CREB1/ATF1 Activation in Photoreceptor Degeneration and Protection

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PURPOSE. The cAMP response element binding protein 1 (CREB1) and activating transcription factor 1 (ATF1) are closely related members of the bZIP superfamily of transcription factors. Both are activated in response to a wide array of stimuli, including cellular stress. This study was conducted to assess the CREB1/ATF1 pathway in photoreceptor disease and protection.

METHODS. The expression levels of p-CREB1, CREB1, and ATF1 were examined by immunoblot and immunohistochemistry in normal canine retina and retinas of several canine models of retinal degeneration (*rcd1*, *rcd2*, *erd*, *prcd*, *XLPR1*, *XLPR2*, *T4R RHO*). Human retinas affected with age-related macular degeneration (AMD) were also examined. p-CREB1/ATF1 immunolabeling was assessed in normal and *rcd1* dogs treated with ciliary neurotrophic factor (CNTF), to examine the effect of a neuroprotective stimulus on activation of CREB1/ATF1.

RESULTS. Native CREB1 and ATF1 as well as phosphorylated CREB1/ATF1 was examined in normal canine retina by immunoblot. The p-CREB1 antibody identified phosphorylated CREB1 and ATF1 and labeled the inner retina only in normal dogs. In degenerate canine and human retinas, strong immunolabeling appeared in rod and cone photoreceptors, indicating increased expression of native CREB1 and ATF1, as well as increased phosphorylation of these proteins. Retinal protection by CNTF in *rcd1* dogs was accompanied by a significant increase in the number of p-CREB1/ATF1-labeled photoreceptor nuclei.

CONCLUSIONS. Positive association of CREB1/ATF1 phosphorylation with photoreceptor protection suggests that it may contribute to an innate protective response. These data identify a

signaling mechanism in rods and cones of potential importance for therapies of RP and AMD. (*Invest Ophthalmol Vis Sci.* 2009; 50:5355–5363) DOI:10.1167/iov.09-3741

The cAMP response element binding protein 1 (CREB1), activating transcription factor 1 (ATF1) and cAMP response element modulator (CREM) are closely related members of the CREB/ATF family. This family belongs to the basic leucine zipper (bZIP) superfamily of transcription factors, which include mammalian c-Fos, c-Jun, and c-Myc.¹ When activated by phosphorylation, these transcription factors bind as homo- or heterodimers to a palindromic consensus sequence known as the cAMP-response element (CRE).^{1–3} CREB1/ATF1 are activated by a wide array of physiological stimuli including peptide hormones, growth factors, intracellular Ca²⁺,¹ and cellular stress.^{4–7}

In adult mammalian retina, p-CREB1 is normally limited to the ganglion cell and inner nuclear layers.^{8–11} It appears that as in other parts of the nervous system,^{12–14} stressful stimuli can induce phosphorylation of CREB1 in retinal neurons.^{8–10,15} In photoreceptors, expression is noted in cat and rabbit after retinal injury by detachment¹⁰ and in rat photoreceptors after penetrating trauma.⁸ In vitro, increased expression of pCREB in mouse photoreceptor-derived 661W cells in response to FGF suggests that CREB1 may be associated with a neuroprotective outcome in photoreceptors.¹⁶

The present study was conducted to determine whether CREB1/ATF1 can be phosphorylated in dog and human photoreceptors and whether this occurs in response to degenerative or protective stimuli. We describe the distribution of phosphorylated CREB1/ATF1 in canine models of retinitis pigmentosa (RP) and in human retinas with age-related macular degeneration (AMD). The association between CREB1/ATF1 phosphorylation and photoreceptor protection induced by ciliary neurotrophic factor (CNTF) was evaluated to assess whether CREB1/ATF1 is influenced by this neuroprotective stimulus.

MATERIALS AND METHODS

Animals and Tissues

Dogs. Tissue sections from 17 dogs with inherited photoreceptor degeneration caused by seven distinct mutations were obtained from archived paraffin blocks. Diseases represented included three early-onset forms of autosomal recessive retinal degeneration, *rcd1*,¹⁷ *rcd2*,¹⁸ and *erd*¹⁹; later onset autosomal recessive retinal degeneration, *prcd*^{20,21}; two allelic forms of X-linked retinal degeneration, *XLPR1* and *XLPR2*²²; and an autosomal dominant form of retinal degeneration, the *T4R RHO* mutant dog.²³ Retinas were selected from animals with pathologic features characteristic of mid-, advanced- and end-stages of disease (Table 1). Eyes of five dogs with normal retinal morphology were included as the control. Paraffin sections were cut at 5 μ m and used for immunohistochemistry and morphology (hematoxylin and eosin [H&E]). Retinas from an additional six dogs (age-matched control and mutant *rcd1* dogs) at 12 weeks were collected for frozen sections, as previously reported.²⁴ Retinas were embedded in OCT medium and frozen, followed by sectioning at 7 μ m. Sections and blocks were stored at -80°C . All experimental animals were

