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Immunolocalization of Ciliary Neurotrophic Factor Receptor α (CNTFRα) in Mammalian Photoreceptor Cells

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Abstract

PURPOSE:

To characterize the site of expression of the α subunit of the receptor for ciliary neurotrophic factor (CNTFRα) in the retina of a variety of mammalian species, and determine whether CNTFRα is localized to photoreceptor cells.

METHODS:

The cellular distribution of CNTFRα (protein) was examined by immunocytochemistry in the adult retinas of several mammalian species that included mouse, rat, dog, cat, sheep, pig, horse, monkey, and human. Developing retinas from 3-day-old and 6-day-old rats were also included in this study. The molecular weight of CNTFRα in rat, dog, cat, pig, and human retinas was determined by immunoblotting.

RESULTS:

CNTFRα immunolabeling was present in the retina of all species. A common pattern was observed in all species, and represented labeling of the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), and outer plexiform layer (OPL). CNTFRα did not immunolocalize to photoreceptor cells in both adult and developing rodent retinas, but was consistently observed in both rods and cones of non-rodent species. The molecular weight of CNTFRα in mammalian retinas was approximately 61-64 kDa.

CONCLUSIONS:

These findings highlight a significant difference in the expression of CNTFRα in the retina of rodent and non-rodent mammalian species. The expression of CNTFRα by rods and cones in non-rodent species may suggest a direct mechanism of action if CNTF administration results in photoreceptor rescue.

Disciplines

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Immunolocalization of ciliary neurotrophic factor receptor α (CNTFRα) in mammalian photoreceptor cells

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Purpose: To characterize the site of expression of the α subunit of the receptor for ciliary neurotrophic factor (CNTFRα) in the retina of a variety of mammalian species, and determine whether CNTFRα is localized to photoreceptor cells.
Methods: The cellular distribution of CNTFRα (protein) was examined by immunocytochemistry in the adult retinas of several mammalian species that included mouse, rat, dog, cat, sheep, pig, horse, monkey, and human. Developing retinas from 3-day-old and 6-day-old rats were also included in this study. The molecular weight of CNTFRα in rat, dog, cat, pig, and human retinas was determined by immunoblotting.
Results: CNTFRα immunolabeling was present in the retina of all species. A common pattern was observed in all species, and represented labeling of the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), and outer plexiform layer (OPL). CNTFRα did not immunolocalize to photoreceptor cells in both adult and developing rodent retinas, but was consistently observed in both rods and cones of non-rodent species. The molecular weight of CNTFRα in mammalian retinas was approximately 61-64 kDa.
Conclusions: These findings highlight a significant difference in the expression of CNTFRα in the retina of rodent and non-rodent mammalian species. The expression of CNTFRα by rods and cones in non-rodent species may suggest a direct mechanism of action if CNTF administration results in photoreceptor rescue.

Ciliary neurotrophic factor (CNTF) rescues photoreceptors in several genetic [1-7] and in light induced models of retinal degeneration [1,8]. Its photoreceptor survival effect was demonstrated in vivo in a variety of animal species that include mouse [1-5], rat [4,8], cat [6], and dog [7], and in mouse retinal explants [9,10]. Although its mechanism of action on photoreceptor cells is not fully understood, CNTF is thought to initiate a survival response by binding to the plasma membrane of retinal cells that express its receptor, ciliary neurotrophic factor receptor (CNTFR). CNTFR is composed of an α subunit (CNTFRα) that specifically binds CNTF, and two β subunits (LIFR, gp-130) that are shared by other members of the IL-6 R family [11]. Binding of CNTF to CNTFRα causes heterodimerization of the α and β subunits and activation of various signaling pathways that promote cell survival [12].

It has been shown that in the rat retina, CNTFRα mRNA is expressed in horizontal cells and subpopulations of amacrine and ganglion cells, but not in photoreceptors [13]. In addition, intravitreal delivery of CNTF to the rodent eye activates signaling pathways predominantly in Müller cells and other inner nuclear layer (INL) cells, ganglion cells, and astrocytes, yet fails to activate signaling pathways in photoreceptors [14-16]. These studies suggest that in the rat and mouse retina, the CNTF photoreceptor rescue effect is mediated through an indirect mechanism of action. It has been proposed that microglia derived CNTF could prevent photoreceptor cells from undergoing degeneration by promoting the release of direct acting photoreceptor survival factors such as bFGF and GDNF by Müller cells [17].

We have recently shown that in the normal adult canine retina both the CNTFRα transcript and protein are expressed by photoreceptors, INL cells, and ganglion cells [18]. The immunolocalization of CNTFRα to rods and cones suggests that, at least in the dog, the photoreceptor rescue effect observed with CNTF in the *rcd1* model of retinal degeneration [7] may be mediated through a direct mechanism of action.

Determining whether photoreceptors are the direct targets of CNTF has become increasingly important since this survival factor is currently being tested in Phase 1 clinical trials in humans with retinitis pigmentosa. To address the differences between dogs and rodents and determine if CNTFRα is localized to photoreceptors in other species, we performed immunocytochemical studies on retinas from a variety of mammalian species.

METHODS

Animals and tissue fixation: Normal adult retinas from the following mammalian species were used for the study: mouse (Balb/c, 6 months), rat (AO derived, 6 months), dog (Beagle, adult), cat (DSH, adult), sheep (adult), horse (Pony of America, 7 years), pig (adult), monkey (cynomolgus and rhesus macaques, adult), and human (52 year old male). In addition, we also collected immature retinas from 3-day-old (PD3) Lewis rats, and 6-day-old (PD6) AO rats. With the exception

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of the sheep, horse, and human for which one single individual was available, retinas from at least two individuals were obtained for each of the other species.

While under anesthesia (mouse, rat) or less than 10 min after euthanasia (dog, cat, sheep, pig, horse), eyes were enucleated. Retinas were then processed at our facility as follows: a slit was made at the level of the ora serrata and the entire globe was fixed for 3 h in 4% paraformaldehyde in 0.1 M phosphate buffered saline at 4 °C. The posterior segment was isolated and fixed for an additional 24 h at 4 °C in 2% paraformaldehyde in 0.1 M phosphate buffered saline. The tissue then was trimmed and cryoprotected sequentially for 24 h in a solution of 15% and 30% sucrose in 0.1 M sodium phosphate and 0.15 M sodium chloride, pH 7.2 [BupH,™ Phosphate Buffered Saline, Pierce, Rockford, IL; (referred in the text as PBS)] at 4 °C, and embedded in optimal cutting temperature (OCT) media.

Cryosections were cut at 7 or 10 µm thickness and stored at -80 °C. The human eye was a surgical specimen that had been enucleated for orbital exenteration of an extraocular tumor. The rhesus macaque and human retina specimens (kindly provided by Drs. Bob Fariss and Ann Milam, respectively) were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffered saline for 4 h followed by 2% paraformaldehyde in 0.1 M phosphate buffered saline for 2.5 years (monkey), or in 4% paraformaldehyde in 0.1 M phosphate buffered saline for several days followed by 2% paraformaldehyde in 0.1 M phosphate buffered saline for approximately 3 years (human). The cynomolgus macaque retina (obtained from the New England Primate Research Center) was fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffered saline. Monkey and human retinas were then cryoprotected and processed as indicated above.

For immunoblot analysis, neuroretinas from several adult species (rat, dog, cat, pig, and human) were dissected following death, and stored at -80 °C until processed for total protein extraction. The human retina came from a 68-year-old Caucasian female that died of multisystem organ failure, and was provided by the Cooperative Human Tissue Network which is funded by the National Cancer Institute. All research conducted was in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the tenets of the Declaration of Helsinki.

