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Acute and Protracted Cell Death in Light-Induced Retinal Degeneration in the Canine Model of Rhodopsin Autosomal Dominant Retinitis Pigmentosa

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Abstract

Purpose
To characterize a light damage paradigm and establish structural and immunocytochemical measures of acute and protracted light-induced retinal degeneration in the rhodopsin (*RHO*) T4R dog model of *RHO*–autosomal dominant retinitis pigmentosa (ADRP).

Methods
Retinal light damage was induced in mutant dogs with a 1-minute exposure to various light intensities (0.1–1.0 mW/cm^2) delivered with a Ganzfeld stimulator, or by fundus photography. Photoreceptor cell death was assessed by TUNEL assay, and alterations in retinal layers were examined by histology and immunohistochemistry 24 hours and 2 weeks after light exposure. Detailed topographic maps were made to document changes in the outer retinal layers of all four retinal quadrants 2 weeks post exposure.

Results
Twenty-four hours post light exposure, the severity of photoreceptor cell death was dose dependent. Immunohistochemical analysis revealed disruption of rod outer segments, focal loss of the RPE integrity, and an increase in expression of endothelin receptor B in Müller cells with the two highest doses of light and fundus photography. Two weeks after light exposure, persistence of photoreceptor death, thinning of the outer nuclear layer, and induction of Müller cell gliosis occurred with the highest doses of light.

Conclusions
We have characterized outcome measures of acute and continuing retinal degeneration in the *RHO* T4R dog following light exposure. These will be used to assess the molecular mechanisms of light-induced damage and rescue strategies in this large animal model of *RHO*-ADRP.

Keywords
retinal degeneration, light damage, rhodopsin, ADRP, canine model

Disciplines
Medicine and Health Sciences | Veterinary Medicine

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Decades of exposure to light is considered to influence retinal damage in AMD,[1] and it may play a role in modifying the disease course in human patients and animal models of RP[2,3] as well as in a subset of inherited retinal diseases in animal models such as Usher's syndrome[4] and Leber congenital amaurosis.[5,6] Autosomal dominant RP accounts for 30% to 40% of all RP patients, and of these, 30% carry a mutation in the rhodopsin gene (*RHO*). These patients have been functionally categorized into class A and class B.[7] Class A patients exhibit early panretinal rod dysfunction; but the onset of disease in class B patients occurs in adulthood and has a slow progression that affects the inferonasal and pericentral areas more severely than the superotemporal retinal region (subclass B1). This patient subclass also exhibits a delay in dark adaptation kinetics after exposure to light that bleaches >95% of available rhodopsin,[8] and a potential for light exposure to trigger and/or contribute to photoreceptor death.[3,9] Experimental evidence for increased susceptibility to light damage has been demonstrated in several animal models carrying class B1 *RHO* mutations, including T4R,[9] T4K,[10,11] T17M,[12] P23H,[13–15] Y102H,[16] I307N,[16] and S334ter.[17] These and other studies have fueled the debate about the detrimental impact of constant light "pollution" in our modern societies,[18] and the need to consider light restriction measures particularly in patients affected with some forms of retinal degeneration.[3]

The naturally occurring *RHO* T4R mutation in the dog[8] presents an extreme model of such light-induced damage, where severe membrane disruption of the rod outer segments (OS) can be observed within minutes of acute clinical light exposure, followed by the onset of photoreceptor cell death 6 hours later.[9,19] The mutant retina undergoes rapid degeneration with short-duration light intensities commonly found under normal environmental conditions. For this reason, it is an optimal large animal model in which to study the molecular pathways that link a photoreceptor-specific gene mutation with light-induced photoreceptor cell death, and to test rescue strategies such as corrective gene therapy or neuroprotective/pharmacologic treatments.

In previous studies, we established doses that cause light damage, examined cell death pathways during the first 6 hours following light exposure, and demonstrated that photoreceptor loss continues for several weeks following the initial insult.[20–23] In the present study, to establish structural outcome measures that can be used to assess the effect of therapeutic intervention for *RHO*-ADRP in this large animal model, we examined the topography of light-induced photoreceptor loss in the first 2 weeks after exposure and identified concomitant and second-

ary alterations in other retinal layers that contribute to the degenerative process.

MATERIALS AND METHODS

Animals and Rearing Conditions

Retinas from 15 mastiff crossbred *RHO* T4R (homozygous or heterozygous) mutant and 2 wild-type (WT) control dogs were used (see Table 1). All dogs (age range, 11–75 weeks) were maintained at the Retinal Disease Studies facility (Kennett Square, PA, USA). The studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The dogs' genotypes were determined either from the known status of their parents, or from genetic testing for the disease-causing mutation.[8]

Animals were housed as previously described[20] in kennels with dim cyclic illumination, consisting of 12 hours of white light per day (175 to 350 lux at the level of the "standard dog eye") and 12 hours of darkness (lights on at 6 AM and off at 6 PM). In the procedure room, light intensities range from 350 to 700 lux. These ambient light levels do not cause retinal damage in normal or homozygous mutant prcd dogs that do not carry the *RHO* T4R mutation.[20] To avoid any potential light-induced damage, none of the animals underwent a clinical ophthalmic or ERG examination.

Retinal Illumination

Animals were dark adapted overnight. On the following morning, the dogs were prepared for retinal illumination as previously described.[22] With two exceptions, the right eye (RE) was shielded and the left eye (LE) was exposed to light.

Two different approaches were used to illuminate the retinas for light-damage experiments. The first approach used the background white light (6500 K) of an Espion electrophysiology system (Diagnosys LLC, Lowell, MA, USA) delivered through a monocular Ganzfeld stimulator (ColorBurst; Diagnosys LLC). The retinas were exposed for 1 minute with different intensities measured with a luminometer (IL1700; International Light Technologies, Peabody, MA, USA), which resulted in corneal irradiance values of 1, 0.5, 0.3, 0.2, and 0.1 mW/cm^2. When measured as illuminance, these light intensities correspond to 1590, 820, 551, 319, and 170 lux, respectively. The second method to light expose the retinas consisted in photographing the retina with a fundus camera. This approach has been previously reported in other studies to induce light damage in the *RHO* T4R model.[9,20,21] In brief, 15 to 17 overlapping retinal photographs of microsecond duration were taken over a period of approximately 5 to 7 minutes with a handheld fundus camera (RC-2; Kowa Ltd., Nagoya, Japan). Light intensities used to view and photograph various retinal regions (tapetal versus nontapetal) were previously described.[9] Both methods used to create light damage in the *RHO* T4R mutant retina do not cause any retinal lesions in normal retinas.[20,22,23]

Following light exposure, dogs were kept under dim-red illumination until the following day when they were either humanely euthanized 24 hours post exposure or returned to the kennel for 2 weeks. Dogs were humanely euthanized in a dark room under dim-red illumination with an intravenous injection of pentobarbital sodium. After enucleation, a slit incision was made at the level of the ora serrata, and the entire globe was fixed in paraformaldehyde, cryoprotected, and embedded in optimal cutting temperature (OCT) medium as previously described.[24]

TUNEL Assay for Cell Death

The occurrence of cell death was assessed on retinal cryosections (10-μm thick) extending from the optic nerve head to the ora serrata along the four meridians (superior, inferior, nasal, and temporal) at 24 hours and 2 weeks following light exposure, by imaging DNA fragmentation using a TUNEL assay as previously reported.[24] In some retinal regions, the overwhelming number of photoreceptors that were TUNEL-labeled precluded any manual or automated cell count; therefore, a semiquantitative grading system (see Table 2; Fig. 1A) was established for the 24-hour postexposure time point and used to produce topographic maps of the distribution and severity of cell death in the outer nuclear layer (ONL).

