Exclusion of the Unfolded Protein Response in Light-Induced Retinal Degeneration in the Canine T4R RHO Model of Autosomal Dominant Retinitis Pigmentosa

Stefania Marsilli

Sem Genini
*University of Pennsylvania*, geninis@vet.upenn.edu

Raghavi Sudharsan
*University of Pennsylvania*

Jeremy Gingrich

Gustavo D. Aguirre
*University of Pennsylvania*, gda@vet.upenn.edu

*See next page for additional authors*

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A brief exposure to clinically-relevant levels of white light causes within minutes acute disruption of the rod outer segment disc membranes, followed by prominent ultrastructural alterations in the inner segments and the initiation of cell death by 6 hours. Activation of the PERK and IRE1 pathways, and downstream targets (BIP, CHOP) of the UPR was not observed. However increased transcription of caspase-12 and hsp70 occurred, as well as calpain activation, but not that of caspase-3.

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Disciplines
Medicine and Health Sciences | Veterinary Medicine | Veterinary Pathology and Pathobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Author(s)
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Stefania Marsili, Sem Genini, Raghavi Sudharsan, Jeremy Gingrich, Gustavo D. Aguirre, William A. Beltran*

Section of Ophthalmology, Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, 19104, United States of America

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Introduction

Retinitis pigmentosa (RP) is a clinically heterogeneous group of inherited retinal degenerative diseases leading to dysfunction and progressive loss of photoreceptor cells characterized by night vision deficits with reduction of peripheral visual field that ultimately evolves into central vision loss [1]. Presently, over 60 genes harboring mutations responsible for RP have been identified [2] (RetNet, http://www.sph.uth.tmc.edu/RetNet/); the primary defect can either occur in the retinal pigment epithelium (RPE) or in rods, with cones typically becoming involved secondarily.

Rhodopsin is the seven trans-membrane G-protein coupled receptor that, together with 11-cis retinal makes up the light-sensing protein of vertebrate rods. Rhodopsin (RHO) was the first gene identified as being causally-associated with RP, and since then more than 140 RHO mutations have been reported (http://www.hgmd.org/). Most of them are inherited in a dominant manner and account for up to 30% of autosomal dominant RP (adRP) [3–6]. In man, mutations have been described in all three domains of the protein: intradiscal, transmembrane and cytoplasmic [7]. For some of these mutations, biochemical and clinical classifications have been proposed based on in vitro characterization [7–11] and in vivo studies in patients [12].

An association between light exposure and the initiation or exacerbation of retinal degeneration has been suggested to occur in a subset of RHO adRP mutations [13–16], and has been experimentally demonstrated in several animal models [14,17–22]. Among them, is the T4R RHO mutant dog, a naturally-occurring animal model of RHO-adRP that shows similar phenotypic features as reported in patients with Class B1 RHO mutations [23]. These include a dramatically slowed time course of recovery of rod photoreceptor function after bleaching, and a distinctive topographic pattern of central retinal degeneration. The extreme sensitivity of this canine model to light has been well documented, and structural alterations have been reported to occur within minutes following acute light exposure at intensities that do not damage the wild-type (WT) retina [14,24,25]. This acute light damage results within hours in biochemical alterations [24], and within 2–4 weeks in complete loss of exposed rods, that are observed in both the tapetal (superior) and non-tapetal (inferior) regions [14,24,25].

The molecular links between RHO mutations and the triggering of rod cell death have been investigated, hypotheses proposed, yet the specific molecular mechanisms for most RHO mutations still unknown.(see for review [7]) One of the proposed mechanisms supported by both in vitro [8–10,26–29] and in vivo [29–34] studies involves misfolding of the mutant rhodopsin protein in the endoplasmic reticulum (ER) lumen as the initial trigger of ER stress, and activation of the unfolded protein response (UPR) that is mediated by three ER signal transducers: PRK-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). The UPR is a physiologic response to ER stress that aims at restoring ER homeostasis by inhibiting protein translation to reduce the accumulation of additional unfolded/misfolded protein; upregulating the expression of chaperones to increase the folding capacity of the ER; and activating an ER-associated degradation (ERAD) to remove unfolded/misfolded proteins from the ER membrane and deliver them to the proteasome for degradation. If ER homeostasis fails to be reestablished, some branches of the UPR may in turn activate apoptotic signals that subsequently lead to cell death (for review see [35,36]).
Although the pathogenic mechanisms of light-induced retinal degeneration in the canine T4R RHO model have been explored [24,25,37], the critical early molecular events that lead to the activation of photoreceptor cell death pathways have yet to be identified. In addition, the role of light as a potential trigger of an ER stress response in animal models of class B1 RHO-adRP has to this date not been assessed. Thus, the purpose of this study was to investigate in the naturally-occurring T4R RHO retinal mutant whether brief light exposure induces an ER stress and/or UPR that could be associated with the acute rod cell death.

Materials and Methods

Cell culture

Madin–Darby Canine Kidney Epithelial Cells [MDCK (NBL-2), ATCC CCL-34], and normal canine fibroblasts (kindly provided by Dr. Charles H Vite, University of Pennsylvania, PA) were grown in DMEM plus 10% FBS and treated with DMSO, tunicamycin (Calbiochem, EMD Chemicals, Gibbstown, NJ) at a final concentration of 2.5 μg/ml for 8 hours, or staurosoporine (Sigma-Aldrich Corp, St Louis, MO) at a final concentration of 1μg/ml for 4 hours.