Immunocytochemistry: To visualize the retinal localization of CNTFRα, both immunoenzymatic and immunofluorescence methods were used. For enzymatic immunocytochemistry, tissue sections were washed three times in a 0.3% hydrogen peroxide solution in 50% ethanol to inhibit endogenous peroxidase. Sections were then treated with 0.25% Triton X-100 in PBS for 5 min followed by 10% normal goat serum (NGS) with 0.25% Triton X-100 and 0.05% sodium azide in PBS for 60 min. They were then incubated overnight at 4 °C with a protein A-purified polyclonal rabbit anti-chick CNTFRα antibody diluted (1:2,000) in PBS with 1.5% NGS, 0.25% Triton X-100 and 0.05% sodium azide. This antibody was raised in a rabbit after immunization with a large fragment of the chick CNTFRα recombinant protein, and has been described previously [19]. After washing in PBS, secondary antibody (biotinylated goat anti-rabbit, 1:200 dilution; Vector Laboratories, Burlingame, CA) was applied for 30 min at room temperature. Antibodies were visualized using the avidin biotin complex (ABC) Elite kit (Vector Laboratories) with diaminobenzidine as a substrate. A nonspecific staining of the photoreceptor layer, and in particular of the outer segments, has been previously reported using this immunoenzymatic method [20], and was observed in this study on the mouse sections and occasionally in sections from other species. To confirm the absence of labeling of mouse photoreceptor cells, and particularly of cones with the CNTFRα antibody, we compared the staining pattern of the photoreceptor layer to that obtained on a sequential section with an antibody directed against mouse cone arrestin (LUMIJ, 1:10,000 [21]; provided by Dr. C. Craft, University of Southern California, Los Angeles, CA). Conversely, to confirm the cone photoreceptor labeling observed with the CNTFRα antibody in the pig, monkey, and human retinas, we used serial sections and immunoreacted each sequential section with CNTFRα antibody or a rabbit affinity-purified antibody directed against human cone arrestin (LUMIf, 1:10,000 [22]; provided by Dr. C. Craft).

In addition to using the anti-chick CNTFRα antibody, we also tested several commercially available antibodies directed against mammalian CNTFRα. The following antibodies were used on retinal cryosections of the collected species: goat polyclonal anti-human CNTFRα (1:100; R&D systems Inc., Minneapolis, MN; catalog number AF303NA), mouse monoclonal anti-human CNTFRα (1:100; R&D systems Inc.; catalog number MAB303), and goat polyclonal anti-rat CNTFRα (1:100; R&D systems Inc.; catalog number AF559NA). The ABC immunoenzymatic method was performed as described above using the appropriate biotinylated secondary antibodies.

To further characterize the subpopulation of cones in the dog, pig, monkey, and human retinas that express CNTFRα, we performed double immunofluorescence labeling using antibodies that identify M/L (COS-1, 1:10 [23]) or S wavelength sensitive cones (OS-2, 1:1,000 [23]). For immunofluorescence, sections were permeabilized with 0.25% Triton X-100 in PBS for 5 min, and then blocked with 10% normal goat serum with 0.25% Triton X-100 and 0.05% sodium azide in PBS for 20 min. The sections were then incubated overnight at 4 °C with the anti-chick CNTFRα antibody (1:500) followed with a red fluorochrome labeled goat anti-rabbit secondary antibody (Alexa Fluor 568, 1:200; Molecular Probes, Eugene, OR) for 90 min, and then with the COS-1 or OS-2 antibodies for approximately 5 h, followed by a FITC labeled horse anti-mouse secondary antibody (1:50; Vector Laboratories) for 90 min.

Sequential retinal sections from rats (PD3, PD6, and 6-month-old) were incubated for immunofluorescence with either the anti-chick CNTFRα antibody followed by a red fluorochrome labeled secondary antibody (as described above) or with polyclonal rabbit anti-rat calbindin D-28 K antibody (catalog number C2724, 1:1,000; Sigma, St. Louis, MO) followed by a green fluorochrome labeled goat anti-rabbit secondary

antibody (Alexa Fluor 488, 1:200; Molecular Probes). To better distinguish the location of the photoreceptor layer, rhodamine labeled peanut agglutinin (1:1,000; Vector Laboratories) was used on some sections as a marker of cone extracellular matrix domain. DAPI nuclear stain (5 µM for 15 min) was used to visualize the nuclear layers. Double immunofluorescence labeling of adult rat retinas with the anti-chick CNTFRα antibody and a mouse monoclonal anti-recombinant CRALBP antibody (provided by Dr. J. Saari; 1: 40,000) was done to determine whether Müller cells express CNTFRα. To determine labeling specificity, control sections were treated in the same way with omission of primary antibodies, and also by substitution with an unrelated primary antibody. Slides were mounted with gelvatol, a medium composed of polyvinyl alcohol and DABCO (Sigma), and examined with an epifluorescent microscope (Axioplan, Carl Zeiss Meditech, Oberkochen, Germany) with or without Differential Interference Contrast (DIC) optics. Images were digitally captured (Spot 3.3 camera, Diagnostic Instruments, Inc., Sterling Heights, MI) and imported into a graphics program (Photoshop; Adobe, Mountain View, CA) for display.

Western blot analysis: Adult rat, dog, cat, pig, and human retinas were homogenized in PBS containing a cocktail of protease inhibitors (catalog number P8340; Sigma), and following sonication the protein levels were determined by the Bradford method (Bio-Rad protein assay, Bio-Rad, Hercules, CA). Protein lysates (60 or 120 µg) were placed in the sample buffer containing 4% glycerol, 0.4% sodium dodecyl sulfate, 1% β-mercaptoethanol, 0.005% bromophenol blue in 12.5 mM Tris-HCl buffer (pH 6.8), and heated at 100 °C for 5 min. Samples and molecular weight standards (catalog number RPN2107; Amersham Biosciences, Piscataway, NJ) were separated by SDS-PAGE (4% stacking gel, 12% separating gel).Transfer of proteins from gels to PVDF membrane (Immobilon, Millipore, Bedford, MA) was performed in chilled transfer buffer (25 mM Tris base, 192 mM glycine, and 15% methanol), and the membranes were then blocked with 10% skim milk in Tris buffered saline containing 0.5% Tween-20 overnight at 4 °C. The membranes were incubated for 1.5 h with either a protein A-purified rabbit anti-chick CNTFRα antibody (1:100,000), a goat polyclonal anti-human CNTFRα antibody (1:500, R&D Systems Inc.; catalog number AF303NA), or a mouse monoclonal anti-human CNTFRα antibody (catalog number 558783, 1:500; BD Pharmingen, San Diego, CA) followed by the appropriate secondary antibody conjugated with horseradish peroxydase (1:10,000, Zymed, San Francisco, CA). The blots were developed using the ECL method according to the manufacturer's recommendations (Amersham Biosciences), and exposed on autoradiograph film (Eastman Kodak, X-oMAT; Rochester, NY).

RESULTS

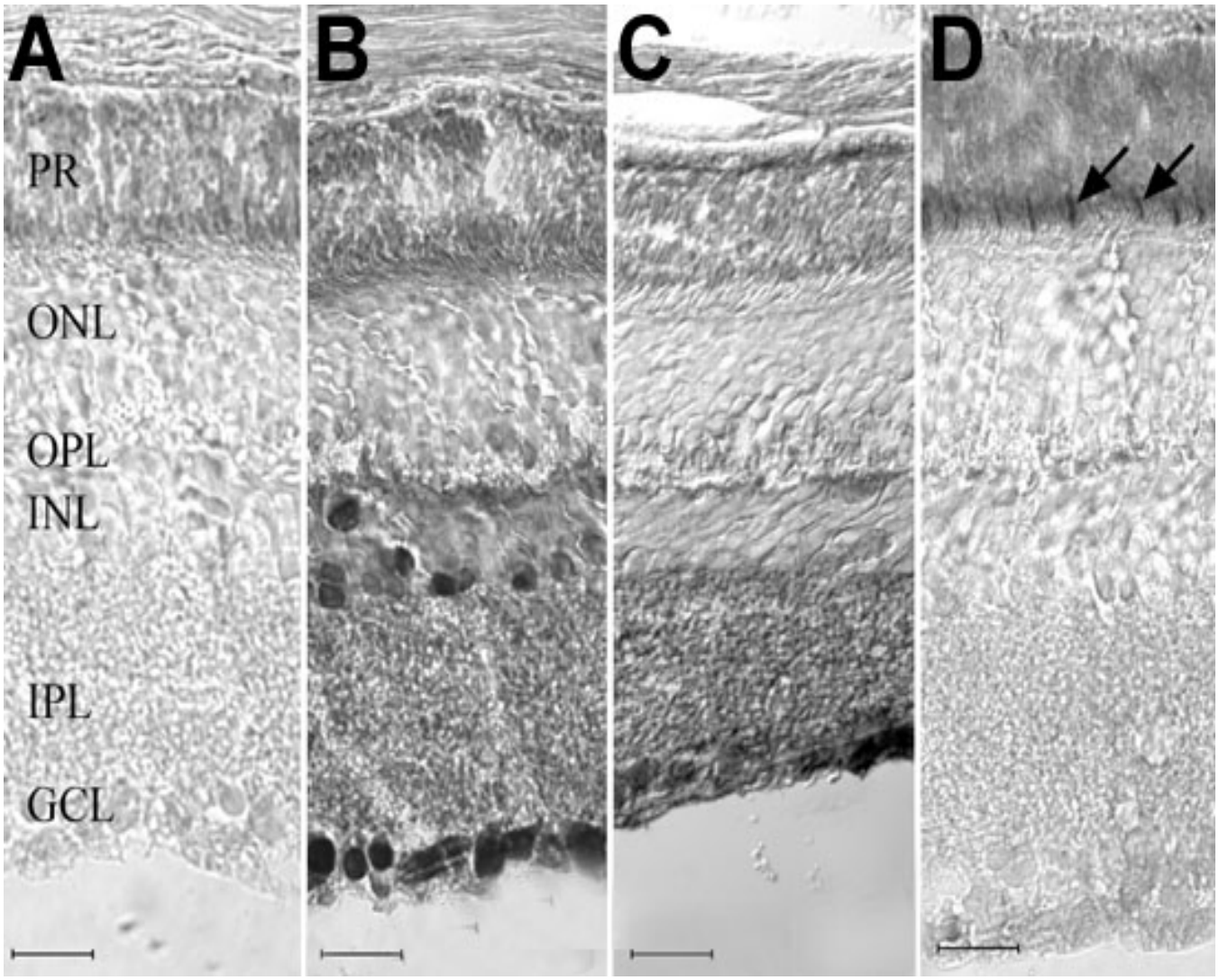
Immunolocalization of CNTFRα in mammalian retinas: Immunolabeling of retinal cells with the polyclonal antibody raised against chick CNTFRα was detected in all species except in the horse, in which case staining was absent in all retinal layers (data not shown). Results obtained with this antibody are summarized in Table 1. A similar pattern of labeling of the nerve fiber layer (NFL), ganglion cell layer (GCL), and INL was observed across all species (Figure 1B; Figure 2H; Figure 3B; Figure 4C; Figure 5B; Figure 6B). Also, less intense staining of the inner plexiform layer (IPL) was observed in all species (Figure 1B; Figure 2H; Figure 3B; Figure 4C; Figure 5B; Figure 6B). In the INL of the adult mouse, rat, and pig, labeling was predominantly observed in cells located at the vitreal and scleral borders of this layer, suggesting that the cells expressing CNTFRα could be amacrine and horizontal cells, respectively (Figure 1B,C; Figure 2H; Figure 4B).

