11-2007

Clinical Light Exposure, Photoreceptor Degeneration, and AP-1 Activation: A Cell Death or Cell Survival Signal in the Rhodopsin Mutant Retina?

Danian Gu
University of Pennsylvania

William Beltran
University of Pennsylvania, wbeltran@vet.upenn.edu

Zexiao Li
University of Pennsylvania

Gregory M. Acland

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

Recommended Citation
Gu, D., Beltran, W., Li, Z., Acland, G. M., & Aguirre, G. D. (2007). Clinical Light Exposure, Photoreceptor Degeneration, and AP-1 Activation: A Cell Death or Cell Survival Signal in the Rhodopsin Mutant Retina?. *Investigative Ophthalmology & Visual Science, 48* (11), 4907-4918. http://dx.doi.org/10.1167/iovs.07-0428

Clinical Light Exposure, Photoreceptor Degeneration, and AP-1 Activation: A Cell Death or Cell Survival Signal in the Rhodopsin Mutant Retina?

Abstract

PURPOSE. The T4R *RHO* mutant dog retina shows retinal degeneration with exposures to light comparable to those used in clinical eye examinations of patients. To define the molecular mechanisms of the degeneration, AP-1 DNA-binding activity, composition, posttranslational modification of the protein complex, and modulation of ERK/MAPK signaling pathways were examined in light-exposed mutant retinas.

METHODS. Dark-adapted retinas were exposed to short-duration light flashes from a retinal camera used clinically for retinal photography and were collected at different time points after exposure. Electrophoretic mobility shift assay (EMSA), supershift EMSA, Western blot analysis, and immunocytochemistry were used to examine AP-1 signaling.

RESULTS. Exposure to light of mutant retinas significantly increased AP-1 DNA-binding activity by 1 hour after exposure, and levels remained elevated for 6 hours. Shielded mutant retinas had similar AP-1 levels to shielded or exposed wild-type retinas. The parallel phosphorylation of c-Fos and activation of ERK1/2 was detected only in exposed mutant retinas. Exposure to light changed the composition of the AP-1 protein complex in the mutant retina from c-Jun/Fra-1/c-Fos to JunB/c-Fos. Immunohistochemistry showed that the components of activated AP-1 (JunB, and phosphorylated c-Fos, and phosphorylated ERK1/2 isoforms) were localized in Müller cells.

CONCLUSIONS. The inner nuclear layer/Müller cell localization of the key proteins induced by light exposure raises the question of the direct involvement of AP-1 in mediating photoreceptor cell death in this model of autosomal dominant retinitis pigmentosa.

Keywords

biochemistry, molecular biology

Disciplines

Biochemistry | Eye Diseases | Medical Biochemistry | Medical Cell Biology | Medical Molecular Biology | Molecular Biology | Ophthalmology | Optometry

Published in final edited form as:
Invest Ophthalmol Vis Sci. 2007 November ; 48(11): 4907–4918.

Clinical Light Exposure, Photoreceptor Degeneration, and AP-1 Activation: A Cell Death or Cell Survival Signal in the Rhodopsin Mutant Retina?

Danian Gu[1], **William A. Beltran**[1], **Zexiao Li**[1], **Gregory M. Acland**[2], and **Gustavo D. Aguirre**[1]

1*Section of Ophthalmology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*

2*J. A. Baker Institute for Animal Health, Cornell University, Ithaca, New York*

Abstract

Purpose—The T4R *RHO* mutant dog retina shows retinal degeneration with exposures to light comparable to those used in clinical eye examinations of patients. To define the molecular mechanisms of the degeneration, AP-1 DNA-binding activity, composition, posttranslational modification of the protein complex, and modulation of ERK/MAPK signaling pathways were examined in light-exposed mutant retinas.

Methods—Dark-adapted retinas were exposed to short-duration light flashes from a retinal camera used clinically for retinal photography and were collected at different time points after exposure. Electrophoretic mobility shift assay (EMSA), super-shift EMSA, Western blot analysis, and immunocytochemistry were used to examine AP-1 signaling.

Results—Exposure to light of mutant retinas significantly increased AP-1 DNA-binding activity by 1 hour after exposure, and levels remained elevated for 6 hours. Shielded mutant retinas had similar AP-1 levels to shielded or exposed wild-type retinas. The parallel phosphorylation of c-Fos and activation of ERK1/2 was detected only in exposed mutant retinas. Exposure to light changed the composition of the AP-1 protein complex in the mutant retina from c-Jun/Fra-1/c-Fos to JunB/c-Fos. Immunohistochemistry showed that the components of activated AP-1 (JunB, and phosphorylated c-Fos, and phosphorylated ERK1/2 isoforms) were localized in Müller cells.

Conclusions—The inner nuclear layer/Müller cell localization of the key proteins induced by light exposure raises the question of the direct involvement of AP-1 in mediating photoreceptor cell death in this model of autosomal dominant retinitis pigmentosa.

Retinitis pigmentosa (RP) is a group of inherited retinal dystrophies characterized by progressive photoreceptor degeneration. It is one of the most common inherited retinal degenerative disorders, affecting an estimated 1.5 million people worldwide.[1,2] Of the genes and mutations causally associated with RP, more than 100 different mutations in rhodopsin (*RHO*) have been reported, and, with few exceptions, cause autosomal dominant RP (adRP) (RetNet: http://www.sph.uth.tmc.edu/RetNet/ provided in the public domain by the University of Texas Houston Health Science Center, Houston, TX). Within the *RHO*-adRP group of diseases, there is a variation in disease severity that is not strictly mutation dependent.[3–5] This finding suggests that genetic background or environmental factors play a role in modifying the phenotype. One such modifier could be environmental light, as rhodopsin-mediated

Corresponding author: Gustavo D. Aguirre, School of Veterinary Medicine, University of Pennsylvania, 3900 Delancey Street, Philadelphia, PA 19104; gda@vet.upenn.edu.

Disclosure: **D. Gu**, None; **W.A. Beltran**, None; **Z. Li**, None; **G.M. Acland**, None; **G.D. Aguirre**, None

photoreceptor degeneration can be readily produced experimentally, particularly in normal laboratory rodents (see Ref. 6 for a review). This possibility raises concern about *RHO* mutations in general, and about Class B1 in particular, as both humans and dogs with this mutation class are very sensitive to bleaching light exposures and show prolonged recovery of dark-adapted sensitivity.[7,8]

We have reported that the T4R *RHO* mutant dog shows exquisite sensitivity to light, and photoreceptor degeneration ensues after exposures to light comparable to those used clinically for eye examinations in humans.[9] The T4R mutant *RHO* is thermally less stable, releases chromophore faster with bleaching, and, in the absence of chromophore, appears more toxic.[10] This finding may explain the photoreceptor degeneration that occurs with exposures to light that bleach most or all of the visual pigment.[9]

A critical intermediary identified in experimental retinal light damage is activation of the activator protein (AP)-1 transcription factor. It has been proposed that light induces photoreceptor apoptosis in wild-type mice by activation of the c-Fos/AP-1 molecular pathway.[11] After exposure to light in rodent eyes, AP-1 activation occurs, and photoreceptor degeneration ensues. Elevation of endogenous cortisol or parenteral dexamethasone administration is associated with AP-1 inhibition and prevents photoreceptor degeneration.[6,12]

As a transcription factor, AP-1 is a key regulator of many biological processes that include cell transformation, proliferation, differentiation, apoptosis, and survival.[13–15] These diverse biological functions are dependent on the tissue and signaling pathways involved. AP-1 can be induced rapidly and transiently in response to various external signals, and mitogen-activated protein kinase (MAPK) cascades, including ERK, JNK, and p38, are recognized as common signaling pathways modulating up- or downregulation of AP-1 activity at the transcriptional and posttranscriptional levels All subgroups of MAPKs are serine/threonine kinases, and Fos and Jun proteins can be phosphorylated directly by activated JNK, ERK1/2, and p38 cascades, or indirectly by their downstream targets.[16,17]

We have reported increased AP-1 DNA-binding activity in the T4R *RHO* mutant retina after clinical exposure to light that damaged the mutant retina.[9] We now characterize the time course, composition, and posttranslational modification of the AP-1 complex after exposure to light, and determine the cellular localization of the signaling pathways. Our findings indicate a close association in the mutant retina between exposure to light and activation of signaling pathways, but a dissociation of this activation from the photoreceptors. This raises the question of whether AP-1 activation in the T4R *RHO* mutant retina is a retinal cell survival response rather than a photoreceptor death signal.

Materials and Methods

Animals

Dogs were maintained at the Retinal Disease Studies (RDS) facility (Kennett Square, PA), and all procedures were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The dogs represent an outbred population with a common genetic background, and independently segregate several retinal degeneration genes.[18] The wild-type control subjects ($n = 8$; age, 7–41 weeks) were homozygous normal (+/+) at the three genetic loci: *RHO*,[8] *RPE65*,[19] and *prcd*.[20] Fifteen T4R/+ dogs (age, 10–41 weeks) were homozygous normal (seven), heterozygous (six), or homozygous mutant (two) at the *RPE65* locus. Three T4R/T4R homozygous mutants were used (age, 23–40 weeks) and were homozygous normal at the *RPE65* locus. As a negative disease control for light-induced damage, four *prcd*-affected dogs (age, 13–91 weeks) that were homozygous normal at *RHO*

and *RPE65* loci were included. These dogs also have a postdevelopmental photoreceptor degeneration that is nonallelic to the *RHO* mutation.[20] A summary of the dogs used and their genotypes is included in Table 1.

At the ages studied, and under the ambient light conditions used in the kennels and procedure rooms (described later), the *RHO* mutants (T4R/+; T4R/T4R) have no photoreceptor degeneration before 1 year of age, as long as ophthalmic examination procedures are not performed.[8,9] The dogs heterozygous for the *RPE65* mutation have a retina that is indistinguishable from homozygous normal subjects in function and structure[21,22] (Acland GM, Aguirre GD, unpublished data, February 2004). Moreover, all dogs, regardless of retinal disease locus, have the conserved codon 450 leucine in the RPE65 protein, and no decrease in light damage susceptibility is expected as occurs in mice with 450 methionine[23] (Aguirre GD, unpublished data, May 2003). When present on an *RHO* mutant background (T4R/+), *RPE65* homozygous mutants show retinal degeneration, and the outer nuclear layer is reduced by ~60% by 6.5 months of age.[10] For *prcd*, the retina does not show evidence of early degeneration until after 6 months of age, and the disease progresses slowly.[18]

Light-Exposure and Tissue Collection

Dogs are kept in kennel runs under cyclic light environment (7 AM on–7 PM off) with light intensities that vary between 175 and 350 lux at the level of the "standard" dog eye. In the procedure room, which is used infrequently, light intensities range from 350 to 700 lux. These ambient light levels do not cause retinal abnormalities in normal or mutant dogs that do not have a *RHO*-based retinal disease.