Animals and light damage paradigms

Dogs were maintained at the Retinal Disease Studies (RDS) facility of the School of Veterinary Medicine, University of Pennsylvania (Kennett Square, PA). The studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the USDA’s Animal Welfare Act and Animal Welfare Regulations, and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The dogs were part of an outbred population with a common genetic background. Six homozygous mutant (RHOT4R/T4R), nine heterozygous (RHOT4R/+), and four wild type (RHO+/+) dogs were used. Details on the allocation of the dogs to the various experiments performed in this study are shown in Table 1. All the procedures carried out in this study, including administration of eyedrops, general anesthesia, retinal light exposure, recovery from anesthesia, euthanasia, and tissue collection, were conducted under dim red light illumination.

Following overnight dark adaptation, the dogs had the pupils of both eyes dilated with 1% tropicamide and 1% phenylephrine, 3 times, every 30 minutes. They were anesthetized with intravenous injection of ketamine (10 mg/kg) and diazepam (0.5 mg/kg), and a retrobulbar saline injection (5–10 ml) was used to prevent the ventral rotation of the globes induced by the general anesthesia, and recenter the eyes in the primary gaze. The left eyes were light-exposed (E) with either a series of sequential overlapping retinal photographs using a hand-held fundus camera (RC-2; Kowa Ltd, Nagoya, Japan) as previously described[24], or using a monocular Ganzfeld (see Table 1). The fundus camera resulted in microsecond duration flashes of a xenon lamp that produced approximate retinal doses/flash of 0.6 and 11 mJ∙cm², respectively, for the tapetal and non-tapetal regions, and resulted in a >95% bleaching [23]. The monocular Ganzfeld was a component of the Espion electrophysiology system (Diagnosys LLC, Lowell, MA, USA), and delivered a constant bright white light (6500 K, corneal irradiance: 1mW/cm²) for 1 min. Light exposures with the fundus camera or the Espion monocular Ganzfeld do not produce any retinal damage in WT retinas or those affected with other inherited retinal degenerations. The contra-lateral right eyes were shielded (S) with a black photographic cloth, and served as unexposed controls. The dogs recovered from anesthesia under dim red light, and at different time-points (15 min, 1hr, 3 hrs, 6 hrs, or 24 hrs) following light exposure they were
euthanized with an intravenous injection of euthanasia solution (Euthasol; Virbac, Ft. Worth, TX) and the eyes enucleated. Retinas were collected as described below.

### Histology / TUNEL assay

The eyes were fixed, trimmed and retinal cryosections were H&E stained or used for TUNEL labeling as previously reported [38].

### Quantitative real-time PCR (qRT-PCR) / Reverse Transcription PCR (RT-PCR)

The neuroretinas were collected from the eyecup under dim red light immediately after enucleation, snap-frozen in liquid nitrogen, stored at −80°C and subsequently processed for RNA studies. Total RNA from left (light exposed) and right (shielded) retinas of three homozygous mutant (*RHO* T4R/T4R) dogs (Table 1) were isolated by standard TRIzol procedure (Invitrogen-Life Technologies, Carlsbad, CA), concentrations measured with a spectrophotometer.

### Table 1. Summary of the experimental procedures performed in the dogs of this study.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Genotype RHO</th>
<th>Age (weeks)</th>
<th>Sex</th>
<th>Light treatment</th>
<th>PE interval (hrs)</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>shielded</td>
<td>exposed</td>
<td></td>
</tr>
<tr>
<td><strong>RHO Mutant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EM335</td>
<td>T4R/+</td>
<td>115</td>
<td>M</td>
<td>RE</td>
<td>LE*</td>
<td>3</td>
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<tr>
<td>EM339</td>
<td>T4R/+</td>
<td>115</td>
<td>F</td>
<td>RE</td>
<td>LE*</td>
<td>6</td>
</tr>
<tr>
<td>EM340</td>
<td>T4R/+</td>
<td>115</td>
<td>F</td>
<td>RE</td>
<td>LE*</td>
<td>24</td>
</tr>
<tr>
<td>EM232</td>
<td>T4R/T4R</td>
<td>17</td>
<td>F</td>
<td>RE</td>
<td>LE*</td>
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<td>EM291</td>
<td>T4R/+</td>
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<td>F</td>
<td>RE</td>
<td>LE*</td>
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<tr>
<td>EM188</td>
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<td>F</td>
<td>RE</td>
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<td>T4R/T4R</td>
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<td>F</td>
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<tr>
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<td>6</td>
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<td>M</td>
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<tr>
<td>EM156</td>
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<td>23</td>
<td>M</td>
<td>RE</td>
<td>LE&lt;sup&gt;−&lt;/sup&gt;</td>
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<td><strong>Normal dogs</strong></td>
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<tr>
<td>P1471</td>
<td>+/+</td>
<td>21</td>
<td>F</td>
<td>RE</td>
<td>LE*</td>
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<tr>
<td>EM262</td>
<td>+/+</td>
<td>16</td>
<td>F</td>
<td>RE</td>
<td>LE*</td>
<td>6</td>
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<tr>
<td>M2367</td>
<td>+/+</td>
<td>278</td>
<td>F</td>
<td>RE</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>M1841</td>
<td>+/+</td>
<td>172</td>
<td>F</td>
<td>RE</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>


LE<sup>−</sup>: Light exposure performed using a hand-held fundus camera and taking a series of sequential overlapping retinal photographs (see methods and [26–27]).

LE<sup>−</sup>: Light exposure performed using a monocular Ganzfeld and delivering a constant bright white light (6500 K, corneal irradiance: 1mW/cm²) for 1 min (see methods).