A significant difference was observed when comparing the labeling pattern of the outer nuclear layer (ONL) and photoreceptor layer in the adult mouse and rat to that observed in the non-rodent mammals. No labeling of adult rodent photoreceptors was detected by either immunoenzymatic or immunofluorescence methods (Figure 1B,C,E; Figure 2G,H), and

Table 1. Pattern of immunolabeling in retinas of mammalian species with the anti-chick CNTFRα antibody

Retinal layer	Mouse	Rat	Dog	Cat	Sheep	Pig	Monkey	Human
RPE	ND	0	+++	+++	+++	ND	ND	ND
OS	0	0	0	0	+	0	0	0
IS	0	0	+++	+++	+++	++	+++	+++
ONL	0	0	+++	++	+	+++	++	+++
OPL	++	++	+++	++	++	+++	+++	+++
INL	+++	+++	+++	+++	+++	+++	+++	++
IPL	++	++	+	++	+	+	+	+
GCL	+++	+++	+++	+++	+++	+++	+++	+++
NFL	+++	++	+++	+++	++	+++	+++	+++

Intensity of labeling was graded as intense (+++), moderate (++), weak (+), or absent (0); RPE staining was not determined (ND) in some species. In mouse RPE, nonspecific labeling could also be observed on negative control sections. Labeling was intense in PD3 and PD6 rats. Data for "Dog" are taken from from a previous study [18]. For dog and human outer nuclear layer (ONL), labeling was limited to cone soma, axon, and pedicle. For cat ONL, labeling was limited to cone soma and some cone axons. For sheep and pig ONL, labeling was limited to cone soma. Retinal pigment epithelium (RPE) pigmentation and autofluorescence precluded assessing the presence of specific labeling in pig, monkey, and human. In pig inner segments (IS), labeling was limited to the inner portion of the IS.



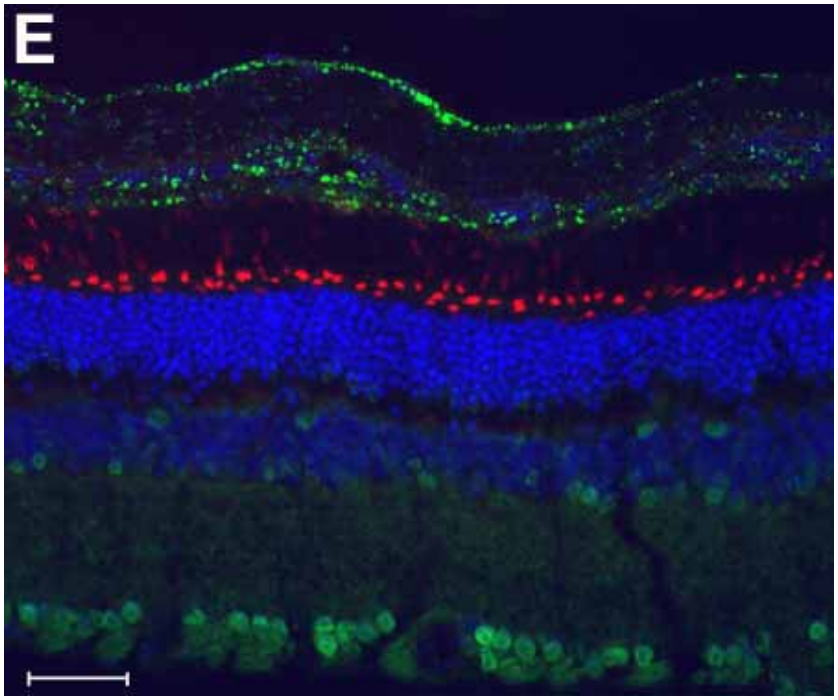


Figure 1. Immunolocalization of CNTFRα in the adult mouse retina. **A**: Negative control. **B**: Pattern of immunoenzymatic labeling with the anti-chick CNTFRα antibody. **C**: Pattern of immunoenzymatic labeling with the anti-rat CNTFRα antibody. **D**: Sequential section (to **B**) labeled with mouse cone arrestin antibody. **E**: Immunofluorescence labeling (overlaid images) with the anti-chick CNTFRα antibody (green), DAPI (blue), and peanut agglutinin (red). Intense labeling with the CNTFRα antibodies (**B**,**E**) was limited to ganglion cells, nerve fibers, and cells located predominantly at the vitreal and scleral borders of the inner nuclear layer (INL). Less intense labeling was also present at the inner plexiform layer (IPL) and outer plexiform layer (OPL). Nonspecific staining was observed at the photoreceptor layer (**A**-**C**) and was distinct from the specific cone inner segment labeling (arrows in **D**) obtained with the mouse cone arrestin antibody. Fluorescence immunocytochemistry confirmed the absence of photoreceptor labeling with the anti-chick CNTFRα antibody (**E**). Scale bars represent 20 μm (**A**-**D**) or 40 μm (**E**).

