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Abstract
Ciliary neurotrophic factor (CNTF) rescues photoreceptors in several animal models of retinal degeneration and is currently being evaluated as a potential treatment for retinitis pigmentosa in humans. This study was conducted to test whether CNTF prevents photoreceptor cell loss in *XLPRA2*, an early onset canine model of X-linked retinitis pigmentosa caused by a frameshift mutation in *RPGR* exon ORF15. Four different treatment regimens of CNTF were tested in *XLPRA2* dogs. Under anesthesia, the animals received at different ages an intravitreal injection of 12 μg of CNTF in the left eye. The right eye served as a control and was injected with a similar volume of phosphate buffered saline (PBS). Ocular examinations were performed regularly during the treatment periods. At termination, the dogs were euthanatized, eyes collected and the retinas were processed for embedding in optimal cutting temperature (OCT) medium. The outer nuclear layer (ONL) thickness was evaluated on H&E sections and values in both CNTF- and PBS-treated eyes were compared. Morphologic alterations in the peripheral retina were characterized by immunohistochemistry using cell-specific markers. Cell proliferation in the retinas was examined on semi-thin plastic sections, and by BrdU pulse-labeling and Ki67 immunohistochemistry on cryosections. All CNTF-treated eyes showed early clinical signs of corneal epitheliopathy, subcapsular cataracts and uveitis. No statistically significant difference in ONL thickness was seen between the CNTF- and PBS-injected eyes. Prominent retinal remodeling that consisted in an abnormal increase in the number of rods, and in misplacement of some rods, cones, bipolar and Müller cells, was observed in the peripheral retina of CNTF-treated eyes. This was only seen when CNTF was in injected before the age at which the canine retina reaches full maturation. In *XLPRA2* dogs, intravitreal injections of CNTF failed to prevent photoreceptors from undergoing cell death in the central and mid-peripheral retina. CNTF also caused ocular side-effects and morphologic alterations in the periphery that were consistent with cell dedifferentiation and proliferation. Our findings suggest that some inherited forms of retinal degeneration may not respond to CNTF's neuroprotective effects.

Keywords
neuroprotection, ciliary neurotrophic factor, retina, photoreceptors, RPGR, X-linked retinitis pigmentosa, remodeling, dog

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Intravitreal injection of ciliary neurotrophic factor (CNTF) causes peripheral remodeling and does not prevent photoreceptor loss in canine *RPGR* mutant retina

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Abstract

Ciliary neurotrophic factor (CNTF) rescues photoreceptors in several animal models of retinal degeneration and is currently being evaluated as a potential treatment for retinitis pigmentosa in humans. This study was conducted to test whether CNTF prevents photoreceptor cell loss in *XLPRA2*, an early onset canine model of X-linked retinitis pigmentosa caused by a frameshift mutation in *RPGR* exon ORF15.

Four different treatment regimens of CNTF were tested in *XLPRA2* dogs. Under anesthesia, the animals received at different ages an intravitreal injection of 12 μg of CNTF in the left eye. The right eye served as a control and was injected with a similar volume of phosphate buffered saline (PBS). Ocular examinations were performed regularly during the treatment periods. At termination, the dogs were euthanatized, eyes collected and the retinas were processed for embedding in optimal cutting temperature (OCT) medium. The outer nuclear layer (ONL) thickness was evaluated on H&E sections and values in both CNTF- and PBS-treated eyes were compared. Morphologic alterations in the peripheral retina were characterized by immunohistochemistry using cell-specific markers. Cell proliferation in the retinas was examined on semi-thin plastic sections, and by BrdU pulse-labeling and Ki67 immunohistochemistry on cryosections.

All CNTF-treated eyes showed early clinical signs of corneal epitheliopathy, subcapsular cataracts and uveitis. No statistically significant difference in ONL thickness was seen between the CNTF- and PBS-injected eyes. Prominent retinal remodeling that consisted in an abnormal increase in the number of rods, and in misplacement of some rods, cones, bipolar and Müller cells, was observed in the peripheral retina of CNTF-treated eyes. This was only seen when CNTF was in injected before the age at which the canine retina reaches full maturation.

In *XLPRA2* dogs, intravitreal injections of CNTF failed to prevent photoreceptors from undergoing cell death in the central and mid-peripheral retina. CNTF also caused ocular side-effects and

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morphologic alterations in the periphery that were consistent with cell dedifferentiation and proliferation. Our findings suggest that some inherited forms of retinal degeneration may not respond to CNTF's neuroprotective effects.

Keywords

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INTRODUCTION

Retinitis pigmentosa (RP) is a genetically heterogeneous group of diseases that constitutes one of the leading causes of blindness worldwide, with an incidence of approximately 1: 4,000 (Boughman et al., 1980; Puech et al., 1991). Despite the identification over the past 20 years of more than 50 genes responsible for RP (http://www.sph.uth.tmc.edu/Retnet/), currently there is no treatment available that can either prevent, or slow-down the course of photoreceptor cell death. A promising therapeutic approach, for which proof of principle has been demonstrated in various animal models, is the use of corrective gene therapy (Acland et al., 2001; Pang et al., 2006). Yet, such a strategy requires the identification of the mutated gene, and is therefore aimed at targeting diseases in a gene-specific manner.

An approach that could potentially bypass the inherent limitation of gene-based therapy is the use of neuroprotective agents that can rescue photoreceptors regardless of the genetic and/or environmental causes of the retinal degeneration. Over the past 15 years, numerous survival factors have been tested in a variety of animal models of RP [e.g. see: (LaVail et al., 1992; Cayouette et al., 1999; Frasson et al., 1999)]. Among these agents, ciliary neurotrophic factor (CNTF) has been shown to rescue photoreceptors in several rodent and large animal models [for review see: (Beltran, 2006)]. Because of the inability for CNTF to cross the blood-retina barrier following systemic administration, the necessity for sustained bioavailablity of the agent in the eye, and the ocular side-effects associated with bolus intravitreal injection, a long-term intraocular delivery system has been developed (Thanos et al., 2004). This encapsulated cell-based technology (ECT) allows for the continuous release of small quantities of CNTF into the vitreous, and was evaluated in experimental animal models (Tao et al., 2002; Bush et al., 2004), and more recently in Phase I clinical trial in humans (Sieving et al., 2006).

Previous work has shown that CNTF delivered through intravitreal injections (Pearce-Kelling, unpublished study), or by means of an ECT device (Tao et al., 2002), rescues photoreceptors in the *rcd1* dog, an early and rapidly progressing large animal model of RP caused by a stop mutation in *PDE6B*. The purpose of the present study was to determine whether intravitreal injections of CNTF could provide a similar neuroprotective effect in *XLPRA2*, an early onset model of X-linked RP caused by a frameshift mutation in *RPGR* exon ORF15 (Zhang et al., 2002; Ferreira, 2005; Khanna et al., 2005). Recently, we reported the morphologic retinal changes, and the kinetics of photoreceptor cell death, that occur during the course of this disease (Beltran et al., 2006). Death of rods occurs in a biphasic manner, beginning as early as 4 weeks of age, and reaching a peak at 6–7 weeks. Following this initial burst, the rate of cell death is considerably slowed down, yet persists at an approximately constant rate for at least 9 months. Based on these findings, the initial phase of cell death was selected as a time-window to evaluate the neuroprotective effect of CNTF. Results show that intravitreal injections of CNTF at the onset and/or peak of cell death do not prevent cell loss in the central and midperipheral *XLPRA2 retina*, but cause prominent remodeling in the periphery.

METHODS

Expression and purification of recombinant CNTF protein

The open reading frame of human *CNTF* cDNA was PCR-cloned into the pQE30 expression vector (Qiagen, Valencia, CA), and fused to a 6x His tag at the amino-terminus to generate plasmid pQE-CNTF. Recombinant human CNTF protein was expressed in *E. coli* (XL-blue, Stratagene, La Jolla, CA), and purified by immobilized-metal affinity chromatography on Ni-NTA Agarose columns (Qiagen) under native conditions. Eluted protein was buffer-exchanged to phosphate buffered saline (PBS), and the protein concentration determined by the BCA protein assay (Pierce, Rockford, IL). The CNTF solution was then diluted with PBS to a concentration of 0.4 μg/μl, sterile filtered (Acrodisc Syringe filter 0.2 μm, Pall Corporation, Ann Harbor, MI), and aliquots of 30 μl (12 μg) were stored at −80°C.

Animals

A total of 16 affected *XLPRA2*, 3 affected *rcd1*, and 1 non-mutant dog was used for this study. All animals were bred and housed at the Retinal Disease Studies Facility (RDSF, University of Pennsylvania, New Bolton Center, Kennett Square, PA). Their genotype was determined either from the known status of their progenitors, or from genetic testing for the disease-causing mutation (Ray et al., 1994; Zhang et al., 2002). All animals underwent an initial ocular examination that confirmed the absence of clinically evident abnormalities.

Twelve *XLPRA2* dogs were initially used in this study, and the animals were allocated to one of the following treatment groups:

- Treatment Group #1 (n = 3): one single injection of CNTF at 4 weeks of age (see below), termination at 8 weeks of age (Inj. 4 wk; Ter. 8 wk).
- Treatment Group #2 (n = 3): injection of CNTF at 4 and 8 weeks of age, termination at 12 weeks of age (Inj. 4 & 8 wk; Ter. 12 wk).
- Treatment Group #3 (n = 3): injection of CNTF at 7 and 10 weeks of age, termination at 14 weeks of age (Inj. 7 & 10 wk; Ter. 14 wk).
- Treatment Group #4 (n = 3): one single injection of CNTF at 12 weeks, termination at 15.6 weeks of age (Inj. 12 wk; Ter. 15.6 wk).

The primary aim of this study was to determine whether intravitreal bolus injections of CNTF could arrest the course of photoreceptor cell death during the very early phase of the disease [ie: when a rapid decline in ONL thickness and a burst of photoreceptor cell death occur (Beltran et al., 2006)]. This was the basis for selecting the time of injection and termination of treatment Groups #1, #2 and #3. We included as positive controls for the biological activity of CNTF, 3 *rcd1* dogs (*rcd1* control group) that were treated following a protocol that achieves photoreceptor rescue in this model (CNTF injection at 7 and 10 weeks of age, termination at 14 weeks of age). This work, presented in abstract form but never published, served as a basis for choosing the dose of CNTF that was administered in this study. Treatment Group #3 was included to determine whether a similar rescue effect would be attained in *XLPRA2* dogs exposed to this same protocol.

The second aim of this study was to determine whether CNTF could cause any alterations in the retinal cyto-architecture of the *XLPRA2* dog. In treatment Groups #1, #2, and #3, CNTF was injected for the first time before the retina had reached full maturation, thus, treatment Group #4 was included to examine the remodeling effects of a single injection of CNTF in the mature*XLPRA2* canine retina. To control for disease-specificity, a single non-mutant dog was treated in the same way as in treatment Group #3 (Inj. 7 & 10 wk; Ter. 14 wk). To further characterize potential CNTF-mediated cellular alterations a different set of studies was carried

out. For this, 4 affected *XLPRA2* dogs were used following protocols detailed below (see under "Histologic procedures", and "Cell proliferation assays" sections).