Three different conditions of exposure to light were used, and will be referred to in the text as unexposed, exposed, and shielded. *Unexposed:* Retinas were obtained from normal or mutant (*RHO* and *prcd*) dogs during the light phase of the cyclic light–dark cycle. In general, the retinas were collected in the procedure room under ambient illumination within 3 hours of light onset (i.e., between 7 and 10 AM). *Exposed and shielded:* for the clinical exposure to light, dogs (normal, *RHO*, and *prcd*) were dark adapted overnight, the pupils dilated (1% tropicamide, 1% atropine, 10% phenylephrine), and the right eye completely shielded from light (*shielded*). Under dim red illumination, the left eye was exposed to a series of overlapping retinal photographs (*exposed*) with a hand-held, manual advance fundus camera (RC-2; Kowa Ltd., Nagoya, Japan) within 5 hours of light onset (i.e., between 7:00 AM and 12 PM). Although this is a standard instrument for clinical or research documentation of the retina, such photography results in end-stage retinal degeneration 2 weeks after exposure to light in the *RHO* mutant retina, but not in normal dogs or those affected with other inherited retinal degenerations (Aguirre GD et al., unpublished data).

Because the dog retina has regions where the pigment epithelium is (inferior, or nontapetal region) and is not (superior, or tapetal region) pigmented, different light intensities were used for viewing and photographing these two regions. These adjustments were made with neutral-density or gray polarizing film filters, and/or adjusting the settings of the photographic flash. For viewing, the approximate retinal illuminances produced by the tungsten bulb were 0.95 and 3.8 $mW \cdot cm^{-2}$, respectively, for the tapetal and nontapetal regions. For photography, microsecond duration flashes of a xenon lamp produced approximate retinal doses/flash of 0.6 and 11 $mJ \cdot cm^{-2}$, respectively, for the tapetal and nontapetal regions.[24,25] These are typical settings used in fundus photography of dogs and result in a >95% bleaching.[8] Fifteen to 17 photographs were taken of the eye during an ~5-minute period, and these were equally distributed between the tapetal and nontapetal zones. After exposure to light, the dogs were returned to the dark until they were killed at 1, 3, 5, 6, or 24 hours after exposure.

For collections of retinas from exposed and shielded eyes, the dogs were anesthetized with intravenous pentobarbital sodium in a dark room with dim red light illumination, the eyes were enucleated, and the dogs were euthanatized with a barbiturate overdose. The globes were opened with a razor blade cut anterior to the ora serrata, the posterior segment was isolated, and the vitreous removed. The retina was then manually separated from the pigment epithelium and frozen at −80°C until use. For immunohistochemistry, the eyes were processed using standard techniques that are described elsewhere[26] and are recounted below.

Preparation of Nuclear Protein Extracts

Nuclear protein extracts were prepared as described.[27] Briefly, a single retinal tissue sample (~50–100 mg) was homogenized in 1.5 mL of homogenization medium, and, after low-speed centrifugation, the supernatant was saved as the postnuclear supernatant fraction. The pellet (crude nuclear fraction) was washed and resuspended in extraction solution, and the supernatant was collected by centrifugation and saved as the nuclear protein fraction. All postnuclear supernatant and nuclear extracts were stored at −80°C until use; protein concentrations were determined with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Probe Labeling

The electrophoresis mobility shift assay (EMSA) probe was a 21-bp double-stranded AP-1 consensus oligonucleotide (5′-CGC TTG ATG AGT CAG CCG GAA-3′, 1.75 pmol/mL; Promega, Madison, WI) labeled with γ-[^{32}P]ATP (10 mCi/mL, 3000 Ci/mM; GE Healthcare, Pittsburgh, PA) according to the phosphorylation reaction protocol in the manufacturer's manual (Gel Shift Assay System; Promega). Unincorporated γ-[^{32}P]ATP was removed by a spin column (Sephadex G-25; Roche Diagnostics, Indianapolis, IN), and radioactivity was determined with a scintillation counter (Beckman LS6500; Beckman Coulter, Fullerton, CA). As a control, a 22-bp double-stranded specificity protein (SP)-1 consensus oligonucleotide (5′-ATT CGA TCG GGG CGG GGC GAGC-3′, 1.75 pmol/mL; Promega) probe was labeled using the same procedure.

Electrophoresis Mobility Shift Assay

EMSA was performed as described in the manufacturer's manual (Gel Shift Assay System; Promega). All binding reactions between nuclear proteins (~1.5 μg of protein/reaction) and labeled probe (0.07 picomoles; ~10,000 cpm/reaction), including the positive control-HeLa cell nuclear extract (>2.4 mg/mL, Promega), were analyzed by 2% to 20% gradient polyacrylamide gels. Dried gels were exposed to a phosphorescence imager (BAS-IP 2040; Fujifilm Life Science, Stamford, CT), a phosphorescence imager screen (GE Healthcare), and/or x-ray film (Fujifilm Life Science). The images were scanned and analyzed (BAS 1000 scanner with Image Gauge software; Fujifilm Life Science, or a Storm 860 scanner and an ImageQuant workstation; GE Healthcare). The relative AP-1 activity was determined based on the intensity of the shifted bands measured (Image Gauge software or ImageQuant workstation; GE Healthcare) Binding specificity was confirmed using unlabeled specific (AP-1) and nonspecific (SP-1) probes. The intensity measurements obtained with the phosphorescence imager or x-ray film were comparable and also with the radiation dose displayed in units of photostimulated luminescence (PSL).

Supershift Assay

Prehybridization between the nuclear extract and antibodies was performed out in the supershift assay. Briefly, ~6 μg of nuclear protein was mixed with 12 μg antibody (Table 2) in EMSA binding system (Promega), incubated at room temperature for 30 minutes, followed by the same EMSA procedure and analysis described earlier.

Immunoblot Analysis

One-dimensional protein electrophoresis and immunoblot analysis were performed with nitrocellulose membranes (Trans-Blot Transfer Medium; Bio-Rad Laboratories). The primary antibodies for this study are listed in Table 2; HRP rabbit anti-mouse IgG and HRP goat anti-rabbit IgG (ZyMax, San Francisco, CA) were used as the secondary antibodies. The immunoreacted bands were detected using the enhanced chemiluminescence (ECL) system (GE Healthcare), scanned, and analyzed (ImageQuant workstation; GE Healthcare).

Phosphorylation Analysis

To identify phosphorylated isoforms, nuclear extracts were treated with protein phosphatase (PP)-1 (New England Biolabs, Ipswich, MA) according to the standard PP1 procedure provided by the manufacturer, and the results were analyzed by immunoblot analysis.

Immunohistochemistry

A slit was made through the enucleated globe at the level of the ora serrata, and the entire globe was fixed for 3 hours in 4% paraformaldehyde in 0.1 M phosphate-buffered saline at 4°C. The posterior segment then was isolated, the vitreous gently removed, and the eyecup fixed for an additional 24 hours at 4°C in 2% paraformaldehyde in 0.1 M phosphate-buffered saline. The tissue then was trimmed, cryoprotected sequentially for 24 hours in a solution of 15% and 30% sucrose in 0.1 M sodium phosphate and 0.15 M sodium chloride (pH 7.2; BupH, phosphate-buffered saline; Pierce, Rockford, IL) at 4°C, and embedded in optimal cutting temperature (OCT) medium. Retinal cryosections along the superior and inferior retinal meridians of both the light exposed and shielded eyes from T4R/+ dogs were collected 1 and 3 hours after unilateral clinical exposure to light, and used to examine the cellular localization of AP-1 complex components (c-Jun, JunB, phosphorylated c-Fos), and phosphorylated ERK1/2 (Table 2) using standard immunofluorescence protocols.[28] Double-immunofluorescence with CRALBP was performed to determine colocalization with Müller cells.

Results

AP-1 DNA-Binding Activity in *RHO* Mutant Retina

To determine whether AP-1 activity was induced by clinical exposure to light, nuclear protein extracts from wild-type and T4R *RHO* mutant retinas were analyzed by EMSA at different time points after exposures that previously were found to cause retinal damage.[9] In normal controls, or unexposed T4R/+ mutant retinas, there was no AP-1 activation (Fig. 1A). In contrast, exposed T4R/+ retinas showed very distinct increased binding of the AP-1 consensus oligonucleotide visualized as a distinct band of labeling, either with the phosphor imager or x-ray film (Fig. 1A). Using groups of T4R/+ and T4R/T4R *RHO* mutants that were homozygous normal (+/+) or heterozygous (−/+) for *RPE65*, we examined the time course of AP-1 activation after exposure. There was an ~3- to 3.5-fold increase in binding activity noted by 1 hour in the exposed mutant retinas which was sustained through 6 hours after exposure (Figs. 1A, 1B). The magnitude and pattern of the activation was the same in T4R/+ *RHO* mutants that were homozygous or heterozygous for *RPE65* or in the T4R/T4R mutants. By 24 hours, the AP-1 levels had decreased, and were comparable to those of the mutant retinas that were shielded, or of the unexposed normal or mutant dogs (Figs. 1B, 1C).

To determine the specificity of increased AP-1 binding in the *RHO* mutant retinas, we performed the same study in homozygous normal dogs at both the *RHO* and *RPE65* loci. Seven dogs were analyzed at 1, 3, 6, and 24 hours after exposure to light, and there was no increase in binding activity (Fig. 1C). Similarly, dogs affected with *prcd*, a nonallelic retinal degeneration,[20] were also studied under the same conditions, and showed no AP-1 induction

with exposure to light (Fig. 1C). Clinical exposure to light similar to the ones used experimentally in this study does not cause or accelerate the retinal degenerative process in *prcd* (data not shown). The control EMSA with an SP-1 probe was performed, and no activation of SP-1 transcription factor could be detected in exposed *RHO* mutant retinas (data not shown). Similarly, pretreatment with an excess of unlabeled AP-1 oligonucleotide eliminated the binding.

