting in the ONL after progressive photoreceptor loss (retinal detachment, age-related macular degeneration in human, and various genetic mutations directly affecting photoreceptor function).^{27,51,57–61} It has also been shown that in cases in which photoreceptors degenerate rapidly, bipolar cell dendrites initially elongate, but then retract (this retraction was shown to occur at a stage where the ONL was lost) or in other cases simply fail to elongate into the ONL at all. In some other cases, horizontal cell dendrites were also shown to invade the INL.^{35,62–65} Throughout the dendritic sprouting into the ONL, pre- and postsynaptic components have been reported to remain closely apposed to each other.^{51,63} Whether the resultant ectopic synapses are functional has yet

TABLE 2. Row Numbers in Retina Center and Periphery in WT and ELOVL4/TG1-2 Mice, as a Function of Age

	3 mo		12 mo	
	Animal (n)	Center: Periphery	Animal (n)	Center: Periphery
WT	1	12:10	7	11:9
	2	9:8	8	12:9
	3	13:11	9	11:9
	4	10:6	10	11:10
	5	13:10	11	10:8
	6	13:11	12	10:9
ELOVL4	1	8:10	7	3:5
	2	8:9	8	5:5
	3	6:7	9	3:5
	4	8:7	10	3:6
	5	7:6	11	7:7
	6	7:8	12	6:7

to be determined; the presence of mGluR6 labeling at postsynaptic structures does support their potential functionality.³⁶ Hence, dendritic sprouting may be a mechanical oddity (epiphenomenon) related to cytoskeletal alteration that could precede apoptosis in photoreceptors; or it could be representative of an as yet unveiled type of plasticity. Since potential therapeutic avenues could involve manipulating such compensatory mechanisms, the reason underlying the absence of dendritic elongation of second-order neurons should be explored.

To summarize our findings, the degenerative processes of second-order neurons in the ELOVL4/TG1-2 mouse retina consist of (1) increased stress-related markers such as GFAP; (2) dendrite shortening; (3) soma disorientation; and (4) cell death. This series of events (first) was variable between age-matched ELOVL4/TG1-2 transgenic mice, but was best correlated with the number of remaining photoreceptors and (second) is not specific to the present model, but rather appears as a scenario common to many degenerative processes involving primarily photoreceptor cells.^{35,59,63,66–68} Only the timing appears to differ with respect to the rate of the degenerative process. Retraction of dendrites is not surprising in cells that progressively lose most of their sensory input.^{68,69}

By 18 months of age, when only sparse photoreceptors remained (Fig. 2A), there was still no obvious change in third-order retinal neurons and inner plexiform layering of the ELOVL4/TG1-2 transgenic mouse as exemplified by ChAT staining. This is in agreement with former observations in various mutants (*rd1* mouse,⁶² *CNGA3*^{−/−} *Rho*^{−/−} mutant mice,⁶³ P347L mutant swine⁷⁰) with the exception of P23H rats.⁵⁹ The seeming lack of amacrine cell remodeling after photoreceptor degeneration is possibly related to the fact that inner retina circuits develop without much photic input.⁶⁸

In contrast, Müller cells were strongly reactive, even at the youngest age examined (3 months) when photoreceptor loss was not still obvious. This is perhaps not surprising considering that Müller cells form adherens junctions with photoreceptor inner segments at the outer limiting membrane (OLM) and display ubiquitous roles that are essential for normal retina physiology.⁷¹ Regardless of its origin, retinal stress induces Müller cell reactivity that is usually best appreciated through an overexpression of neurofilament proteins. Elevated levels of GFAP and vimentin in astrocytes and Müller cells have been observed in several rodent models of retinal degeneration.^{32,62,66,72,73} It has also been documented in humans with AMD,^{49,74,75} X-linked retinitis pigmentosa,⁷⁶ diabetic retinopathy,⁷⁷ and retinal detachment,⁷⁸ to list a few examples.

The Ultimate Collapse of the Inner Retina Network

The loss of the ONL and the exacerbated Müller cell reactivity (both occurring around 18 months of age in the present model) denote the onset of the deconstruction of the inner retina (phase 3 of the degenerative process⁶⁸). Strong distortion of the INL occurred in the central retina of the present model starting at 24 months, with cells in the INL oriented downward, invasion of the retina by RPE cells, dense neovascularization, and disruption of the IPL sublayers.

The invasion of the retina by RPE cells has been extensively studied in cases of advanced retinitis pigmentosa.⁷⁹ In the ELOVL4/TG1-2 mice, this invasion occurred around vessels and extended, in some instances, to the innermost retina (Fig 7B). The role of these migrating RPE cells at late degenerative steps is currently unknown. These cells could be involved, at least to some degree, in the phagocytosis of retinal cell debris. However, RPE cells are also able to transdifferentiate, both in vivo and on injury, into fibroblasts^{80,81}; therefore, migrated RPE cells may exacerbate the degenerative process through the

formation of scarlike tissue and the production of pathologic extracellular matrix components.