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TABLE 1. Genotype, Retinal Morphology, and p-CREB Immunolabeling in Dogs

Animal ID (Genotype)	Age	Stage of Disease	ONL Width (No. of PR Nuclei)	p-CREB in ONL Layer
Control Eyes				
H87 (+/+)	1.5 y	Normal retina	11	None
B15 (+/+)	6.8 y	Normal retina (peripheral sections)	7 (peripheral retina)	Single cones, very faint
EP7 (+/+)	1.0 y	Normal retina	10	None
IP8 (+/+)	8.2 mo	Normal retina	10	None
IW105 (+/+)	2.4 mo	Normal retina	11	Single cones, very faint
rcd1-Affected (-/-; PDE6B)				
1542 (-/-)	6 mo	Advanced disease	2-3	Most
1267 (-/-)	1.1 y	End-stage disease	0-1	Few
1234 (-/-)	2.3 y	End-stage disease	0-1	Few
rcd2-Affected (-/-; RD3)				
RC92 (-/-)	3.4 mo	Mid-stage disease	5-6	Moderate
RC153 (-/-)	1.1 y	End-stage disease	1-2	Moderate
RC53 (-/-)	2.2 y	End-stage disease	0-1	Moderate
XLPR1 (X-linked; RPGR)				
H29 (M, affected)	1.1 y	End-stage disease	0-1	None
H77 (M, affected)	4.1 y	End-stage disease	2-4	None
XLPR2 (X-linked; RPGR)				
Z35 (M, affected)	3.2 y	End-stage disease	0-1	None
PomPom (F, carrier)	7 y	Peripheral degeneration	4-6	Most
erd-Affected (-/-; STK38L)				
E715 (-/-)	3.6 mo	Mid-stage disease	5-7	Moderate
E585 (-/-)	11 mo	Advanced disease	2-4	Few
E530 (-/-)	1.8 y	End-stage disease	0-2	Few
T4RHO-Affected (+/-; RHO)				
Pete Sanchez (+/-)	10.8 y	Heterogenous disease, moderate	10 central, 0-4 periphery	Many, only in degenerate areas
Dusty (+/-)	4.5 y	Heterogenous disease, severe	0-2	Few
prcd-Affected (-/-; PRCD)				
P1141 (-/-)	5.2 mo	Mid-stage disease	3-5 central, 7-10 periphery	Most
P879 (-/-)	1.7 y	Advanced disease	2-4 central; 3-5 periphery	Most

Identities, genotypes, and age of morphologically normal control dogs and retinal degenerate animals are given in columns 1 and 2. Retinal disease is characterized as mid, advanced, and end stage. These are defined by the number of remaining nuclei in the ONL (7-12 nuclei, normal; 5-7 nuclei, advanced disease; 2-5 nuclei, end-stage disease; 0-2 nuclei, end-stage disease). Examples of these are illustrated in Figure 5. In both normal and diseased dogs, retinal thickness declined toward the periphery. Two exceptions are noted in the table: *T4RHO* mutant dog, in which retinal degeneration was patchy and heterogenous, and the *prcd* dog, in which the rate of central retinal degeneration exceeded that in the periphery. A rough estimate of the number of p-CREB immunopositive photoreceptors is given in the last column. PR, photoreceptor.

handled in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Human Retinas. Human retinal samples from three anonymous patients (two women, 78 years and 80 years old, and one man 90 years old) were obtained through the National Retinitis Pigmentosa Foundation Donor Program (Hunt Valley, MD) in accordance with the privacy guideline in the Declaration of Helsinki. All had clinical diagnoses of advanced AMD with various degrees of geographic atrophy and central retinal scarring. On enucleation, eyes were fixed in 0.5% glutaraldehyde/4% paraformaldehyde in 0.1 M phosphate buffer for several days. The eyes were then transferred to 2% paraformaldehyde for storage. Six 0.5-cm diameter circular punches were taken from each donor eye: three from the central retina at the junction of atrophic and more normal retina and three from the grossly normal peripheral retina. Retinal trephines were embedded in paraffin and sectioned at 5 μ m for morphology (H&E) and immunohistochemistry.

