Cloning, Mapping, and Retinal Expression of the Canine Ciliary Neurotrophic Factor Receptor α (CNTFRα)

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PURPOSE. To clone, map, and determine the site of expression (mRNA and protein) of the α subunit of the receptor for ciliary neurotrophic factor (CNTFRα) in the normal adult canine retina.

METHODS. The complete coding sequence of the canine CNTFRα cDNA was cloned, and radiation hybrid (RH) mapping was used to determine the chromosomal localization of the gene. CNTFRα mRNA expression in retina and other tissues was examined by reverse transcription–polymerase chain reaction. The cellular distribution of CNTFRα in the canine retina was studied by in situ hybridization and immunocytochemistry.

RESULTS. Canine CNTFRα shares a high degree of homology with the human, mouse, and rat coding sequences, both at the nucleotide and amino acid level, but has lower homology with the chicken. CNTFRα was RH mapped to CFA 11 (Cantis familiaris autosome 11) and showed homology to the short arm of human chromosome 9 (Op13). The gene is transcribed in retina, brain, spleen, lung, liver, and kidney. In the retina, CNTFRα was highly expressed by photoreceptors, but both the transcript and protein were also found in the RPE, inner nuclear layer, and ganglion cells.

CONCLUSIONS. These findings demonstrate that CNTFRα is expressed by rods and cones in the normal adult canine retina and suggest that ciliary neurotrophic factor (CNTF) could have a direct photoreceptor rescue effect by binding to CNTFRα in these cells. This could open novel pathways for the treatment of retinal degeneration in animal models and humans. (Invest Ophthalmol Vis Sci. 2003;44:3642–3649) DOI:10.1167/iovs.02-0765

Retinal degenerations are a major cause of blindness for which no treatment is currently available. Different therapeutic approaches are being investigated—among these, the use of survival factors that may slow the rate of photoreceptor death and delay the onset of vision loss in various forms of retinal degeneration. Over the past decade, a variety of survival factors have been tested in several animal models of retinal degeneration.1–4 Ciliary neurotrophic factor (CNTF), a survival factor originally isolated from the chick ciliary ganglion, has been shown to promote photoreceptor rescue when delivered intravitreally or subretinally to the rat (Song Y, et al. IOVS 2000;41:ARVO Abstract 964), mouse, or cat eye.5–8 Our laboratory has shown a similar neuroprotective effect when CNTF is injected into the vitreous of rd11 dogs, a canine model of early-onset and rapidly progressing retinal degeneration caused by a mutation in the PDE6B gene. Similar to observations made in the rd mouse,9,10 a disease model also caused by a mutation in PDE6B, a neuroprotective effect was observed in 14-week-old rd1 dogs when intravitreal injections of CNTF were performed at 7 and 10 weeks of age (Pearce-Kelling S, et al. IOVS 1998;39:ARVO Abstract 2645). However, side effects such as cataracts were present after intravitreal CNTF injections, but these complications were eliminated when CNTF was delivered by means of an encapsulated cell-based delivery system. In addition, this long-term delivery device allowed prolonged rescue of photoreceptors over a period of 7 to 14 weeks.11 CNTF is thought to trigger a survival signal by binding to the ciliary neurotrophic factor receptor (CNTF). This receptor is a member of the cytokine receptor superfamily, and is composed of three subunits: an α subunit (CNTFRα),9 which carries the specific CNTF binding site, and two different β subunits (gp-130 and leukemia inhibitory factor receptor [LIFR])10 that are preassociated with members of the Jak/Tyk family of cytoplasmic tyrosine kinases. The binding of CNTF to CNTFRα causes heterodimerization of gp-130 and LIFR, and activation of the Jak/Tyk kinases. This, in turn, recruits and activates a variety of downstream signaling molecules, turning on different signaling pathways,11 that promote a cell-survival response. CNTFRα is an extracellular protein that is attached to the plasma membrane by a glycosyl-phosphatidylinositol link. Cleavage of this link by phosphatidylinositol-specific phospholipase C (PI-PLC) releases a soluble form of CNTFRα.9 CNTFRα has been isolated from a variety of tissues, including the retina, central nervous system, peripheral nervous system, muscle, skin, lung, liver, kidney, and testes.12–14 Although the neuroprotective effect of CNTF in the retina has been demonstrated in a variety of animal models of retinal degeneration,2,5,3–5–7 the site of expression of its receptor and the mechanism of action by which it rescues photoreceptors is unknown in mammalian species. Several studies have suggested that CNTFRα is not expressed by photoreceptor cells and that the neuroprotective effect of CNTF is mediated by Müller cells.15,16 Because of the dramatic rescue effect of CNTF on rd11-affected photoreceptors and the lack of knowledge of its cellular targets in the retina, we decided to clone CNTFRα and study its expression in the normal adult canine retina.