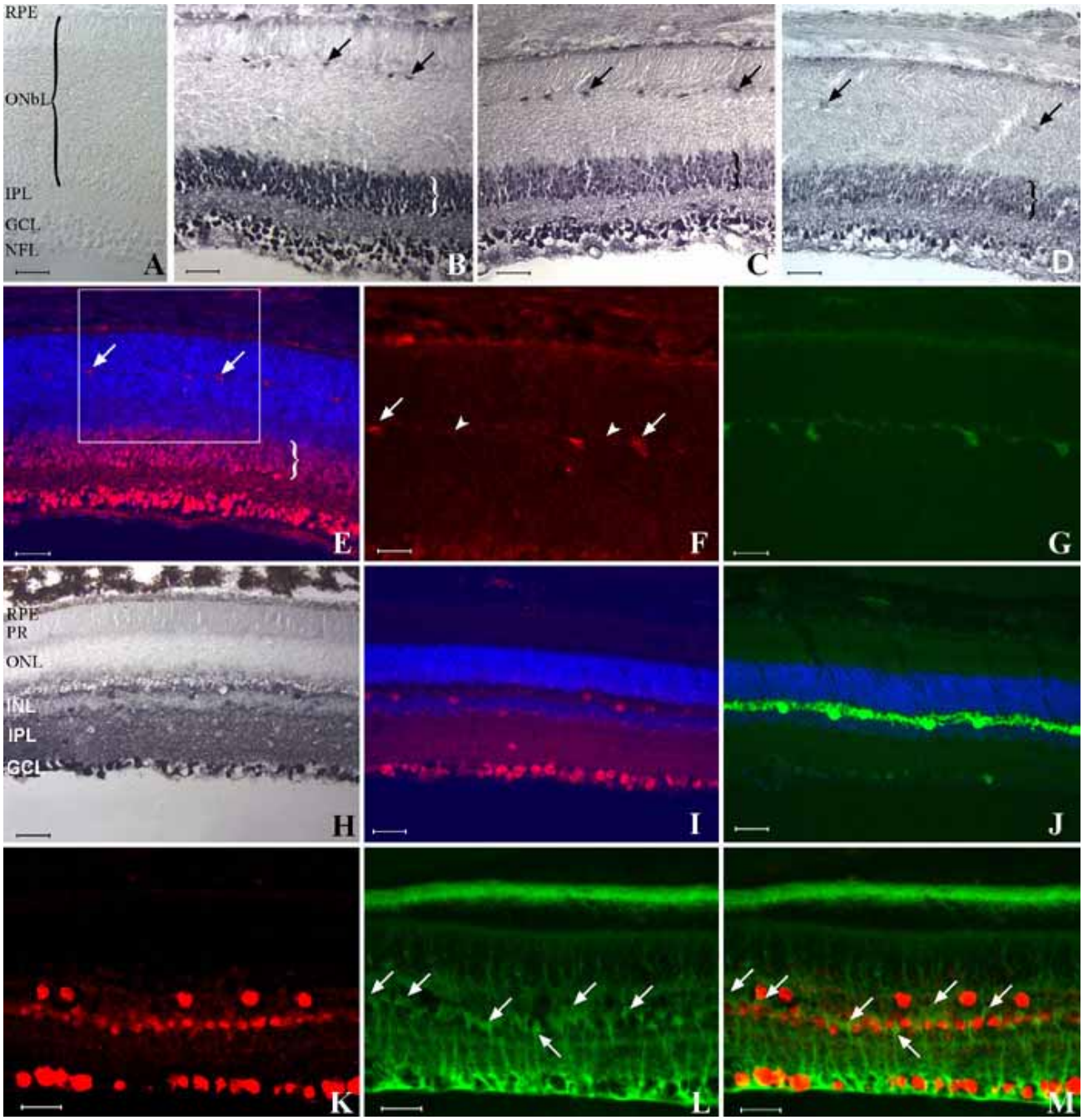


Figure 2. Immunolocalization of CNTFRα in the developing and adult rat retina. **A**: Negative control. **B**,**C**: Pattern of immunoenzymatic labeling with the anti-chick CNTFRα antibody in 3-day-old (**B**) and 6-day-old (**C**) rat retinas. **D**: Pattern of immunoenzymatic labeling with the anti-human CNTFRα antibody in a 6-day-old retina. **E**: Immunofluorescence labeling (overlaid images) with the anti-chick CNTFRα antibody (red) and DAPI (blue) on a 6-day-old retina. Intense labeling with the CNTFRα antibodies (**B**-**E**) was observed in the developing retina at the level of the ganglion cell layer (GCL), retinal pigment epithelium (RPE), and in two distinct areas of the outer neuroblastic layer (ONbL): the inner 6-7 rows of nuclei (braces in **B**-**E**) and in horizontal cells (arrows). **F**: Boxed region in **E** showing only CNTFRα labeling. Distinct horizontal processes (arrowheads) were seen extending from the labeled cell bodies (arrows) of the horizontal cells. **G**: Immunofluorescence labeling of horizontal cells with the calbindin antibody showing a similar pattern as observed in **F**. **H**: Pattern of immunoenzymatic labeling with the anti-chick CNTFRα antibody in an adult rat retina. Intense labeling was present at the level of the GCL and cells at the innermost and outermost border of the inner nuclear layer (INL). No labeling of photoreceptor cells was observed. **I**: Immunofluorescence labeling (overlaid images) with the anti-chick CNTFRα antibody (red) and DAPI (blue) on the adult retina seen in **H**. Absence of staining in photoreceptors and RPE was observed. Note that this rat's RPE is pigmented (seen in **H**), but no labeling of the RPE is observed by immunofluorescence. **J**: Sequential section labeled with the calbindin antibody. **K**: Immunofluorescence from staining with the anti-chick CNTFRα antibody. **L**: Immunofluorescence from staining with the rCRALBP antibody. **M**: Double immunofluorescence (overlaid images) with the anti-chick CNTFRα and rCRALBP antibodies. There is no co-localization of CNTFRα immunoreactive cells in the INL with the soma of Müller cells (arrows in **L**,**M**). Scale bars represent 40 μm (**A**-**E**,**H**-**J**) or 20 μm (**F**,**G**,**K**-**M**).

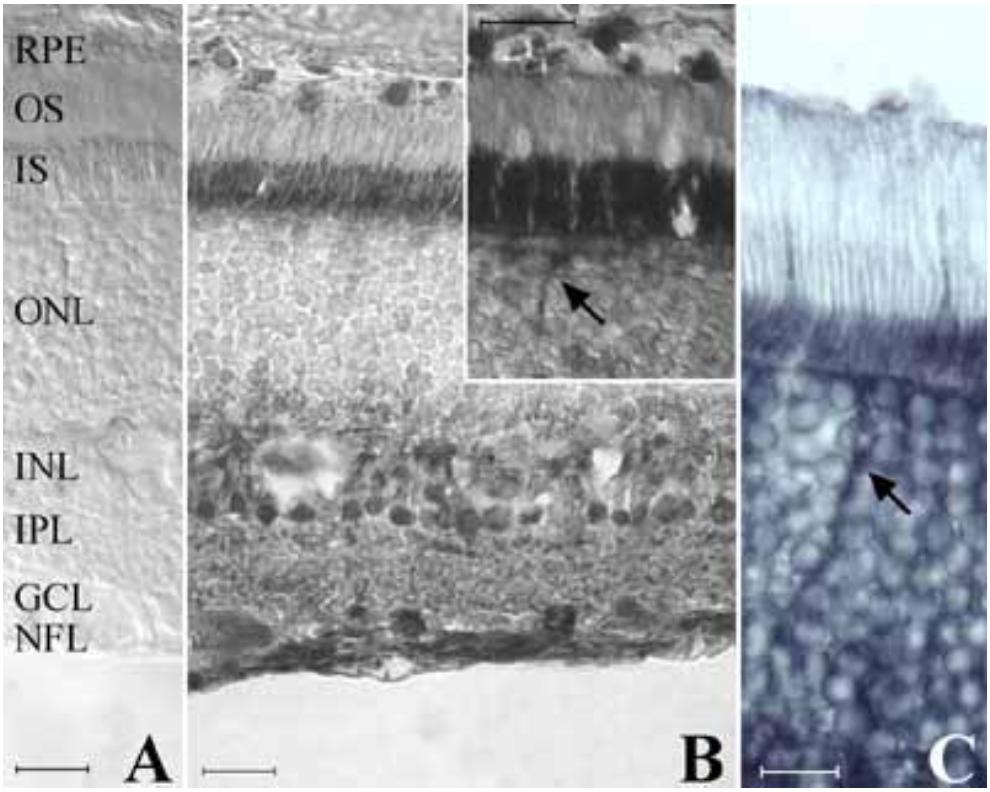


Figure 3. Immunolocalization of CNTFRα in the adult cat retina. **A**: Negative control. **B**: Pattern of immunoenzymatic labeling with the anti-chick CNTFRα antibody. **C**: Immunoenzymatic labeling of photoreceptors with the anti-human CNTFRα antibody. Intense labeling with the anti-chick CNTFRα antibody was seen at the retinal pigment epithelium (RPE), inner segments (IS), inner nuclear layer (INL), ganglion cell layer (GCL), and nerve fiber layer (NFL; **B**). With longer incubation times in the enzyme substrate (DAB), labeling of cone cell bodies and their extending axons (arrow) could be detected (inset to **B**, **C**). Scale bars represent 20 µm (**A**,**B**) or 10 µm (**C**).