Blood vessels within the rodent retina are normally distributed within the optic nerve fiber layer (superficial plexus), at the proximal part (intermediate plexus) and distal part (deep plexus) of the INL. At late degenerative stages, intermediate and deep retinal capillaries have been reported to invade the RPE where they formed abnormal vascular patterns^{82,83} supplied by redirected superficial vessels. Although not studied in detail, this phenomenon most likely occurs in ELOVL4/TG1-2 transgenic mice once the photoreceptors are lost (Fig. 7B). As in the RCS rat,⁸⁴ hypertrophied superficial vessels may also push (“garrote”) RGC axons into the inner retina (Fig. 7C), leading ultimately to axotomy and RGC death. Factors leading to vascular changes in this model are unknown, but reduced oxygen availability and upregulation of vascular endothelial growth factor induced by strongly reactive astrocytes^{85,86} (Figs. 7D–F) and excessive A2E levels⁸⁷ may be involved. Finally, the few cones remaining, despite any ONL, showed highly abnormal opsin distribution and aberrant axonal sprouting. These two features were respectively reported in the retina of aged humans and patients with AMD⁸⁸ and in humans with advanced retinitis pigmentosa.⁸⁹

Relevance of the Model for Retinal Degeneration Research

Although the ELOVL4 mouse model has analogies with dry AMD, it is, strictly speaking, not a model of this condition. Since the *ELOVL4* transgene is expressed in both rods and cones, mutations in this gene must affect all the photoreceptors; as rods represent close to 99% of all photoreceptors in mice, rod degeneration dominates in this model. Even if we consider a primary defect specific to cones, on a strictly anatomic point of view, rodents cannot be used as models for macular degeneration, since they do not have a macula. Although cones only represent approximately 5% of all photoreceptors in the human retina, the macula contains 100% cones. In mice, cones represent only 1% to 3% of the photoreceptors, with a major distribution in the ventral retina, at least in the ubiquitously used C57BL6 mouse. In these animals, cones express both M- and S-opsins (although at different levels, depending on their location) leading in fact to the conclusion that there is a single cone type in this animal. A first, rather inexpensive step to modeling human disease would be to use rodents with cone-rich retinas, dual-type cones, and uniform cone distribution. Unfortunately, there is to date no mutant model in such animals. A potential complication of the ELOVL4 mutation, in addition to its primary defect, has to do with the progressive deterioration of the photoreceptor outer membrane (as a consequence of hampered synthesis of VLC-PUFA, C28 and longer carbon chains¹¹). This deterioration is likely to affect ABCR function, which is directly dependent on the membrane lipid environment.⁹⁰ ABCR, a photoreceptor-specific adenosine triphosphate (ATP)-binding cassette (ABC) transporter, is causal in arSTGD2 and in several other retinal diseases, such as recessive cone-rod dystrophy (CRD) and some forms of autosomal recessive retinitis pigmentosa (RP19). At advanced stages of degeneration, the initial phenotype specific to ELOVL4 mutation may therefore be confounded with some variants of retinitis pigmentosa. Furthermore, ELOVL4 mutations have also been suggested to have negative effects on rhodopsin trafficking.⁹¹ These defects have been associated with models of autosomal dominant retinitis pigmentosa such as P23H and GHL mice.⁶⁸

CONCLUSIONS

Altogether, our results indicate that photoreceptor-driven retinal degeneration in ELOVL4/TG1-2 transgenic mice proceeds into three major phases: The primary one (from birth to $\approx$ 9 months of age) consists of subtle changes sensed by Müller glia, without obvious photoreceptor loss or retinal dysfunction as assessed with the ERG. The secondary phase (from $\approx$ 9 to 18 months of age) is dominated by a dramatic loss of photoreceptors and a large negative remodeling of second-order neurons. At the end of this phase, ERGs are not recordable and photoreceptor rescue is impossible. The third phase takes place once the ONL is lost. The remnant retina displays abnormally wired networks; ultimately, it presents itself as a sheet of necrotic tissue that is ostensibly unable to subserve vision. With the exception of its time course, this scheme of events is very much analogous and pertinent to the understanding and treatment of several types of retinal dystrophies (retinitis pigmentosa, Stargardt macular dystrophy, cone rod dystrophy, choroideremia, Leber congenital amaurosis, and other retinopathies). It seems therefore unmistakable that, independent of the strategy used to cure or prevent degenerative retinal diseases, success could not be expected without preserving a functional inner retina network.⁹² The ELOVL4/TG1-2 mouse may be particularly attractive for examining such strategies. First, although mice lack a macula, the retinal dystrophy seen in this transgenic mouse line proceeds from the center to the periphery, adding to its pertinence as a tool for STGD3 dystrophy, as well as to be a general model for most degenerative retinal diseases. Second, and most important, the slow rate of the degeneration process (with an approximate halfway point at 1 year of age) makes these mice an ideal model for the study of the physiological reactions accompanying the primary genetic defects and for examining the ability of potential therapeutic treatments to slow or to prevent the cascade of events leading to retinal collapse. One such treatment is currently under investigation.

Acknowledgments

The authors thank the University of Alberta Faculty of Medicine and Dentistry Confocal Microscopy Facility for allowing us to use their Zeiss LSM 510 laser-scanning microscope; Honey Chan for technical assistance; Gregory Gilmour and Shuo Chen for helping with the ERG recordings; Monique Munro for assistance in counting cells in histologic sections; Shigetada Nakanishi (Kyoto University, Kyoto) for donating the mGluR6 antibody; and Fred Berry for guidance in aspects related to the molecular genetics of our transgenic mouse colony.

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