METHODS

Primer Design

The human and mouse CNTFRα complete coding sequences (GenBank accession nos. M73238, NM0166673, respectively; available by ftp at...
Reverse Transcription–Polymerase Chain Reaction and cDNA Cloning

Brain tissue from the frontal cerebral cortex of a 17-week-old male beagle was used as a source of total RNA. The tissue was homogenized in extraction reagent (TRizol; Invitrogen, Carlsbad, CA) and total RNA extracted from the aqueous phase with chloroform. First-strand cDNA was synthesized with an RNA PCR kit (GeneAmp; Applied Biosystems, Inc., Foster City, CA). The 20-μL reaction volume contained 1 μg total RNA, 2.5 μM random hexamers, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 5 mM MgCl2, 1 mM of each dNTP, and 0.5 U RNase inhibitor, and 50 U murine leukemia virus (MuLV) reverse transcriptase. After 10 minutes at room temperature, the reaction mixture was incubated at 42°C for 15 minutes and terminated by incubation at 70°C for 15 minutes at room temperature, the reaction mixture was incubated at 42°C for 10 minutes and terminated by incubation at 70°C for 15 minutes. The reaction mixture was incubated at 42°C for 10 minutes after addition of 5 μL of cDNA synthesis reaction mix and 45 μL of PCR reaction mix containing 0.25 U Taq DNA polymerase (Perkin-Elmer, Foster City, CA), 0.4 μM of primers CNTFR 6F and CNTFR 2R (Table 1), 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM dNTP, and 0.5 U Taq polymerase. PCR amplifications with primers CNTFR 1F and CNTFR 1R are expected to generate a 1272-bp DNA fragment that contains the complete canine CNTFRα coding sequence. Reactions were performed for 36 cycles at an annealing temperature of 55°C for 20 seconds, a polymerization temperature of 72°C for 40 seconds, and a heat-denaturation temperature of 94°C for 20 seconds in a thermal cycler (model PTC-200; MJ Research, Waltham, MA). The PCR-amplified DNA fragment was cloned in a commercial vector using a TA-cloning kit (pCR2.1; Invitrogen, San Diego, CA) according to the manufacturer’s recommendations. Sequencing was done by Taq cycle with terminators (DyeDeoxy) in an automated sequencer (Prism 3700; Applied Biosystems Inc., Foster City, CA) at the core sequencing facility of Cornell University. The MEGA program (Molecular Evolutionary Genetics Analysis, ver. 2.0, www.megasoftware.net; available in the public domain; developed by Kumar S et al.) was used to establish the degree of homology between the canine CNTFRα coding sequence (nucleotide and amino acid) and that of the human (M73238), mouse (NM016673), rat (NM216673), and hamster cell line with a retention estimate of 28%. CNTFRα maps to the short arm of human chromosome 9 (9p13). We therefore selected six markers (REN142009, REN275M05, IFNA3, IFNA1, REN174D18, and REN147002) located on the canine homologous region on canine chromosome 11 (CFA 11) in the RHDF5000 map19 to generate a framework map and establish the map position of this gene in the dog. Primers CNTFR 9F and CNTFR 9R (Table 1) were used to amplify a 112-bp fragment of canine-specific CNTFRα. The map was constructed using MultiMap software20 based on best two-point analysis, placing markers at a lod score of 3.0 for overall order (http://www.mgc.har.mrc.ac.uk/ provided in the public domain by the UK Mouse Genome Centre and Mammalian Genetics Unit, Harwell, UK). Distances are referred to as centimorgans (cR3000), in reference to the 3000-rad value used to construct the panel.