Composition of AP-1 Complex in *RHO* Mutant Retina

To characterize the composition of AP-1 protein complex in the mutant retina, we used a homozygous T4R/T4R mutant that was +/+ at the *RPE65* locus and performed the analysis on nuclear protein extracts from retinas collected 3 hours after exposure. The composition was determined by supershift-EMSA analysis with antibodies directed against Fos and Jun proteins (Table 2). In the shielded mutant retina, the supershift assay showed that c-Fos, Fra-1, and c-Jun are the major AP-1 components. In contrast, the exposed retina showed that AP-1 consisted of c-Fos and JunB (Fig. 2).

Phosphorylated c-Fos in Light-Exposed *RHO* Mutant Retina

Immunoblot analysis performed to identify the individual AP-1 components in nuclear protein extracts showed an additional c-Fos immunoreactive band of higher molecular weight (~56 kDa) in the exposed T4R/+ retinas. This band was present between 1 and 6 hours after exposure, but was not detected at 24 hours. The shielded mutant retinas, or wild-type control, did not show this additional c-Fos band (Fig. 3A). Animals with different genotypes at the *RHO* (T4R/+, T4R/T4R) or *RPE65* (+/+, −/+) loci gave the same results (Figs. 3A, 3B). Dephosphorylation with PP1 before electrophoresis and immunoblotting eliminated the higher molecular size band, and confirmed that it represented a phosphorylated isoform of c-Fos (Fig. 4).

Exposure to Light and Extracellular Regulated Kinase Activation in *RHO* Mutant Retina

We examined three of the upstream activators of AP-1 (c-Jun N-terminal kinase [JNK], p38 and Extracellular Regulated Kinase [ERK] in postnuclear supernatant fractions, to determine the signaling pathway(s) that result in increased AP-1-binding activity after clinical exposure to light. Total JNK and phosphorylated JNK were detectable in immunoblots, and no changes were found in shielded or exposed mutant retinas. Intense labeling of total p38 was present, but phosphorylated p38 levels were low. As with phosphorylated JNK, there were no changes observed between shielded and exposed mutant retinas (Figs. 5A, 5B).

In the normal control retinas, either shielded or exposed, the total and phosphorylated levels of ERK1/2 isoforms were the same (Fig. 6A). As well, both the unexposed and shielded T4R/+ retinas showed levels comparable to those in the normal controls (Figs. 6B–D). In contrast, the exposed *RHO* mutant retinas showed a marked increase in phosphorylated ERK1 and -2 isoforms without an increase in total ERK1/2 levels (Fig. 6). This occurred within 1 hour of exposure to light, and levels remained elevated at the 3 and 6 hour postexposure time periods. By 24 hours, phosphorylated ERK1/2 levels were comparable to normal control, or the shielded mutant retinas. The time course and magnitude of increased ERK1/2 phosphorylation was similar for the ERK1 and -2 isoforms, and no differences were found in dogs that were T4R/+ or T4R/T4R at the *RHO* or +/+ or −/+ at the *RPE65* loci (Fig. 6).

Different Signaling Pathway in *RHO* Mutants with *RPE65* Functional Knockout

We used T4R/+ *RHO* mutants who were homozygous null at the *RPE65* locus to determine the effect of clinical exposure to light on AP-1 activation. The exposed double mutant (T4R/+; RPE −/−) retinas showed an ~2.5- to 3-fold elevation in AP-1-binding activity at 3 hours (Figs. 7A, 7B), but this activation was not associated with c-Fos phosphorylation (Fig. 7C;

compare with Figs. 3A, 3B). As well, although there were no differences between the paired shielded and exposed retinas in the phosphorylation of the ERK1 and -2 isoforms, in both, the levels were elevated and comparable to those found in the exposed *RHO* mutant retinas that were normal at the *RPE65* locus (Figs. 6C, 6D). Last, the results obtained in the analysis of JNK and p38, both in total levels and phosphorylated isoforms in shielded and exposed retinas, were the same as in the other animals discussed in the preceding sections (data not shown).

Effect of Exposure to Light on Phosphorylation of ERK1/2 and AP-1 Activation in Müller Cells of the T4R/+ *RHO* Mutant Retina

Fluorescence immunohistochemistry was used to localize the site of ERK1/2 phosphorylation and AP-1 activation in the T4R/+ *RHO* retina. Sections from the superior and inferior meridians were examined in shielded and exposed retinas, and the results were the same, both for the meridians and for different regions within the meridians. Three hours after exposure to light (Figs. 8A–C) antibodies against the phosphorylated ERK1/2 labeled the somatas of cell bodies that were located at the vitreous side of the inner nuclear layer (INL; Fig. 8A2, arrows). Double labeling showed that the phospho-ERK1/2 antibody labeled CRALBP-positive Müller cells (Fig. 8A2 + A3). Antibodies directed against components of the activated AP-1 protein complex (phosphorylated c-Fos and JunB) also labeled cells in the INL (Figs. 8B2, 8C2, arrows). Colocalization of phosphorylated c-Fos and CRALBP antibodies confirmed that these were Müller cells (Fig. 8B2 + B3). A similar pattern of immunoreactivity was seen with JunB, but double immunofluorescence could not be done with this antibody (Figs. 8C1, 8C2). In addition, JunB labeling was observed also at the level of the outer segments (OS), as well as the outer and inner plexiform layers (OPL, IPL) in both exposed and shielded retinas. However, a distinct punctate pattern of staining in the OPL, consistent with that of cone pedicles, was seen predominantly in the exposed retina. Neither phospho-ERK1/2 nor phospho-cFos immunoreactivity was found in photoreceptor cells. With the exception of a very faint JunB immunoreactivity in some cells of the INL (data not shown), a similar pattern of labeling was observed with phospho-ERK1/2 and phospho-cFos antibodies in the T4R/+ RHO retina 1 hour after exposure to light (Figs. 8D, 8E).

Discussion

Because of the potential clinical relevance of damaging exposures to light in patients with adRP who have *RHO* mutations, we have taken advantage of the experimental light-damage paradigm developed in rodents to examine the association between exposure to light and photoreceptor degeneration in the *RHO* mutant dog model.[9] The purpose was to identify the signaling pathways that link the two events. As a first step, we focused on AP-1, the proposed key intermediary of light-induced damage that induces photoreceptor apoptosis in wild-type mouse retinas by activation of the c-Fos/AP-1 molecular pathway.[11] We characterized the time course of AP-1 activation and the protein complex components and identified ERK1/2 as potential upstream activators of AP-1. In parallel, we localized the site of expression of these putative key players to the retinal Müller cells in the canine T4R *RHO* model of light-induced damage.

For these studies, we used multiple microsecond-duration light flashes produced by a hand-held fundus camera that is used in routine clinical ophthalmic practice for recording of retinal images in human patients or experimental animals. In normal dogs, or in dogs with non-*RHO*–associated retinal diseases, these exposures do not cause any retinal abnormalities, thus ruling out thermal damage or other causes of photoreceptor degeneration secondary to exposure to light. EMSAs determined that AP-1 DNA-binding activity was elevated in light-exposed *RHO* mutant retinas. Similar results were obtained in animals that were T4R/+ or T4R/T4R, confirming the results of prior studies indicating that predegenerate retinas of homozygous or

heterozygous mutants had no differences in retinal structure and function, or in the sensitivity to damage from clinical exposure to light.[8–10] As well, the same increases in AP-1-binding activity were present in *RHO* mutants that were +/+ or −/+ at the *RPE65* locus. The *RPE65* heterozygotes have a retina that is indistinguishable from that of the homozygous normal dog in function and structure[21,22] (Acland GM, Aguirre GD, unpublished results, February 2004).

Elevation in AP-1 DNA-binding activity was found only in the *RHO* mutant retina after clinical exposure to light. Wild-type controls, as well as dogs with *prcd*, a nonallelic cause of photoreceptor degeneration,[20] showed no light-associated elevation in retinal AP-1 activity, nor light-induced retinal degeneration. The results with *prcd* emphasize that it is the *RHO* mutation, rather than inherited photoreceptor disease per se, that predisposes the visual cells to damage from clinical exposure to light. These findings provide evidence that photoreceptor cell death is triggered by the light-activated T4R mutant *RHO*.

Our light-damage results in the *RHO* mutant dog retina warrant comparison with the experimental models of light-induced damage, particularly in mice.[6,29] In both, damage is mediated by rhodopsin and is prevented by absence of a chromophore.[30] In the double-mutant dog (T4R/+; *RPE65*−/−) the retina degenerates early (described later), but the degeneration is not accelerated by the exposure to light used in this study[10] (unpublished results, May 2005). Both models demonstrate activation of AP-1 associated with the damaging exposure to light (Refs. 9, [31] and the present study). In mice, there is a requirement for repetitive photon absorption to cause damage,[6] but in the dog, damage occurs with short-duration exposures that bleach >95% of the visual pigment.[8,9] The most salient difference between the two, however, is that in the dog, there is a mutation in opsin that predisposes the photoreceptors to degenerate with light intensities that normally cause no damage in this species.[9] Further support comes from studies in pigmented mice that are hemizygous null for wild-type mouse *Rho*, and have a T17M mutation affecting the second glycosylation site. In these animals, short 2.5-minute exposures to 5000 lux cause extensive retinal degeneration; as well, two fundus photographs through a stereo fundus lens cause loss of ERG a- and b-wave amplitudes within 24 hours.[32]

Elegant experimental studies, particularly in normal albino mice, have begun to characterize the pathways and molecular events that link exposure to light to photoreceptor degeneration and have implicated AP-1 activation as a key player in this process.[6] However, localization of AP-1 constituents, effectors or downstream targets has not been reported in the murine light-damage model. In the present study, immunocytochemistry showed that the key proteins in the pathway induced by exposure to light were localized to the INL. Using a marker for Müller glial cells, we found colocalization of phosphorylated c-Fos and phosphorylated ERK1/2 to this cell class; JunB was also present in the same INL cells, but confirmation of Müller cell identity was not possible because the primary antibodies were from the same species.