Immunohistochemistry

Paraffin-embedded canine retinas from archived blocks were sectioned at 5 μ m. After deparaffinization, antigen retrieval was performed by

boiling the sections in a microwave oven for 10 minutes in 10 mM sodium citrate. The sections were allowed to cool for 20 minutes before a 5-minute endogenous peroxidase block in 5% H₂O₂. Immunohistochemistry was performed by overnight labeling at 4°C with anti-p-CREB^{ser 133} polyclonal antibody (cat. no. 9191, dilution 1:100; Cell Signaling Technology, Inc., Beverly, MA), which has been used in retinal studies of rat,^{25,26} cat,¹⁰ and mouse.^{9,27} Sections were then labeled with goat anti-rabbit (for p-CREB) secondary antibody (cat no. BA 1000; 1:400; Vector Laboratories, Burlingame, CA) followed by visualization with nickel-enhanced diaminobenzidine (cat no. SK4100; Vector Laboratories). To determine whether antigen retrieval had altered the pattern of immunolabeling, we repeated anti-p-CREB1 immunolabeling on frozen canine retinal sections, without antigen retrieval (Supplementary Fig. S1, <http://www.iovs.org/cgi/content/full/50/11/5355/DC1>). Negative controls for nonspecific binding of anti-p-CREB1 were performed in all species by two methods. First, the p-CREB1 antibody was preincubated with a specific blocking peptide (cat. no. 1090, Cell Signaling Technology, Inc.; Supplementary Fig. S1). Next, to confirm that the p-CREB antibody specifically bound a phospho-residue, we preincubated retinal tissue with λ -protein phosphatase (cat.

no. P07535S; New England Biolabs, Ipswich, MA) before immunolabeling with the antibody (see Figs. 1, 2). To assess nonspecific binding of the secondary antibody, tissues were incubated with normal rabbit serum instead of the primary antibodies (not shown).

Double immunohistochemistry was performed to determine whether p-CREB1 was localized to rods (mouse anti-rhodopsin; RET-P1, 1:25; Sigma-Aldrich, St. Louis, MO) and dog and human cones (rabbit anti-human cone arrestin; hCAR; 1:5000; a generous gift from Cheryl Craft, University of Southern California, Los Angeles). Cytoplasmic staining of these proteins was visualized using DAB (brown) or VIP (purple; cat. no. 2200; Vector Laboratories).

Immunoblot Analysis

The expression of total and phosphorylated forms of ATF1 and CREB1 in normal and *rcd1* canine retinas was examined by using the following antibodies: anti-ATF1 (1:200 dilution, cat. no. sc-270; Santa Cruz Biotechnology, Santa Cruz, CA); anti-CREB1 (1:1000 dilution, cat. no. 9197; Cell Signaling Technology); and anti-pCREB1/pATF1 (1:1000 dilution, cat. no. 9191, Cell Signaling Technology). Retinas from normal (7 and 16 weeks of age) and *rcd1*-affected (16 weeks of age) eyes were collected and homogenized in RIPA lysis buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 150 mM NaF, 0.5 mM Na_3VO_4 , 5 mM EDTA, 50 mM β -glycerophosphate, 5% glycerol, 1% SDS, 20 mM DTT, 1% Triton X-100, 1% deoxycholate, and 1 protease inhibitor tablet [Roche Diagnostics, Indianapolis, IN] per 1 mL of buffer), incubated on ice for 15 minutes, sonicated, and centrifuged at 13,000 rpm for 15 minutes at 4°C. The protein content of the supernatant was quantified with a protein assay (RC DC; Bio-Rad) with BSA as a protein standard (Bio-Rad). Protein (20 μg) was loaded on sodium dodecyl sulfate-polyacrylamide gels (12%). After electrophoresis, the proteins were blotted onto polyvinylidene difluoride membranes and incubated overnight at 4°C in blocking solution (10% milk in TBST). The membranes were then cut into three strips that were each incubated with the ATF1, CREB1, or pCREB1 antibody. Immunopositive signals were developed by chemiluminescence detection (ECL kit; GE Healthcare, Piscataway, NJ). After development, the blots were re probed with anti- α -tubulin antibody (1:20,000 dilution; cat. no. cs-2144; Cell Signaling Technology) to verify loading of the wells with equal amounts of total retinal protein. Blots that compared ATF1, CREB1, pCREB1, and pATF1 levels in the 16-week normal and *rcd1* retinas were also re probed with photoreceptor specific anti-GRK1a antibody (cat. no. MA1-720; Affinity Bioreagents, Thermo Fisher Scientific, Rockford, IL). Two different types of molecular weight markers were used: a biotinylated protein marker based on strep-HRP detection (cat. nos. 7727 and 7075; Cell Signaling Technology) and precision plus protein standards (cat. no. 161-0374; Bio-Rad). A personal densitometer (Molecular Dynamics, Sunnyvale, CA) was used to scan the x-ray film. The target signals were then quantified (cat. no. 1709600; Quantity One software; Bio-Rad), normalized against the housekeeping protein α -tubulin and against the photoreceptor-specific protein GRK1a. Subsequently, graphic representation of the results was performed.

CNTF Treatment of *rcd1* Dogs

All tissue sections used in this study were obtained from archived paraffin blocks²⁸ of dogs with homozygous mutation at the *rcd1* locus. The methods used in CNTF treatment of this study population are described in Tao et al.²⁸

Selection of Retinas for p-CREB1 Immunohistochemistry

For immunohistochemistry, a subset of the CNTF-treated population was used.²⁸ Left and right eyes of six dogs that demonstrated the greatest protection were chosen (12 eyes). Dose rates of CNTF ranged from 2 to 15 ng/d. Eight dogs with morphologically normal retinas were used as the control (seven wild-type dogs and one *rcd1* heterozygote). Four of these were untreated (four eyes), and four received

intraocular CNTF implants (five eyes). Dose rates for treated control animals ranged from 1.3 to 20.35 ng/d.