All intravitreal injections were performed in eyes that had their pupils previously dilated by topical application of atropine, phenylephrine and tropicamide. Under general anesthesia (isoflurane), dogs underwent a 1 minute massage of their globes to reduce the intraocular pressure (IOP). A 29-gauge needle mounted on an insulin syringe was then inserted in the supero-temporal quadrant approximately 5 mm behind the limbus, and the tip was directed towards the center of the vitreous to inject in the left eye (OS) 12 μg of CNTF in 0.1 M PBS (Total volume injected: 30 μl). The right eye (OD) served as a control, and was injected with 30 μl of the PBS diluent. Immediately following the injections, both eyes were examined by slit-lamp biomicroscopy and indirect ophthalmoscopy to verify that no lesions were caused to the lens and/or retina during the procedure. All animals underwent additional ocular examinations 2–4 days following the intravitreal injections and subsequently, at least once a week, for the remaining of the treatment period. This included IOP measurements by applanation tonometry (TonoPen XL, Medtronic Ophthalmics, Jacksonville, FL). At the end of the treatment period, the animals were euthanatized by intravenous injection of pentobarbital sodium, enucleated, and the eyes processed as indicated below. All procedures involving animals were done in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histologic procedures

The eyes of the 16 dogs (12 *XLPRA2*, 3 *rcd1*, 1 non-mutant) used in the initial part of the study were processed after enucleation as previously published (Beltran et al., 2006). A slit was made at the level of the *ora serrata*, and the entire globe was fixed for 3 hours in 4% paraformaldehyde in 0.1 M PBS at 4°C. The posterior segment was isolated and fixed for an additional 24 hours at 4°C in 2% paraformaldehyde. The tissue then was trimmed into four pieces that extended from the optic disc to the *ora serrata* along the superior, inferior, nasal, and temporal meridians. Following sequential cryoprotection for 24 hours in solutions of 15% and 30% sucrose in PBS at 4°C, the tissues were embedded in optimal cutting temperature (OCT) medium. Cryosections were cut at 7 or 10 μm thickness, air-dried, and stained with hematoxylin & eosin (H&E), or used for immunohistochemistry (see below). The anterior segments of some animals were post-fixed in Bouin's solution, and paraffin embedded. Paraffin sections were cut at 6 μm thickness, H&E stained, and used to examine the pathology in the cornea, iris and lens.

Retinal cryosections stained with H&E were examined by light microscopy (Axioplan; Carl Zeiss Meditec GmbH, Oberkochen, Germany) in contiguous fields extending from the optic disc to the *ora serrata* with 10X and 40X objectives. For each animal, quantitative evaluation of the outer nuclear layer (ONL) thickness was done on sections from the 4 meridians of both eyes at 3 specific locations: S1, S2, and S3 (See Figure 1). At each of these sites, the number of rows of nuclei in the ONL was counted in at least 3 representative areas and averaged. The field that was viewed with the 40X objective covered a retinal length of 290 μm. Because alterations in the retinal architecture were frequently seen involving site S3 (see Results), we excluded ONL data collected at this site, and restricted the analysis of photoreceptor rescue to the central (S1) and mid-peripheral (S2) regions of the retina. For each quadrant, the average ONL thickness (mean of ONL thickness at S1 and S2) was calculated, and compared to that of the contralateral eye. The paired Student t-test was used to analyze the differences in ONL thickness between the CNTF- and the PBS-injected eyes. Statistical analysis was performed using a commercial software (Statistix 8; Analytical Software, Tallahassee, FL).

To better detect and characterize potential retinal alterations induced by CNTF, we examined on semi-thin plastic sections the retinal morphology of two additional *XLPRA2* dogs. One dog

was treated following the same protocol as for Group #1 (Inj. 4 wk, Ter. 8 wk); the second dog received the same treatment as Group #2 (Inj. 4 & 8 wk; Ter 12 wk). Eyes were collected, fixed, and trimmed as described above, and processed for epoxy resin embedding as previously reported (Beltran et al., 2006). Retinal sections extending along all 4 meridians were cut at 1 μm with glass knives using a supercut microtome (Reichert Jung model 2065; Leica, Deerfield, IL), and stained with azure II-methylene blue with or without paraphenylenediamine counterstain. For each meridian, the site of highest ONL thickness in the peripheral retina of the CNTF-injected eyes was located, and its distance from the *ora serrata* measured. This was used to locate the corresponding site in the contralateral PBS-injected eye. Using the 40X objective, digitally captured images (Spot 4.0 camera; Diagnostic Instruments, Inc., Sterling Heights, MI) that encompassed a 290 μm length of retina, were printed to manually count the total number of photoreceptor nuclei at these two sites. For each meridian, the counts were performed in triplicate on sequential sections, and the values averaged.

Immunohistochemistry

Fluorescence immunohistochemistry was used on 7 or 10 μm retinal cryosections of dogs from all four treatment groups to examine CNTF-mediated modifications with cell-specific markers. Cryosections were air-dried, blocked in a solution containing 10% normal serum from the appropriate species, and incubated overnight at 4°C with the primary antibody. Primary antibodies used in this study are listed in Table 1. Species-specific secondary antibodies conjugated to fluorochromes (Alexa Fluor, Invitrogen, Carlsbad, CA, 1: 200 dilution) were then applied to the sections for 1 hour. The antibodies were used at the appropriate dilution in 1.5% normal serum, 0.25% Triton X-100, 0.05% sodium azide in PBS. DAPI stain was used to label cell nuclei. Slides were mounted with a medium composed of polyvinyl alcohol and DABCO (1,4 diazabicyclo-[2.2.2]octane) (Gelvatol; Sigma-Aldrich, St. Louis, MO), and examined with an epifluorescence microscope (Axioplan; Carl Zeiss Meditec). Digitally captured images were imported into a graphics program (Photoshop; Adobe, Mountain View, CA) for display. When double fluorescence immunohistochemistry was done, primary antibodies (followed by their secondary) were applied sequentially.

In order to determine whether the α subunit of the CNTF receptor (CNTFRα) is expressed in normal and mutant photoreceptors during postnatal retinal maturation, as well as during the course of retinal degeneration, archival collections of normal and mutant retinas developed by our lab were used. These included retinas of normal, *rcd1*, and *XLPRA2* dogs (age: 4–24 weeks); some of these tissues have been used in other previous studies (Beltran et al., 2006). Enzymatic immunohistochemistry was performed on 7 μm thick cryosections from the superior meridian of these retinas as previously described (Beltran et al., 2003). A rabbit protein A-purified polyclonal antibody raised against a large fragment of the chicken CNTFRα recombinant protein [1:2,000 dilution; (Holst et al., 1997)] was used for this study. This antibody cross reacts in the dog as well as in other mammalian species (Beltran et al., 2003; Beltran et al., 2005).

Cell proliferation assays

To determine whether intravitreally-injected CNTF could cause cell proliferation, we conducted BrdU pulse-labeling experiments in two *XLPRA2* dogs. At 4 weeks of age both dogs were anesthetized with isoflurane and injected intravitreally in the left eye with 30 μl of a 0.1 M PBS solution containing 12 μg of CNTF and 10 μg of 5-Bromo-2′-Deoxyuridine (Sigma, St-Louis, MO); the contralateral eye was injected with 30 μl of a 0.1 M PBS solution containing 10 μg of BrdU. The dose of BrdU injected was extrapolated from the dose used in chick eyes (Fischer and Reh, 2000). Twenty four hours later, one dog was euthanatized and the eyes processed as described below. The second dog was re-injected under isoflurane anesthesia in both eyes with 30 μl of a 0.1 M PBS solution containing 10 μg of BrdU at 24 and 96 hours

following the initial injection. At 5 weeks of age, this animal was killed, and the eyes collected. Following enucleation, the anterior and posterior segments were separated and the vitreous was gently removed from the posterior eyecup. The tissues were then fixed for 30 min in 4% paraformaldehyde with 3% sucrose in 0.1 M PBS at 4°C. Following three washes in PBS, tissues were cryoprotected, trimmed and embedded in OCT as described above. Immunohistochemical detection of BrdU incorporation was done on 7 μm cryosections that were pretreated in 4 M HCl for 10 min, followed by overnight incubation with the primary monoclonal BrdU antibody (see Table 1). BrdU-positive cells were counted throughout the entire length (i.e. from optic disc to *ora serrata*) on a least three retinal sections for each of the superior, inferior and temporal meridians. Values for each meridian were averaged and expressed as the number of BrdU-positive cells per unit length of retina. The unit length was set at 10,000μm. Expression of the nuclear cell proliferation marker Ki67 (Table 1) was also examined in the retinas of these two dogs by immunohistochemistry.

RESULTS

Clinical findings and CNTF-mediated side effects

Ocular examinations performed immediately after the intravitreal injections did not reveal any lesions caused by the procedure. Yet, several ocular abnormalities were visible clinically within a few days following intravitreal injection of CNTF in both*XLPRA2* and *rcd1* dogs (Table 2). None of these changes were seen in any of the PBS-injected eyes. Therefore, the following description only applies to the CNTF-treated eyes.

Clinical signs of uveitis were the first to appear, and consisted mainly of miosis, intraocular hypotension, and occasional iridal changes (see below). Aqueous humor flare was not detected by slit lamp biomicroscopy during the first week following the injection. Later, examination of the aqueous humor transparency was hampered by development of corneal haze (see below). Miosis that was refractory to dilatation with topical mydriatics was seen in all dogs that were reexamined 2 days after the first CNTF injection, and persisted for approximately 2 weeks.

In 2 *XLPRA2* dogs (treatment Group # 4) that had lightly pigmented irides, vascular engorgement was seen 48 hours following injection. Anterior displacement of the iris, causing a reduction in anterior chamber depth, was also seen 2 days post-injection in 2 *rcd1* dogs. An increase in iris pigmentation was observed in 1 dog (treatment Group #3), and in 3 dogs from (treatment Group #2), respectively, at 17 and 51 days after the 1st injection of CNTF. Combined IOP values of *XLPRA2* (all treatment groups) and *rcd1* dogs, measured between post-injection days 2–4, were significantly lower ($P<0.0001$, paired Student t test) in CNTF- injected (mean: 7.1 mm Hg, min:4 mm Hg, max: 12 mm Hg; n= 15) versus PBS-injected (mean: 13.5 mm Hg, min:8 mm Hg, max:22 mm Hg; n= 15) eyes. All CNTF-injected eyes were hypotensive (IOP < 8 mm Hg, or IOP at least 5 mm Hg lower than in the PBS-treated eye) after the first or second injection. A steep decrease in IOP was seen during the first 4–7 days following CNTF injection, but values returned to pre-injection levels within 20 days. In PBS-injected eyes, a slight increase in IOP occurred that remained within normal levels [19.2 ± 5.9; (Gelatt and MacKay, 1998)] was observed for 10–14 days following injection. Similarly, IOP values returned to pre-injection values within approximately 20 days. Figure 2 illustrates the IOP changes observed in a subset of the experimental dogs.

Corneal epithelial abnormalities were observed by external and slit-lamp examination in 15/16 of the eyes injected with CNTF (Figure 3A). Discrete lesions that caused a hazy patch in the corneal epithelium could be observed in some animals as early as 3–4 days following CNTF injection. By post-injection day 11, the corneal epithelial haze was present in all 15 affected animals. These epithelial changes progressed with time, and could involve more than 75% of the corneal surface. However, a peripheral band located in the superior part of the cornea was

never affected, and remained optically clear. The lesions persisted throughout the entire treatment period, and were never associated with corneal ulceration. Histological examination revealed thinning of the epithelium with attenuation of the basal and suprabasilar (wing) cell layers (Figure 4A,B). No corneal abnormalities were observed in any of the PBS-injected eyes.

Cataracts were commonly observed following CNTF injection (15/16 eyes). Examination of the lens was hampered during the first 2 weeks by the intense miosis and difficulty in fully dilating the pupil. By post-injection day 14, posterior sutural cataracts could be observed in CNTF-injected eyes of *XLPRA2* (8/12 eyes), *rcd1* (3/3 eyes), and non-affected (1/1 eye) dogs (Figure 3B). Cataracts often progressed by involving the posterior and anterior cortex (Figure 3C). Histological examination of some cataractous lenses confirmed the predominant involvement of the posterior suture lines and cortex (Figure 4C, D). No lenticular opacities were detected in any of the PBS-injected eyes.