Immunohistochemistry

Assessment of the morphology and immunoreactivity of several retinal cell populations was done at 24 hours and 2 weeks following light exposure, by fluorescence immunohistochemistry (IHC) using a battery of cell-specific antibodies (see Table 3) on 10-μm-thick cryosections that extended along the superior (tapetal) and inferior (nontapetal) meridians. Specificity of immunostaining with endothelin receptor B (EdnRB) was demonstrated by incubating 1 μg EdnRB antibody with 1 μg commercially available blocking peptide for 1 hour at room temperature prior to incubation on the sections. DAPI stain was used to label nuclei. Sections were viewed under a widefield epifluorescent microscope (Axioplan; Carl Zeiss Meditec GmbH, Oberkochen, Germany), and images were digitally captured (Spot 4.0 camera; Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and imported into a graphics program (Photoshop; Adobe, Mountain View, CA, USA) for display. A detailed protocol has been described previously.[24]

Retinal Morphology and Morphometry

Retinal cryosections (10-μm thick) extending along the four meridians were stained with hematoxylin and eosin (H&E) and examined under bright field microscopy (Axioplan; Carl Zeiss Meditec) with a 40× objective. Outer nuclear layer thickness was based on the number of ONL nuclei counted every 1000 μm. In addition, the location and morphology of the photoreceptor inner segments (IS) and OS, the RPE, and the tapetum lucidum (a cellular multilayered reflective structure located in the superior region of the choroid) were observed, manually recorded, and reported in a graphic format. Specific criteria examined included density and/or length of IS and OS, pigmented status of the RPE and altered morphology (e.g., hypertrophy), and presence or absence of a tapetum.

RESULTS

Light Exposure Causes Acute Dose-Dependent Photoreceptor Cell Death in the RHO T4R Retina

A dose-dependent cell death response was observed in the ONL of *RHO* T4R mutant retinas 24 hours after a 1-minute exposure period of varying intensities of white light delivered by means of a Ganzfeld stimulator (Figs. $1B_1$–B_4). With the highest dose of light (1 mW/cm^2 for 1 minute), there was massive TUNEL-labeling of photoreceptors (grade 4) in the central region of the retina with a decreasing gradient of TUNEL-positive cells from center to periphery (Fig. $1B_1$). With a 50% decrease in light dose (0.5 mW/cm^2 for 1 minute), the most severe damage (grade 4) was limited to the central region of both the superior and temporal meridians, while there were

TABLE 1. Summary of Dogs and Light-Exposure Conditions

Animal ID*	RHO Status	Age, wk	Light Exposure	PE Interval	Analysis (Morphology)
EM189	T4R/T4R	67	LE: 0.2 mW/cm^2 for 60 s RE: shielded	24 h	OCT
EM187†	T4R/T4R	67	LE: 0.3 mW/cm^2 for 60 s RE: shielded	24 h	OCT
EM186†	T4R/T4R	67	LE: 0.5 mW/cm^2 for 60 s RE: shielded	24 h	OCT
EM248	T4R/+	38	RE: 1 mW/cm^2 for 60 s	24 h	OCT
EM165	T4R/+	68	RE: 1 mW/cm^2 for 60 s	24 h	OCT
WB-E239	+/+	13	LE: 1 mW/cm^2 for 60 s RE: shielded	24 h	OCT
EM210	T4R/+	11	LE: fundus photographs RE: shielded	24 h	OCT
EM169	T4R/+	75	LE: 0.1 mW/cm^2 for 60 s RE: shielded	2 wk	OCT
EM198	T4R/T4R	55	LE: 0.3 mW/cm^2 for 60 s RE: shielded	2 wk	OCT
EM194†	T4R/+	55	LE: 0.5 mW/cm^2 for 60 s RE: shielded	2 wk	OCT
EM181	T4R/T4R	73	LE: 0.5 mW/cm^2 for 60 s RE: shielded	2 wk	OCT
EM179	T4R/T4R	73	LE: 1 mW/cm^2 for 60 s RE: shielded	2 wk	OCT
WB-E240	+/+	13	LE: 1 mW/cm^2 for 60 s RE: shielded	2 wk	OCT
EM112	T4R/+	19	LE: fundus photographs RE: shielded	2 wk	OCT
EM109	T4R/+	19	LE: fundus photographs RE: shielded	2 wk	PB
EMB37†‡	T4R/+	16	LE: fundus photographs RE: shielded	2 wk	PB
EMB38‡	T4R/+	16	LE: fundus photographs RE: shielded	2 wk	PB

PE, postexposure interval; PB, plastic embedding medium (PolyBed; Polysciences, Warrington, PA, USA).
* Tissues from dogs whose IDs are underlined were used in previous studies.[20,21]
† All dogs were +/+ at the VMD2 (CMR1) locus unless an † is placed after their ID, which indicates asymptomatic heterozygous status (VMD2$^{-/+}$).
‡ All dogs were +/+ at the RPE65 locus unless an ‡ is placed after their ID, which indicates asymptomatic heterozygous status (RPE65$^{-/+}$).

fewer TUNEL-labeled cells (grades 3 and 2) in the nasal and inferior meridians (Fig. 1B$_2$). A dose of 0.3 mW/cm^2 for 1 minute caused moderate cell death in the central retina that did not exceed grade 2 (Fig. 1B$_3$). After exposure with the lowest

TABLE 2. Grading System Used to Establish the Incidence of TUNEL-Labeling in 10-µm–Thick Retinal Sections 24 Hours Following Light Exposure

Grade	Definition/Criteria
0	ONL contains 0–2 TUNEL-labeled cells per 300 µm ONL length. This is considered a background finding.
1	ONL contains 3–20 TUNEL-labeled cells per 300 µm ONL length. Cell death is associated with light exposure as grade 1 and higher grades are not observed in shielded and control retinas.
2	ONL contains 21–50 TUNEL-labeled cells per 300 µm ONL length.
3	ONL contains >50 TUNEL-labeled cells per 300 µm ONL length. There are limited areas with clusters of TUNEL-labeled cells, but the density of these cells remains quantifiable. Some waviness of the ONL can be present.
4	a. ONL contains >50 TUNEL-labeled cells per 300 µm ONL length or density is unquantifiable due to clustering. b. Presence of irregular features of nuclear staining. c. This grade is always associated with waviness of the ONL.