Morphometry

Morphometric measurements and images were collected at four consistent locations in all eyes. These were 5000 μm from the optic nerve and 1000 μm from the ora serrata in both inferior and superior retina. Immunohistochemical staining tended to be inconsistent in the central 1000 μm of the superior retina, so this region was excluded from analysis. Sections were examined with a light microscope (Axioplan; Carl Zeiss Meditec, Inc., Dublin, CA) and digital images were collected at each location at 400 $\times$ magnification with a digital camera (AxioCam; Carl Zeiss Meditec, Inc.). These were printed and the hard copies used for morphometric analysis.

CREB1 Immunoreactive Photoreceptors. To quantify CREB1/ATF1 immunostaining of photoreceptor nuclei, we counted densely stained p-CREB1/ATF1-positive nuclei (nuclei in which staining was so dark that no nuclear morphology was detectable) in a 1000- μm^2 rectangle at each location. To avoid bias, the observer was blinded as to identity and treatment status of each eye.

Outer Nuclear Layer Thickness. To express p-CREB1/ATF1 immunoreactivity as a function of photoreceptor preservation, p-CREB1/ATF1 data were compared with outer nuclear layer (ONL) thickness. ONL thickness was assessed at each location by counting the number of photoreceptor nuclei in three adjacent columns of the ONL on a single plane of focus and averaging them.

ONL Density. Photoreceptor preservation in CNTF-treated eyes could be accompanied by increased ONL density compared with untreated eyes. This increase in density could result in a false perception of the number of p-CREB1/ATF1-immunoreactive nuclei in CNTF treated eyes. To assess cell density within the ONL, we counted all nuclei within a 1000- μm^2 rectangle of the ONL at each location. Cell density was used to standardize results of p-CREB1/ATF1 immunostaining by dividing the number of positive-stained cells by the number of total nuclei within a 1000- μm^2 rectangle.

Data Analysis

First, we compared the effect of CNTF treatment on the number of p-CREB1/ATF1-positive nuclei in control (morphologically normal) untreated and CNTF-treated retinas. The data were analyzed by using a mixed-effects model^{29,30} that used each location on the retina as a repeated measure nested within each dog.³¹ The mixed model accounts for the covariance between location measures in the same dog. A variety of covariance structures were tested³²—specifically, compound symmetry and unstructured and first-order autoregressive (AR(1)). The AR(1) covariance structure allowed the dog to be modeled as a random effect, thus accounting for the inherent variation of each dog. The covariance structures were compared by the finite-sample corrected Akaike information criteria (AICc),³³ from fitting the full model by using restricted maximum likelihood. These models test for the fixed effect of phenotype and location on the retina. For all outcomes an AR(1) covariance structure had the lowest AICc, thus the best fit. Least-squares means were estimated for the fixed effects of phenotype, and the differences between phenotypes were tested.

Next, we estimated the effect of CNTF treatment on the number of CREB1/ATF1-positive nuclei in *rcd1* dogs. In these models, the untreated eye within each dog is the reference measure. These were modeled as just described. Again, based on the lowest AICc, it was found that all outcomes were best modeled with an AR(1) covariance structure. Next, the interaction term was tested by re-estimating the model using the maximum-likelihood method. The final model for each outcome was then estimated with a restricted maximum likelihood. Least-squares means were estimated for the fixed effects of dose, and the differences between doses were tested. All statistical tests were two-tailed, and $P < 0.05$ was considered to indicate statistical significance (analysis software: SAS ver. 9.1; SAS Institute, Cary, NC).

RESULTS

p-CREB1/ATF1 in Normal Canine Retina

Immunoblot analyses of 7- and 16-week-old canine retinas with the anti-p-CREB1 antibody identified three bands: a strong band at approximately 44 kDa and two weaker bands at 36 and 42 kDa (Fig. 1A). As these corresponded to the expected sizes of ATF1 (36 kDa) and alternatively spliced isoforms of CREB1 (42–44 kDa)³⁴ respectively, we proceeded to assess whether the native forms of ATF1 and CREB1 could be identified on immunoblot. Immunoblot analysis with an antibody raised against full-length human CREB1 identified a strong single band at 44 kDa. Similarly, the anti-ATF1 antibody identified this 44-kDa band, as well as a much weaker 36-kDa band. From these results we concluded that both native and phosphorylated forms of CREB1 and ATF1 are expressed in canine retina, and as indicated by the supplier, the anti-p-CREB1 antibody