A thorough funduscopic examination was difficult to conduct in the CNTF-injected eyes, initially because of the associated miosis, and later on due to the loss of transparency of the ocular media caused by corneal epitheliopathy and lens opacities. In those animals in which areas of the fundus were visible, no abnormalities were detected. One exception was seen in the non-mutant control dog that had multiple small “doughnut” shaped foci scattered throughout both the tapetal and non-tapetal regions of the fundus (data not shown). Although this was an isolated observation in this study, we saw a similar type of lesion in a normal dog injected with a similar dose of CNTF in a previous pilot study.

Gross examination of the globes at the time of enucleation did not reveal any differences in size between PBS- and CNTF-injected eyes.

CNTF fails to prevent photoreceptor cell loss in *XLPRA2*

The average ONL thickness in each quadrant was determined as a means of assessing the photoreceptor survival effect of CNTF. Using this measure, there was no statistically significant difference in any of the 4 treatment groups between the CNTF and PBS-injected eyes (Figure 5 and 6). The mean difference in ONL thickness of 0.90 rows of nuclei (Group #1: 0.98 ± 0.52; Group #2: 0.77 ± 0.69; Group #3: 0.94 ± 0.63) between the CNTF versus PBS-treated eyes also was not biologically relevant. Based on the kinetics of photoreceptor cell loss, (Beltran et al., 2006) a difference of at least 2 rows of nuclei (treatment Groups #1 and #3), and 3 rows of nuclei (Group #2), would have been expected in both the superior (Figure 7) and inferior meridians if CNTF had caused a complete rescue of photoreceptors from the time of the first injection. Further confirming the absence of any significant neuroprotective effect of CNTF in the *XLPRA2* retina was the presence of morphological alterations at the level of the photoreceptor inner (IS) and outer (IS) segments that were characteristic of the disease. Disruption of the OS, shortening and broadening of the IS, and reduction of the subretinal space was seen in all groups, either CNTF- or PBS-treated, suggesting that the natural course of the disease had not been arrested or modified.

In the *rcd1* control group, we observed a statistically significant rescue of photoreceptors with CNTF (Figure 5 and 6) The mean (± SD) difference in ONL thickness between the CNTF and PBS injected retinas was of 1.5 ± 0.56 rows of nuclei, and consistent with findings previously observed (Pearce-Kelling, unpublished). In the 7 week-old *rcd1* retina, the ONL has already lost approximately 40% – 50% of its photoreceptors, and has an average thickness of 5 to 6 rows of nuclei along the superior meridian (Schmidt and Aguirre, 1985). In this study, the mean ONL thickness of the superior retina at 14 weeks of age was 4.4 and 2.5 rows of nuclei in the CNTF- and PBS-injected eyes, respectively. This indicates that although statistically significant, there is not a total rescue of photoreceptor cells in this model following an intravitreal injection of CNTF at 7 and 10 weeks of age.

To determine whether the lack of CNTF-mediated rescue in *XLPRA2* could be explained by the absence of expression of the α subunit of the CNTF receptor, immunolocalization of CNTFRα was done on retinas from *XLPRA2* as well as in *rcd1* and normal dogs, at ages ranging from 4 to 24 weeks. The pattern of labeling was similar to that previously reported for the normal adult canine retina (Beltran et al., 2003), and was characterized by an intense staining of the IS, inner nuclear layer (INL), ganglion cell layer (GCL) and nerve fiber layer (NFL). At the earliest age examined (4 weeks; Figure 8 A_1–A_3), CNTFRα-immunoreactivity was present in both rod and cone IS. With progression of both diseases, the shortening of rod IS, and their subsequent disappearance, photoreceptor labeling was restricted to the remaining cone IS (Figure 8 B_2, C_2, C_3).

CNTF causes peripheral remodeling in *XLPRA2* retinas

A consistent finding in all 4 quadrants of CNTF-injected eyes of *XLPRA2* dogs enrolled in treatment Groups #1, #2, and #3 was abnormal changes in the retinal periphery. These alterations included a loss of IS and OS, an increase in ONL thickness, and misplaced rod-like nuclei in the INL (Figure 9 and Table 3). These changes were disease- (*XLPRA2*) and age-specific, since they were not present in either the *rcd1* control group, the single non-mutant dog, or the *XLPRA2* mutants treated for the first time at 12 weeks of age (Group #4).

A remarkable feature associated with peripheral ONL remodeling was the loss of both photoreceptor IS and OS that caused, in some areas, the retinal pigment epithelium (RPE) to be in direct apposition with the external limiting membrane (ELM) (Figure 9 A_2, B_2, C_2). This loss of the photoreceptor layer (PRL) extended over distances (labeled "*l*" in Table 3) that were usually shorter, although occasionally equal, than the lengths (labeled "L" in Table 3) over which an increase in ONL was noted. In animals from Group #4, there was rarely a complete loss of the PRL, yet loss of OS and significant shortening of IS was occasionally seen (Figure 9 D_2).

When combining results from all 4 meridians of treatment Groups #1, #2, and #3 (Table 3), the mean distance (L) from the *ora serrata* over which an increase in ONL thickness could be observed in the CNTF-treated eye was L:2,075 μm (L min: 500 μm; L max: 5800 μm). The longest expanse of altered ONL was seen in the superior meridian, and then in the inferior meridian. The peripheral ONL remodeling was most extensive in animals from treatment Group #2. The increase in ONL thickness was associated with an increase in spacing between nuclei, such that the density of photoreceptors appeared to be reduced following CNTF injection. Because of this, and due to the misalignment of layers of photoreceptor nuclei, the thickness of the ONL was measured in micrometers rather than in number of rows of nuclei. This was done at the site of highest ONL thickness (Labeled "D" in Table 3). If, an equal increase in ONL thickness was observed throughout an extended length of ONL, the area that was selected for analysis was the one located the furthest distance from the *ora serrata*. At that site, there was a 1.94 fold average increase (R min: 1.22; R max: 3) in ONL thickness following CNTF admistration in comparison to the corresponding region of the PBS-treated eye [Figure 9 and Table 3 ("R" ratio)].

Finally, misplacement of rod-like nuclei in the outer plexiform layer (OPL) and INL caused a disorganization of the typical layering of the retina. A wavy aspect of the vitreal edge of the ONL was responsible for the frequent loss of a clear delimitation by the OPL of both nuclear layers (Figure 9 A_2, B_2).

To determine the nature of the cell population(s) responsible for this apparent increase in cell number in the ONL, immunohistochemical studies were performed using several cell-specific markers on *XLPRA2* retinas from all 4 treatment groups. The results confirm that the overwhelming majority of cells present in the peripheral ONL, and migrating to the INL of

CNTF-treated eyes (Groups #1–3), are rod opsin-positive (Figure 10 A_2). Although we have previously shown in *XLPRA2* (Beltran et al., 2006) a mislocalization of rod opsin to the soma and axons of rods (see Figure 10 A_1) similar to that reported by others (Li et al., 1995), this was more pronounced in retinas examined 4 to 8 weeks after CNTF treatment. In addition, in contrast to what is seen in the untreated *XLPRA2* retina (Beltran et al., 2006), there was absence of rod opsin labeling in rod OS and IS in the periphery, thus, further confirming the loss of these structures following CNTF treatment (Figure 9 A_2). The use of a cone arrestin antibody also confirmed the presence of heterotopic cone somas in the middle and inner portions of the ONL. These cells had neither an axon nor an IS (Figure 10 A_2). One week following the injection of CNTF in a 4 week-old *XLPRA2* retina, the migration of cone somas down their axon was seen in the periphery (Figure 10 B_3).

Rod opsin and cone arrestin immunoreactivities were also examined in *XLPRA2* retinas 1 and 7 days following an injection of CNTF, and a significant decrease in intensity of labeling was seen in comparison to the PBS-injected eye (Figure 10 B_1, B_2). This decrease in rod opsin and cone arrestin expression which was more pronounced 1 week post-CNTF injection was seen throughout the entire length of the retinal sections, but was most prominent in the periphery. This reduction in immunoreactivity was not present 4 weeks after the injection.

Peripheral remodeling of the retina also involved cells of the INL. The use of cell markers for bipolar cells showed significant dendritic sprouting into the peripheral ONL, as well as heterotopia of PKCα/Goα-positive cells (two markers for rod bipolar cells) (Figure 10 C_1, C_2). Similarly, radial extension into the ONL of processes originating from calretinin-immunoreactive horizontal cells was observed in the peripheral retina (Figure 10 D_1, D_2). CNTF treatment also caused misplacement in the ONL of the somas of some CRALBP-positive Müller cells (Figure 10 E_1, E_2). Glial fibrillary acid protein (GFAP) immunoreactivity was also examined and showed a similar labeling of the entire cell body of Müller cells (data not shown) in both CNTF- and PBS-treated eyes. This is not surprising since GFAP activation of Müller cells is known to occur at the ages examined (Beltran et al., 2006).

CNTF causes an increase in cell number in the peripheral *XLPRA2* retina

To determine whether the thickening of the ONL was caused solely by increased internuclear spacing, or by a higher number of ONL cells, plastic embedded retinal sections of two *XLPRA2* dogs were examined; each dog was treated using the same protocols as for Group #1 (Inj. 4 wk; Ter. 8 wk), and Group #2 (Inj. 4 & 8 wk; Ter. 12 wk), respectively. These 1-μm thick sections permitted individual cell counts (Figure 11) which could not be done on the thicker (7 or10 μm) cryosections where substantial overlap of nuclei occurred. Examination of the peripheral retina of the CNTF-treated eyes confirmed that there was more space between the photoreceptor nuclei, although it could not be determined whether this resulted from intra- or extracellular swelling. Elevation of the external limiting membrane, suggesting cytoplasmic swelling of photoreceptors or Müller cell processes, has been reported in both *rcd1* and non-mutant dogs implanted with a long-term CNTF release device (Zeiss et al., 2006). This was not observed in the current study. In addition to heterotopic rod nuclei in the INL (Figure 11 C_2; arrows), there were cells with larger nuclei within the thickness of the ONL (arrowheads). These nuclei contained fewer chromatin clumps than rods, and resembled that of cones. Nuclear count in both CNTF- and PBS-injected eyes was done at the site of highest ONL thickness as described above. Although statistical analysis could not be done due to the limited number of observations, we observed a consistently higher number of cells in the CNTF-treated peripheral retina (Table 4).

To determine how early following an injection of CNTF an increase in ONL thickness is observed in the peripheral *XLPRA2* retina, and to determine if this is caused by cell proliferation, BrdU pulse labeling and Ki67 immunohistochemical studies were performed in

two dogs. No major differences in ONL thickness, BrdU incorporation, or Ki67 immunolabeling were observed 24 hours following CNTF injection (data not shown). Yet, a major increase in ONL thickness was observed in the dog whose retina was collected one week post-CNTF administration (Figure 12A, B). Although BrdU incorporation was seen in cells located at the peripheral retinal margin (Figure 12 D_1, D_2), and in the NFL, GCL, and INL throughout the entire length of the retina of both CNTF- and vehicle-treated eyes, there was a higher number of BrdU-positive nuclei with CNTF (Table 5). Indeed, combining results from three meridians, a mean of 47 and 135 cells per 10,000 μm of retina length, was found in the PBS- and CNTF-injected eyes, respectively. In addition, rare BrdU-positive cells were found exclusively in the ONL of the CNTF-treated retina (Figure 12 D_3). A similar finding was observed with Ki67 (Figure 12 E_2).

DISCUSSION

XLPRA2 is an early onset model of X-linked retinitis pigmentosa characterized by an early burst of photoreceptor cell death that begins at 4 weeks of age and reaches a peak at 7 weeks. This event is followed by a more gradual loss of rods and later of cones that persists for over 9 months (Beltran et al., 2006). An attempt to rescue photoreceptors from this initial phase of cell death with intravitreal injections of CNTF failed to promote in *XLPRA2* a similar neuroprotective effect as seen in the *rcd1* dog (Tao et al., 2002). A peculiar set of findings that were exclusively observed in *XLPRA2* consisted of remodeling of the retinal periphery and an increase in cell number in the ONL. In addition, ocular toxic effects that involved the cornea, lens, and uveal tract were seen in both mutants and normal dogs.