Müller cell reactivity after a diverse array of retinal injuries is thought to promote initially a survival response through the release of neurotrophic factors and antioxidants.[33–35] Early nonspecific responses of Müller cells involve ERK activation and upregulation of GFAP expression.[36] Several studies also have shown that c-Fos expression in Müller cells is activated shortly after exogenous administration of neurotrophic factors,[37,38] and there is endogenous upregulation of CNTF and FGF-2 in Müller cells after damaging exposure to light.[39] Based on findings in these studies, we suggest that the events associated with AP-1 activation by clinical exposure to light in the T4R *RHO* retina may represent a neuroprotective response that is insufficient to prevent the subsequent degeneration of photoreceptors. Supporting this hypothesis is a recent report on the neuroprotective effect of the cellular prion protein (PrP^c) isoform in which the authors suggest that PrP^c acts downstream of AP-1 to prevent photoreceptor apoptosis in a model of light-induced retinal degeneration.[40] Additional biochemical and cytochemical studies in mutant dogs, particularly between exposure to light

and the 1-hour period, are needed to define the time course of these changes, and, as well, to identify other signaling molecules and downstream targets of AP-1.

Based on the present results, it is appropriate to re-examine the putative role of AP-1 as the key intermediary in the signaling pathway that links damaging exposure to light with photoreceptor degeneration. Other than the close association between exposure to light and AP-1 activation, the main support for the proapoptotic role of AP-1 is based on the prevention of photoreceptor degeneration through the inhibition of AP-1, either with c-Fos knockouts[31,41] or by activation of the glucocorticoid receptor.[12] There are now an increasing number of studies that highlight how both prodeath and prosurvival signals are activated shortly after exposure to light.[42,43] Thus identifying the activation of a signaling pathway after exposure to light cannot merely be considered as causally associated with the cell death process. In regard to the prevention of degeneration with c-Fos knockouts, the protection occurs only in mice with the methionine substitution at RPE65 codon 450,[23] and this protection fails in those having leucine at this position.[44] As far as AP-1 inhibition through activation of the glucocorticoid receptor is concerned, inhibition requires a dose of 52 mg/kg of dexamethasone, and lower doses (e.g., 22 and 37 mg/kg) offer only partial protection.[12] Such doses are not pharmacologically relevant, lack specificity, and probably result in off-target effects.

Our results showed significant activation only of ERK1/2 with clinical exposure to light in the T4R *RHO* mutant retina, but not in normal control eyes. This finding is in contrast to that in a recent study in which ERK activation after exposure to light was reported in mice that are susceptible or resistant to light-induced damage.[45] ERK1/2 activation also occurs in dogs with mutations at both the *RHO* (T4R/+) and *RPE65* (−/−) loci, but this activation is independent of exposure to light. In these dogs, the same clinical protocol of exposure to light results in a modest elevation of AP-1, but no phosphorylation of c-Fos. Such response to light is not surprising, given that retinal responses can be recorded by electroretinography with suprathreshold stimuli, even though there is no detectable chromophore.[21,22] However, phosphorylated ERK1/2 levels were elevated and similar in both the exposed or shielded double-mutant retinas. The results suggest that ERK1/2 become constitutively activated as a consequence of the ongoing photoreceptor degeneration by a signal that is not associated with the damaging exposure to light. Thus, the mechanism of cell death in *RHO* mutant dogs after clinical exposure to light differs between animals that have a functional or a nonfunctional retinoid cycle in the retinal pigment epithelium.

If AP-1 activation is a prosurvival rather than cell death signal in the T4R *RHO* retina, what then is the effector that executes the cell death signal? Elegant studies in which a heterologous cell expression system was used suggest that abnormal trafficking or protein misfolding and entrapment in Golgi/endoplasmic reticulum (ER) are causally associated with photoreceptor degeneration in some rhodopsin mutations.[46–49] However, the expression systems use nonphotoreceptor cells (e.g., the 293S line of human embryonal kidney cells), and these cells lack an outer segment and the key components for the elaborate vectorial transport of opsin and other proteins destined for outer segment morphogenesis and renewal. In contrast, for the small number of cases examined in humans patients (T17M, Q64ter)[50–52] or dogs (T4R)[10] with naturally occurring *RHO* mutations, there is no evidence of abnormal trafficking that would cause opsin to accumulate in the inner segment; as well, there is no evidence of opsin accumulation in the inner segments of dogs before or after damaging exposure to light.[9] Thus, the molecular mechanism(s) linking *RHO* mutation to disease are complex, poorly understood, and likely to be mutation class specific, and they require additional investigation.

Our results indicate that three early biochemical events, activation of ERK1/2, c-Fos phosphorylation, and induction of AP-1 DNA-binding activity, occur in Müller cells after clinical exposure to light that subsequently results in photoreceptor degeneration (summarized

in Fig. 9). These changes had a similar magnitude and time course and suggest the following working model. When T4R mutant *RHO* is activated by clinical exposure to light, the ERK pathway is triggered by activated T4R *RHO*, and ERK1/2 are phosphorylated. c-Fos protein, as one of the major nuclear protein targets of ERK, is phosphorylated by activated ERK1/2 directly, or indirectly by downstream signaling molecules. Thus, the increased AP-1 DNA-binding activity is induced by c-Fos phosphorylation, which promotes transactivation of downstream targets. Because the key proteins in the AP-1 signaling pathway are located in Müller cells/INL in the T4R *RHO* mutant retina, we posit that AP-1 activation is a survival response. Thus, the cell death signal in the *RHO* mutant model and in patients with adRP remains to be identified.

Acknowledgements

The authors thank the RDS facility staff for excellent technical assistance, Sue Pearce-Kelling for helpful suggestions, Artur Cideciyan and Andras Komaromy for the light-intensity measurements, Mary Leonard for the figures, and John Saari (University of Washington, Seattle, WA) for the CRALBP antibody.

Supported by National Eye Institute Grants EY-06855 and EY-13132; The Foundation Fighting Blindness; the Van Sloun Fund for Canine Genetic Research; Vision Research Center Grant P30 EY-001583; The ONCE International Prize for R&D in Biomedicine and New Technologies for the Blind. WAB is a recipient of an FFB/Square D/Schneider Electric Advanced Science Research Career Development Award and of a grant from the University of Pennsylvania Research Foundation.

References

1. Berson EL. Retinitis pigmentosa. The Friedenwald Lecture. Invest Ophthalmol Vis Sci 1993;34:1659–1676. [PubMed: 8473105]
2. Dryja TP, Berson EL. Retinitis pigmentosa and allied diseases: implications of genetic heterogeneity. Invest Ophthalmol Vis Sci 1995;36:1197–1200. [PubMed: 7775097]
3. Berson EL, Rosner B, Weigel-DiFranco C, Dryja TP, Sandberg MA. Disease progression in patients with dominant retinitis pigmentosa and rhodopsin mutations. Invest Ophthalmol Vis Sci 2002;43:3027–3036. [PubMed: 12202526]
4. van den Born LI, van Schooneveld MJ, de Jong LAMS, et al. Thr4Lys rhodopsin mutation is associated with autosomal dominant retinitis pigmentosa of the cone-rod type in a small Dutch family. Ophthalmic Genet 1994;15:51–60. [PubMed: 7850269]
5. To K, Adamian M, Dryja TP, Berson EL. Histopathologic study of variation in severity of retinitis pigmentosa due to the dominant rhodopsin mutation Pro23His. Am J Ophthalmol 2002;134:290–293. [PubMed: 12140048]
6. Wenzel A, Grimm C, Samardzija M, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. Prog Retin Eye Res 2005;24:275–306. [PubMed: 15610977]
7. Cideciyan AV, Hood DC, Huang Y, et al. Disease sequence from mutant rhodopsin allele to rod and cone photoreceptor degeneration in man. Proc Natl Acad Sci USA 1998;95:7103–7108. [PubMed: 9618546]
8. Kijas JW, Cideciyan AV, Aleman TS, et al. Naturally occurring rhodopsin mutation in the dog causes retinal dysfunction and degeneration mimicking human dominant retinitis pigmentosa. Proc Natl Acad Sci USA 2002;99:6328–6333. [PubMed: 11972042]
9. Cideciyan AV, Jacobson SG, Aleman TS, et al. In vivo dynamics of retinal injury and repair in the rhodopsin mutant dog model of human retinitis pigmentosa. Proc Natl Acad Sci USA 2005;102:5233–5238. [PubMed: 15784735]
10. Zhu L, Jang GF, Jastrzebska B, et al. A naturally occurring mutation of the opsin gene (T4R) in dogs affects glycosylation and stability of the G protein-coupled receptor. J Biol Chem 2004;279:53828–53839. [PubMed: 15459196]
11. Hao W, Wenzel A, Obin MS, et al. Evidence for two apoptotic pathways in light-induced retinal degeneration. Nat Genet 2002;32:254–260. [PubMed: 12219089]