A

FIGURE 1. Immunoblot analysis and immunohistochemistry for CREB1/ATF1 in wild-type canine retina. **(A)** Immunoblots of p-CREB1 and native forms of CREB1 and ATF1 in canine retina at 7 and 16 weeks. At both ages, the anti-p-CREB1 antibody identified three bands: a strong band at approximately 44 kDa and two weaker bands at 36 and 42 kDa. The anti-CREB1 antibody identified a strong single band at approximately 44 kDa. Similarly, the anti-ATF1 antibody identified this 44-kDa band, as well as a much weaker (36-kDa) band. Equivalent immunolabeling for photoreceptor-specific GRK1 and pan-retinal α -tubulin confirm equal loading of all lanes. **(B)** Immunohistochemical staining for p-CREB1 in paraffin-embedded retinas from a wild-type 16-week-old dog. p-CREB1 was present in nuclei of the INL and GCL, but not in the ONL. **(C)** Preincubation of duplicate tissue with λ -phosphatase before immunohistochemistry for p-CREB1. Immunolabeling was abolished, indicating that the p-CREB1 antibody was specific for a phosphorylated residue. Scale bar: **(B, C)** 20 μ m.

does not differentiate between the phosphorylated forms of these proteins. In 16-week-old wild-type dogs, p-CREB1/ATF1 immunoreactivity was present in the inner nuclear layer (INL) and ganglion cell layer (GCL; Fig. 1B). This observation resembles the previously reported pattern of p-CREB staining in adult retina of mouse,^{9,27} rat,²⁵ and cat.¹⁰ The pattern of p-CREB1/ATF1 immunoreactivity was identical in both frozen and Bouin's-fixed, paraffin-embedded (BFPE) sections (Supplementary Fig. S1), indicating that antigen retrieval in BFPE sections did not alter the pattern of immunoreactivity typical of frozen sections. Preincubation of tissue with λ -phosphatase abolished p-CREB1/ATF1 immunoreactivity, indicating that immunolabeling is specific for a phospho-residue (Fig. 1B).

p-CREB1/ATF1 in *rcd1*-Affected Canine Retina

Affected *rcd1* dogs that are homozygous for a mutation in the *PDE6B* gene³⁵ exhibit progressive photoreceptor death during the neonatal period.³⁶ Immunoblots of p-CREB1/ATF1 and native forms of CREB1 and ATF1 were performed in *rcd1*-affected and normal canine retina at 16 weeks. The blot was normalized to lanes labeled for a photoreceptor-specific protein (GRK1a) and a widely expressed protein (α -tubulin). α -Tubulin expression was comparable across animals, confirming equal loading of whole retinal protein amounts. Expression of the photoreceptor-specific GRK1 was reduced in *rcd1*-affected retina, consistent with the photoreceptor loss in these animals. A similar pattern of expression was noted in *rcd1*-affected and normal retinas probed with antibodies to ATF-1, CREB1, and p-CREB1 (Fig. 2A). When normalized for the photoreceptor-specific protein GRK1a, expression of the phosphorylated forms of both CREB1 and ATF1 were increased in the *rcd1*-affected retina compared with the normal retina (Fig. 2B). In addition, expression of native CREB1 and ATF1 were increased in *rcd1*, implying that transcription of these proteins increases, as well as their phosphorylation (Figs. 2A, 2B). In contrast to the pattern noted in wild-type dogs, strong p-CREB1/ATF1 labeling was noted at all levels of the ONL in *rcd1* retinas. This immunolabeling was abolished after removal of phospho-residues by preincubation with λ -phosphatase (Figs. 2C, 2D). Taken together, these data indicate that the ATF1/CREB1 pathway is activated in degenerating photoreceptors in *rcd1*.

p-CREB1/ATF1 in Rods and Cones

Double immunolabeling with RET-P1 and hCAR confirmed that both rods and cones expressed p-CREB1/ATF1, despite limitation of the *rcd1* mutation to rods (Fig. 3). ATF1 immunohistochemistry revealed a similar pattern of labeling, with strongest labeling in the INL and GCL and weaker labeling in the ONL of degenerate *rcd1* retinas (data not shown).

p-CREB1/ATF1 in Other Canine Models of Retinitis Pigmentosa

Next, to determine whether CREB1/ATF1 phosphorylation in photoreceptors occurred only in *rcd1* dogs or in dogs with other forms of retinal degeneration, we examined an additional 14 dogs with forms of inherited retinal degeneration caused by six different mutations. Eleven of the 14 dogs with retinal degeneration exhibited strong p-CREB1/ATF1 labeling in the ONL, whereas all five control retinas did not (Table 1, Fig. 4). The three animals failing to exhibit p-CREB1/ATF1 immunoreactivity in the ONL were all males affected with X-linked progressive retinal atrophy (*XLPR1* and -2). In contrast, the *XLPR2* carrier female displayed p-CREB1/ATF1 labeling in photoreceptors. In animals with disease that was not uniformly distributed (e.g., the T4R