Intravitreal injection of survival factors in animal models of RP has been used routinely to establish proof of principle of their photoreceptor rescue properties (LaVail et al., 1992). Yet, a recognized limitation to this route of administration for the treatment of retinal degenerations is the short half-life of these agents (Cayouette et al., 1999), and their associated ocular side-effects (Faktorovich et al., 1990; Faktorovich et al., 1992; Lewis et al., 1992; Perry et al., 1995). In this study, corneal epitheliopathy, cataracts, and clinical signs of uveitis were observed within a few days following a single intravitreal injection of 12 μg of CNTF. Similar clinical findings were observed in the *rcd1* dog (Pearce-Kelling, unpublished) and *Rdy* cat (Chong et al., 1999) treated with Axokine® (Regeneron, Tarrytown, NY), a recombinant mutein of human CNTF. These side-effects appear to be dose-related because they are not seen in *rcd1* dogs that are intravitreally implanted with a long-term delivery device that releases a daily dose approximately 1000 fold lower (Tao et al., 2002).

Several hypotheses may explain the absence of a positive rescue effect in photoreceptors of the *XLPRA2* dog: 1) A lack of activation of intracellular signaling pathways may be responsible for the absence of a survival response. This study showed that the α subunit of the CNTF receptor (CNTFRα) was expressed throughout the course of retinal degeneration in *rcd1* and *XLPRA2*, but we did not investigate whether CNTF triggered any cell signaling pathways. Therefore, it is not known whether the binding of CNTF to CNTFRα activates the same molecular signaling cascades in both diseases. 2) The activation of anti-apoptotic molecules may not interfere with the molecular mechanisms of cell death that are involved in *XLPRA2*. Identifying the key molecular events that occur in different models of photoreceptor degeneration may reveal a spectrum of cell death pathways that respond differentially to survival factors. This may be the basis for the difference in response between *rcd1* and *XLPRA2*, and could explain CNTF's positive rescue effect in the *rd/rd, nr/nr* and Q344 ter mice, and its absence in the *rds/rds, pcd/pcd*, P23H and VPP mice (LaVail et al., 1998). 3) In the mutant, there may be activation of a stimulus so detrimental to the cell's homeostasis that its effects can not be countered by the survival factor. We intentionally chose to target the initial burst of photoreceptor cell death that occurs between 4 and 7 weeks of age in

XLPRA2 (Beltran et al., 2006) as an approach to inhibit as early as possible the onset of the disease, and rescue the highest number of cells. However, this may not have been the optimal time-window for therapy, if the putative toxic gain of function caused by the *RPGR* frameshift mutation is at its highest level during this period. 4) Finally, we cannot exclude the possibility that bolus intravitreal injections of CNTF may not be the optimal route of administration to detect a potent rescue effect in this model. The rate of cell loss in *XLPRA2* is rapid, yet slightly slower than that observed in *rcd1*; and a rapid elimination of CNTF from the eye may have prevented the prolonged activation necessary to elicit a full rescue response in this model. The treatment of *XLPRA2* retinas with CNTF caused a mild increase in ONL thickness (~ 1 row of nuclei) in comparison to that obtained with PBS. Yet, this difference was below that expected from a total rescue, and, within the time-window of this study, may not be biologically relevant. Confirmation of a potential neuroprotective effect in *XLPRA2* would require the use of a long-term delivery device to achieve sustained bioavailability of CNTF over a longer period of time.

Quantitative methods were not used to measure the levels of expression of rod opsin and cone arrestin, yet immunohistochemical results suggested that CNTF caused a rapid but transient decrease in the expression of these two proteins. It was seen as early as 24 hours following the injection, and was more pronounced 7 days later. Several groups have reported that CNTF causes a transient and reversible inhibition of rhodopsin in postmitotic opsin-negative rod precursor cells in rodents (Ezzeddine et al., 1997; Neophytou et al., 1997; Kirsch et al., 1998; Caffe et al., 2001; Schulz-Key et al., 2002; Zahir et al., 2005). However, a significant difference between these studies and our, is that we tested the effect of CNTF on photoreceptors that are at a more advanced stage of development. At 4 weeks of age in the canine retina, photoreceptors although not fully mature (IS are budding) already express rod opsin and cone arrestin. Thus, the effect of CNTF reported in the present study appears to be a down-regulation of phototransduction proteins rather than an arrest in rod development. This negative regulation in the expression of phototransduction proteins has been also reported in mature photoreceptors of the normal rat (Song et al., 2005), and *rcd1* dog (Zeiss et al., 2006) following intravitreal administration of CNTF. Such an effect may be responsible for the electroretinographic alterations reported in rat and mouse models of retinal degeneration, as well as in the normal albino rabbit following intraocular delivery of CNTF (Bush et al., 2000; Liang et al., 2001; Bok et al., 2002; Schlichtenbrede et al., 2003).

Retinal remodeling associated with photorececeptor degeneration as been reported by several groups in humans with RP (Fariss et al., 2000; Jones et al., 2003), as well as in animal models (Strettoi and Pignatelli, 2000; Fisher and Lewis, 2003; Marc et al., 2003; Pignatelli et al., 2004; Jones et al., 2005; Beltran et al., 2006). In this study, a striking finding was the prominent remodeling that occurred in the peripheral retina of *XLPRA2* dogs following intravitreal injection of CNTF. These retinal alterations consisted of loss of IS and OS, neuronal sprouting, heterotopia of retinal cells, and increase in the number of rods that contributed to the thickening of the ONL. The changes were disease- and age-specific. Indeed, they were only observed in *XLPRA2* dogs, and only if the first intravitreal injection of CNTF was done no later than at 7 weeks, an age at which the normal canine retina is reaching full maturation (Farber et al., 1992).

The disorganization in the normal retinal layering was associated with the loss of inner and outer segments, which has been also observed by others (Schlichtenbrede et al., 2003; Song et al., 2005), Similar alterations were found in the retinas of transgenic mice that constitutively express leukemia inhibitory factor (Graham et al., 2005; Sherry et al., 2005). Although IS are beginning to bud in the 4 week-old canine peripheral retina, IS and OS are fully formed when the retina has reached maturity at 7–8 weeks of age. CNTF, therefore, may have prevented the formation of IS and OS when injected at 4 weeks of age (treatment Groups #1 and #2), and caused their loss when injected later in 7 week-old dogs (treatment Group #3). Our findings

suggest that CNTF may not only arrest photoreceptor maturation when administered at 4 weeks of age in the *XLPRA2* dog, but also cause them to loose structural and molecular markers that are characteristics of a mature photoreceptor. We propose that the loss of these phenotypic markers may represent a form of dedifferentiation. To the best of our knowledge we are unaware of reports of such a process in photoreceptor cells, yet, this is not unexpected since CNTF has recently been shown to induce the dedifferentiation of adult human myoblasts into multipotent progenitor cells (Chen et al., 2005), as well as the transformation of striatal astrocytes toward a more immature and activated phenotype (Escartin et al., 2006).

CNTF is known to elicit the formation of new neuritic processes in rodent motor neurons and this process is currently being investigated as a potential approach to treat partial denervation and neuromuscular paralysis (Gurney et al., 1992; Siegel et al., 2000). More recently, it was reported that gene delivery of CNTF in a feline model causes neurite extension from rods, bipolar, and horizontal cells, and such a process may be seen as a detrimental effect (Sethi et al., 2005). We observed in this study a similar effect in the *XLPRA2* dog. This may be caused by an alteration in neurotransmission at the synaptic terminal between rods, horizontal, and bipolar cells, as a result of a negative regulation on the phototransduction pathway. Another feature of the retinal remodeling that took place in the periphery was heterotopia of some rod, cone, bipolar, and Müller cell somas. These findings share some similarities with observations made in mouse and rat retinas exposed *in vitro* to CNTF. In these species there is currently still some controversy as to whether CNTF causes a phenotypic “switch” by turning rod precursor cells into bipolar cells (Ezzeddine et al., 1997; Zahir et al., 2005), or whether it transiently inhibits rod opsin expression and increases the expression of bipolar cell markers in rod precursor cells (Neophytou et al., 1997; Kirsch et al., 1998; Schulz-Key et al., 2002). In our study the origin of the PKCα/Goα and CRALBP positive cells located in the ONL remains unknown. These could be rod precursor cells that have undergone a change in cell fate, mature rods that have lost some of their specific photoreceptor markers and now express bipolar or glial cell markers, or alternatively, mature bipolar neurons and Müller cells that have migrated to an ectopic location. We do provide evidence that misplaced cones represented somas that had moved down the cone axon.

The most prominent feature of the peripheral remodeling was the abnormal increase in ONL thickness that has also been reported in normal rabbits implanted with ECT devices secreting a high dose (22ng/day) of CNTF (Bush et al., 2004). In the present study, the thickened ONL could be seen extending several millimeters away from the*ora serrata*, and was detected as early as one week following CNTF administration. Although this was undoubtedly associated with some degree of intra- and/or extracellular swelling, an increase in the number of rod opsin-positive cells was the major contributing factor. The extent of the increase in cell number could not be explained only by a neuroprotective effect. The use of cell proliferation markers confirmed that CNTF caused a higher number of cells to enter the cell cycle. Yet, the number of BrdU- or Ki67-positive cells that were found in the ONL was limited, and could not account completely for the supernumerary nuclei seen in this layer. The most plausible explanation for this discrepancy has to do with the period of cell proliferation, and the time-points at which tissues were collected. It is indeed possible that, despite doing repeated injections of BrdU, a high enough concentration may not have been attained at the optimal time for its incorporation. Similarly, one week following CNTF injection, the great majority of these cells may have exited the cell cycle and were no longer Ki67-positive. Addressing this issue would require determining the precise time of onset of the cell proliferation, and performing additional BrdU pulse-labeling experiments. This will be the subject of future studies.

The increased number of cells in the ONL raises the question of their origin. Although there is recent evidence for the presence of retinal progenitor cells at the retinal margin of $ptc^{+/-}$ mice (Moshiri and Reh, 2004), monkeys, and humans (Cuenca et al., 2005), it is unlikely that

neurogenesis in this zone may account for all the additional cells that are seen over distances as far as 5,800 μm from the *ora serrata*. Recently, a pool of mitotic retinal progenitor cells was found distributed in the central retina of the adult Chx10$^{-/-}$ mouse (Dhomen et al., 2006). Therefore, it may be possible that in *XLPRA2*, CNTF induces proliferation of such precursor cells (Zhang et al., 2005). Finally, another possibility is that CNTF causes rods to dedifferentiate and subsequently re-enter the cell cycle. The elimination of CNTF from the eye would cause the cells to stop proliferating, and assume a more differentiated phenotype.

The increase in cell number in the retinal periphery raises the question as to whether this response represents a rescue effect. We do not consider it to be neuroprotection because such a mechanism of cell survival implies the preservation of existing cells in a differentiated state and without proliferation. If proliferation could be considered beneficial, it would require that the proliferated cells differentiate; a situation that was not observed in this study.

In summary, this study shows that intravitreal injection of CNTF does not prevent the early phase of cell death that occurs in a canine model of X-linked retinitis pigmentosa caused by a frameshift mutation in *RPGR* exon ORF15. Whether CNTF can rescue photoreceptors from the later phase of degeneration now needs to be addressed. This should provide further evidence as to whether human patients with similar mutations in *RPGR* would be expected to respond to this specific neuroprotective agent. CNTF also caused substantial remodeling in the peripheral retina of *XLPRA2* dogs. These peripheral alterations are most likely age-, dose-, and disease-specific. They were only observed when CNTF was injected before the canine retina had reached full maturation. Therefore, although this may not be an issue for adult human patients treated with sustained release of CNTF (Sieving et al., 2006), peripheral retinal changes observed in normal adult rabbits (Bush et al., 2004), and the suggestion that endogenous secretion of CNTF may be the cause of cell proliferation in humans with mutations in the *NR2E3* gene (Jacobson et al., 2004) warrants further investigation.