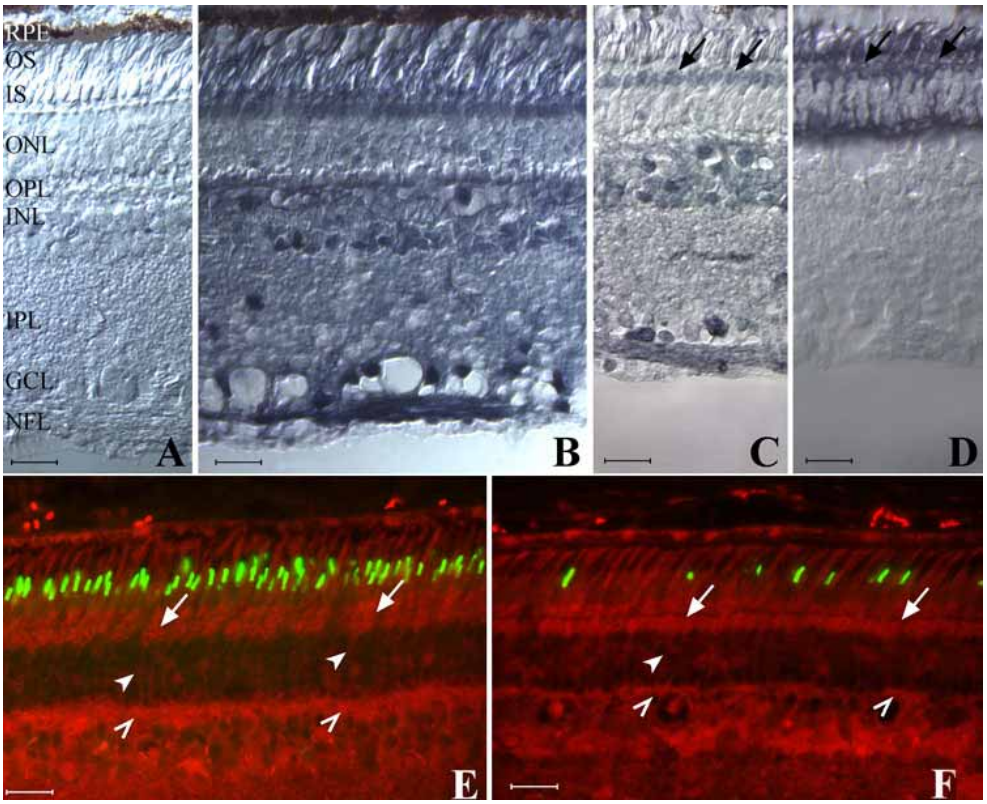


Figure 4. Immunolocalization of CNTFRα in the adult pig retina. **A**: Negative control (pigmented RPE). **B**,**C**: Pattern of immunoenzymatic labeling with the anti-chick CNTFRα antibody at 1:2,000 (**B**) or 1: 10,000 (**C**) dilutions. **D**: Sequential section labeled with human cone arrestin antibody. **E**,**F**: Double immunofluorescence labeling (overlaid images) with the anti-chick CNTFRα (red) and COS1 (green, **E**) or OS2 (green, **F**) antibodies. Intense immunoenzymatic labeling with the CNTFRα antibody (1:2,000 dilution) was seen at the inner segments (IS), outer nuclear layer (ONL; cone cell bodies), outer plexiform layer (OPL), inner nuclear layer (INL), ganglion cell layer (GCL), and nerve fiber layer (NFL; **B**). Labeling with the CNTFRα antibody at a 1:10,000 dilution was still present in the cone cell bodies (arrows, **C**), and resembled that obtained with the human cone arrestin antibody (arrows, **D**). CNTFRα immunolabeling of M/L (**E**) and S (**F**) cones was localized to their inner segments, cell bodies (arrows), axons (arrowheads), and pedicles (open arrowheads). Scale bars represent 20 µm.

this was observed with all four CNTFRα antibodies (see Table 2). In the developing rat retina (PD3 and PD6), all cells located in the GCL were labeled (Figure 2B-D). In addition, the CNTFRα antibodies labeled the inner six to severn rows of cells of the outer neuroblastic layer (Figure 2B-D). A population of cells located at the edge of the presumptive outer plexiform layer (OPL), was also distinctively labeled. These cells were regularly spaced and disposed in a linear fashion from the ora serrata to the optic nerve at approximately 50 µm from the external limiting membrane (ELM; Figure 2B-D). Immunofluorescence studies showed that these cells had extended horizontal processes, suggesting that they could be horizontal cells (Figure 2E,F). To confirm this hypothesis, sequential serial sections were labeled with either the anti-chick CNTFRα antibody or with an antibody directed against calbindin D-28 kDa, an epitope located on horizontal, amacrine, and ganglion cells in the adult and developing rat retina [24,25]. We observed a similar pattern of labeling with both antibodies (Figure 2F,G), suggesting that horizontal cells express CNTFRα as early as PD3. This expression persists in the adult (Figure 2I,J).

In the developing and mature rat retina and in the adult mouse retina, labeling of CNTFRα was not detected in the photoreceptor cell bodies in the ONL, in the inner segments or in the outer segments. Since CNTFRα labeling was not absent in rodents (it was observed in other retinal layers such as NFL, GCL, IPL, INL) the most likely conclusion is that rodent photoreceptors do not express CNTFRα. In the adult rat, double immunofluorescence studies did not show any colocalization of CNTFRα with CRALBP suggesting that Müller cells do not express CNTFRα either (Figure 2K-M).

Recently, we reported that CNTFRα transcript and pro-

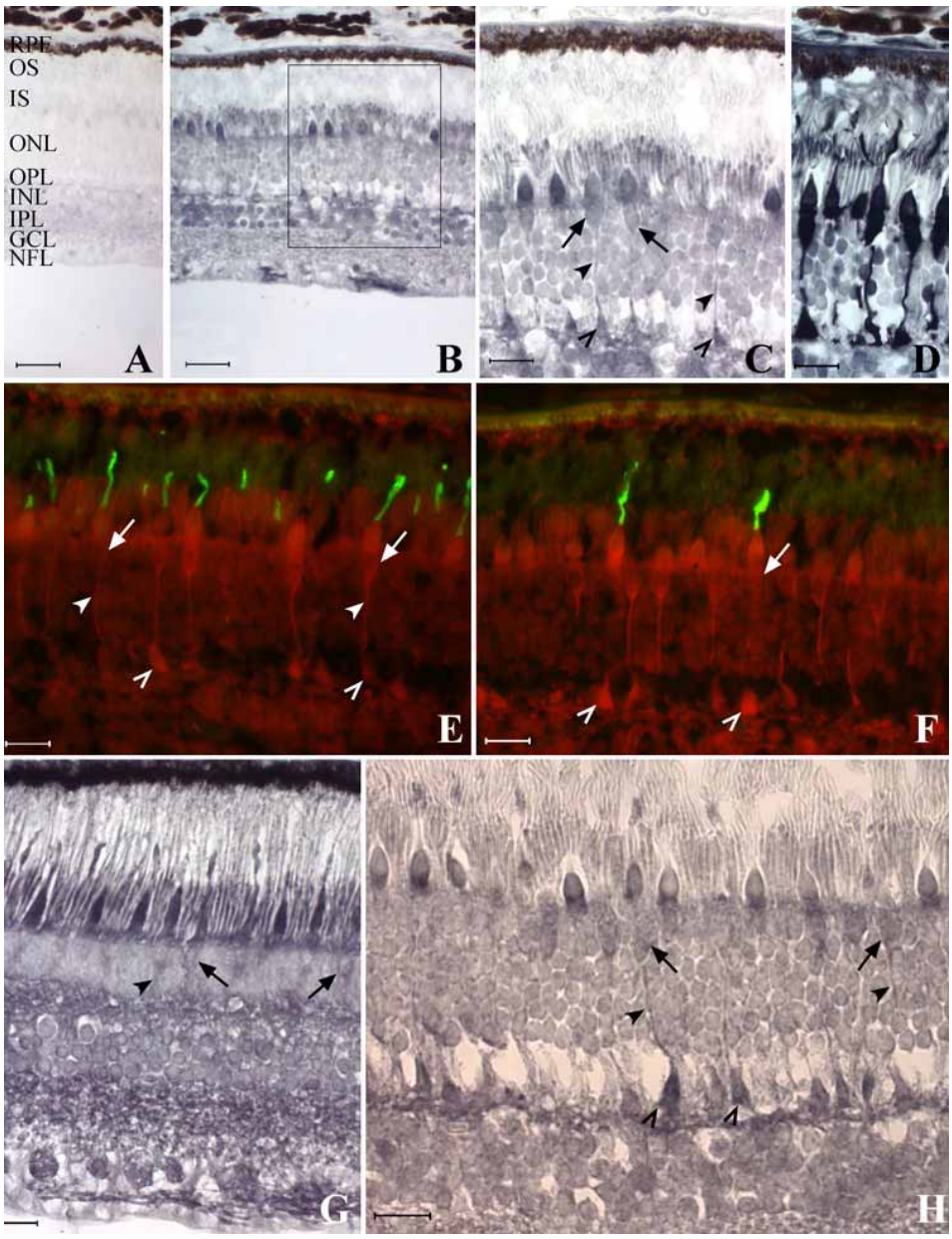


Figure 5. Immunolocalization of CNTFRα in the adult monkey retina. **A**: Negative control (pigmented RPE). **B**: Pattern of immunoenzymatic labeling in the rhesus macaque with the anti-chick CNTFRα antibody. **C**: Boxed region in **B**. **D**: Sequential section labeled with human cone arrestin antibody. **E**,**F**: Double immunofluorescence labeling (overlaid images) in the rhesus macaque with the anti-chick CNTFRα (red) and COS1 (green, **E**) or OS2 (green, **F**) antibodies. **G**: Pattern of immunoenzymatic labeling in the cynomolgus macaque with the monoclonal anti-human CNTFRα antibody. **H**: Pattern of immunoenzymatic labeling in the rhesus macaque with the anti-rat CNTFRα antibody. Intense labeling with the CNTFRα antibodies was seen at the inner segments (IS), outer nuclear layer (ONL; cone cell bodies), outer plexiform layer (OPL), inner nuclear layer (INL), ganglion cell layer (GCL), and nerve fiber layer (NFL; **B**,**G**). Labeling was present at both rod and cone IS, and at cone cell bodies (arrows), axons (arrowheads), and pedicles (open arrowheads; **C**,**E**-**H**). Both M/L (**E**) and S (**F**) cones were labeled by the CNTFRα antibody. Scale bars represent 40 µm (**A**,**B**) or 20 µm (**C**-**H**).