FIGURE 2. Immunoblot and immunohistochemistry for native ATF1, native CREB1 and p-CREB1 in *rcd1*-affected and wild-type canine retina. (A) Immunoblots of p-CREB1 and native forms of CREB1 and ATF1 in *rcd1*-affected and normal canine retina at 16 weeks. The blot was normalized to lanes labeled for a photoreceptor-specific protein (GRK1) and a widely expressed protein (α -tubulin). Expression levels of the 44-kDa band corresponding to native CREB1 and p-CREB1 were comparable in whole retinas of *rcd1*-affected and normal dogs. Expression of the 36-kDa band, corresponding to native ATF1, and p-ATF1 was reduced in *rcd1*-affected retinas. α -Tubulin expression was comparable across animals, confirming equal loading of whole retinal protein amounts. Expression of the photoreceptor-specific GRK1 was reduced in *rcd1*-affected retina, consistent with the photoreceptor loss in these animals. (B) Bar graphs indicate the ratio of expression of the indicated protein in *rcd1*-affected retina compared with wild-type retina (horizontal line = 1) following normalization to photoreceptor-specific GRK1. Expression of both the 44-kDa bands (consistent with CREB1 and p-CREB1) and 36-kDa bands (consistent with ATF1 and p-ATF1) was elevated in the *rcd1* retina compared with that in the wild-type retina, indicating that both transcription and phosphorylation of ATF1 and CREB1 were increased in *rcd1*. (C, D) Immunohistochemical staining for p-CREB1 in BFPE retinas from 14-week *rcd1*-affected and normal dogs. p-CREB1 was present only in the inner retina in the normal dog. In the *rcd1*-affected dog, strong immunolabeling was present in photoreceptor nuclei. *Insets*: identical reactions performed with preincubation of the tissue with λ -phosphatase. Immunolabeling was abolished, indicating that the p-CREB1 antibody specifically labeled a phospho-residue. Scale bar, 20 μ m.

rhodopsin mutation), p-CREB1/ATF1 labeling was strongest where there was the most photoreceptor damage. As in *rcd1* dogs, double labeling with p-CREB1/ATF1 and either RET-P1

FIGURE 3. Double immunostaining in *rcd1* retinas for p-CREB1 and rod (RET-P1) or human cone arrestin (hCAR). (A) Rods in the inner portion of the ONL coexpressed p-CREB1 (gray) and RET-P1 (purple; black arrows). (B) hCAR positive cones (brown) coexpressed p-CREB1 (gray; black arrows). Scale bar, 5 μ m.

FIGURE 4. p-CREB1 labeling in additional canine models of RP. No labeling for p-CREB1 was noted in the ONL of the wild-type dog (A). Strong expression of p-CREB1 was present throughout the ONL in mid, advanced, and end stages of photoreceptor degeneration in *erd* (B), *prcd* (C), and *rcd2* (D). In the *T4RHO* mutant dog, no p-CREB1 labeling of the ONL was present in morphologically unaffected areas (E), but it was evident in the ONL of degenerate regions (F). Scale bar, 20 μ m.

FIGURE 5. p-CREB1 labeling in AMD retinas. (A–F) Images from a donor eye (78-year-old female); (G–I) images from another donor eye (90 year-old male). A large area of geographic atrophy and scarring was present in this postmortem fundus (arrow; A). The central retina demonstrated the presence of RPE atrophy and hypertrophy and photoreceptor loss (B). The photoreceptor layer was intact in the peripheral retina (C). p-CREB1 labeling was strong in the ONL in regions of the central retina with photoreceptor loss (D). In contrast, p-CREB1 labeling was limited to the inner layers of relatively normal areas of central retina (E) and peripheral retina (F). p-CREB1 labels remaining ONL photoreceptor nuclei in the central retina of a different patient (G). This labeling was abolished by preincubation with the blocking peptide (H). In regions of central retina with photoreceptor loss, p-CREB1 labeling occurred in cones (hCAR, purple cytoplasm; p-CREB1, black nucleus; I). Scale bar: (B–H) 20 μ m; (I) 5 μ m.

or hCAR identified p-CREB1/ATF1 labeling in both rods and cones (data not shown).

p-CREB1/ATF1 in Human Retinas

We then explored whether phosphorylated CREB1/ATF1 could be detected in human photoreceptors (Fig. 5). We examined retinas from central and peripheral regions of three human donors with a clinical diagnosis of AMD. Most central sections demonstrated focal to diffuse lesions characteristic of AMD (large drusen, retinal pigment epithelial atrophy/hypertrophy, and loss of adjacent photoreceptors). Peripheral sections demonstrated normal retinal pigment epithelial and photoreceptor morphology. p-CREB1/ATF1 immunoreactivity was limited to inner retinal layers in peripheral retina and in portions of morphologically normal central retina. However, in areas with photoreceptor loss, p-CREB1/ATF1 antibody strongly labeled both rod and cone nuclei.