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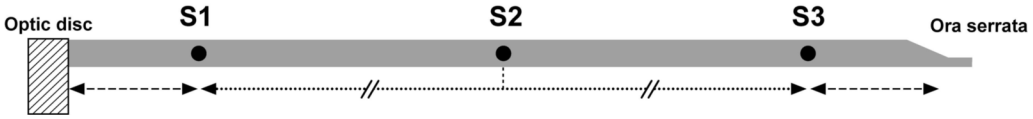

Figure 1.
Schematic of a retinal section showing the location of the sites S1, S2, and S3. S1: 2000 ± 500 μm from the optic disc; S3: 2000 ± 500 μm from the ora serrata; S2: equidistant (± 500 μm) from S1 and S3.

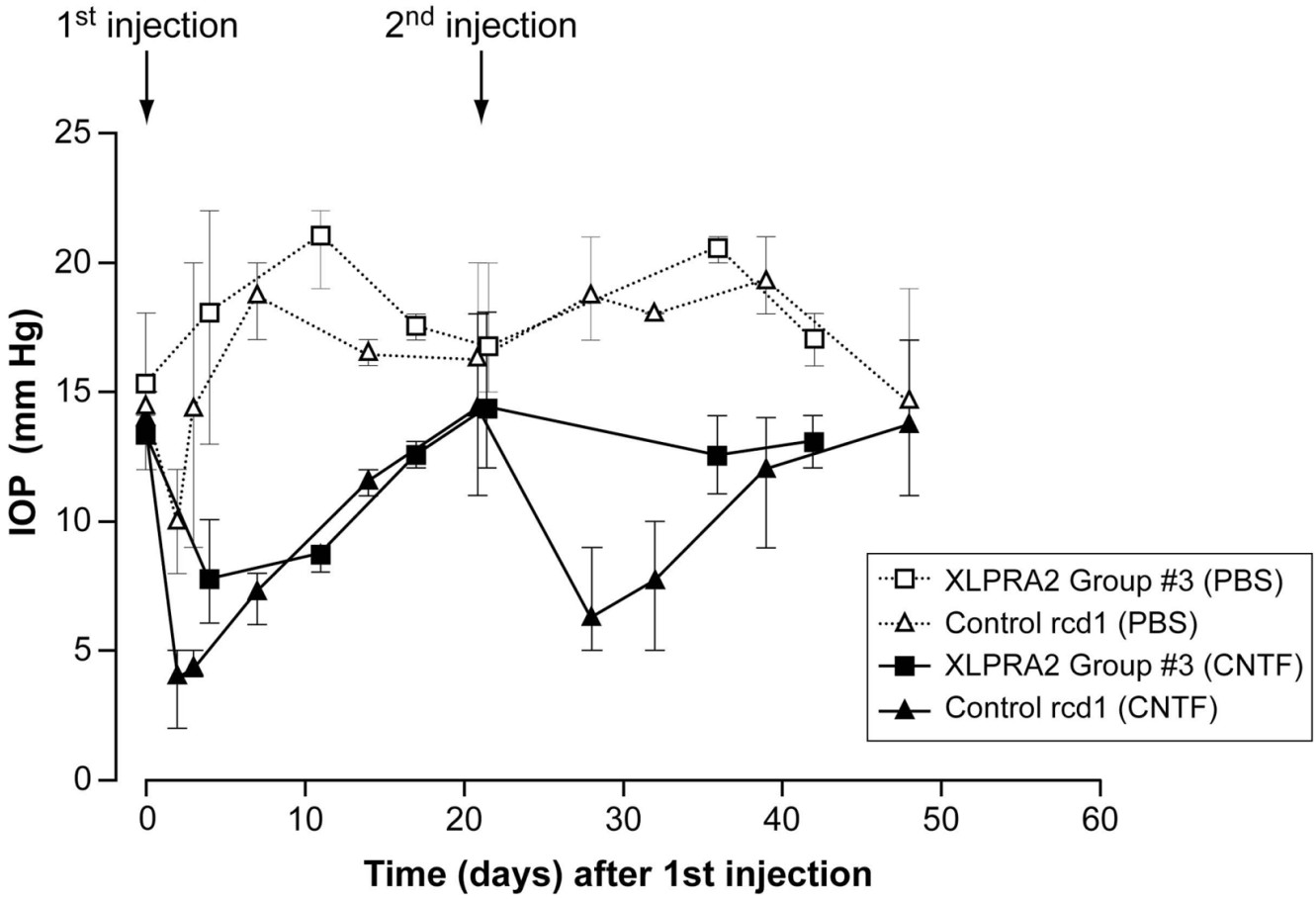

Figure 2.

Values (mean, and range) of intraocular pressure in CNTF- and PBS-treated eyes. Data is provided for dogs from treatment Group #3 (*XLPRA2*; n=3), and *rcd1* control group (n=3) that were injected at 7 and 10 weeks of age. Pre-injection values of intraocular pressure are indicated at time 0. In the days following both CNTF injections a decrease in IOP was observed. The intraocular hypotension was transient, since IOP values returned to pre-injection values within approximately 20 days.

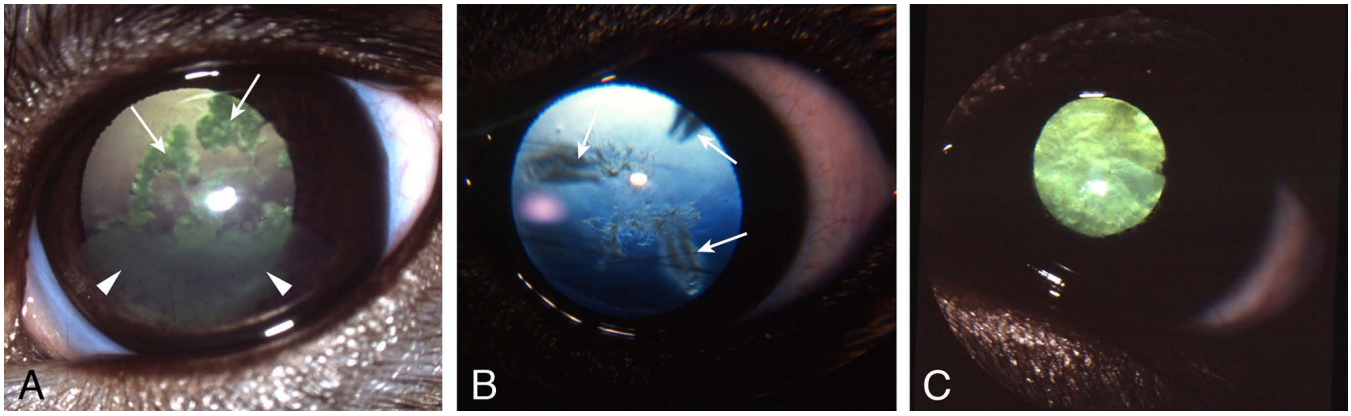

Figure 3.
Ocular lesions caused by intravitreal injection of CNTF. (**A**) Corneal epithelial haze (*arrowheads*), and posterior subcapsular cataracts (*arrows*) 7 weeks post-injection. A corneal haze and sutural cataract had been detected in this dog 11 days following the first CNTF injection. (**B**) The earliest lens changes involved the extremities of the posterior Y sutures (arrows). (**C**) Immature cataract with anterior and posterior cortical opacification.

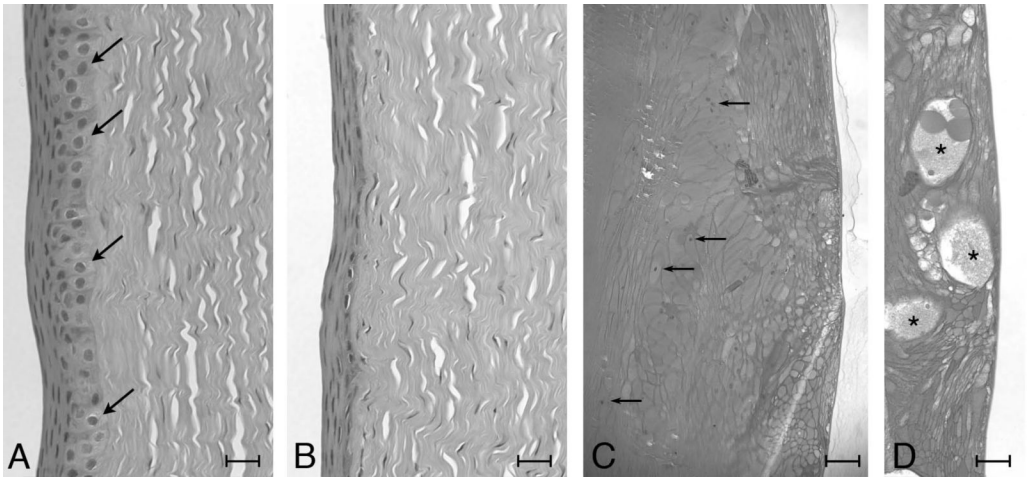

Figure 4.

Histological lesions of the cornea and lens present after intravitreal injection of CNTF. (**A**) Normal aspect of the corneal epithelium in a PBS-injected eye. Note the presence of normal basal cells (*arrows*). (**B**) Cornea of the contralateral eye that was injected with CNTF. Note the thinning of the corneal epithelium due to the loss of the basal and suprabasilar (wing) cells. (**C**) Cataract caused by CNTF. Swelling and disorganization of lens fibers was observed in the posterior cortex, and was associated with the retention of lens fiber nuclei (*arrows*). (**D**) High magnification of the posterior subcapsular region of the same lens as in (C). Note the presence of large vacuoles that contain eosinophilic material (*asterisks*), and swollen lens fibers. H&E stain; scale bars: (**A**, **B**) 20 μm; (**C**) 80 μm; (**D**) 40 μm.

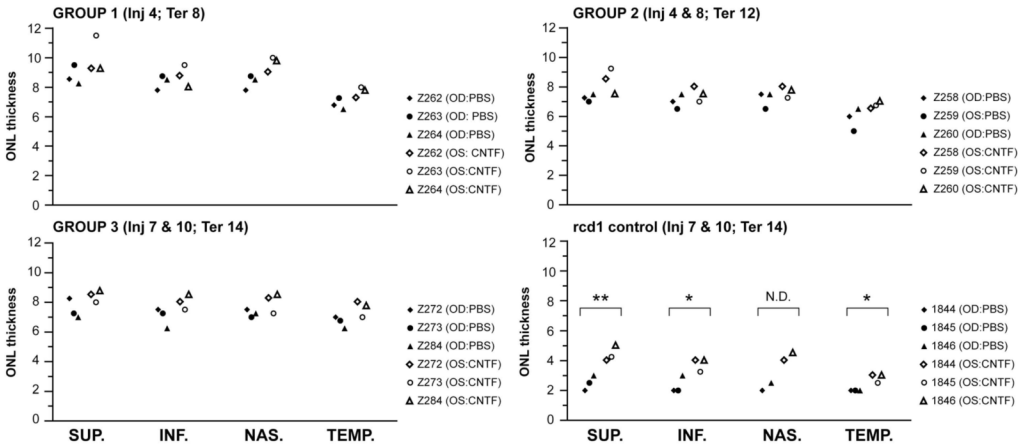

Figure 5.

Scatter plots comparing the ONL thickness (mean number of rows of nuclei at S1 and S2 sites combined) along the four meridians of eyes injected with CNTF or PBS. No statistically significant difference in ONL thickness was seen in any of the *XLPRA2* treatment Groups #1 – #3. CNTF caused a statistically significant increase in ONL thickness in *rcd1*. *: < 0.05; **: < 0.001; ND: not determined; OS: left eye; OD: right eye.