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(Nanodrop 1000, Thermo Fisher Scientific, Wilmington, DE), and quality verified by microcapillary electrophoresis on Agilent Bioanalyzer (Agilent, Santa Clara, CA). Only high quality (RNA A260/280 > 1.8 and RIN > 9) was used. RNA samples were treated with RNase-free DNase (Applied Biosystems (ABI), Foster City, CA) and 2 μg RNA was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcriptase Kit (ABI). qRT-PCR was performed on a 7500 Real Time PCR System and software v2.0 (ABI) using 20 ng cDNA for each sample to examine the expression of 18 selected canine genes involved in ER stress: ASK1, ATF4, BIP, CASP12, CHOP, DNAJA1, DNAJB1, DNAJB11, EDEM1, EDEM2, EDEM3, HRD1, HSP70, HSP90AA1, HSP90AB1, HSP90B1, VCP, and XBP1. In addition, RNA levels of CASP3 were also examined. Details on the genes are presented in Table 2 including names, descriptions and primer sequences. TaqMan reagents were used for GAPDH while SYBR green was used for the remaining genes. The specificity of every SYBR green assay was confirmed by dissociation curve procedures. Single melting temperatures were observed for each gene, thus excluding the presence of secondary non-specific gene products and primer dimers. For RT-PCR analysis of XBP1 splicing, cDNA of exposed and shielded retinas of three homozygous mutants (RHO T4R/T4R) (Table 1) was used as template for PCR amplification across the fragment of the XBP1 cDNA bearing the unconventional intron target of IRE1α ribonuclease activity (see Table 2 for the sequences of the primers). cDNA of normal canine fibroblasts and MDCK cells that were treated with tunicamycin, dimethyl sulfoxide (DMSO), or left untreated, served as controls. PCR products were resolved on an 8% polyacrylamide/1x TBE gel.

Statistical analysis of qRT-PCR data

All samples were run in duplicates. CT values of each gene were normalized with those of the housekeeping gene GAPDH and the ratio of exposed vs. shielded retinas determined with the ΔΔCT method [39]. Mean fold change (FC) differences were calculated as FC = 2-⁴ΔΔCT. The range of FC values (FC min to FC max) were reported for each gene. Statistical significance between gene expression profiles in exposed and shielded retinas was assessed with a paired t-test.

Protein analysis (Western Blot)

Retinal protein extracts were obtained by sonication in a buffer containing 50 mM Tris-Cl, 10 mM EGTA, 10 mM EDTA, 250 mM sucrose, 1% Triton together with a cocktail of protease inhibitors (Complete EDTA-free, Roche Applied Science, Indianapolis, IN) and phosphatase inhibitors (EMD Millipore/Calbiochem, Billerica, MA) followed by centrifugation at approximately 14,000 g for 15 min to pellet the debris. Canine fibroblasts and MDCK total cell lysates were extracted using RIPA buffer. Total protein concentration was quantified (Bradford Protein Assay, Pierce Biotechnology, Rockford, IL) and 40 μg of protein lysate for each sample was resolved on a 4–10% gradient gel and transferred to a nitrocellulose membrane (iBlot, Life Technology, Grand Island, NY). The blotted membrane was then blocked in TBST (10 mM Tris-Cl [pH 7.5], 100 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk at room temperature for 1 hour and incubated with the specific primary antibody overnight at 4°C to detect the level of stress-induced proteins (BIP/Grp78, calnexin, GRP94, HSP70, HSP90, XBP1, eIF2α, P-eIF2α, α-II spectrin, m-calpain, cleaved caspase-3). Either β-actin or α-tubulin were used as internal controls for normalization. Details on the primary antibodies are reported in Tables 3 & 4. After washing 3x with TBST, the membrane was incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (1:2,000, Zymed, San Francisco, CA) at room temperature for 1 hour. Following washing with TBST, protein signals were visualized using the ECL method according to the manufacturer’s recommendations (ECL Western Blotting Detection Kit, GE Healthcare, Little Chalfont, UK).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene description [NCBI Reference Sequence]</th>
<th>Sequence (5'-3') or expression assay (ABI) number</th>
</tr>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase [NM_002046.3]</td>
<td>TaqMan gene expression assay: Hs02786624_g1</td>
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<tr>
<td>ASK1</td>
<td>MAP3K5 mitogen-activated protein kinase kinase 5 [XM_533420.5]</td>
<td>F: TCCCCAGAGAGAGATAGATAGCATAC R: CTCACCTGAAGAGCCCGAGCATAC</td>
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<tr>
<td>ATF4</td>
<td>activating transcription factor 4 (tax-responsive enhancer element B67), transcript variant 2 [X5_854584]</td>
<td>F: CGAATGGTGGCTTGGTTGGA R: GTCCCCGAGAGGCACTCTT</td>
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<tr>
<td>BIP</td>
<td>heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa), transcript variant 5 ([HSPA5] [X5_858292.2])</td>
<td>F: TGAAGTCACCTTTGAGATAGATGTGA R: GTGGCCCGCTACCTTTGGTTT</td>
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<tr>
<td>CASP3</td>
<td>Caspase 3 [NM_001003042.1]</td>
<td>F: TCGAAGCGGACTTCTTGTATG R: ACTCAGCGCTGTGAGCGTATAG</td>
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<tr>
<td>CASP12</td>
<td>Caspase 12 [NM_001077236.1]</td>
<td>F: GGCCGCTCGGTTGAGCTGATAG R: ACTGCAGGGCTGCTACAT</td>
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<td>CHOP</td>
<td>DNA-damage-inducible transcript 3 (DDIT3) [X5_844109.2]</td>
<td>F: CCCCGGTGGCCCTACTCTA R: TCGTTGGACCTGGAAGATG</td>
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<td>DNAJA1</td>
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<td>F: GGAGAAGTGACTGCTATGTT G: ATTTGCTGCTGACAACTTTGA</td>
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<td>EDEM1</td>
<td>ER degradation enhancer, mannosidase alpha-like 1 [XM_533753.4]</td>
<td>F: GTCGAGAGGCTGCTGAAAG R: GCCATCTCCATCACCTCATTC</td>
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<td>EDEM2</td>
<td>ER degradation enhancer, mannosidase alpha-like 2 [XM_859274.3]</td>
<td>F: CTTTGAGTACCTGTTGAAAGGA R: CAGTCATCGAAGCCGAGTGA</td>
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<tr>
<td>EDEM3</td>
<td>ER degradation enhancer, mannosidase alpha-like 3 [XM_537162.4]</td>
<td>F: GAGAGGCGAGAGAGACAGAAG R: ATGAGGTCATACGCTGAGGTA</td>
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<td>HRD1</td>
<td>synovial apoptosis inhibitor 1, synoviolin (SYVN1) [XM_540867]</td>
<td>F: GCCGTTGTCATGCTCATACAGA R: CTGTTGCACCTTGATG</td>
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<td>HSP70</td>
<td>heat shock protein 70 [NM_001003067.1]</td>
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<td>HSP90AA1</td>
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<td>VCP</td>
<td>valosin containing protein [XM_847533.3]</td>
<td>F: CAAAGGAGAGAGAGGAGGATG R: GCCTTAAAGAGAGGGAGATG</td>
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<td>XBP1total</td>
<td>X-box binding protein 1 [XM_849540.2]</td>
<td>F: ATGGATACCTGGTCTAGAGA R: CACGGGCTCACTCATT</td>
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<td>X-box binding protein 1 Unspliced form</td>
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<td>XBP1s</td>
<td>X-box binding protein 1 Spliced form</td>
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**Note:** GAPDH was analyzed with TaqMan reagents, while all other genes with SYBR green.