p-CREB1/ATF1 Expression in Response to CNTF

Finally, we wanted to determine whether CREB1/ATF1 phosphorylation in photoreceptor nuclei was affected by a stimulus known to protect photoreceptors (Table 2, Fig. 6). To do this, we assessed whether treatment with CNTF, an agent known to provide morphologic preservation of rods in several animal models of RP,^{28,37–39} affects the number of p-CREB1/ATF1-

immunopositive nuclei in normal canine retinas, as well as those undergoing photoreceptor degeneration due to the *rcd1* mutation. As expected, normal untreated eyes did not express p-CREB1/ATF1 in the ONL. CNTF treatment of normal retinas enhanced p-CREB1/ATF1 immunolabeling of the INL compared with untreated control eyes (Fig. 6B). In addition, CNTF treatment significantly increased the number of CREB1/ATF1-immunopositive nuclei in the ONL ($P = 0.0063$). CNTF treatment had no significant effect on ONL thickness or density in WT dogs (Table 2). In untreated *rcd1* retinas, extensive photoreceptor nuclear labeling for p-CREB1/ATF1 was present. At 14 weeks, these animals were the youngest *rcd1* dogs examined; at this stage, significant cone loss has not yet occurred.⁴⁰ Double immunohistochemistry established that both rods and cones expressed p-CREB1/ATF1 (data not shown). In CNTF-treated *rcd1* retinas, photoreceptor staining appeared more extensive. Morphometric analysis of p-CREB1/ATF1 immunoreactivity was performed to determine whether CNTF treatment increased the number of p-CREB1/ATF1-positive nuclei. Using the mixed-effects model, we confirmed that CNTF treatment of *rcd1* retinas preserved ONL thickness ($P = 0.0002$). This preservation was accompanied by increased, but statistically nonsignificant ($P = 0.06$) ONL density, and a significant increase in the number of p-CREB1/ATF1 labeled photoreceptor nuclei ($P = 0.0083$). We also identified a significant asso-

TABLE 2. ONL Thickness, ONL Cell Density, and p-CREB Immunopositive Photoreceptor Nuclei in Untreated and CNTF-Treated WT and *rcd1* Dogs

Genotype/ Parameter	No Treatment	CNTF Treatment	<i>P</i>
Wild-type			
<i>n</i>	4	5	
ONL thickness	8.6 (1.5)	9.3 (1.3)	0.51
ONL cell density (1000 μm^2)	104.9 (16.6)	91.2 (7.9)	0.66
p-CREB +ve (1000 μm^2)	1.0 (1.0)	8.6 (7.4)	0.0063*
<i>rcd1</i>			
<i>n</i>	6	6	
ONL thickness	3.4 (0.9)	5.5 (1.0)	0.0002*
ONL cell density (1000 μm^2)	97.3 (13.5)	103.9 (8.9)	0.06
p-CREB +ve (1000 μm^2)	10.4 (4.8)	19.5 (9.7)	0.0083*

Numerical data are given as the mean of cell counts at all four locations. Standard deviations are given in parentheses. Probabilities are from mixed-effects models. CNTF treatment had no significant effect on ONL thickness or density in WT dogs, but increased the number of p-CREB1-positive photoreceptor nuclei. In *rcd1*, CNTF treatment preserved ONL thickness, accompanied by increased, but statistically nonsignificant, ONL density, and a significant increase in the number of p-CREB1-labeled photoreceptor nuclei.

* Significant difference.

ciation between the proportion of CREB1/ATF1-positive nuclei cells and the ONL thickness ($P < 0.0001$). These data provide evidence for a positive association between a neuroprotective stimulus (CNTF) and the extent of p-CREB1/ATF1 labeling.

DISCUSSION

In this study, we identified native CREB1 and ATF1 in normal inner canine retina. We establish that photoreceptor degeneration due to the *rcd1* mutation results in increased expression of both proteins, as well as phosphorylation of CREB1/ATF1 in photoreceptors. The latter finding was also identified in canine retinal degenerations of diverse genetic causes, as well as in human AMD retinas. Last, we associated increased phosphorylation of photoreceptors in normal and *rcd1* dogs with a neuroprotective stimulus, CNTF.

Our data confirm that both ATF1 and CREB1 are present in normal canine retina, but that, as predicted by the manufacturer and other studies,^{41,42} the anti-p-CREB1 antibody cannot distinguish between the phosphorylated forms of these proteins. Although CREB1 and ATF1 share structural similarity, the function of ATF1 is far less understood. Both are widely expressed and are activated by phosphorylation of Ser¹³³ (CREB1) or Ser⁶³ (ATF1).^{1,41,42} Both can bind to the CRE as homodimers or CREB1/ATF1 heterodimers.^{43,44} ATF1 and CREB1 are activated by similar upstream stimuli, such as cAMP-dependent protein kinase A⁴⁵ and Ca²⁺.⁴¹ However, the magnitude and context of their responses to these stimuli differ,^{41,42} implying that they integrate stimuli to differentially regulate transcription.