dose (0.2 mW/cm^2 for 1 minute), a limited number of TUNEL-labeled photoreceptors (grade 1) were found, predominantly in the tapetal region (superior and temporal meridians) (Fig. 1B$_4$). No cell death was seen in a WT retina exposed to the highest dose (1 mW/cm^2 for 1 minute) of light (Supplementary Figs. S1A–C).
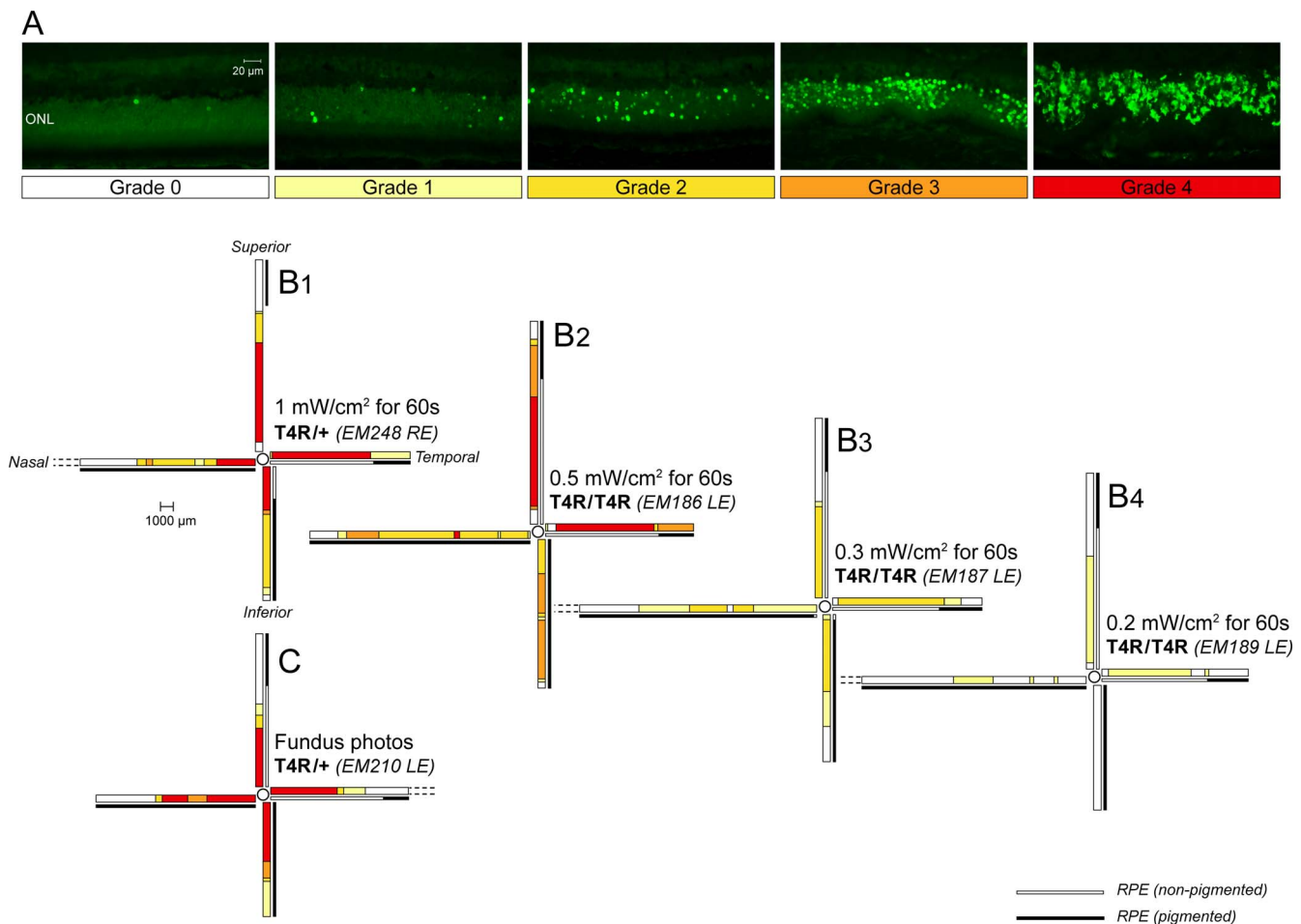
Light damage triggered by a series of overlapping fundus photographs caused a pattern of cell death severity that was similar to that obtained after Ganzfeld illumination at 1 mW/cm^2 for 1 minute. Grade 4 was observed in the central retina throughout the four meridians (Fig. 1C). One difference between the two light-damage paradigms appeared to be the more limited distribution of cell death throughout the retinal expanse, which likely resulted from anatomic limitations because it is more difficult to visualize and photograph the more peripheral regions with a handheld fundus camera in the awake dog.

Some morphologic variation in the pattern of TUNEL-labeling could be seen when comparing areas with grade 4 to areas less severely affected. In central regions with grade 4, many TUNEL-labeled figures in the ONL appeared swollen, with an irregular contour, and decreased fluorescence in contrast to the more typical TUNEL-positive nuclei that were seen more peripherally (Figs. 2A$_1$, 2A$_2$). In addition, TUNEL-positive RPE cells were seen with grade 4 in the central retina after fundus photography (Fig. 2A$_3$).

FIGURE 1. Spatial distribution of photoreceptor cell death 24 hours following light exposure in *RHO* T4R mutant dogs. (**A**) Grading system used to map the incidence of TUNEL-positive cells in the ONL. (**B₁–B₄**, **C**) Schematic color-coded representation of grades of TUNEL-labeling in retinal sections extending from the edge of the optic nerve head to the superior, inferior, nasal, and temporal ora serrata: (**B₁–B₄**) four *RHO* T4R dogs exposed to decreasing doses of light by means of a Ganzfeld stimulator; (**C**) a *RHO* T4R dog exposed to a series of overlapping fundus photographs. LE, left eye; RE, right eye.

TABLE 3. List of Antibodies Used for IHC

Antigen	Source and Catalog No.	Antibody Dilution for IHC
RPE65	Novus Biologicals, NB100-355	1:500
Rhodopsin	Millipore, MAB5316	1:200
Human Cone Arrestin	C. Craft (LUMIF)	1:10,000
Peripherin	R. Molday (3B6)	1:100
GRK1	Affinity Bioreagents, MA1-721	1:1,000
PKCα	BD Biosciences, BD610107	1:100
Goα	Millipore, MAB3037	1:5,000
Vimentin	Agilent, M0725	1:2,000
GFAP	Agilent, Z0334	1:1,000
EdnRB	Alomone Labs, AER-002	1:200

Novus Biologicals (Littleton, CO, USA); Millipore (Billerica, MA, USA); Affinity Bioreagents (Golden, CO, USA); BD Biosciences (San Jose, CA, USA); Agilent (Santa Clara, CA, USA); Alomone Labs (Jerusalem, Israel).