12. Wenzel A, Grimm C, Seeliger MW, et al. Prevention of photoreceptor apoptosis by activation of the glucocorticoid receptor. Invest Ophthalmol Vis Sci 2001;42:1653–1659. [PubMed: 11381074]
13. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1991;1072:129–157. [PubMed: 1751545]
14. Reddy SP, Mossman BT. Role and regulation of activator protein-1 in toxicant-induced responses of the lung. Am J Physiol 2002;283:L1161–L1178.
15. Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997;9:240–246. [PubMed: 9069263]
16. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153–183. [PubMed: 11294822]
17. Rubinfeld H, Seger R. The ERK cascade: a prototype of MAPK signaling. Mol Biotechnol 2005;31:151–174. [PubMed: 16170216]
18. Aguirre, GD.; Acland, GM. Models, mutants and man: searching for unique phenotypes and genes in the dog model of inherited retinal degeneration. In: Ostrander, EA.; Giger, U.; Lindblad-Toh, K., editors. The Dog and Its Genome. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2006. p. 291-325.
19. Aguirre GD, Baldwin V, Pearce-Kelling S, Narfstrom K, Ray K, Acland GM. Congenital stationary night blindness in the dog: common mutation in the RPE65 gene indicates founder effect. Mol Vis 1998;4:23–29. [PubMed: 9808841]
20. Zangerl B, Goldstein O, Philp AR, et al. Identical mutation in a novel retinal gene causes progressive rod-cone degeneration in dogs and retinitis pigmentosa in humans. Genomics 2006;88:551–563. [PubMed: 16938425]
21. Acland GM, Aguirre GD, Bennett J, et al. Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. Mol Ther 2005;12:1072–1082. [PubMed: 16226919]
22. Acland GM, Aguirre GD, Ray J, et al. Gene therapy restores vision in a canine model of childhood blindness. Nat Genet 2001;28:92–95. [PubMed: 11326284]
23. Wenzel A, Remé CE, Williams TP, Hafezi F, Grimm C. The *RPE65* Leu450Met variation increases retinal resistance against light-induced degeneration by slowing rhodopsin regeneration. J Neurosci 2001;21:53–58. [PubMed: 11150319]
24. Kremers JJ, van Norren D. Retinal damage in macaque after white exposures to light lasting ten minutes to twelve hours. Invest Ophthalmol Vis Sci 1989;30:1032–1040. [PubMed: 2732019]
25. Nygaard RW, Frumkes TE. Calibration of the retinal illuminance provided by Maxwellian views. Vision Res 1982;22:433–434. [PubMed: 7112941]
26. Beltran WA, Rohrer H, Aguirre GD. Immunolocalization of ciliary neurotrophic factor receptor alpha (CNTFRalpha) in mammalian photoreceptor cells. Mol Vis 2005;11:232–244. [PubMed: 15827545]
27. Gorski K, Carneiro M, Schibler U. Tissue-specific in vitro transcription from the mouse albumin promoter. Cell 1986;47:767–776. [PubMed: 3779841]
28. Beltran WA, Hammond P, Acland GM, Aguirre GD. A frameshift mutation in RPGR exon ORF15 causes photoreceptor degeneration and inner retina remodeling in a model of X-linked retinitis pigmentosa. Invest Ophthalmol Vis Sci 2006;47:1669–1681. [PubMed: 16565408]
29. Remé CE. The dark side of light: rhodopsin and the silent death of vision. The Proctor Lecture. Invest Ophthalmol Vis Sci 2005;46:2671–2682. [PubMed: 16043837]
30. Grimm C, Wenzel A, Hafezi F, Yu S, Redmond TM, Reme CE. Protection of Rpe65-deficient mice identifies rhodopsin as a mediator of light-induced retinal degeneration. Nat Genet 2000;25:63–66. [PubMed: 10802658]
31. Wenzel A, Grimm C, Marti A, et al. c-Fos controls the “private pathway” of light-induced apoptosis of retinal photoreceptors. J Neurosci 2000;20:81–88. [PubMed: 10627584]
32. White DA, Hauswirth WW, Kaushal S, Lewin AS. Increased sensitivity to light-induced damage in a mouse model of autosomal dominant retinal disease. Invest Ophthalmol Vis Sci 2007;48:1942–1951. [PubMed: 17460245]
33. Oku H, Ikeda T, Honma Y, et al. Gene expression of neurotrophins and their high-affinity Trk receptors in cultured human Muller cells. Ophthalmic Res 2002;34:38–42. [PubMed: 11834883]

34. Honjo M, Tanihara H, Kido N, Inatani M, Okazaki K, Honda Y. Expression of ciliary neurotrophic factor activated by retinal Müller cells in eyes with NMDA- and kainic acid-induced neuronal death. Invest Ophthalmol Vis Sci 2000;41:552–560. [PubMed: 10670488]
35. Schutte M, Werner P. Redistribution of glutathione in the ischemic rat retina. Neurosci Lett 1998;246:53–56. [PubMed: 9622206]
36. Bringmann A, Pannicke T, Grosche J, et al. Müller cells in the healthy and diseased retina. Prog Retin Eye Res 2006;25:397–424. [PubMed: 16839797]
37. Wahlin KJ, Adler R, Zack DJ, Campochiaro PA. Neurotrophic signaling in normal and degenerating rodent retinas. Exp Eye Res 2001;73:693–701. [PubMed: 11747369]
38. Wahlin KJ, Campochiaro PA, Zack DJ, Adler R. Neurotrophic factors cause activation of intracellular signaling pathways in Muller cells and other cells of the inner retina, but not photoreceptors. Invest Ophthalmol Vis Sci 2000;41:927–936. [PubMed: 10711715]
39. Joly S, Pernet V, Chemtob S, Di Polo A, Lachapelle P. Neuroprotection in the juvenile rat model of light-induced retinopathy: evidence suggesting a role for FGF-2 and CNTF. Invest Ophthalmol Vis Sci 2007;48:2311–2320. [PubMed: 17460296]
40. Frigg R, Wenzel A, Samardzija M, et al. The prion protein is neuroprotective against retinal degeneration in vivo. Exp Eye Res 2006;83:1350–1358. [PubMed: 16952355]
41. Hafezi F, Steinbach JP, Marti A, et al. The absence of c-fos prevents light-induced apoptotic cell death of photoreceptors in retinal degeneration in vivo. Nat Med 1997;3:346–349. [PubMed: 9055866]
42. Li G, Anderson RE, Tomita H, et al. Nonredundant role of Akt2 for neuroprotection of rod photoreceptor cells from light-induced cell death. J Neurosci 2007;27:203–211. [PubMed: 17202487]
43. Huang H, Frank MB, Dozmorov I, et al. Identification of mouse retinal genes differentially regulated by dim and bright cyclic light rearing. Exp Eye Res 2005;80:727–739. [PubMed: 15862179]
44. Wenzel A, Grimm C, Samardzija M, Remé CE. The genetic modifier Rpe65Leu(450): effect on light damage susceptibility in c-Fos-deficient mice. Invest Ophthalmol Vis Sci 2003;44:2798–2802. [PubMed: 12766089]
45. Samardzija M, Wenzel A, Aufenberg S, Thiersch M, Reme C, Grimm C. Differential role of Jak-STAT signaling in retinal degenerations. FASEB J 2006;20:2411–2413. [PubMed: 16966486]
46. Sung CH, Davenport CM, Nathans J. Rhodopsin mutations responsible for autosomal dominant retinitis pigmentosa: clustering of functional classes along the polypeptide chain. J Biol Chem 1993;268:26645–26649. [PubMed: 8253795]
47. Sung CH, Schneider BG, Agarwal N, Papermaster DS, Nathans J. Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. Proc Natl Acad Sci USA 1991;88:8840–8844. [PubMed: 1924344]
48. Noorwez SM, Malhotra R, McDowell JH, Smith KA, Krebs MP, Kaushal S. Retinoids assist the cellular folding of the autosomal dominant retinitis pigmentosa opsin mutant P23H. J Biol Chem 2004;279:16278–16284. [PubMed: 14769795]
49. Mendes HF, van der Spuy J, Chapple JP, Cheetham ME. Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy. Trends Mol Med 2005;11:177–185. [PubMed: 15823756]
50. John SK, Smith JE, Aguirre GD, Milam AH. Loss of cone molecular markers in rhodopsin-mutant human retinas with retinitis pigmentosa. Mol Vis 2000;6:204–215. [PubMed: 11063754]
51. Li Z-Y, Jacobson SG, Milam AH. Autosomal dominant retinitis pigmentosa caused by the threonine-17-methionine rhodopsin mutation: retinal histopathology and immunocytochemistry. Exp Eye Res 1994;58:397–408. [PubMed: 7925677]
52. Milam AH, Li Z-Y, Fariss RN. Histopathology of the human retina in retinitis pigmentosa. Prog Retin Eye Res 1998;17:175–205. [PubMed: 9695792]

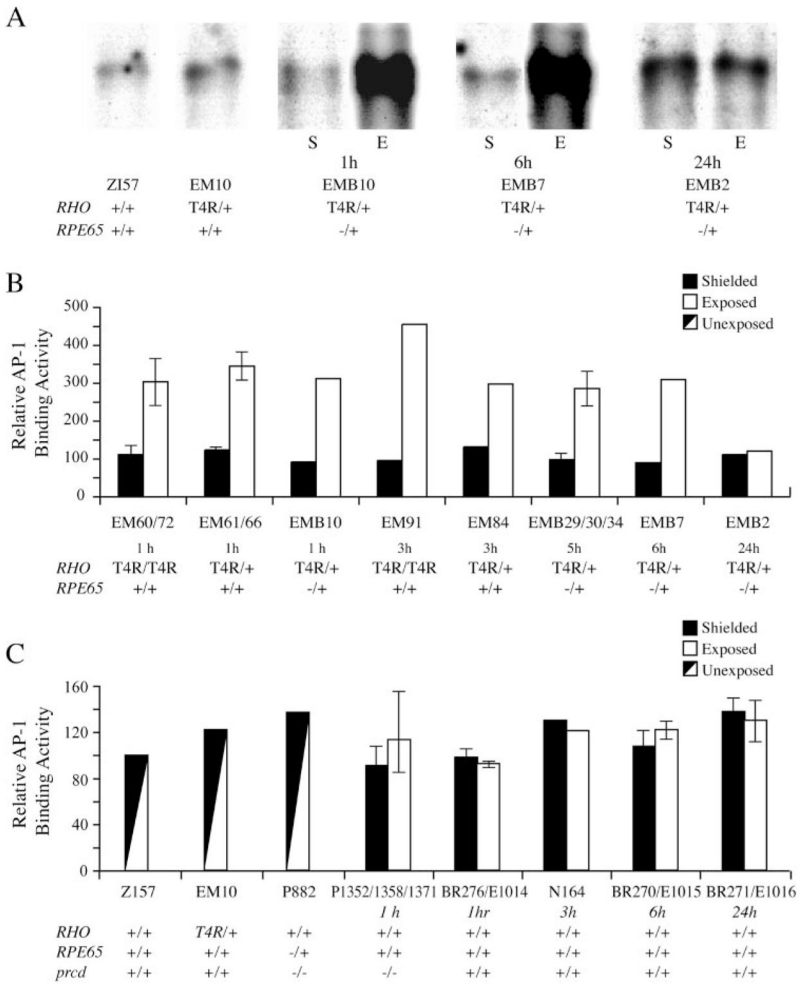

Figure 1.