Consistent with previously reported findings,^{8–11} p-CREB1/ATF1 is detected by immunohistochemistry in the INL and GCL. However, in degenerating canine photoreceptors, p-CREB1/ATF1 immunostaining is also prominent in the photoreceptors. By immunoblot, increased expression of both p-CREB1 (44kDa) and p-ATF1 (36 kDa) was detected in *rcd1*-affected retina after normalization against a photoreceptor-specific protein, GRK1. Similarly, *rcd1* retinas also experienced

increased expression of the native forms of CREB1 and ATF-1, suggesting that photoreceptor degeneration is accompanied by increased CREB1/ATF1 transcription and phosphorylation.

We identified CREB1/ATF1 phosphorylation in photoreceptors of genetically distinct forms of canine inherited retinal degeneration, as well as in human retinas with AMD. This finding suggests that CREB1/ATF1 phosphorylation is a generalized response to photoreceptor insult, regardless of whether it arises within the photoreceptor itself (as in canine models of RP) or affects photoreceptors secondary to retinal pigment epithelial damage (as in AMD). In canine and human retinas, we identified p-CREB1/ATF1 in both rods and cones. In *rcd1*, the product of the mutated gene is expressed only in rods, and causes initial rod death followed by later cone death.³⁶ We identified p-CREB1/ATF1 expression in cones at a stage of the disease in which altered cone morphology is apparent, but significant cone loss has not yet occurred.^{17,40} Cone loss invariably follows rod loss in RP, even in cases where the product of the mutant gene is limited to rods. This finding results in recognition of a poorly understood mechanism of rod-dependent cone loss in RP (reviewed in Ripps⁴⁶). Cone expression of p-CREB1/ATF1 in *rcd1* dogs is clear evidence of a signaling event in cones initiated either by direct rod-cone interaction or by the altered retinal environment accompanying rod loss.

This study provides new evidence of a signaling pathway in photoreceptors that is associated with both degenerative and neuroprotective stimuli. Whether the CREB1/ATF1 pathway contributes to photoreceptor degeneration cannot be determined by this study. In contrast, we established that CNTF, a known photoreceptor protective stimulus in mice^{37–39} and dogs,²⁸ induces CREB1/ATF1 phosphorylation in normal retinas and is associated with increased p-CREB1/ATF1 expression in *rcd1* retinas. These data suggest that p-CREB1/ATF1 is associated with a neuroprotective process in photoreceptors. Confirming this hypothesis requires additional work to determine whether activation of the p-CREB1/ATF1 pathway can retard photoreceptor death.

CNTF may exert its photoreceptor protective effects via indirect or direct means, with the greater body of evidence supporting the former. Reports of signaling responses to CNTF

FIGURE 6. p-CREB1 immunohistochemistry in WT and *rcd1* retinas with and without CNTF treatment. Untreated WT retinas display immunoreactivity predominantly in Müller cell nuclei and INL neuronal nuclei, but not in the ONL (A). CNTF treatment of WT retinas resulted in p-CREB1 staining in photoreceptor nuclei (B). *rcd1* retinas displayed marked photoreceptor immunoreactivity (C), which was enhanced by CNTF treatment (D). Scale bar, 20 μm .

in normal and degenerating mouse retina^{47,48} demonstrate increased expression of p-CREB in Müller glia and inner retina, but not in photoreceptors. Downstream signaling intermediates known to respond to CNTF include STAT1/3 and ERK1/2.⁶ CREB1/ATF1 are downstream targets for ERK1/2.⁴¹ Short-term application of CNTF to explanted or in vivo mouse retinas results in increased p-STAT and p-ERK1/2 expression in Müller glia and inner retina,^{11,47,48} implying that CNTF promotes photoreceptor protection by indirect means. In wild-type and mutant dogs, demonstration of the receptor for CNTF in rods and cones^{24,49} suggests that CNTF may also afford photoreceptor protection via a direct mechanism of action. Furthermore, long-term rAAV-driven CNTF expression has been shown to increase expression of p-ERK1/2, an upstream activator of CREB1,⁵⁰ in peripherin/*rds*^{+/−} photoreceptors.⁵¹

Observations in other studies have suggested a model by which cellular insults are capable of initiating two parallel signals: one that promotes cell death and a second that activates a CREB1-directed prosurvival program.⁵² If a similar mechanism exists in photoreceptors, activation of the CREB1/ATF1 pathway may represent an innate neuroprotective response to photoreceptor degeneration, which, if augmented by pharmacologic means, may prolong photoreceptor life. Last, as very few signaling events have been identified in cones, exploration of this hypothesis may identify potential mechanisms of cone support in RP and AMD.

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