Light Exposure Causes Acute Outer Retinal Alterations and Müller Cell Response

We first examined the effect of light at the 24-hour time point following exposure and assessed the effects on the retina using cryosections stained with H&E or immunolabeled. Following both fundus photography and Ganzfeld exposure with the two highest doses of light (0.5 and 1 mW/cm² for 1 minute), an increase in photoreceptor internuclear spacing was seen centrally, causing an apparent increase in ONL thickness (Figs. 2B₁–B₃). The RPE layer also showed damage and apparent cell loss centrally as reflected by focal loss of RPE65 immunolabeling (Figs. 3A₂, 3A₄). Staining with rod-OS specific markers (rhodopsin, peripherin, and GRK1) showed significant disruption of the OS centrally (Figs. 3B₂, 3B₄, 3C₂, 3C₄, 3D₂, 3D₄) but not in a WT dog (Supplementary Fig. S1C). Under the same exposure protocols, the ONL, OS, and RPE were well preserved in the periphery (Figs. 3B₃, 3C₃, 3D₃), and the pattern of immunolabeling was like that seen in the central region of a shielded *RHO* T4R retina (Figs. 3A₁, 3B₁, 3C₁, 3D₁) or in the exposed retinas of WT controls (Supplementary Fig. S1D₂, data not shown). At this time point, no obvious changes in the structure of bipolar cells were seen, although some distortion of the lamination of the inner nuclear layer (INL) and shrinkage of the outer plexiform layer (OPL) was observed in
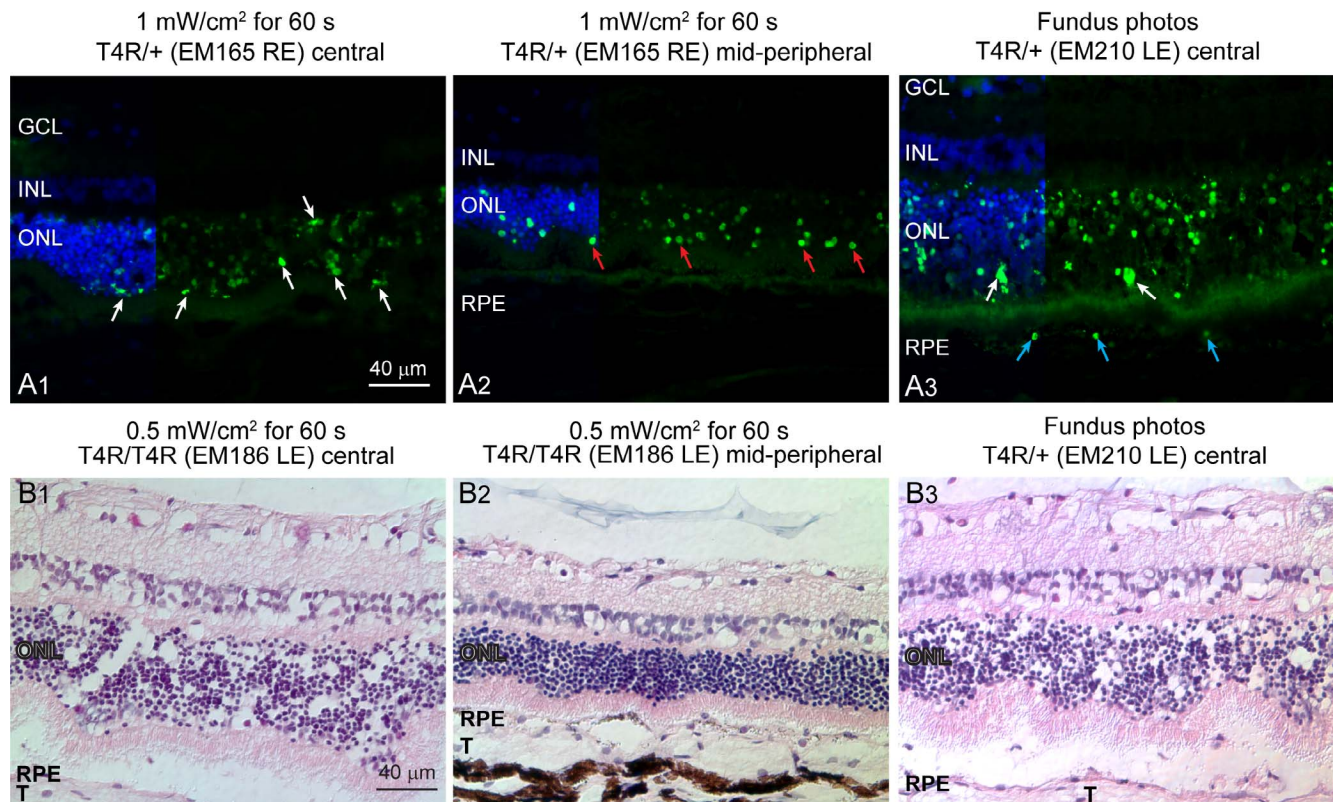
FIGURE 2. TUNEL-labeling patterns and ONL swelling. (A_1) Irregular TUNEL-labeled nuclear morphology of photoreceptors (*white arrows*) in the central versus midperipheral retina 24 hours after 1-minute Ganzfeld light exposure. (A_2) Regular nuclear morphology of TUNEL-labeled photoreceptors (*red arrows*) in the midperipheral retina 24 hours after 1-minute Ganzfeld light exposure. (A_3) Irregular TUNEL-labeled nuclear morphology of photoreceptors (*white arrows*) and TUNEL-labeled RPE cells (*blue arrows*) in central retina 24 hours after fundus photography. (B_1, B_2) ONL swelling in central versus midperipheral retina 24 hours after 1-minute Ganzfeld light exposure. (B_3) ONL swelling in central retina 24 hours after fundus photography. In both cases, the irregular swelling caused undulations of the ONL. T, tapetum lucidum.

the central/exposed *RHO* T4R retina (Figs. $3E_1$–E_4). These alterations were likely the consequence of the swelling of the overlying ONL. Müller cells, whose radial extension through the retinal thickness was clearly immunolabeled with vimentin, showed no increase in glial fibrillary acid protein (GFAP) staining, a marker of reactive gliosis; however, there was an increase in expression of EdnRB in the central exposed retinas (Figs. $3F_1$–F_4, $3G_1$–G_4). Immunohistochemical alterations were also found in *RHO* T4R retinas exposed to lower doses of light (0.5 mW/cm² and 0.3 mW/cm²) for 1 minute, but no obvious changes were seen with exposure to 0.2 mW/cm² (Table 4).

Light Exposure Causes Rapid Retinal Degeneration in the *RHO* T4R Dog

To examine the effect of light at 2 weeks post exposure, retinas from dogs exposed to similar light-damage protocols were processed for histologic/IHC evaluation. Biaxial spider graphs that extended along the horizontal (nasal-temporal) and vertical (inferior-superior) axes were constructed. Careful attention to the structure of the IS, OS, and RPE was documented in sections extending from the optic disc to the ora serrata in both tapetal and nontapetal areas. By 2 weeks post exposure, the retina that had received the highest dose of light (1 mW/cm² for 1 minute) had an ONL thickness reduced to 1 to 2 rows of nuclei over a distance extending more than 10 mm from the optic nerve head and that included the central and midperipheral regions of all four quadrants (Fig. $4A_1$). This was associated with a loss of photoreceptor IS and OS. Loss of RPE integrity was found particularly in the superior tapetal

area, whereas RPE hypertrophy was commonly seen in nontapetal areas (Fig. 5). Exposure to half of the highest dose (0.5 mW/cm² for 1 minute) caused ONL thinning, particularly in sections extending through tapetal areas (superior and temporal). Loss or shortening of IS and OS was also seen, but the RPE was preserved (Figs. $4A_2$, $4A_3$). Exposure to 0.3 mW/cm² of light for 1 minute caused photoreceptor damage and some ONL thinning that was limited to the central to midperipheral tapetal areas (Fig. $4A_4$). No evidence of photoreceptor loss was seen in a retina exposed to 0.1 mW/cm² for 1 minute, corresponding to one-tenth of the highest dose of light, although loss of OS was found in one focal area (Fig. $4A_5$). Spider graphs of retinas that were exposed to a series of overlapping fundus photographs (Figs. $4B_1$–B_4) showed a loss of ONL, IS, OS, and RPE that was similar to that observed following Ganzfeld exposure to 1 mW/cm² of light for 1 minute and was uniformly present in the three animals with this exposure paradigm. However, damage was limited to the central retina (approximately 5 mm from the optic nerve head) as more peripheral regions were difficult to light expose using fundus photography as the exposure paradigm.