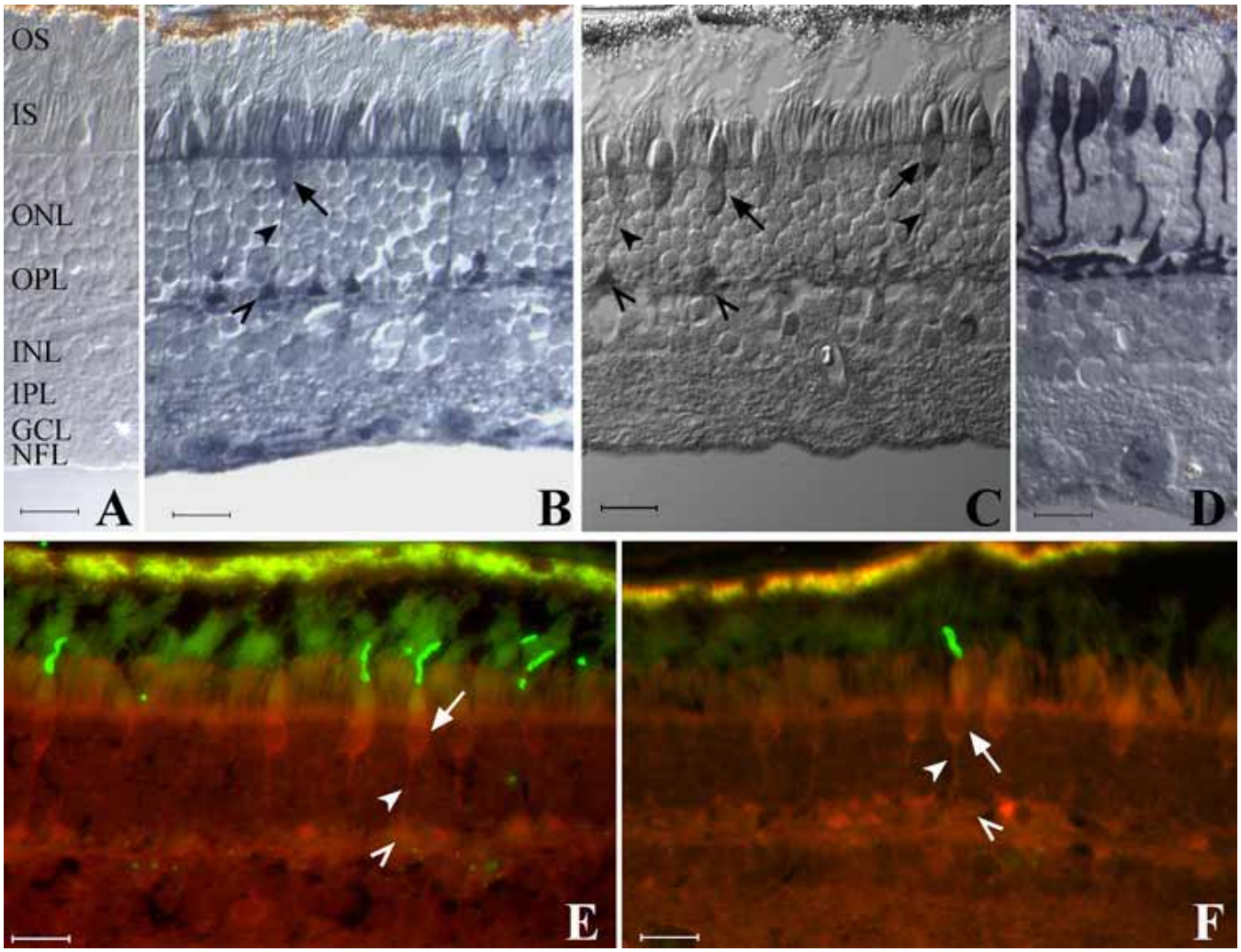


Figure 6. Immunolocalization of CNTFRα in the adult human retina. **A**: Negative control (pigmented RPE). **B**: Pattern of immunoenzymatic labeling with the anti-chick CNTFRα antibody. **C**: Pattern of immunoenzymatic labeling with the anti-rat CNTFRα antibody. **D**: Sequential section (to **B**) labeled with human cone arrestin antibody. **E**,**F**: Double immunofluorescence labeling (overlaid images) with the anti-chick CNTFRα (red) and COS1 (green, **E**) or OS2 (green, **F**) antibodies. Intense labeling with the CNTFRα antibody (**B**) was seen at the inner segments (IS), outer nuclear layer (ONL; cone cell bodies), outer plexiform layer (OPL), ganglion cell layer (GCL), and nerve fiber layer (NFL). Labeling was present (**B**,**C**,**E**,**F**) at rod and cone IS, cone cell bodies (arrows), axons (arrowheads), and pedicles (open arrowheads). Both M/L (**E**) and S (**F**) cones were labeled by the anti-chick CNTFRα antibody. Scale bars represent 20 μm.

Table 2. Photoreceptor immunolabeling with different CNTFRα antibodies in mammalian retina

CNTFRα antibodies	Mouse	Rat 6 d	Rat adult	Dog	Cat	Sheep	Pig	Horse	Cynomolgus macaque	Rhesus macaque	Human
rabbit anti-chick	0	0	0	+++	+++	+++	+++	NR	BG	+++	+++
goat anti-human	0	0	0	++	+++	IN	++	++	+	+	+
mouse anti-human	IN	0	0	BG	+	++	+	NR	+++	+	+
goat anti-rat	0	0	0	BG	++	IN	++	BG	+	+++	+++

The table summarizes the level of immunodetection of CNTFRα in the photoreceptor cells of several mammalian species, with the four antibodies used in this study. Intensity of photoreceptor labeling was characterized as strong (+++), moderate (++), weak (+), absent but with labeling in other retinal layers (0), nonspecific background labeling (BG), nonreactive with an absence of labeling throughout the retina (NR), or indeterminate due to binding of secondary antibody to the tissue (IN).

tein are expressed in dog photoreceptor cells [18]. Here, using a different antibody directed against human CNTFRα, we obtained a similar pattern of labeling (Figure 7F). In addition, we show by double immunofluorescence studies that CNTFRα co-localized to both M/L and S wavelength sensitive cones of the dog retina (Figure 7D,E). Similarly to what we have observed in the dog, labeling of sequential sections with CNTFRα or human cone-arrestin antibodies in pig, monkey, and human, confirmed that both rod and cone photoreceptors express CNTFRα, and that the cellular distribution of the receptor for CNTF differs between the two classes of photoreceptors (Figure 4C,D; Figure 5C,D; Figure 6B-D). In non-rodent mammals, labeling of rods was limited to their inner segments (IS), while cones showed a distinct pattern of staining that involved their IS, and soma, with variable labeling of their axon and pedicle (Figure 7F, Figure 3B; Figure 4B; Figure 5C,G,H; Figure 6B,C). As in the dog, double immunofluorescence studies, showed that CNTFRα co-localized to the two subpopulation of cones in pig, monkey, and human retinas (Figure 4E,F; Figure 5E,F; Figure 6E,F).

Western blot analysis of CNTFRα in mammalian retinas: Using a polyclonal goat anti-human CNTFRα antibody on total retinal protein extracts of several mammalian species (rat, dog, cat, pig, and human), we detected under reducing conditions a common band at a molecular weight varying between 61 and 64 kDa (Figure 8). A similar band was detected in the rat and human when immunoblots were analyzed using the polyclonal rabbit anti-chick CNTFRα, and the monoclonal anti-human CNTFRα antibodies (data not shown). However, with these two antibodies, several additional nonspecific bands were detected.