The asterisk (*) indicates the set of primers used to detect total XBP1 (spliced and unspliced transcripts by RT-PCR).

doi:10.1371/journal.pone.0115723.t002
Table 3. List of primary antibodies successfully used for western blotting in the current study.

<table>
<thead>
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<th>Antigen / (species)</th>
<th>Host</th>
<th>Source, Catalog No. or Name</th>
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Table 4. List of primary antibodies that were tested but failed to detect by western blotting the specific antigen in canine retina lysates when used overnight at the indicated dilution.

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Results

Rod cell death begins 6 hours after light exposure in T4R RHO retinas

At 3 hours post-exposure, there were no observable morphologic abnormalities by light microscopy on H&E stained sections from both the tapetal (superior) and non-tapetal (inferior) regions of the fundus (Fig. 1A). Earliest light microscopic changes, consisting in shortening, disorganization and fragmentation of rod outer segments, were present at the 6 hour time-
point, and were more prominent at 24 hours. Consistent with these early morphological abnormalities, cell death was first detected by TUNEL labeling at 6 hours post light exposure both in the tapetal and non-tapetal regions, and was more prominent, particularly in the central retina, at 24 hours (Fig. 1B). At that time point there was greater damage in the photoreceptor layer and ONL of the tapetal than of the non-tapetal retina. This difference likely results from lack of RPE pigmentation and increased reflected light from the tapetum lucidum in the superior part of the fundus.

Acute disruption of rod outer segment discs and inner segment organelles following light exposure in T4R RHO retinas

To further characterize the early stages and course of morphologic alterations that lead to the death of mutant T4R RHO rods following light exposure, retinas from RHO<sup>T4R<sup>T4R</sup></sup>, RHO<sup>T4R/+<sup>+</sup></sup> and RHO<sup>T4R<sup>T4R</sup></sup> dogs were examined by transmission electron microscopy. As previously reported...
and confirmed in this study, young RHO T4R mutants raised under standard kennel illumination conditions and not exposed to bright lights had normal retinal ultrastructure (Fig. 2A-B). However, as early as 15 min after bright light exposure, there was vesiculation and misalignment of rod outer segment discs in the mutants, but not in the WT retinas (compare Fig. 2C and 2D). Similar vesiculo-tubular structures were seen in ROS of mutant dogs at 1 hr post exposure; however at this later time-point prominent alterations were also seen in the rod inner segments (RIS). These consisted in disruption of the plasma membrane, presence of single-membrane vesicles, and swelling of mitochondria (Fig. 2F-H). No such changes were seen in neighboring cones.

Based on the time course of TUNEL labeling following light exposure, and the ultrastructural studies that confirmed early structural alterations before the onset of cell death, we carried out a series of molecular and biochemical studies that focused on the ER stress response at the 6 hour post-exposure time period. This time point shows a small but significant increase in TUNEL-positive cells, an indication that cells are in the process of committing to cell death that involves many more cells by 24 hours, and continues unabated until there is extensive loss of rod photoreceptors by 2–4 weeks following exposure [14,24,25].