With the exception of the 0.1 mW/cm² exposure, TUNEL-labeled nuclei were present in the central ONL of all retinas 2 weeks after light exposure (Figs. $6A_1$–A_6) but were not observed in the peripheral retina at any light dose. Significant autofluorescent material was observed in the RPE layer (Figs. $6A_2$–A_4, white asterisk) in retinas exposed to damaging doses of light (0.3–1 mW/cm²). RPE65 staining was preserved in all retinas except in that exposed to the highest dose of light, or
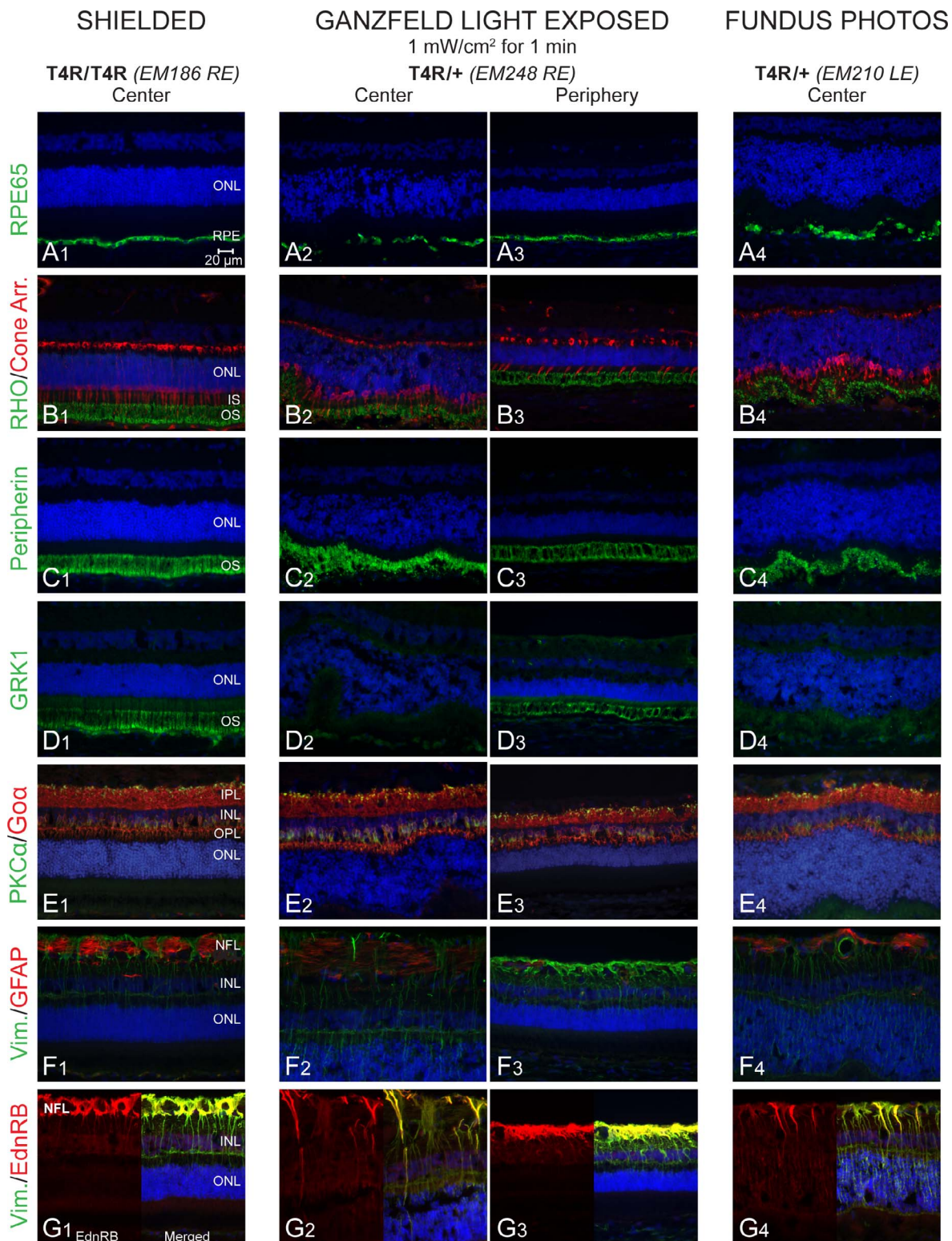
FIGURE 3. Immunohistochemical characterization of light-induced damage 24 hours post exposure in *RHO* T4R mutant dogs. (**A₁–A₄**) RPE-specific protein 65 kDa (RPE65). (**B₁–B₄**) RHO and human Cone Arr. (**C₁–C₄**) Peripherin. (**D₁–D₄**) GRK1. (**E₁–E₄**) PKCα and (Goα). (**F₁–F₄**) Vim. and GFAP. (**G₁–G₄**) Vim. and EdnRB. IPL, inner plexiform layer; NFL, nerve fiber layer; Cone Arr., cone arrestin; PKCα, protein kinase C alpha; GFAP, glial fibrillary acidic protein; Goα, Go-alpha.

fundus photography where there was a loss of RPE cells (Figs. 6B₁–B₆). Complete loss of rhodopsin (Figs. 6C₂, C₃) was observed at the two highest doses of light, and there was a marked shortening of rod OS in the retina exposed to 0.3 mW/ cm² (Fig. 6C₄). At the lowest dose (0.1 mW/cm²), mild shortening of rod OS and rhodopsin mislocalization to the ONL were seen (Fig. 6C₅). Cone-arrestin labeling revealed prominent alterations of cone structure (loss of IS and OS) following