DISCUSSION

We have shown that CNTFRα protein is expressed in the retina in a variety of mammalian species including primates. While a similar pattern of CNTFRα expression could be observed across species in the inner retina, a distinct difference between rodent and non-rodent species was observed in photoreceptors.

Our results (summarized in Table 1 and Table 2) suggest that the mechanism by which CNTF stimulates photoreceptor survival may depend on the expression of CNTFRα by photoreceptor cells, and thus on the animal species examined.

Recently, we reported that normal adult canine photoreceptor cells express CNTFRα when using a polyclonal antibody directed against chick CNTFRα [18]. Here, we have

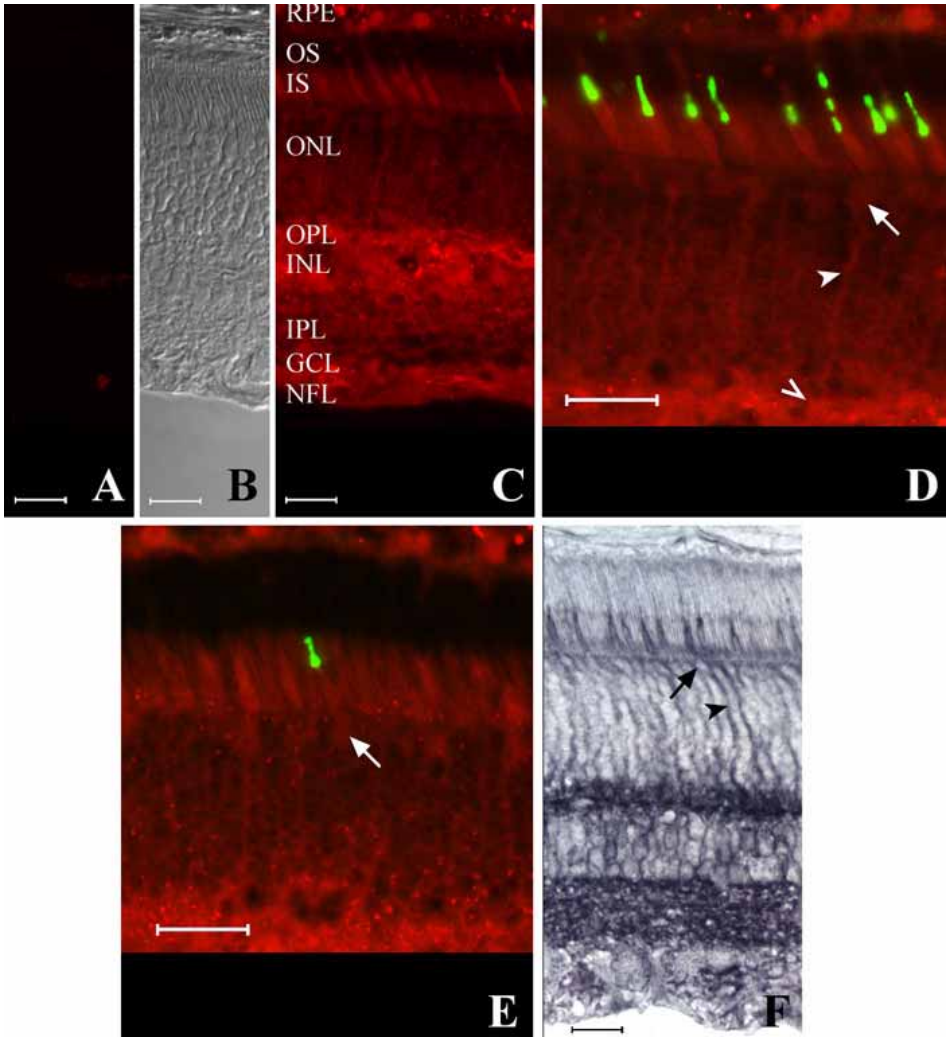


Figure 7. Immunolocalization of CNTFRα in cone photoreceptor cells of the dog. **A**: Negative control. **B**: Negative control with DIC optics. **C**: Immunofluorescence labeling with the anti-chick CNTFRα antibody. **D**,**E**: Double immunofluorescence labeling (overlaid images) with the anti-chick CNTFRα (red) and COS1 (green, **D**) or OS2 (green, **E**) antibodies. **F**: Pattern of immunoenzymatic labeling with the anti-human CNTFRα antibody. Intense labeling was present at the level of the inner segments (IS) of both rod and cones, outer plexiform layer (OPL), inner nuclear layer (INL), ganglion cell layer (GCL), and nerve fiber layer (NFL). CNTFRα immunolabeling of M/L (**D**) and S (**E**) cones was localized to their inner segments, cell bodies (arrows), axons (arrowheads), and pedicles (open arrowhead). Scale bars represent 20 μm.

extended our work using this same antibody to examine the expression of CNTFRα in the retina of several other mammals. Since the degree of homology between the amino acid sequence of chicken CNTFRα and that of dog and other mammals is relatively low [18], we also used three other antibodies raised against mammalian CNTFRα to verify our results. While the intensity and the quality of the immunolabeling were higher with the anti-chick CNTFRα antibody, a similar pattern could be observed with the other antibodies, thus confirming the specificity of the labeling detected in mammalian species with the anti-chick CNTFRα antibody. It must be noted that the manufacturer of these three CNTFRα antibodies (goat anti-human, mouse anti-human, goat anti-rat) does not advertise them for immunocytochemical use, yet we were able to detect some labeling by using the immunoenzymatic method described above.

Our findings support previous observations that suggest that CNTFRα is not expressed by rodent photoreceptors. In both mouse and rat adult retinas, CNTFRα immunolabeling was restricted to some INL and ganglion cells. This pattern resembled that observed for CNTFRα mRNA in the rat [13]. We showed that photoreceptor expression of CNTFRα was absent in both the mature normal rodent retina and in the developing rat retina at postnatal day 3 (PD3) and PD6. This is in contrast to a study that claimed that CNTFRα expression could be detected in rat photoreceptors at PD5 and PD8 [26]. In that report, the authors analyzed on immunoblots the expression of CNTFRα in both the outer and inner retina. Since the horizontal cut made to isolate the outer retina (on flatmounted retinas) was done at a thickness of 140 µm from the vitreal surface, it is likely that the "outer retina" defined by the authors contained a portion of the developing INL that included CNTFRα expressing horizontal cells. Indeed, on our sections, the distance separating the horizontal cells from the internal limiting membrane was approximately 200-250 µm in the PD3 rat, and 170-200 µm in the PD6 rat.

Another study has suggested that rat photoreceptor cells express CNTFRα [27]. While high magnification views showed punctate immunolabeling exclusively limited to the photoreceptor outers segments, no labeling was observed in ganglion, and INL cells. Since negative controls were not performed using a pre-immunized, non-immunized serum, or an unrelated primary antibody from the same species in which the CNTFRα antibody was raised, it cannot be excluded that outer segment staining was nonspecific and due to "antibody stickiness" to the photoreceptor outer segments. A similar phenomenon has been reported when performing enzymatic immunocytochemistry on the retina [20], and can be a source of false positive immunolabeling when appropriate antibody controls are not used. We were not able to verify the specificity of the commercial CNTFRα antibody used in that study, as it has not been commercially available for over three years (Research Diagnostics Inc., Flanders, NJ; personal communication in November 2003).