Fig 2. Ultrastructural alterations in rods following acute light exposure in T4R RHO canine retinas. Transmission electron micrographs of photoreceptors from T4R RHO mutant and WT canine retinas at 15 min, 1 hour, and 6 hours after light exposure (LE) to a 1 min duration of white light (corneal irradiance: 1mW/cm²). Black arrowheads point to vesiculo-tubular structures located in the rod outer segments (ROS) and rod inner segments (RIS) of light exposed mutant retinas. Note that the CIS and COS remain normal even though there is extensive rod degeneration. CIS; cone inner segment; m: mitochondria.

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Absence of ER stress and UPR activation in T4R RHO retinas at the onset of light-induced rod photoreceptor cell death

Although ER stress associated with retinal degeneration in some animal models of RHO-ADRP is likely the result of chronic accumulation of misfolded rhodopsin [29,32–34,40,41], some studies have demonstrated acute ER stress being triggered within hours following exposure to a toxic chemical [42], or to light [41,43]. This led us to examine whether the acute cell death observed at 6 hours after light exposure in the RHO T4R retina could be associated with disruption of ER homeostasis, and activation of an ER stress response.

We began by examining the levels of expression of intraluminal chaperones involved in the maintenance of ER homeostasis. Heat shock protein 90 kDa beta member 1 (HSP90B1, also known as GRP94, GP96, ERP99) is an ER paralog of heat shock protein 90 (HSP90) that plays a role in stabilizing and folding proteins in the ER. Like other members of the HSP family, its levels of expression are increased with the accumulation of misfolded proteins [44]. qRT-PCR analysis did not show any statistically significant changes in expression between exposed and shielded eyes of RHOT4R/T4R dogs (Fig. 3A). Similarly, no differences in protein levels were seen 6 hours following light exposure in mutant (homozygous, and heterozygous) and WT dogs (Fig. 3B). As well, no statistically significant differences were seen at the RNA level for DNAJ (Hsp40) and Homolog subfamily B member (DNAJB11, also known as HEDJ, ERdj3) (Fig. 3A), a soluble glycoprotein of the ER lumen that serves as a co-chaperone for BIP (also known as GRP78/HSPA5) which is the central regulator of ER stress, by stimulating its ATPase activity [45,46]. No changes were also seen in transcript levels of EDEM1, EDEM2, and EDM3, three ER-stress-induced members of the glycosyl hydrolase 47 family that play a role in degradation of folding defective glycoproteins [47]. In addition, western blot analysis of calnexin, an integral protein of the ER that assists in protein folding and quality control by retaining in the ER unfolded or unassembled N-linked glycoproteins [48], revealed that protein levels were not

Fig 3. Luminal ER chaperones in T4R RHO and WT canine retinas 6 hours after light exposure. (A) Differential expression of genes HSP90B1/GRP94, DNAJB11, EDEM1, EDEM2, and EDEM3 in the retinas of three RHO T4R/T4R mutant dogs following light exposure. Displayed are the mean fold change (FC) differences compared to the contralateral shielded retinas. Error bars represent the FC range (FC min to FC max). (B) Immunoblots showing the protein level of ER luminal chaperones GRP94 and Calnexin in light exposed (E) compared to shielded (S) retinas of mutant (RHO T4R/T4R, and RHO T4R/+), and wild-type RHO (+/+) dogs. A single retina from a wild-type dog kept under standard ambient kennel illumination (K) was included as a control of basal levels of GRP94, and calnexin proteins. There is no change in protein levels associated with light exposure.

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altered following light exposure in the mutant (homozygous and heterozygous) retina (Fig. 3B).

To determine whether an UPR occurred following light exposure in the T4R RHO mutant retina we examined the three branches of the response that can be activated following accumulation of a misfolded protein, and the subsequent dissociation of BIP from the three ER stress transducers (PERK, IRE1, and ATF6).

Activation of the PERK pathway is initiated after the dimerization and autophosphorylation of PERK which subsequently phosphorylates the eukaryotic initiation factor eIF2α. Phosphorylation of eIF2α leads to global reduction in protein synthesis to reduce ER overload. However eIF2α also can promote transcription of activating transcriptional factor 4 (ATF4), which, in turn, can increase the expression of the central ER chaperone BIP/GRP94. ATF4 is also known to activate the expression of apoptosis-related genes such as C/EBP-homologous protein (CHOP, also known as GADD153, DDIT3) (For review see [35,36,49]).

Western blot analysis revealed similar levels of eIF2α in shielded and light exposed retinas from mutant (homozygous and heterozygous) T4R RHO and WT dogs (Fig. 4A, upper portion). A very faint band corresponding to the phosphorylated form of eIF2α was similarly detected in both exposed and shielded retinas suggesting that there was no activation of eIF2α beyond the low basal levels. Detection of a single band at the correct molecular weight in protein extracts from MDCK cells treated with the ER-stress inducer tunicamycin confirmed the specificity of the P-eIF2α antibody against the canine amino-acid sequence (Fig. 4A, lower portion). Consistent with the absence of activation of eIF2α we did not detect by qRT-PCR any increased expression of the downstream ATF4 transcript following light exposure (Fig 4B). The results, therefore, did not show any evidence for activation of the PERK pathway 6 hours after a light exposure that results in rod degeneration in the T4R RHO retina.