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TABLE 4. Immunohistochemical Alterations in *RHO* T4R Retina 24 Hours After a 1-Minute Exposure to Light

| Marker	Retinal Structure Labeled in WT Retina	EM248-RE T4R/+ 1 mW/cm²	EM186-LE T4R/T4R 0.5 mW/cm²	EM187-LE T4R/T4R 0.3 mW/cm²	EM189-LE T4R/T4R 0.2 mW/cm²	EM210-LE T4R/+ Fundus Photos
DAPI	RPE, ONL, INL, GCL nucleus	ONL: thickened/disrupted/undulated	ONL: thickened/disrupted/undulated	Same as in WT	Same as in WT	ONL: thickened/disrupted/undulated
RPE65	RPE	Hypertrophic and disrupted	Hypertrophic	Hypertrophic	Same as in WT	Hypertrophic and disrupted
Rhodopsin	Rod OS	Fragmented/misaligned/blunted	Fragmented/misaligned/blunted	Patchy disruption	Same as in WT	Fragmented/misaligned/blunted
Cone arrestin	Cone cells	Fragmented OS; enlarged IS; small pedicles	Misalignment in undulations	Patchy OS misalignment	Same as in WT	Fragmented OS; enlarged IS; small pedicles
Peripherin	Rod OS	Fragmented/misaligned/blunted	Fragmented/misaligned/blunted	Patchy disruption	Same as in WT	Fragmented rod OS
GRK1	Rod OS (faint IS)	No labeling	No labeling	Patchy labeling	Same as in WT	No labeling
PKCα	Rod bipolar cells	Shortened/flattened dendrites in undulations	Shortened/flattened dendrites in undulations	Same as in WT	Same as in WT	Shortened/flattened dendrites in undulations
Goα	ON bipolar cells	Shortened/flattened dendrites in undulations	Shortened/flattened dendrites in undulations	Same as in WT	Same as in WT	Shortened/flattened dendrites in undulations
Vimentin	Müller cells	Same as in WT	Same as in WT	Same as in WT	Same as in WT	Same as in WT
GFAP	Astrocytes/Müller cell end feet	Same as in WT	Same as in WT	Same as in WT	Same as in WT	Same as in WT
EdnRB	Limited to Müller cell end feet	Expression along full radial length	Expression along full radial length	Same as in WT	Same as in WT	Expression along full radial length

GCL, retinal ganglion cell layer.

exposure to the two highest doses of light as well as after fundus photography, with only cone-arrestin labeled remnants remaining (Figs. 6C$_2$, 6C$_3$, 6C$_6$). Although the INL appeared to be well preserved, significant reduction in OPL thickness because of retraction of bipolar cell dendrites was seen in the light-damaged retinas (Figs. 6D$_2$–D$_4$, 6D$_6$). Increase in vimentin and GFAP staining, indicative of reactive gliosis (Figs. 6E$_2$, 6E$_3$), as well as sustained expression of EdnRB in ONL, INL, and retinal ganglion cell layer (GCL) (Figs. 6F$_2$–F$_6$) was observed in light-damaged retinas.

DISCUSSION

The occurrence of nonsynchronized and protracted cell loss that takes place over years or decades presents a significant challenge in studying the molecular mechanisms of photoreceptor degeneration in RP patients and most animal models. Indeed, at any given time, the low percentage of dying photoreceptors frequently limits the use of tissue-based assays that may not be sensitive enough to detect rare cell death events in a whole retina. Thus, even in models of RP that undergo a transient increased rate of photoreceptor death, single-cell assays or methods that provide cellular resolution have been proposed.[25] Light-induced retinal damage, however, can trigger a synchronized and massive burst of photoreceptor cell death that facilitates the characterization of activated cell signaling pathways.[26] Among the subset of *RHO*-ADRP models with increased light sensitivity (for review, see Ref. 22, Supplementary Table S1), the *RHO* T4R dog retina undergoes rapid and synchronized cell death following a 1-minute duration exposure to white light at levels encountered under normal environmental or clinical conditions.

We have previously shown that clinical fundus photography of *RHO* T4R dogs triggers an acute retinal degeneration[9] that is not activator protein 1 (AP-1) dependent.[20,21] In subsequent publications, we have used a 1-minute exposure to 1 mW/cm² of white light delivered by a Ganzfeld dome to initiate rapid photoreceptor cell death[22,23] and ruled out the involvement of an endoplasmic-reticulum (ER) stress response.[23] Here, we show by TUNEL assay that the cell-death pattern in the ONL using a Ganzfeld stimulator at 1 mW/cm² was very similar to that initiated after fundus photography. However, we were able to achieve more widespread retinal damage with the Ganzfeld stimulator compared with the fundus camera and in a much shorter period of time (1 minute by Ganzfeld illumination); in contrast, comparable damage was obtained by fundus photography where ~10-μsec duration flashes were delivered over a 5- to 7-minute time period for a total cumulative exposure duration of ~150 to 170 μsec. As previously shown,[22] light exposure caused more ONL cell death in the tapetal region versus the nontapetal area. This topographic difference is expected as the tapetum lucidum, a biologic reflector system found in various vertebrates that are active under dim light environment, increases photoreceptor photon catch by reflecting light back onto the OS.[27] At 24 hours post light exposure, the central TUNEL-stained nuclei presented an irregular contour and variable labeling intensities, in contrast to the peripheral TUNEL-positive nuclei that were circular with uniform staining. The spatial variability in TUNEL-labeling may indicate differences in the modes of rod cell death in the central versus peripheral retina, or alternatively, the irregularly labeled nuclei may represent those undergoing fragmentation. Since TUNEL staining is not sufficient to discriminate between apoptosis, necrosis, or autolytic cell death,[28] additional assays may be needed to identify the possible concomitant occurrence of multiple cell death processes.