EMSA detection of AP-1 DNA-binding activity in retinal nuclear proteins from normal and mutant dogs. (**A**) AP-1 DNA-binding activity was detected with an AP-1 consensus probe in normal and *RHO* mutants (T4R/+) at different time points after exposure to light. *RPE65* status (+/+, homozygous normal; −/+, heterozygous) is indicated for each animal. Retinas of unexposed normal (Z157) and *RHO* mutants (EM10) collected under ambient illumination showed comparable AP-1 levels to shielded (S) mutant retinas. Exposed eyes (E) showed increased AP-1-binding activity at 1 (EMB10) and 6 (EMB7) hours after exposure. By 24 hours, the levels were the same in exposed and shielded eyes (EMB2). (**B**) Graphic representation of relative AP-1-binding activity in mutant retinas. One hour after exposure to light, the AP-1 levels increased and remained elevated for 6 hours. There was no AP-1 elevation in the shielded eyes. By 24 hours, the relative AP-1-binding activity levels were the same in shielded and exposed eyes. There were no apparent differences in AP-1-binding activity between *RHO* mutant dogs that are heterozygous (−/+) or homozygous normal (+/+) at the *RPE65* locus. When more than one animal of the same genotype was sampled at one time point, the bar and lines represent the mean and range of values. (**C**) Graphic representation of relative retinal AP-1-binding activity in nuclear protein extracts of unexposed normal (Z157), *RHO* mutant (T4R/+; EM10), and *prcd* mutant (P882) dogs; there was no increase in AP-1-binding activity. Exposed retinas of genetically normal dogs and dogs affected with *prcd* (P1352, P1358, P1371) showed no significant increase in AP-1-binding activity in comparison to the shielded retinas. When more than one animal of the same genotype was sampled at one time

point, the data represent the mean and range of values. (**B**, **C**, *bottom*) The genotypes at the *RHO* (T4R/T4R or T4R/+), *RPE65* (+/+ or −/+), and *prcd* (+/+ or −/−) gene loci.

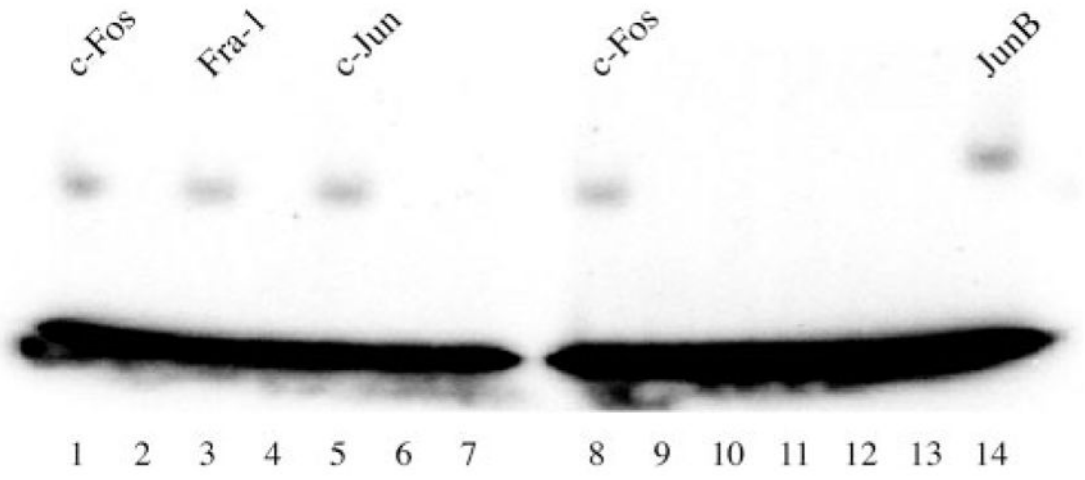

Figure 2.
A supershift assay identified composition of the AP-1 complex in T4R/T4R *RHO* retina (dog EM91). Retinal nuclear protein extracts from shielded (*lanes 1–7*) and exposed (*lanes 8–14*) T4R/T4R *RHO* mutants 3 hours after unilateral clinical exposure to light. In the shielded retina, the AP-1 complex was composed of c-Fos (*lane 1*), Fra-1 (*lane 3*), and c-Jun (*lane 5*). There was no binding to FosB (*lane 2*), Fra-2 (*lane 4*), JunD, or JunB (*lanes 6* and *7*). In the exposed retina, the AP-1 complex changes, and only c-Fos (*lane 8*) and JunB (*lane 14*) were bound. There is no binding with FosB, Fra-1, Fra-2, c-Jun, or JunD (*lanes 9–13*).

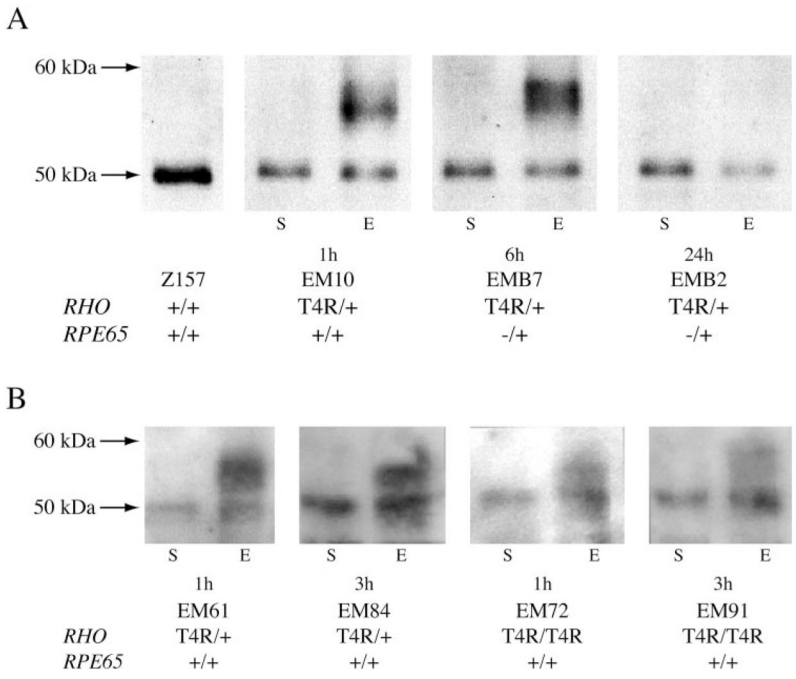

Figure 3.

c-Fos immunoreactive pattern in nuclear proteins of light exposed T4R/+ retina. (**A**) The unexposed normal retina (Z157) or the shielded (S) T4R/+ retina had a single c-Fos immunoreactive band of 50 kDa. At 1 and 6 hours after clinical exposure to light, the exposed (E) retina showed a second band of ~56 kDa. By 24 hours, only the 50-kDa band was present in both the shielded (S) and exposed retinas. (**B**) The same c-Fos pattern was present in exposed (E) T4R/+ or T4R/T4R retinas that were homozygous normal at the *RPE65* locus. The shielded (S) retinas only have the 50-kDa immunoreactive c-Fos band.

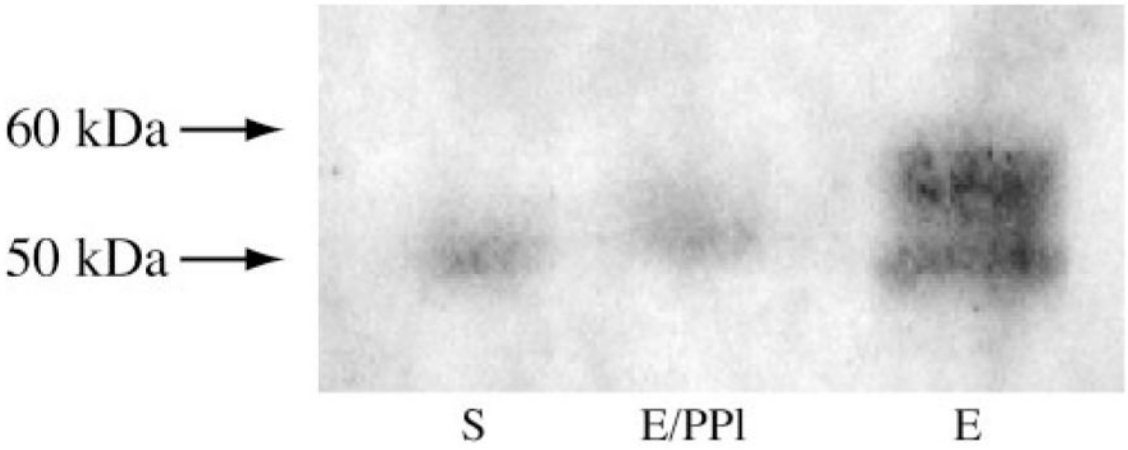

Figure 4.
Protein phosphatase 1 (PP1) eliminated the higher-molecular-weight c-Fos band. Gels from shielded (S) and exposed (E) *RHO* mutant retina (dog EM84) show different patterns of c-Fos immunore-activity. The exposed retina had 2 bands of ~50 and ~56 kDa. Pre-treatment with protein phosphatase 1 eliminated the higher-molecular-weight band.

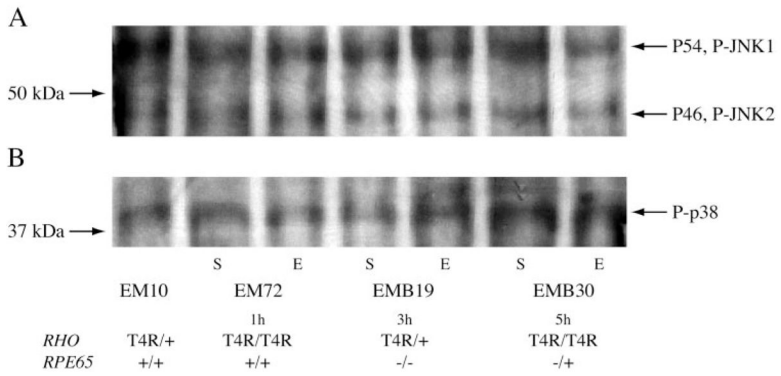

Figure 5.

Phosphorylated JNK and phosphorylated p38 in retinal post-nuclear supernatant fractions. (**A**) P-JNK antibody identified two different phosphorylated JNK isoforms (P-JNK1, P-JNK2) of different molecular size. There was no difference between T4R/+ *RHO* mutant (EM10) that was unexposed or *RHO* mutants (T4R/T4R, T4R/+) that were shielded (S) or exposed (E) and had different genotypes at the *RPE65* locus (+/+, −/+, −/−). (**B**) P-p38 antibody detected a single band, and there was no difference between animals that are shielded (S) or exposed (E). *Bottom*: genotypes at the RHO and RPE65 loci.