Fig 4. PERK-eIF2α-ATF4 pathway in mutant T4R RHO and WT canine retinas 6 hours after light exposure. (A) Immunoblots showing the protein levels of total and phosphorylated forms of eIF2α in light exposed (E) compared to shielded (S) retinas of mutant (RHO T4R/T4R, and RHO T4R/+), and WT (+/+) dogs. A single retina from a WT dog kept under standard ambient kennel illumination (K) was included as a control of basal levels of total and phosphorylated eIF2α. MDCK cells either treated with DMSO or Tunicamycin (Tun) were used as controls of P-eIF2α expression and antibody specificity. (B) Differential expression of gene ATF4 in the retinas of three RHO T4R/T4R mutant dogs following light exposure. Displayed is the mean fold change (FC) difference compared to the contralateral shielded retinas. Error bars represent the FC range (FC min to FC max).

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The IRE1 branch of the UPR is activated after oligomerization and autophosphorylation of the IRE1α sensor, a ubiquitously expressed Ser/Thr protein kinase that also harbors an endoribonuclease domain. Activated IRE1 catalyzes the unconventional splicing of the mRNA of X-box binding protein 1 (XBP1). The 263 bp fragment, which represents the spliced form of canine XBP1 was not observed except in the tunicamycin treated normal canine fibroblasts (NCF). A retina from a wild-type dog kept under standard ambient kennel illumination (K) was used as a control of basal XBP1 expression and splicing. 

In order to evaluate the activation of IRE-1, we analyzed the unconventional XBP1 mRNA splicing by RT-PCR. Our results showed that the unconventional XBP1 mRNA splicing does not occur in the T4R
t4R mutant retinas 6 hours after light exposure (Fig. 5A). This was further confirmed by qRT-PCR analysis using primers that specifically detect the unspliced and unconventionally spliced XBP1 transcripts (Fig. 5B). In addition, there were no significant differences at the protein levels (Fig. 5C) between exposed and shielded eyes. ASK1 transcript levels did not significantly vary either (Fig. 5B) but state of activation of the protein could not be assessed due to lack of antibodies that would recognize total and phosphorylated forms of ASK1 (Table 4). These results still suggest however that the IRE1 branch of the UPR is not activated in the light exposed T4R RHO mutant retina. In contrast, normal canine fibroblast cultures treated with the ER stress inducer tunicamycin did show unconventional splicing of XBP1 mRNA (Fig. 5A).

The third branch of the UPR involves cleavage in the Golgi by site-1 and site-2 proteases of the activating transcription factor 6 (ATF6). The N-terminal 50 kDa fragment of ATF6 (p50ATF6) translocates to the nucleus and upregulates the expression of BIP, and CHOP (For review see [35,36,49]). Despite testing several antibodies directed against ATF6 (see Table 4)
we did not identify one that recognized canine ATF6, and thus were not able to assess the cleavage of ATF6. However, downstream targets of the ATF6 pathway, BIP and CHOP, could be examined (see below), and the results indirectly rule out the activation of this branch of the UPR.

We analyzed the expression of BIP/GRP78 and CHOP, two target genes of the three branches of the UPR. BIP/GRP78 is a key chaperone induced by UPR signaling. It is an ER luminal protein that binds to each of the transducers of ER stress and serves as a sensor of alteration of ER homeostasis. Up-regulation of BIP expression promotes protein folding and reestablishment of ER homeostasis, and increased levels have been reported in genetic and light-induced models of retinal degeneration [32,33,40,41]. CHOP, also known as Growth-Arrest and DNA damage-inducible gene 153 (GADD153), is a key mediator of ER-stress induced apoptosis, and all three branches of the UPR, either independently or cooperatively, regulate its activation. Under physiological conditions, CHOP is expressed at low levels, but expression increase significantly in the presence of severe and persistent ER stress [40]. Our results showed no significant differences in RNA expression of BIP and CHOP (Fig. 6A), and protein levels of BIP (Fig. 6B) were similar between the shielded and exposed mutant retinas (homozygous and heterozygous) 6 hours after light exposure. The levels of CHOP protein could not be evaluated as three commercially-available antibodies that were tested failed to recognize canine CHOP (Table 4).

HSP70 cytosolic chaperone is up-regulated in T4R RHO retinas after light exposure

To determine whether light exposure is associated with the activation of cytosolic chaperones that prevent misfolded protein aggregation and ultimately favor degradation via the proteasome, we examined the RNA levels in exposed and shielded mutant retinas of the following genes: VCP (Valosin Containing Protein), HRD1 (ERAD-associated E3 ubiquitin protein ligase; also known as synoviolin), DNAJA1 [DnaJ(hsp40) homolog subfamily A, member 1],
Expression of ER-resident caspase-12 mRNA is up-regulated after light exposure

The involvement of ER-resident caspase-12 in retinal degeneration has been described [51–53]. To better understand the cell death mechanisms involved in photoreceptor degeneration in this large animal model, we examined the expression of the ER associated caspase-12 at the transcription level. Although there was some inter-individual variability (FC min = 1.7; FC max = 4.5), qRT-PCR analysis showed a 2.8 mean fold increase in levels of HSP90 and HSP70 in light exposed (E) compared to shielded (S) retinas of mutant (RHO T4R/T4R, and RHO T4R/+) and WT (+/+). A single retina from a wild-type dog kept under standard ambient kennel illumination (K) was included as a control of basal levels of HSP90 and HSP70. There are no changes in HSP90 and HSP70 protein levels associated with light exposure. Note: the HSP90 antibody used recognizes the products of both the HSP90AA1 and HSP90AB1 genes.