GANZFELD LIGHT EXPOSURE   FUNDUS PHOTOGRAPHY
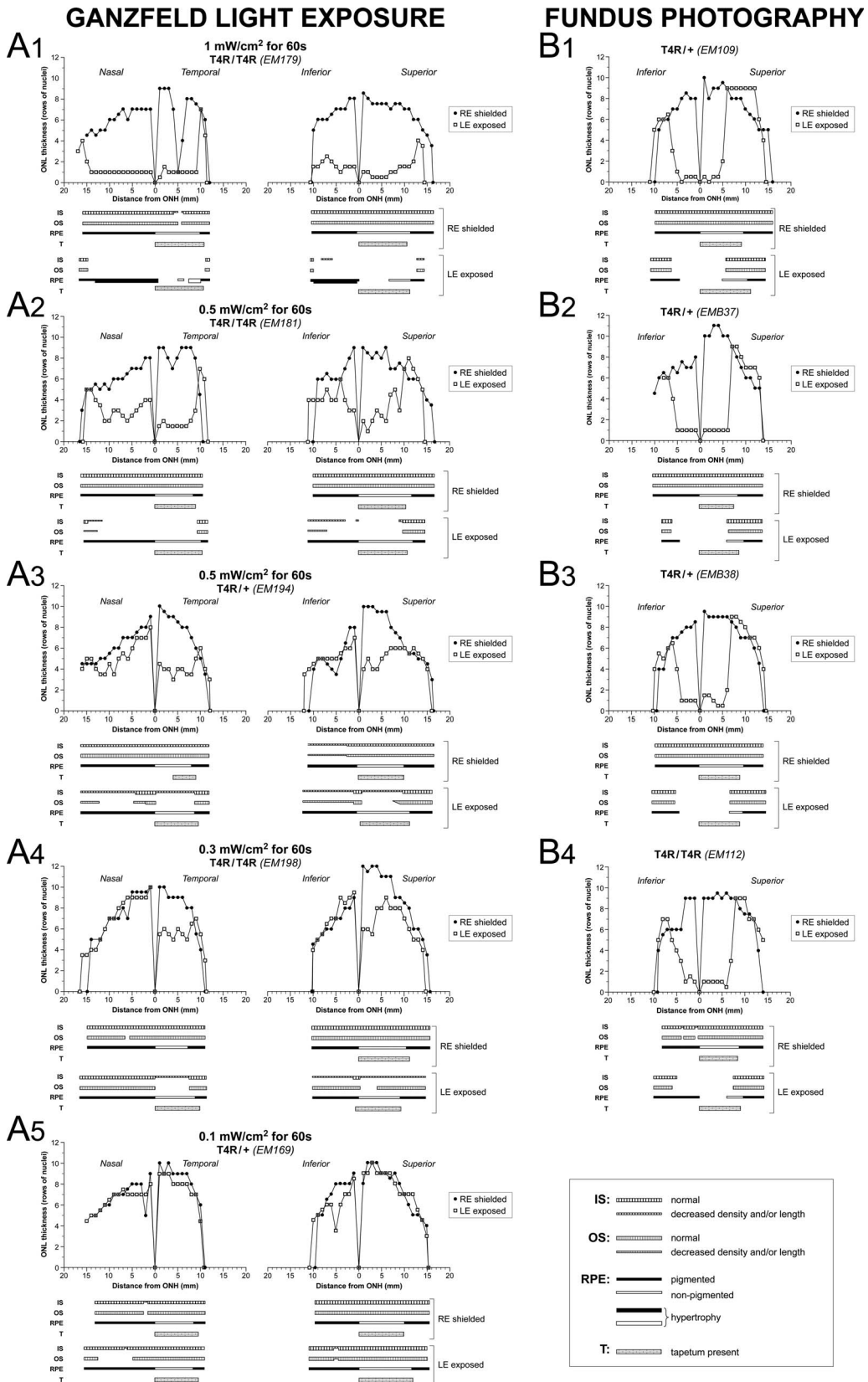
FIGURE 4. Morphometric analysis of retinal changes 2 weeks after light exposure. Spider graphs of ONL thickness and schematic representation of IS, OS, and RPE structure 2 weeks post light exposure of the LE of *RHO* T4R mutant dogs. The RE was shielded. Data derived from retinal histologic sections extending from the edge of the optic nerve head to the superior, inferior, nasal, and temporal ora serrata. T, tapetum.

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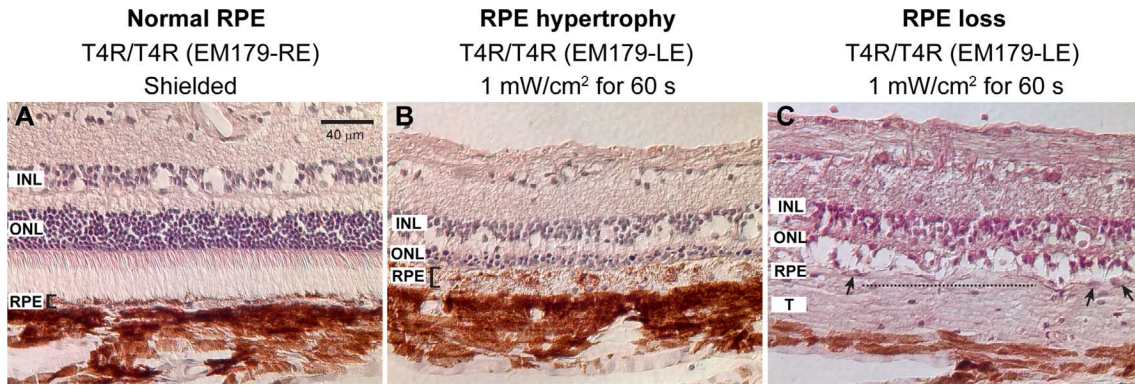
FIGURE 5. Retinal sections (H&E stained) showing light-induced alterations in the RPE of the *RHO* T4R mutant dog. (**A**) Preserved structure and morphology of the RPE in the shielded eye (*bracket* shows RPE thickness). Microphotograph was taken in the nontapetal region, where the RPE is pigmented. (**B**) Light-induced RPE hypertrophy (*bracket* shows RPE thickness) in the nontapetal region of the contralateral eye exposed 2 weeks earlier. (**C**) Light-induced disruption and loss of RPE monolayer in the tapetal region of the same eye as shown in (**B**). *Dotted line* indicates area devoid of RPE nuclei and *arrows* point to single RPE nuclei. The RPE is nonpigmented in the tapetal region.
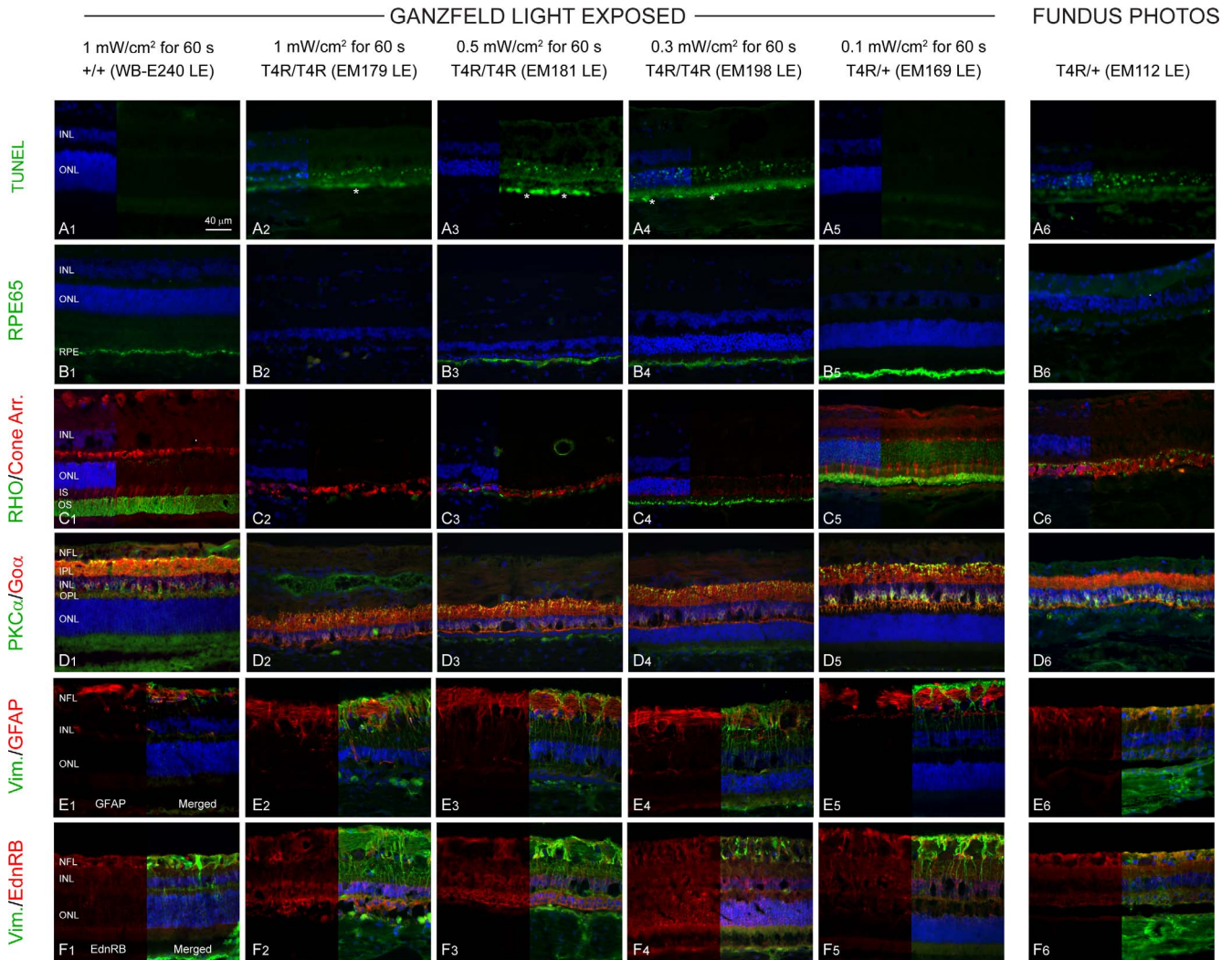
FIGURE 6. TUNEL and immunohistochemical characterization of light-induced damage 2 weeks post exposure in *RHO* T4R mutant dogs. (**A₁–A₆**) TUNEL-labeling. (**B₁–B₆**) RPE-specific 65 kDa protein (RPE65). (**C₁–C₆**) RHO and human Cone Arr. (**D₁–D₆**) PKCα and Goα. (**E₁–E₆**) Vim. and GFAP. (**F₁–F₆**) Vim. and EdnRB. All images were acquired in central retina. *Asterisks* (**A₂–A₄**) indicate RPE autofluorescence.