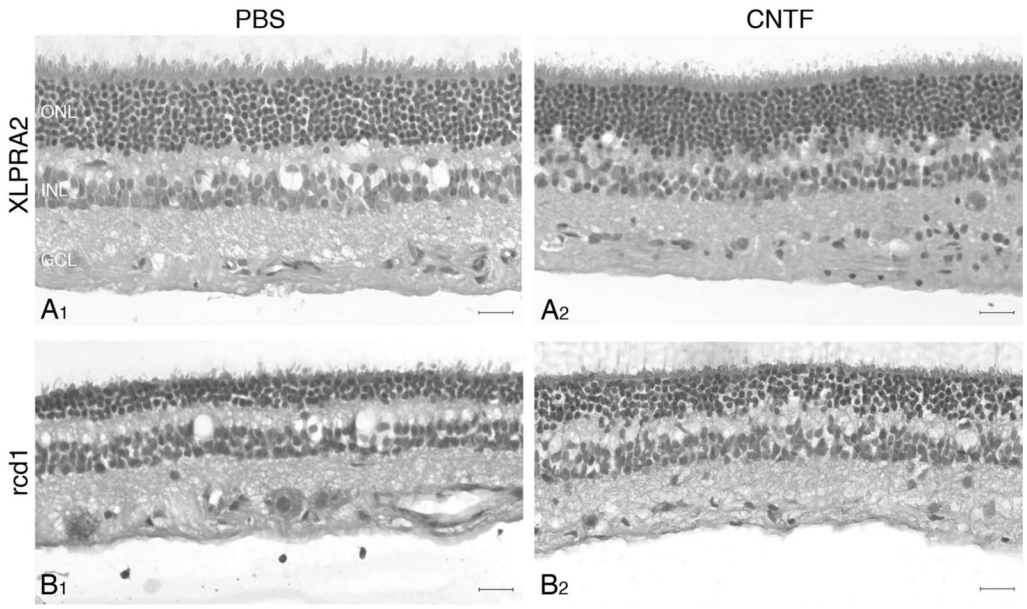

Figure 6.

Illustration of the effect of CNTF on outer nuclear layer thickness in the mid-peripheral retina (Site S2) of a 14 week-old *XLPRA2* dog (treatment Group #3) (**A_2**), and a 14 week-old *rcd1* dog (*rcd1* control group)(**B_2**). Contralateral eyes served as controls and were injected with PBS (**A_1**, **B_1**). CNTF caused an increase in the number of photoreceptor nuclei in *rcd1* (compare **B_2** to **B_1**), but no differences were seen in *XLPRA2* (compare **A_2** with **A_1**). CNTF often caused the vitreal border of the ONL to appear wavy (**A_2**, **B_2**). On these cryosections, RPE separation from the neuroretina was an artifact. H&E stain; scale bars: 20 μm.

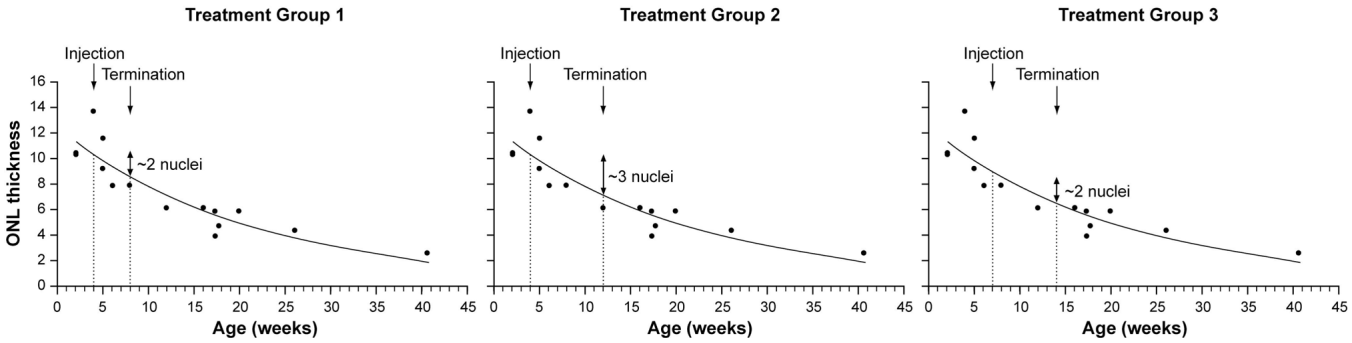

Figure 7.

Rate of photoreceptor cell loss in the *XLPRA2* retina as a function of age. ONL thickness was measured as the number of rows of photoreceptor nuclei. The figure illustrates for the superior meridian the expected decline in ONL thickness in the absence of a photoreceptor rescue effect for each treatment Group #1, #2, and #3 between the time of 1st injection and termination. Double headed arrows and numbers show the expected change in number of nuclei between these 2 time points for each treatment group. Note that treatment Groups #2 and #3 received a second injection, respectively, at 8 and 10 weeks of age. Modified (with permission) from Figure 2A in Beltran et al. *IOVS*, 2006; 47: 1669–1681.

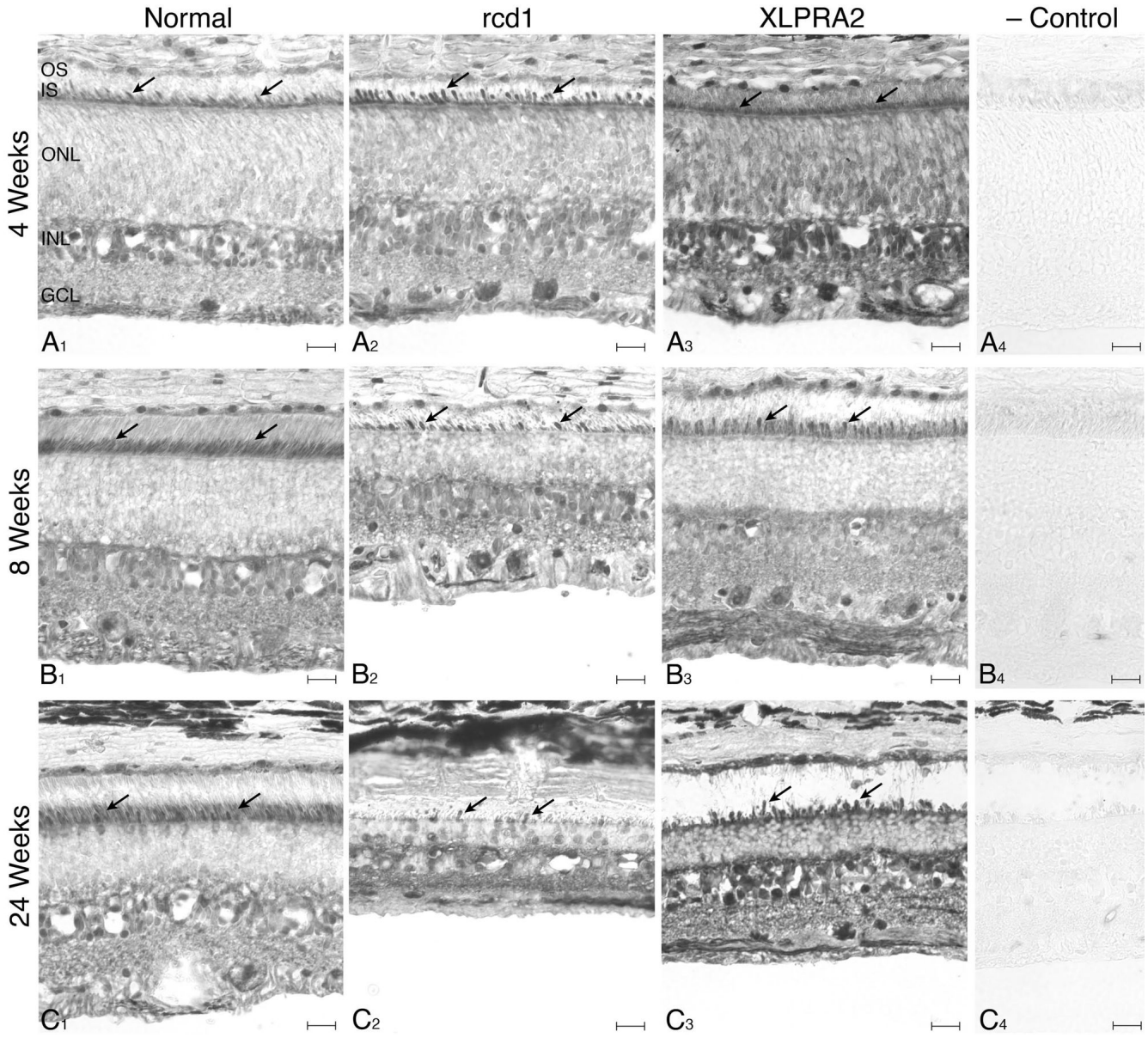

Figure 8.

Immunohistochemical localization of CNTFRα in the retinas of normal, *rcd1*, and *XLPRA2* dogs of different ages. Strong immunoreactivity for CNTFRα was seen in the GCL, INL and inner segments (IS, *arrows*) in the normal and mutant retinas at 4 weeks of age (**A_1–A_3**). At 8 weeks of age, intense labeling is seen throughout the IS of rods and cones in the normal retina (**B_1**). In the mutant retinas, labeling persists in the IS of both rods and cones in *XLPRA2* (**B_3**), but is essentially limited to cone IS in *rcd1* which is the predominant cell class remaining in the PRL at this stage of disease (**B_2**). (**C_1–C_3**) At 24 weeks of age, CNTFRα-positive cone IS and somas are still seen in *rcd1* and *XLPRA2* despite substantial loss of photoreceptors. Negative controls (**A_4**, **B_4**, **C_4**) were *XLPRA2* retinas that were treated with the omission of the primary antibody. Scale bars: 20 μm.

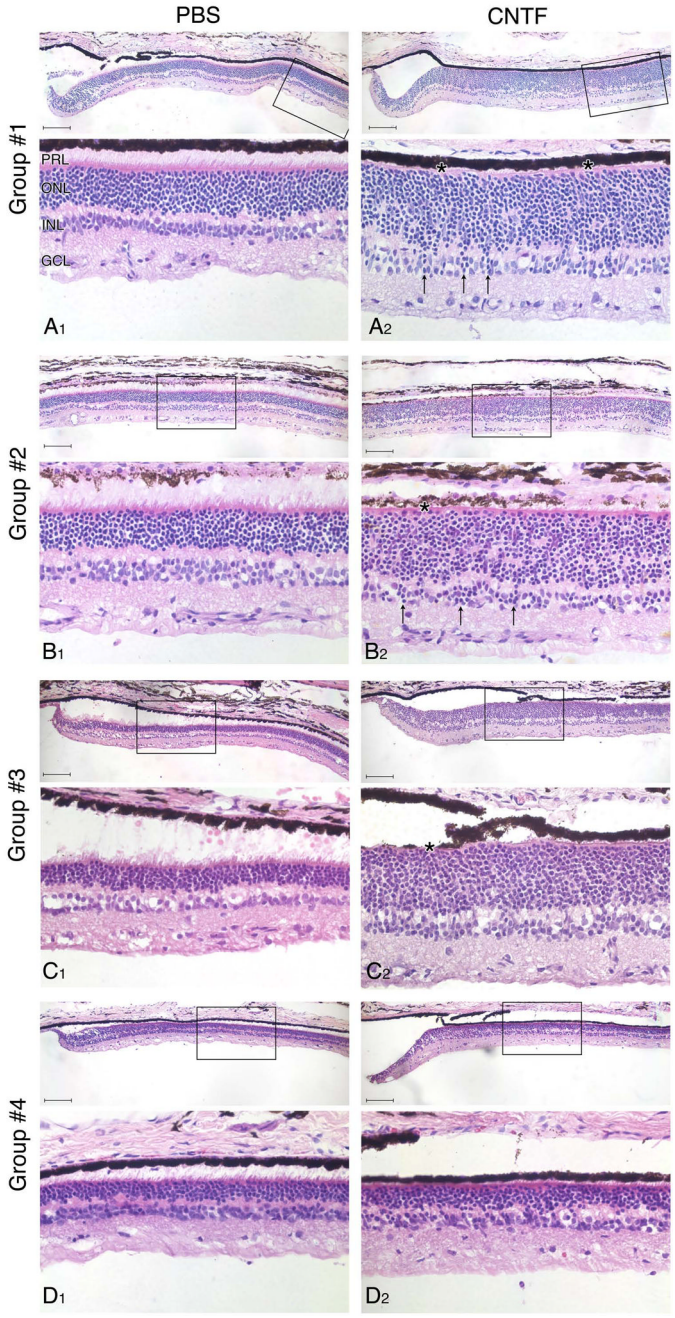

Figure 9.