Absence of UPR in the T4R RHO Canine Retina

DNAJB1 [DnaJ(hsp40) homolog subfamily B, member 1], HSP70, HSP90AA1 (Heat shock protein 90 kDa alpha, class A, member 1), and HSP90AB1 (Heat shock protein 90 kDa alpha, class B, member 1). Results showed an up-regulation of HSP70 transcription in the light-exposed retinas (p = 0.03; FC = 2.5), while no significant differences were observed for the other 7 genes (Fig. 7A). However, no differences in the protein levels of HSP70 (nor HSP90) were observed (Fig. 7B).
Evidence of calpain-activation as early as 1 hour post-light exposure in the T4R RHO mutant retina

Accumulating evidence has been pointing out to the role of calpains in photoreceptor cell death [32,54–57]. These Ca^{2+}-dependent cysteine proteases are rapidly activated following an increase in concentration of cytosolic Ca^{2+} that can be released from intracellular stores such as the ER, mitochondria, or photoreceptor discs [58]. Thus, we examined the involvement of calpain activation at 3 time points (1, 3, and 6 hours) following acute light exposure in RHOT4R/+ dogs by assessing alpha-II spectrin (also known as alpha-fodrin) signature breakdown products (SBDP). Both m-calpain (= calpain 1) and μ-calpain (= calpain 2) are known to induce proteolysis of alpha-II spectrin at specific sites that result in 145 and 150 kDa SBDP, while caspase 3 cleaves α-II spectrin at an additional site resulting in a 120 kDa SBDP [59,60]. Our results showed that m-calpain was expressed in both shielded and exposed retinas at all 3 time points following light exposure. α-II spectrin protein levels increased with light exposure, and a 150 kDa SBDP was found only in the exposed retinas (Fig. 8A). Absence of a 120 kDa SBDP (seen in canine MDCK cells treated with staurosporine; Fig. 8B) indicates calpain but not caspase 3 activation in the T4R RHO retina following acute light exposure. This was further confirmed by western blot which failed to detect any cleaved/activated caspase 3 protein in the T4R RHO retinas following light exposure (Fig 8C). No evidence of increased CASP3 expression was either detected by qRT-PCR (Fig 8D). Thus, in the absence of results examining the occurrence of cell death at the single cell level [57], there is no evidence to suggest any involvement of Caspase 3 in this model system.

Discussion

Transgenic animal models of RHO-adRP have been a common resource to investigate the cell signaling pathways that lead to photoreceptor cell death in this form of retinal degeneration. Among the mechanisms examined, the involvement of ER stress has been proposed as a common pathway in rod photoreceptor cell death in several animal models of retinal degeneration that carry different RHO mutations [29–33,40,41]. In this study, we examined whether ER stress, and the UPR in particular, were temporally associated with the onset of rod cell death that occurs following a short clinical light exposure in a naturally-occurring canine model of class B1 RHO-adRP. Our results did not identify any UPR activation concomitant with the severe ultrastructural alterations and early cell death events that occur within hours following the light exposure; instead, they point out to the extreme instability of rod disc membranes containing the mutant T4R opsin protein.

Mis-trafficking of mutant rhodopsin to the cell membrane has been shown in cultured cells [8–10,26,27,29,61,62], and in some transgenic animal models of RHO-adRP there is evidence of rhodopsin accumulation in rod IS [31,33,63] as well as co-localization with ER markers [30,31]. This has led several groups to hypothesize that misfolded mutant rhodopsin could induce an ER stress response. Evidence for the activation of the UPR and other ER stress markers has recently been reported in different models including: the transgenic P23H rat (lines 1 and 3) [29,34,40,41], the transgenic S334ter rat (lines 3,4 and 5) [32,41], and the T17M transgenic mouse [33].

Whether activation of the branches of the UPR reflects a compensatory mechanism to maintain ER homeostasis and promote cell survival, or on the contrary, constitutes an initial molecular event that leads to rod photoreceptor death currently is still not clear. Indeed, while increased expression of pro-apoptotic downstream targets of the UPR such as CHOP and ASK1 have been reported in retinas of RHO-adRP models, ablation of these genes has either not modified the course of disease or negatively influenced cell survival [64,65].
To assess the involvement of ER stress in a naturally-occurring model of RHO-adRP we selected the T4R RHO dog. Besides avoiding the increase in RHO gene dosage that is inherent to some transgenic animals, this model provides the opportunity to trigger a synchronized, acute rod photoreceptor degeneration following short term exposure to doses of light that are not damaging to the WT retina; the light exposures used are approximately 1000 fold or more lower in intensity than the retinal damage threshold intensities for white or medium-wavelength light in different species (see [14,24]). In this study, we detected TUNEL-labeled rods as
early as 6 hours post exposure both in the tapetal and non-tapetal fundi, and by 24 hours extensive cell death was present, particularly in the central retina. Thus, to identify the early cell signalling events that are initiated following light exposure in the RHO-T4R retina, and that ultimately lead to cell death commitment by rods, we focused on the 6 hour time point as the majority of the photoreceptors had not yet undergone DNA cleavage and fragmentation. The analysis of the expression profile of ER markers involved in the three branches of the UPR (Fig. 9) indicates: a) the absence of chronic ER stress in the unexposed/shielded mutant retina, and b) that these pathways are not activated in the acute light-induced death of rods. During ER stress, the three associated UPR signaling pathways, PERK, IRE1 and ATF6, are typically activated [35,36,49]. In the present study only two UPR signaling pathways were examined directly, the PERK and the IRE1 branches. The third signaling pathway, the ATF6 branch, was not investigated due to lack of antibodies that recognize canine p50ATF6. However, we are confident that ATF6 pathway was not activated as we did not see any up-regulation of the two downstream targets: BIP and CHOP.