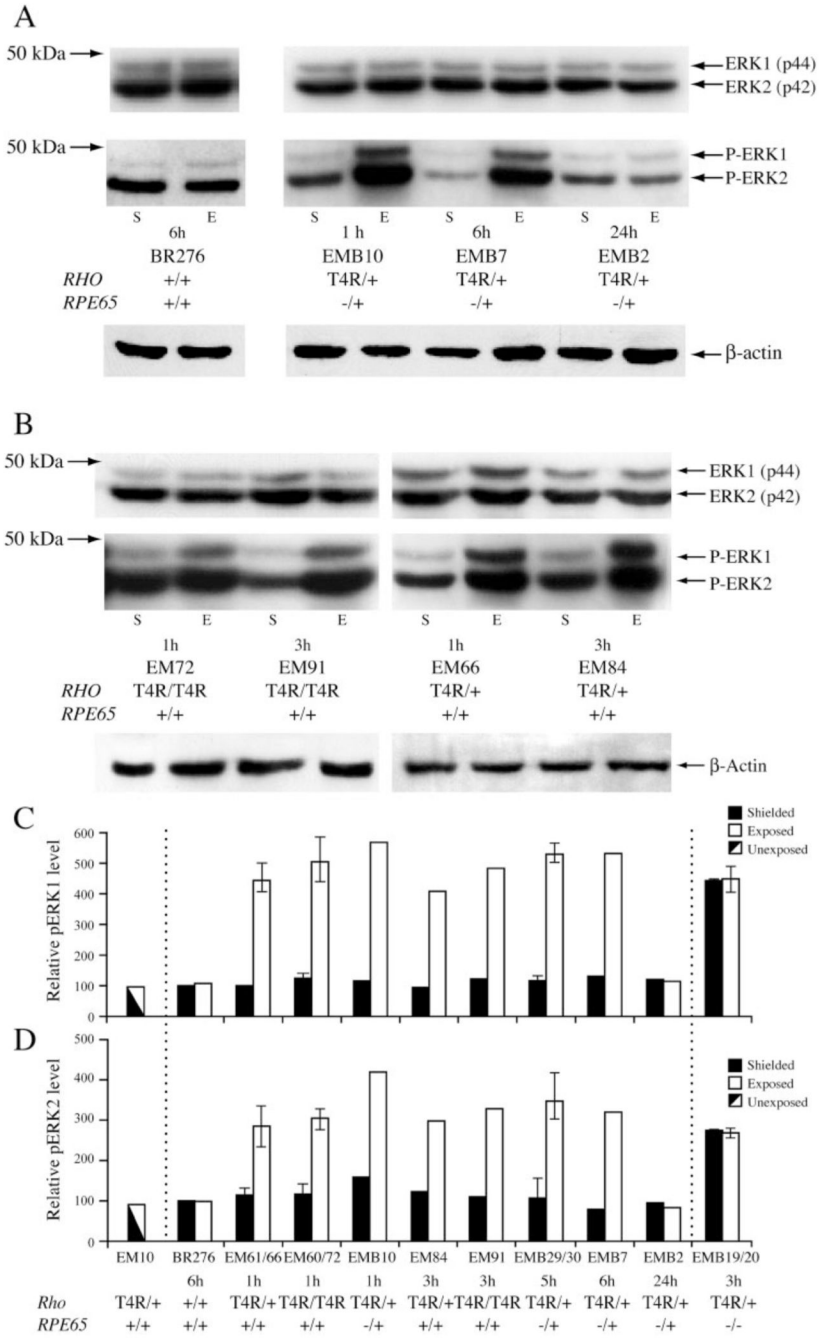

Figure 6.

Total and phosphorylated ERK1/2 in retinal postnuclear supernatant fractions from normal and mutant dogs. (**A**) Immunoblot analyses with antibodies against ERK1, ERK2, P-ERK1, and P-ERK2 in samples from normal and mutant (T4R/+; *RPE65* −/+) dogs that were shielded (S) or exposed (E). Levels of total ERK1/2 isoforms were similar to normal (BR276) and did not change with exposure to light (*top row*). Similarly, P-ERK1/2 isoforms (*middle row*) did not change with exposure to light in normal animals, and the pattern was similar to that of shielded (S) mutant retina. In exposed (E) *RHO* mutant retinas, there was a marked increase in P-ERK1/2 at 1 and 6 hours after exposure, but levels returned to normal by 24 hours. *Bottom row*: *β*-actin control. (**B**) Immunoblots from *RHO* mutant (T4R/T4R or T4R/+) homozygous normal at the

RPE65 locus that were shielded (S) or exposed (E). Levels of total ERK1/2 isoforms do not change with exposure to light (*top row*), but levels of P-ERK1/2 isoforms (*middle row*) increase at 1 and 3 hours after exposure. *Bottom row*: *β*-actin control. (**C**, **D**) Graphic representation of the relative levels of P-ERK1 (**C**) and P-ERK2 (**D**) (normalized against *β*-actin) in dogs of different genotypes at the *RHO* and *RPE65* loci. In unexposed mutant (EM10) retina, P-ERK1/2 levels were low. With exposure to light, P-ERK1/2 levels increased by 1 hour in the exposed mutant retinas and remain elevated for 6 hours. There was no difference in the levels of P-ERK1/2 in animals that were homozygous mutant or heterozygous at the *RHO* locus, or +/+ or −/+ at *RPE65*. By 24 hours, the levels in the exposed *RHO* mutant retinas decreased to normal. Dogs that are T4R/+ and homozygous mutant at *RPE65* (−/−) (*right*, EMB19/20) showed increased levels of P-ERK1/2 that were the same in exposed or shielded retinas. When more than one animal of the same genotype was sampled at one time point, the data represent the mean and range of values.

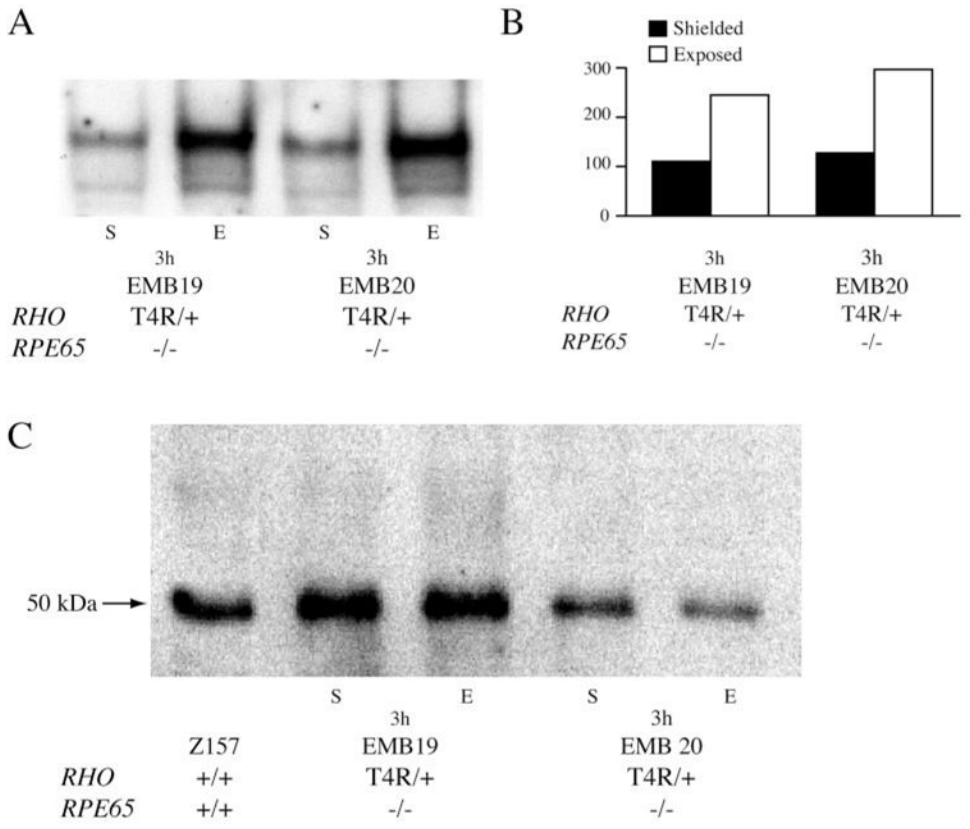

Figure 7.

AP-1 activation, but lack of c-Fos phosphorylation, with clinical exposure to light in T4R/+, *RPE65*−/− mutant retinas. (**A**, **B**) Three hours after exposure to light there was an increase in AP-1-binding activity in the exposed (E) in comparison to the shielded (S) retina. (**C**) Nuclear protein extracted labeled with a c-Fos antibody. In normal (Z157) and double-mutant retinas, shielded (S) or exposed (E), a single immunoreactive band is recognized by the c-Fos antibody.

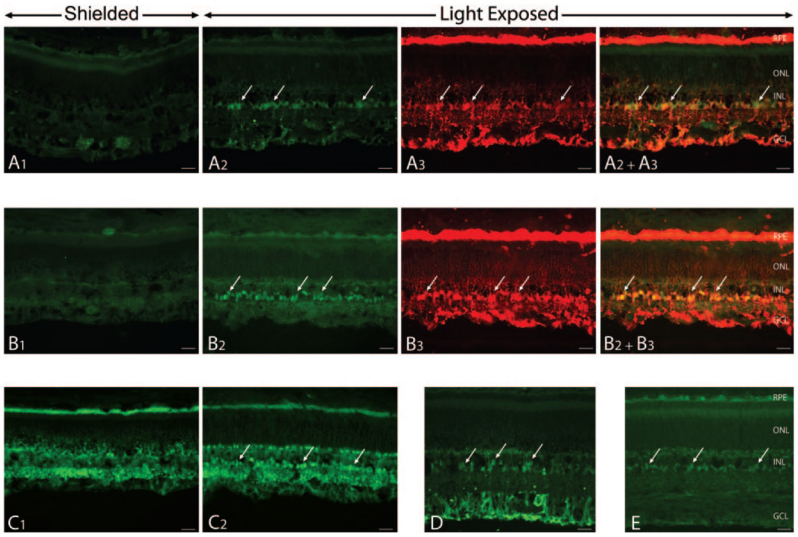

Figure 8.