Illustration of the morphologic changes observed in the peripheral retina of *XLPRA2* dogs following intravitreal injection with CNTF. An example from each of the 4 treatment groups is shown at the site where the measured ONL thickness was maximal. This same location in the contralateral (PBS-injected eye) is shown for comparison. The boxed areas in the low power photographs are shown in higher magnification. (**A_1**, **A_2**) Inferior periphery (1,000 μm from the *ora serrata*) in an 8 week-old dog (Z263; treatment Group #1). (**B_1**, **B_2**) Superior periphery (3,900 μm from the *ora serrata*) in a 12 week-old dog (Z259; treatment Group #2). (**C_1**, **C_2**) Temporal periphery (550 μm from the *ora serrata*) in a 14 week-old dog (Z272; treatment Group #3). (**D_1**, **D_2**) Superior periphery (680 μm from the *ora serrata*) in a 15.6 week-old dog

(Z288; treatment Group #4). Note the prominent increase in ONL thickness in dogs from Groups #1–3 (**A_2**, **B_2**, **C_2**), and the misplacement of rod-like nuclei in the INL (**A_2**, **B_2**; *arrows*). No significant difference in ONL is seen in Group #4 (**D_1**, **D_2**). A loss of the photoreceptor layer (PRL) is seen in Groups #1–3, which causes the external limiting membrane to be in direct contact with the retinal pigment epithelium (**A_2**, **B_2**, **C_2**; *asterisks*). Cryosections; H&E stain; scale bars: 100 μm.

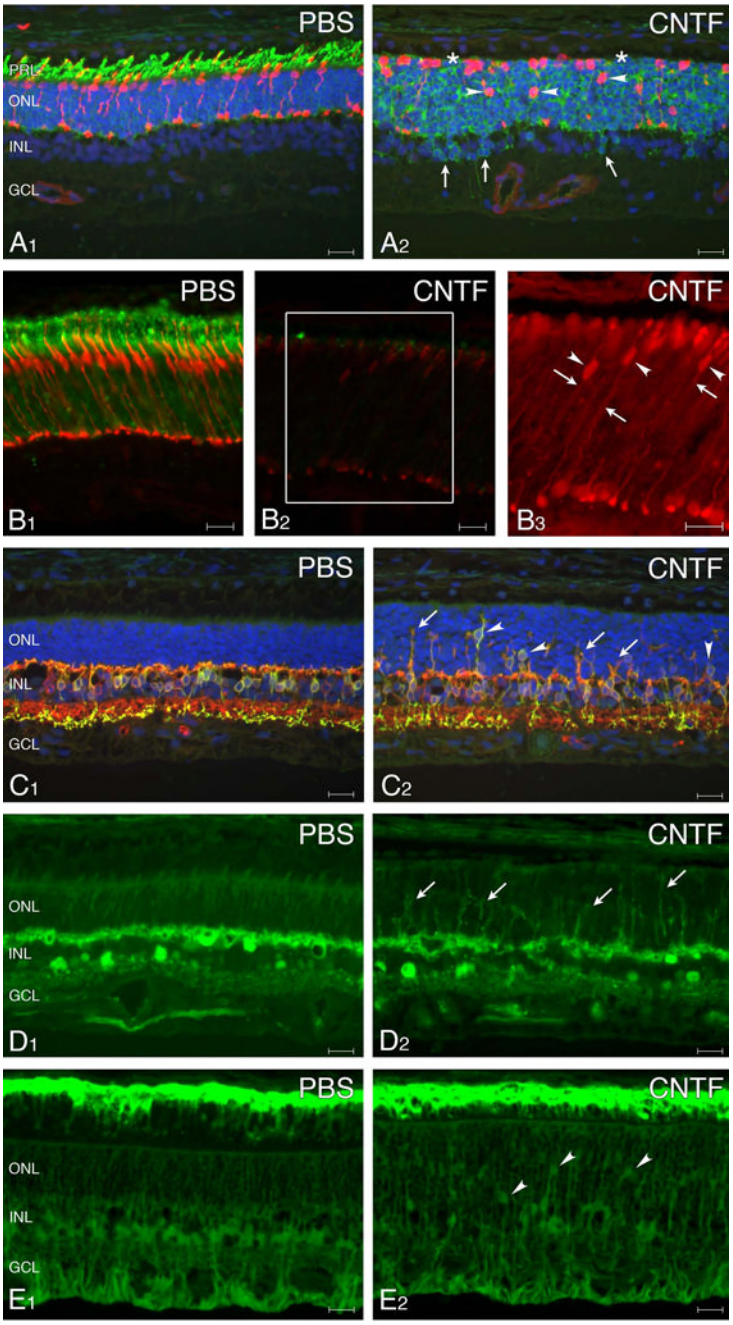

Figure 10.

Immunohistochemical characterization of CNTF-mediated remodeling of the peripheral retina of *XLPRA2*. (**A_1**, **A_2**, **B_1**, **B_2**) Double immunofluorescence labeling of rods and cones with, respectively, anti-rod opsin (*green*) and anti-cone arrestin (*red*), in a 12 week-old dog, 8 weeks after the first CNTF injection (Group # 2; **A_1**, **A_2**), and in a 5 week-old animal one week post-CNTF injection (**B_1**, **B_2**). No labeling is seen at the level of the PRL due to the loss of inner and outer segments (**A_2**; *asterisks*). The majority of the cells found in the ONL are rod opsin-positive due to the increased mislocalization caused by CNTF (**A_2**). Rod opsin immunoreactive cells are seen misplaced in the INL (*arrows*), and cone arrestin-labeled somas are present within the thickened ONL (*arrowheads*). One week following CNTF injection, there is a significant

decrease in rod opsin and cone arrestin expression (compare **B_2** with **B_1** which were taken at the same exposure settings). Digitally increasing the red fluorescent signal in an area of (**B_2**) shows the displacement of cone somas (*arrowheads*) down their respective axons (*arrows*; **B_3**). (**C_1**, **C_2**) Double immunofluorescence labeling of ON bipolar cells with anti-Goα (*red*), and rod bipolar cells with anti-PKCα (*green*) in a 12 week-old dog 8 weeks after the first CNTF injection (Group # 2). DAPI (*blue*) was used as a nuclear counterstain. Rod bipolar cells are co-labeled with both antibodies and appeared yellow-orange, whereas ON cone bipolar cells were only labeled with anti-Goα and appeared *red*. Note the neuritic sprouting extending into the ONL (*arrows*), and the presence of Goα- and PKCα-positive somas in the ONL (*arrowheads*) of the CNTF-treated eye. (**D_1**, **D_2**) Immunofluorecence labeling of horizontal and amacrine cells with anti-calretinin in a 12 week-old dog (Group #2) 8 weeks after the first CNTF injection. There are radial extensions into the ONL of neurite sprouts originating from the horizontal cells (*arrows*) in the CNTF-treated eye. (**E_1**, **E_2**) Immunofluorescence labeling of Müller cells with anti-CRALBP in a 12 week-old dog (Group #2) 8 weeks after the first CNTF injection. Müller cell somas are seen misplaced into the ONL *(arrowheads*) in the CNTF-treated eye. Note the intense normal CRALBP labeling in the RPE. Scale bars: 20 μm.

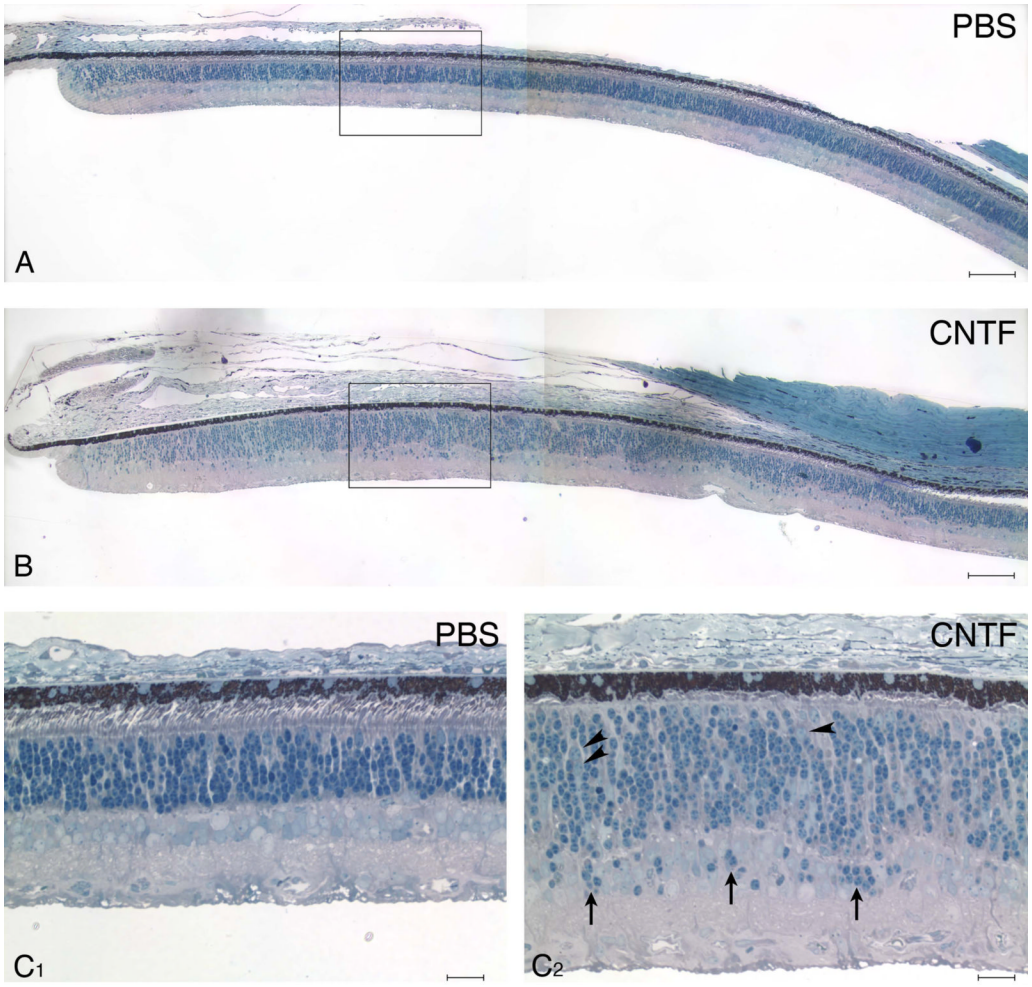

Figure 11.
Changes in the ONL of the inferior periphery in an 8 week-old *XLPRA2* dog following intravitreal injection of CNTF (PBS in the contralateral eye) at 4 weeks of age. Note the increase in ONL thickness (Compare **A** and **B**). The 1 μm thin epoxy resin sections enabled counts of individual cells in the ONL (**C_1**, **C_2**). Rod-like nuclei were seen in the INL (*arrows*), and larger euchromatic nuclei with a cone-type morphology were found in deeper layers of the ONL (*arrowheads*). Azure II-methylene blue stain with PPDA counterstain; scale bars: (**A**, **B**) 100 μm; (**C_1**, **C_2**) 20 μm.