Rhodopsin in the T4R RHO mutant retina is located in rod OS and by immunohistochemistry is not retained in the ER nor aggregates in the IS [23,37]. The absence of a UPR further supports the claim that neither the lack of glycosylation at Asn(2) [37], nor the T4R mutation cause accumulation in the ER or impaired trafficking to the OS. These results resemble those
recently reported for the P23H-opsin knock in mouse [66], and for the T4K and T17M transgenic Xenopus laevis where mutant RHO protein was not retained in the ER and localized normally to the rod OS [67]. The discrepancy between these findings, and that reported in P23H transgenic animals where opsin is found to accumulate in the ER, may be explained by the expression of higher levels of opsin mRNA in the transgenic models. This leads to question whether the reported occurrence of ER stress in transgenic RHO-adRP animals is a combination of the mutation and an increased gene dosage effect, rather than strictly the effect of the RHO mutation in photoreceptors. Recent evidence for an absence of increased BIP expression in rods of the T4K transgenic X. laevis following light-exposure [67] also calls for further investigation of the mechanism of action of other RHO mutations.

Besides activating pro-apoptotic downstream targets of the UPR such as CHOP and ASK1, ER stress can induce other signaling pathways that lead to cell death. Among them is the activation of the ER-associated caspase-12 which was found to be overexpressed in the light exposed T4R RHO retina. Different mechanisms for caspase-12 activation have been proposed. Pro-caspase-12 which is located on the cytoplasmic side of the ER membrane has been reported to interact with IRE1 through the adaptor molecule TRAF2 [68]. Upon ER stress, pro-caspase-12 can be released from TRAF2 to translocate from the ER to the cytosol where it directly cleaves pro-caspase-9, which in turn activates the effector caspase, caspase-3. Another proposed mechanism for pro-caspase-12 activation is via calpain cleavage [69], a pathway that has been identified in the rd1 mouse [53]. In our study, we observed in the T4R RHO retina an increase in calpain activation as early as one hour after light exposure, suggesting a rapid increase in cytosolic concentrations of Ca^{2+}. What are then the possible sources for such a raise in calcium levels?

Electron microscopy analysis of T4R RHO retinas showed prominent disruption of rod OS discs and plasma membrane as early as 15 min after a one minute period of light exposure. As the intradiscal and extracellular environments have higher concentrations of Ca^{2+} than the cytosol [58], disruption of these compartments could, within minutes, alter the intracellular calcium homeostasis. At 6 hours post light exposure there also were severe ultrastructural alterations in the rod IS with numerous single-membrane vacuoles and dilated mitochondria. Similar morphologic features have been observed in cells undergoing ER stress, where the ER swells and ribosomes dissociate from the rough ER [70,71]. As both the ER and mitochondria are major intracellular stores of Ca^{2+}, loss of their membrane integrity could further contribute to the raise in cytosolic calcium. Based on our results that exclude an ER stress response as the initiating cause for the cell death process, we posit that an increase in the concentrations of cytosolic Ca^{2+} via its release from the rod intradiscal space and/or extracellular space through disruptions in the cell membranes shortly after the light exposure could subsequently affect adversely the mitochondria, and initiate the cascade of events that culminate in rod cell death.

A critical question that remains to be answered is how photobleaching of mutant T4R opsin with intensities of white light (corneal irradiance of 1mW/cm^2; equivalent to ~1,500 lux) and exposure durations that are not toxic to the WT retina leads to the severe disruption of discal and plasma membranes. The T4R mutation which is located in the intradiscal domain affects the chromophore-binding site causing it to release the chromophore faster than WT opsin [37]. In addition, T4R opsin alone is more toxic than T4R opsin bound to 11cis-retinal as evidenced by the much accelerated course of retinal degeneration observed in double mutant dogs that also carry the RPE65 mutation depriving them from the ability to produce the 11-cis retinal chromophore [37]. One could then speculate that in the absence of chromophore, or following intense photobleaching, a change in the conformation of mutant T4R opsin alters its mobility within the lipid bilayer of the discal and cytoplasmic membranes. Similar disruption of rod OS discs as seen in our study have been reported in models of P23H RHO adRP
including the P23H transgenic *Xenopus laevis* [63], the VPP mouse [34,72], the P23H-3 rat [34], the P23H knock in mouse [73], and more recently in the T4K transgenic *Xenopus laevis* following light exposure [67]. These ultrastructural alterations in discs may be explained by the recent evidence that P23H opsin tends to aggregate in the photoreceptor discs of transgenic P23H *Xenopus laevis* [63], and in the nervous system of transgenic *C. elegans* [74]. Similar aggregation and impaired diffusion within the lipid bilayer [63] may lead photobleached mutant T4R opsin to disturb the membrane structure, leading it to vesiculate and ultimately break down.

In summary, this study did not show any evidence of activation of the UPR in the canine T4R RHO model and thus does not support modulation of ER stress sensor activation [29] as a potential therapeutic venue. Besides an allele-independent corrective gene therapy approach that combines the knockdown of mutant rhodopsin mRNA and replacement with a hardened wild-type copy, pharmacological strategies aimed at stabilizing mutant opsin with locked forms of retinoids that cannot isomerize [27], or the use of cell-membrane stabilizers [75] may be beneficial for light sensitive Class B1 RHO-ADRP mutations that lead to disruption of discs.

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**Author Contributions**

Conceived and designed the experiments: SM SG WAB. Performed the experiments: SM SG RS JG. Analyzed the data: SM SG RS GDA WAB. Wrote the paper: SM SG RS GDA WAB.

**References**