In a recent study, Rhee and Yang reported that CNTFRα immunolabeling of the inner and outer segments of mouse photoreceptors could be observed as early as postnatal day 10 (PD10), and persisted in the mature retina [28]. The commercial goat polyclonal antibody raised against CNTFRα (R-20, sc-1914, Santa Cruz Biotechnology, Santa Cruz, CA) that the authors used in their study was one that we had tested previously in the dog, rat, mouse, and other mammalian retinas [18]. We had observed (data not shown) in both the mature mouse and rat retinas a similar pattern of labeling as that de-

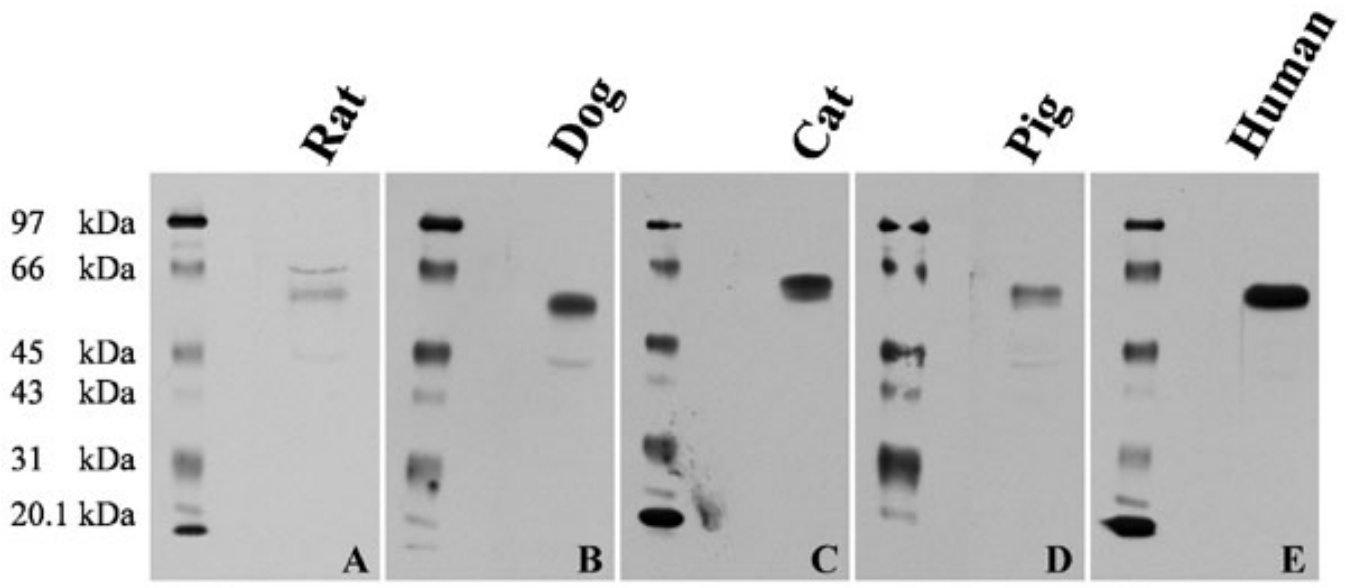


Figure 8. Western blot analysis of CNTFRα in retinas of mammalian species. Total protein extracts were run on a reducing 12% SDS-PAGE gel and immunoblotted with a polyclonal antibody raised against human CNTFRα. **A**: Detection of a major band at approximately 63 kDa in the rat. **B**: Detection of a major band at approximately 61 kDa in the dog. **C**: Detection of a band at approximately 64 kDa in the cat. **D**: Detection of a major band at approximately 63 kDa in the pig. **E**: Detection of a band at approximately 62 kDa in the human.

scribed by the authors. In addition, by double immunofluorescence, we observed a colocalization of both PNA and the CNTFRα antibody (sc-1914) in the mouse retina, suggesting that murine cones express CNTFRα (data not shown). Yet, as we have reported previously [18], we were not able to block the labeling obtained with the sc-1914 antibody by preincubating it with its blocking peptide (sc-1914P) on either immunoblots or immunohistochemical sections. We tested two different lots of the blocking peptide and a lyophilized peptide preparation provided by the company, at concentrations 5-100 folds higher (10-200 μg/ml) than that of the antibody, and for several different preincubation periods (2-14 h). While we cannot fully exclude the possibility of a manufacturing defect in the preparation of the blocking peptide, we have questioned the specificity of the sc-1914 antibody. In their study, Rhee and Yang [28] have omitted to report the results of the negative controls used to verify the specificity of the sc-1914 antibody while providing thorough details for the other antibodies tested.

RT-PCR analysis of CNTFRα expression in the ONL of normal and light reared PD35 rat retinas failed to detect CNTFRα gene expression [17]. Similarly, single cell RT-PCR detected CNTFRα transcript in a fraction of mouse Müller cells but never in rod photoreceptor cells [29]. These results contrast with those from Ju et al. [30] that report increased CNTFRα mRNA expression in rat photoreceptors following retinal ischemia and reperfusion. These apparently contradicting observations may suggest that under normal conditions, both mature and developing rodent photoreceptors do not express CNTFRα, but that under conditions of stress such as ischemia and reperfusion, CNTFRα expression occurs.

Several reports have failed to show any CNTF mediated activation of signaling pathways in juvenile and adult rodent photoreceptor cells [14-16], and our results support the idea that direct activation of these cells may not occur in the absence of CNTFRα. Yet, very recent studies show that CNTF can trigger the activation of cytokine signaling events in photoreceptor precursor cells and differentiating rods in the early postnatal rodent retina [31-33]. It is therefore possible, that immature photoreceptor cells express low levels of CNTFRα that escape detection by immunocytochemistry, yet are critical to the development of these cells. Alternatively, CNTF may act directly on these photoreceptor precursor cells by binding a different member of the cytokine receptor family [34].

A striking difference in the retinal expression of CNTFRα in non-rodent mammals has been the observation that CNTFRα immunolocalizes to photoreceptors. Similarly to our previous findings in the normal adult canine retina [18], photoreceptors of the adult cat, sheep, horse, pig, monkey and human express CNTFRα. A similar pattern of photoreceptor staining was observed across these species, and is characterized by the labeling of rod and cone IS, cone soma, axon and pedicle. Labeling with the anti-chick CNTFRα did not appear to be restricted to the cell membrane, particularly at the level of the cone IS and soma. Recently, it was reported that CNTFRα immunostaining of neurons in the rat cochlear nucleus revealed its presence at the level of the cell membrane and at the soma; ultrastructural analysis localized it to the endoplasmic reticulum [35]. The localization of CNTFRα to photoreceptors would suggest that endogenous or exogenously administered CNTF could bind directly to CNTFRα expressed at the surface of these cells. Further studies are needed to determine whether CNTFRα is capable of transducing a survival signal to photoreceptors or whether it plays the role of a CNTF sink. This will require characterizing the expression of the other two β components (LIFR and gp-130) of the complete CNTF receptor and its downstream signaling pathways in photoreceptors of non-rodent mammals. This would allow characterization of a potential direct mechanism of photoreceptor rescue by CNTF. In parallel, investigations on the indirect mechanism of photoreceptor rescue by CNTF in rodent species need to be pursued. Indeed, at this stage, it cannot be excluded that CNTF protects non-rodent mammalian photoreceptors through both a direct and indirect mechanism of action.

There is evidence that CNTF triggers the activation of signaling pathways in Müller cells of the rodent retina [14-16,36]. Yet, our results fail to identify adult rat Müller cells as a site of CNTFRα expression. In addition, in both the adult rat and mouse, immunolabeling of the INL was mainly restricted to cells located at the vitreal and scleral borders, consistent with the location of horizontal and amacrine cells, respectively. A similar observation has been made when assessing the sites of expression of CNTFRα mRNA in the INL by in situ hybridization [13]. This may suggest that Müller cells are also activated indirectly by CNTF or, as proposed by Peterson et al. [14], that Müller cells have the potential to bind the soluble form of CNTFRα. Yet, by single cell RT-PCR, CNTFRα mRNA was detected recently in no more than 30% of isolated mouse Müller cells [29]. In our study, repeated double immunofluorecence labeling experiments have failed to show any localization of CNTFRα to rat Müller cells. Although this discrepancy may be explained by a species specificity issue, it is also possible that a low level of CNTFRα expression in Müller cells of normal adult rats is not detected by our current immunofluorecence method. In all other non-rodent mammals, with the exception of the pig in which distinct cell populations predominantly located at the scleral and vitreal borders of the INL were labeled, CNTFRα was found throughout most of the INL. This precluded distinguishing particular cell populations located in the INL, and determining in these species whether Müller cells express CNTFRα.

We previously reported that the anti-chick CNTFRα antibody used in this study detected on immunoblots of canine and chick retina a protein of approximately 62 kDa [18]. This value was slightly lower than that previously reported [13,19], and may be due to different electrophoresis conditions. In the present study, we have confirmed our initial finding using antibodies raised against mammalian CNTFRα, and now show that in other mammalian species a protein with a similar molecular weight is detected. Our results are consistent with those of others on rat [37] and bovine [38] tissues.

In summary, we have shown that CNTFRα is expressed as a protein with an apparent molecular weight of approximately 61-64 kDa in the retina of a wide variety of mamma-

lian species. To the best of our knowledge we provide the first immunocytochemical evidence that CNTFRα is not expressed by rodent photoreceptor cells, while it is found on photoreceptors of non-rodent mammalian species. A previous study by one of the co-authors had shown in the chicken retina that CNTFRα expression was present in photoreceptor cells [39], and it was recently shown to be restricted to violet sensitive cones [40]. This further illustrates that caution needs to be used when extrapolating results from one animal species to another.

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