We have recently shown continued ONL loss occurring for weeks after acute light exposure.[22] In the current study, we now have confirmed that the light-induced death of rods is not just an acute event but continues for at least 2 weeks post exposure. This finding strongly supports the occurrence of a distinct process of cell death happening long after the acute light insult. Possible causes for this prolonged cell death may include both passive and active processes such as loss of structural integrity of the tissue, "bystander" effect,[29,30] and hyperoxia[31,32] as well as immune responses to primary cell death.[33,34]

The two lowest light intensities (170 lux/0.1 mW/cm^2 and 319 lux/0.2 mW/cm^2) used in this study for acute exposure overlap with the range (175–350 lux) of ambient illuminations in the kennels where these dogs are housed. In our previous publication,[22] we suggest that the mild ONL thinning observed at 36 weeks (but not at 2 weeks) post exposure to 1 minute of the lowest light intensity (170 lux/0.1 mW/cm^2) may have been triggered by the acute light insult (continuous exposure for 1 minute in a dilated eye). However, we did not exclude the possibility that this might also have been caused by environmental exposure to the standard levels of white light used in the kennels. In this current study, we confirmed the absence of photoreceptor loss 2 weeks after a 1-minute exposure to 170 lux/0.1 mW/cm^2 but did observe some TUNEL-labeling in the ONL 24 hours after a 1-minute exposure to 319 lux/0.2 mW/cm^2. We are confident that these short-term (24 hours and 2 weeks) post–light-exposure changes are secondary to the experimental acute exposure and not to environmental light, as these were seen exclusively in the exposed but not the shielded eyes. However, these combined results do suggest that prolonged housing under ambient illuminations ranging between 175 and 350 lux may trigger a sustained and slow rate of retinal degeneration. Ongoing studies in which *RHO* T4R mutant dogs are being housed exclusively under dim-red illumination are being conducted to experimentally confirm the likely contribution of low levels of environmental white light to the slow "natural" course of disease.

Several studies have reported that the disruption of rod OS is one of the very first events of retinal degeneration following light exposure.[35,36] In the *RHO* T4R model, disruption of rod OS (but not cones) occurs within 15 minutes of light exposure at the highest dose (1 mW/cm^2 for 1 minute).[23] In the current study, there was disruption of rod OS 24 hours after light exposure with the two highest doses of light, as evidenced by the altered labeling of two rod OS integral membrane proteins, rhodopsin and peripherin, and the loss of immunostaining of G protein-coupled receptor kinase 1 (GRK1), a peripheral membrane-associated protein that binds to discs by isoprenylation[37] and/or through interaction with phosphodiesterase 6D (PDE6D).[38] Two weeks following exposure to the lowest dose of light (0.1 mW/cm^2 for 1 minute), there was shortening of rod OS length and rhodopsin mislocalization without any evidence of photoreceptor cell loss. These structural alterations may limit the amounts of RHO protein packaged in discs and subsequently lead to an overflow in other cellular compartments. Transient alterations in IS/OS structure has been previously demonstrated by OCT imaging in *RHO* T4R dogs exposed to similar low doses of light, suggesting that a repair mechanism of OS may take place over time after a sublethal insult.[9]

This initial disruption was specific to rods, while cones appeared to be initially preserved. There was also focal loss of the integrity of the RPE layer, particularly in the central retina, and an increase in internuclear spacing in the ONL, both processes indicative of a collapse of structural integrity of the outer retinal layers. Breakdown of the outer blood retinal barrier, which is formed by the tight junctions between RPE cells, has been associated with edema in retinas exposed to blue light.[39] As the levels of white light used in this current study did not cause similar alterations in the RPE of WT dogs, it remains to be determined whether RPE susceptibility to light in the *RHO* T4R dogs is mediated via a direct phototoxic effect or whether it is secondary to the acute damage of mutant rods.

While the initial alterations were seen in the outer retina, an increase in EdnRB expression in Müller cells was also observed at 24 hours post light exposure. At two weeks post exposure, EdnRB immunoreactivity was present in the INL, in particular in cell bodies of Müller cells, as well as in the ONL (Supplementary Fig. S2). To the best of our knowledge, this is the first time that expression of EdnRB in photoreceptors has been reported, and it may suggest an interspecies difference as also found for the receptor of ciliary neurotrophic factor.[40] Although increased GFAP immunoreactivity was also seen in Müller cells, the level of reactive gliosis appeared to be milder in the T4R light-induced model of retinal degeneration than in P23H rats or following light damage in BALB/C mice.[41,42] Regardless, our observations are consistent with other reports of increased EdnRB expression after acute light damage[42,43] and induction of Müller cell gliosis by a wide variety of retinal injuries including photoreceptor degeneration.[44,45] While we have not examined the expression of endothelin itself, it has been shown to be increased in photoreceptors after light damage as well as in inherited retinal degeneration.[42] Endothelin-2 secretion by photoreceptors and its binding to EdnRB on the surface of Müller cells can induce reactive gliosis.[46]

In summary, we have further characterized a light-damage paradigm using a Ganzfeld stimulator that extends retinal damage to all four quadrants and induces an acute onset of widespread rod cell death in a large animal model of *RHO*-ADRP. Death of rods begins by 6 hours,[23] is very pronounced at 24 hours, and persists at least up to 2 weeks post light exposure. We also identified various immunohistochemical markers to assess acute as well as progressive photoreceptor loss following a single light-exposure event. This sets the stage to further our efforts[20,21,23] at identifying pathogenic mechanisms of light-induced damage as well as to evaluate outcomes of therapeutic interventions in the *RHO* T4R model of ADRP.

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