Localization of phosphorylated ERK1/2, phosphorylated c-Fos and JunB in T4R/+ *RHO* mutant retinas after clinical exposure to light. Images shown are from the superior central and equatorial regions which gave the same results. The same findings were observed in comparable regions of the inferior meridian. (**A**) Cellular location of phosphorylated ERK1/2 in T4R/+ *RHO* retina 3 hours after exposure to light. (**A1**) Phospho-ERK1/2 (*green*) in a shielded eye; (**A2**, **A3**) Phospho-ERK1/2 (*green*) and CRALBP (*red*), in an exposed eye. (**A2 +A3**) Merged image. Phospho-ERK1/2 and CRALBP colocalized in Müller cells. (**B**) Cellular location of phosphorylated c-Fos in T4R/+ *RHO* retina 3 hours after exposure to light. (**B1**) Phospho-c-Fos (*green*) in a shielded eye; (**B2**, **B3**) Phospho-c-Fos (*green*) and CRALBP (*red*), in an exposed eye. (**B2+B3**) Merged image. phospho-c-Fos and CRALBP colocalized in Müller cells. (**C**) Cellular location of JunB in T4R/+ *RHO* retina 3 hours after exposure to light. (**C1**) JunB (*green*) in a shielded eye; (**C2**) JunB (*green*) in an exposed eye. Labeled INL cells had cytological characteristics of Müller cells. (**D**) Cellular location of phosphorylated ERK1/2 in T4R/+ *RHO* retina 1 hour after exposure to light. (**E**) Cellular location of phosphorylated c-Fos in T4R/+ *RHO* retina 1 hour after exposure to light. RPE, retinal pigment epithelium; ONL, outer nuclear layer; IS, inner segments; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar, 20 μm.

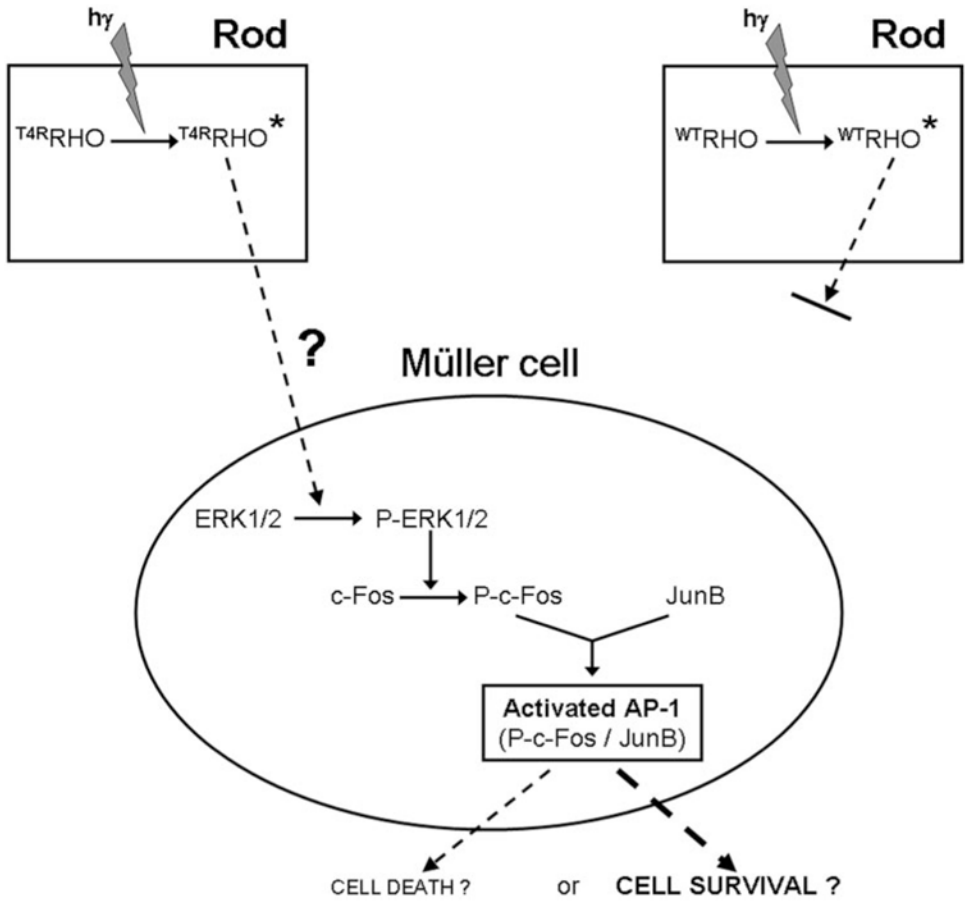

Figure 9.

The events occurring in the T4R mutant retina after clinical exposure to damaging light. Exposure to light of mutant rods results in signaling through the G-coupled receptor pathway.[8] Signaling to Müller cells, either directly or as a secondary stress response (*dashed arrow* with ?), results in ERK1/2 phosphorylation which activates AP-1 by phosphorylating c-Fos, and changing the AP-1complex to c-Fos/JunB. Previous studies have suggested that AP-1 activation results in cell death, but the current results indicate that AP-1 activation may represent a cell survival signal. Exposure to light of rods with wild-type *RHO* does not cause activation of downstream pathways in Müller cells.

Table 1

Summary of the Experimental Dogs Used in the Studies

Animal ID	Genotype			Age (wk)	Light Exposure			PE Interval (h)
	RHO	*RPE65*	*PRCD*		Exposed	Shielded	Unexposed	
Control								
Z157	+/+	+/+	+/+	11.0			B	—
BR276	+/+	+/+	+/+	7.0	L	R		1
E1014	+/+	+/+	+/+	18.6	L	R		1
N164	+/+	+/+	+/+	41.1	L	R		3
BR270	+/+	+/+	+/+	7.1	L	R		6
E1015	+/+	+/+	+/+	18.6	L	R		6
BR271	+/+	+/+	+/+	7.3	L	R		24
E1016	+/+	+/+	+/+	18.7	L	R		24
P882	+/+	+/+	−/−	91			R	—
P1352	+/+	+/+	−/−	23.1	L	R		1
P1358	+/+	+/+	−/−	19.6	L	R		1
P1371	+/+	+/+	−/−	13.0	L	R		1
RHO Mutant								
EM10	−/+	+/+	+/+	10.1			B	—
EM61	−/+	+/+	+/+	26.1	L	R		1
EM66	−/+	+/+	+/+	15.6	L	R		1
EM84	−/+	+/+	+/+	41.1	L	R		3
EM141	−/+	+/+	+/+	22.9	L			1
EM142	−/+	+/+	+/+	22.9		R		1
EM158	−/+	+/+	+/+	23.4	L	R		3
EMB10	−/+	−/+	+/+	34.3	L	R		1
EMB29	−/+	−/+	+/+	14.1	L	R		5
EMB30	−/+	−/+	+/+	14.1	L	R		5
EMB34	−/+	−/+	+/+	14.1	L	R		5
EMB7	−/+	−/+	+/+	34.3	L	R		6
EMB2	−/+	−/+	+/+	37.3	L	R		24
EM60	−/−	+/+	+/+	39.7	L	R		1
EM72	−/−	+/+	+/+	22.7	L	R		1
EM91	−/−	+/+	+/+	34.4	L	R		3
EMB19	−/+	−/−	+/+	27.0	L	R		3
EMB20	−/+	−/−	+/+	27.0	L	R		3

L, left eye; R, right eye; B, both eyes; PE interval, postexposure interval.

Table 2

List of Primary Antibodies

Antigen	Host	Source*	Working Concentration		
			IB	IHC	SSA
c-Fos (4)	Rabbit pc	S.C.B.: sc-52 (0.2 μg/μL)	1:500	(NL at 1:50)	
c-Fos (4)	Rabbit pc	S.C.B.: sc-52X (2 μg/μL)			1:3
Fra-1 (R-20)	Rabbit pc	S.C.B.: sc-605 (0.2 μg/μL)	1:500		
Fra-1 (R-20)	Rabbit pc	S.C.B.: sc-605X (2 μg/μL)			1:3
Fra-2 (L-15)	Rabbit pc	S.C.B.: sc-171 (0.2 μg/μL)	1:500		
Fra-2 (L-15)	Rabbit pc	S.C.B.: sc-171X (2 μg/μL)			1:3
FosB (102)	Rabbit pc	S.C.B.: sc-48 (0.2 μg/μL)	1:500		
FosB (102)	Rabbit pc	S.C.B.: sc-48X (2 μg/μL)			1:3
c-Jun (H-79)	Rabbit pc	S.C.B.: sc-1694 (0.2 μg/μL)	1:500	(NL at 1:50)	
c-Jun (H-79)	Rabbit pc	S.C.B.: sc-1694X (2 μg/μL)			1:3
JunB (210)	Rabbit pc	S.C.B.: sc-73 (0.2 μg/μL)	1:500	1:10	
JunB (210)	Rabbit pc	S.C.B.: sc-73X (2 μg/μL)			1:3
JunD (329)	Rabbit pc	S.C.B.: sc-74 (0.2 μg/μL)	1:500		
JunD (329)	Rabbit pc	S.C.B.: sc-74X (2 μg/μL)			1:3
P-c-Fos (pS374)	Mouse mc	Biomol: SA-345		1:10	
JNK 1 (FL)	Rabbit pc	S.C.B.: sc-571 (0.2 μg/μL)	1:500		
P-JNK (G-7)	Mouse mc	S.C.B.: sc-6254 (0.2 μg/μL)	1:500		
p38 (C-20)	Rabbit pc	S.C.B.: sc-535 (0.2 μg/μL)	1:500		
P-p38 (D-8)	Mouse mc	S.C.B.: sc-7973 (0.2 μg/μL)	1:500		
ERK 1 (K-23)	Rabbit pc	S.C.B.: sc-94 (0.2 μg/μL)	1:2,000	1:50	
P-ERK (E-4)	Mouse mc	S.C.B.: sc-7383 (0.2 μg/μL)	1:1,000	(NL at 1:10)	
P-ERK1/ERK2	Mouse mc	C.S.T.: #9106		1:30	
P-ERK1/ERK2	Rabbit pc	C.S.T.: #4377		1:50	
β-Actin	Mouse mc	Chemicon: #MAB1501	1:1,000		
CRALBP	Rabbit pc	John Saari, University of Washington, Seattle, WA		1:5,000–40,000	

P-, phosphorylated; pc, polyclonal antibody; IB, immunoblot; SSA, supershift assay; mc, monoclonal antibody; IHC, immunohistochemistry; NL, no labeling; Biomol, Biomol International LP, Plymouth, PA; S.C.B., Santa Cruz Biotechnology, Santa Cruz, CA; C.S.T., Cell Signaling Technology, Charlottesville, VA.

*Catalog numbers for commercially available antibodies are listed as a reference to their specificity.