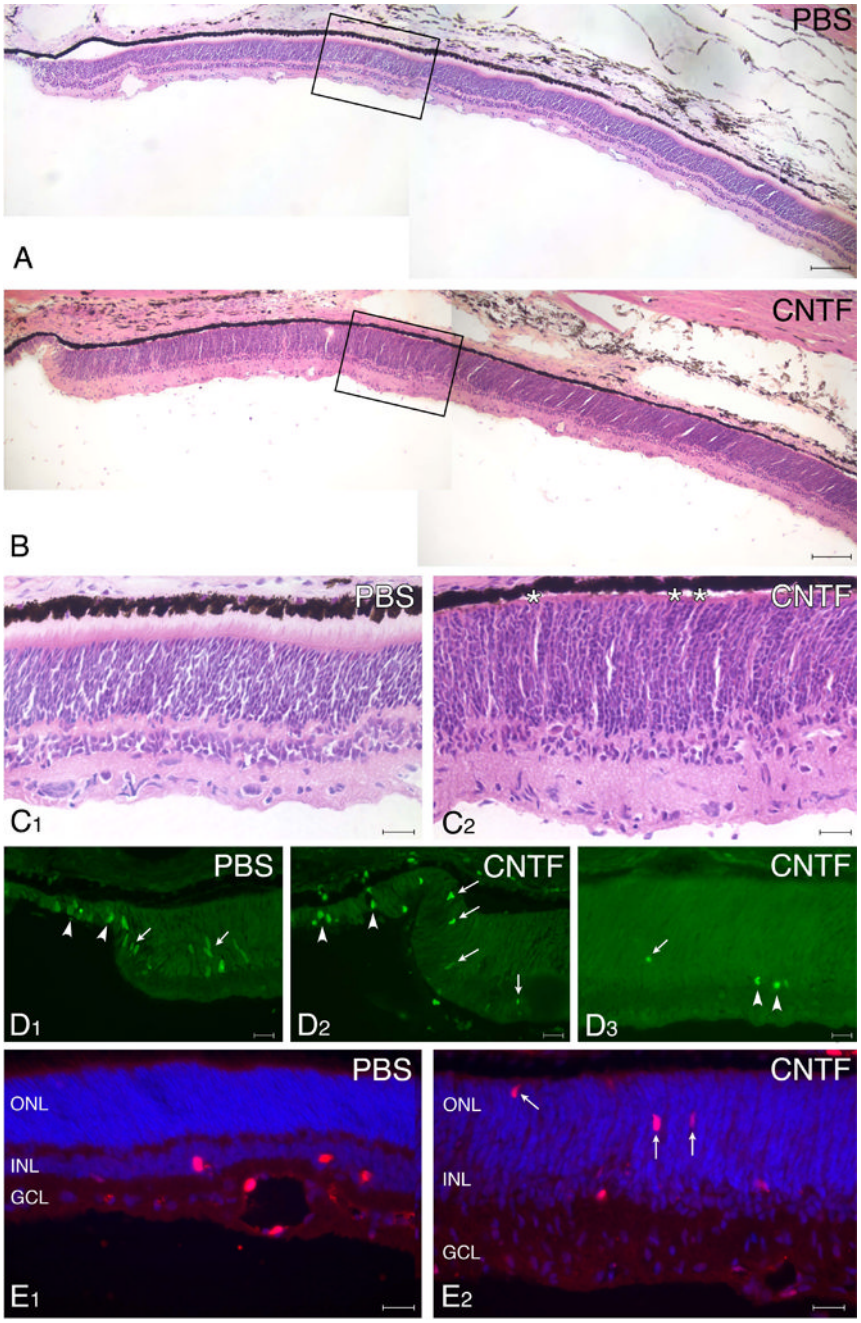

Figure 12.

Evidence for cell proliferative events in the peripheral ONL of *XLPRA2* following intravitreal injection of CNTF. (**A–D**) One week after CNTF injection there was increased ONL thickness in a 5 week-old dog. Note also the extended loss of the PRL (**B**, and **C_2**, *asterisks*). (**D_1–D_3**) Fluorescence immunohistochemical labeling for BrdU (*green*) shows incorporation in the ciliary epithelium of the *pars plana* (**D_1**, **D_2**, *arrowheads*), and in cells located at the retinal margin (**D_1**, **D_2**, *arrows*) in both the CNTF- and PBS-injected eye. (**D_3**) BrdU incorporation was detected in cells located in the INL (*arrowhead*), GCL and NFL in both eyes, but few BrdU-positive cells were seen only in the ONL of the CNTF-treated eye (*arrow*).

(**E_1**, **E_2**) Fluorescence immunohistochemical labeling for the nuclear cell marker of proliferation Ki67 (*red*). (**E_2**) Ki67-positive cells were seen exclusively in the ONL of the CNTF-treated eye (arrows), but Ki67 positive cells were found in other retinal layers in both PBS-and CNTF-injected eyes. Scale bars: (**A**, **B**) 100 μm; (**C_1**-**E_2**) 20 μm.

Table 1

List of primary antibodies used.

Antigen	Host	Source, catalog # or name	Working concentration	Specificity
Rod opsin	Mouse monoclonal	Paul Hargrave, R2-12N	1:300	OS of rods
Rod opsin	Mouse monoclonal IgG1	Chemicon, MAB5316	1:1,000	OS of rods
Human cone arrestin	Rabbit polyclonal	Cheryl Craft, LUMIF	1:10,000	Cone photoreceptors
Protein Kinase C (PKCα)	Mouse monoclonal IgG2b	BD Biosciences, 610107	1:100	Rod bipolar cells
Goα	Mouse monoclonal IgG1	Chemicon, MAB3073	1:5,000	ON (rod and cone) bipolar cells
Calretinin	Rabbit polyclonal	Sigma, C7479	1:500	Horizontal, amacrine, ganglion cells
CRALBP	Rabbit polyclonal	John Saari	1:1,500	Müller cells, RPE
Glial fibrillary acidic protein (GFAP)	Rabbit polyclonal	DakoCytomation, Z0334	1:1,000	Astrocytes, Müller cells (reactive)
CNTFRα	Rabbit polyclonal	Hermann Rohrer	1:2,000	IS of rods & cones, INL, GCL
Bromodeoxyuridine (BrdU)	Mouse monoclonal IgG1	Chemicon, MAB4072	1:100	Cells that have incorporated BrdU
Ki67	Mouse monoclonal IgG1	BD Biosciences, 550609	1:20	Proliferating cells

Table 2

Summary of clinical findings observed in eyes following intravitreal injection with CNTF (OS) or PBS (OD). Numbers for each category reflect the number of dogs affected in each treatment group.

	Group 1		Group 2		Group 3		Group 4		rcd1 control	
Clinical observations	**OD (n = 3)**	**OS (n = 3)**	**OD (n = 3)**	**OS (n = 3)**	**OD (n = 3)**	**OS (n = 3)**	**OD (n = 3)**	**OS (n = 3)**	**OD (n = 3)**	**OS (n = 3)**
Miosis	0	3	0	3	0	3	0	3	0	3
Iris vascular congestion	0	0	0	0	0	0	0	2	0	0
Iris hyperpigmentation	0	3	0	3	0	0	0	0	0	0
Intraocular hypotension	0	3	0	3	0	3	0	3	0	3
Corneal epitheliopathy	0	3	0	3	0	3	0	3	0	3
Cataract	0	3	0	3	0	3	0	2	0	3

Table 3

Increase in ONL thickness and loss of IS and OS in the *XLPRA2* peripheral retina after CNTF treatment.

		Group 1 (Inj 4; Ter 8)			Group 2 (Inj 4&8; Ter 12)			Group 3 (Inj 7&10; Ter 14)			Group 4 (Inj 12; Ter 15.6)		
	ID	**Z262**	**Z263**	**Z264**	**Z258**	**Z259**	**Z260**	**Z272**	**Z273**	**Z274**	**Z286**	**Z287**	**Z288**
	L	3,200	4,700	1,300	5,200	5,800	4,500	2,650	2,200	2,700	0	0	0
	D	2,350	3,400	1,000	3,550	3,900	3,800	1,500	2,000	2,150	None	None	None
SUP	R	1.48	1.67	1.85	1.59	1.87	1.85	2.03	1.22	2.77	None	None	None
		(68/46)	(87/52)	(72/39)	(62/39)	(71/38)	(63/34)	(67/33)	(44/36)	(72/26)			
	l	2,300	3,300	700	3,800	3,900	3,800	1,900	2,200	2,700	0	0	0
	L	2,000	1,800	1,000	3,000	3,200	1,400	1,900	1,400	1,500	0	0	0
	D	1,250	1,000	400	2,000	1,350	550	650	1,100	1,350	None	None	None
INF	R	2.05	1.83	1.69	1.61	2.40	2.27	2.35	1.41	2.26	None	None	None
		(80/39)	(75/41)	(44/26)	(61/38)	(72/30)	(68/30)	(61/26)	(41/29)	(43/19)			
	l	1,350	1,200	300	2,100	1,350	600	400	1,400	1,200	0	0	0
	L	/	/	/	/	/	/	1,300	1,200	1,150	/	/	0
	D	/	/	/	/	/	/	700	1,100	900	/	/	None
NAS	R	/	/	/	/	/	/	2.77	1.53	2.24	/	/	None
								(61/22)	(46/30)	(56/25)			
	l	/	/	/	/	/	/	800	1,200	950	/	/	0
	L	800	500	/	750	900	850	1,000	1,400	900	0	0	0
	D	600	450	/	500	650	600	550	1,100	600	None	None	None
TEMP	R	1.53	1.44	/	1.24	2.28	3	2.5	1.46	2.17	None	None	None
		(52/34)	(46/32)		(36/29)	(57/25)	(69/23)	(60/24)	(38/26)	(39/18)			
	l	650	300	/	600	500	550	700	700	600	0	0	0

SUP: superior meridian; INF: Inferior meridian; NAS: Nasal meridian; TEMP: Temporal meridian; ONL: outer nuclear layer; IS: inner segment; OS: outer segment.

L: length of retina (measured in μm from the ora serrata) over which there is an increase in ONL thickness in the CNTF-treated eye in comparison to the PBS-treated eye.

D: distance from the ora serrata (measured in μm) of the site of highest ONL thickness in the peripheral retina of the CNTF-treated eye.

R: ratio of ONL thickness in the CNTF-treated eye over the ONL thickness in the PBS-treated eye at distance D from the ora serrata.

l:Length of retina (measured in μm from the ora serrata) over which there is a loss of IS and OS in the CNTF-treated eye in comparison to the PBS-treated eye.

Table 4

ONL nuclear count (mean [min; max]) in a 40X microscope field (290 μm in length) of the peripheral *XLPRA2* retina treated with CNTF or PBS. (Note: rod-like nuclei present in the INL were also included in this count)

Dog:meridian	PBS-injected	CNTF-injected	% increase
Z265:	(Inj. 4 wk; Ter. 8 wk)	(Inj. 4 wk; Ter. 8 wk)	
Superior	483 [469; 498]	552 [546; 556]	14
Inferior	362 [360; 366]	544 [533; 553]	50
Temporal	398 [391; 406]	530 [520; 548]	33
Z261:	(Inj. 4 & 8 wk; Ter. 12 wk)	(Inj. 4 & 8 wk; Ter. 12 wk)	
Superior	363 [341; 384]	424 [411; 431]	17
Inferior	298 [280; 318]	421 [400; 445]	41
Temporal	321 [313; 331]	393 [376; 416]	22

Table 5

Number of BrdU-positive cells per unit length of retina (10,000 μm) following CNTF or PBS injection in a 5week-old *XLPRA2* dog. Mean [minimum; maximum] values were obtained from at least three sections for each of the meridians.

Meridian	PBS-injected	CNTF-injected
Superior	43 [39; 47]	138 [122; 161]
Inferior	54 [41; 65]	101 [97; 104]
Temporal	45 [34; 55]	165 [140; 187]