Animals and Histologic Procedures

Retinas from normal adult beagles were used for both the in situ hybridization and immunocytochemistry studies. Dogs were anesthetized with intravenous pentobarbital and the eyes rapidly enucleated in the light. After a 3-hour fixation of the entire globe at 4°C in 4% paraformaldehyde (PAF) in 0.1 M phosphate-buffered saline, the posterior segment was isolated and fixed for an additional 24 hours at 4°C in 2% PAF in 0.1 M phosphate-buffered saline. The tissue then was trimmed, cryoprotected in a solution of 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 for 48 hours, and embedded in optimal cutting temperature (OCT) compound. Cryosections were cut at a 7-, 10-, or 15-μm thickness. All research conducted was in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In Situ Hybridization

A 369-bp fragment of canine CNTFRα cDNA encoding exons 7 and 8 was amplified with primers CNTFR 6F and CNTFR 2R (Table 1) and subcloned in a dual promoter vector (pCRII-TOPO; Invitrogen). After purification using a kit (QIAprep Miniprep; Qiagen, Valencia, CA), the plasmid was linearized using HindIII and EcoRV restriction enzymes, and single-strand sense and antisense digoxigenin (DIG)-labeled RNA probes were generated by T7 and Sp6 RNA polymerases, respectively, using a DIG RNA labeling kit (Roche Diagnostics, Inc., Mannheim, Germany). The slides were air dried overnight at 40°C, then washed twice for 5 minutes in PBS, 100 mM glycine in PBS, 0.3% Triton X-100 in PBS, and rinsed with PBS. The 15-μm-thick sections were then permeabilized with 500 ng/ml proteinase K in 100 mM Tris-HCl, and 50 mM EDTA (pH 8.0), for 30 minutes at 37°C, and postfixed with 4% PAF in PBS. After two rinses in PBS, the sections were acetylated twice for 5 minutes with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) and incubated for 10 minutes with deionized formamide in 2× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.2). The sections were then hybridized with 100 ng of RNA probe in hybridization buffer (In Situ Hyb Buffer; Ambion, Austin, TX) for 16 hours at 55°C for 15 minutes and terminated by incubation at 70°C for 15 minutes.
50°C in a humid chamber. After hybridization, the slides were washed twice in 2×H100 SSC and twice in 1×H100 SSC at 37°C. They were then treated with RNase A (20 μg/mL in 500 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 8.0]) for 30 minutes at 37°C and washed twice in 0.1×H100 SSC for 30 minutes at 37°C. RNA hybrids were detected by incubation for 30 minutes with an alkaline-phosphatase–conjugated anti-DIG antibody (1:500) and then for 16 hours with the chromogenic substrates 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP; DIG Nucleic Acid Detection Kit, Roche Diagnostics). Slides were mounted with antifade mounting medium (Aqua Poly-mount; Polysciences, Warrington, PA) and examined by 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 for display (Photoshop; Adobe, Mountain View, CA).

FIGURE 1. Comparison of the dog CNTFRα amino acid sequence with that of the human, mouse, rat, and chicken. (A) Alignment of amino acid sequences. Underscored sequences: conserved cysteine residues and the conserved cytokine receptor motif (WSXSW box); shaded boxes: putative N-glycosylation sites (ArgXSer/Thr) in the dog sequence. (B) Levels of homology between amino acid sequences are indicated as a percentage.
Cloning of Canine CNTFRα cDNA

RT-PCR, using primers that hybridize in the 3′ and 5′ untranslated region (UTR) of the CNTFRα gene, amplified a single 1272-bp product from brain-derived mRNA. Sequence analysis revealed the CNTFRα coding sequence (1119 bp), 26 bp of 5′ UTR, and 86 bp of 3′ UTR (GenBank no. AF529215). Alignment of the nucleotide coding sequence (data not shown) showed a cDNA of identical length with that of human, mouse, and rat, and high sequence identity of 93.5%, 89.5%, and 88.7%, respectively. The alignment of the predicted amino acid sequence of canine CNTFRα with that of the human, mouse, and rat also showed a high degree of homology between these species (Fig. 1). The canine CNTFRα amino acid sequence is longer than that of the chicken (372 vs. 362 amino acids), and the chicken sequence shares a lower degree of homology with the dog (69.1%) and other mammals. The amino acid identity was

Immunoblot Analysis

For Western blot analysis, adult canine retina was homogenized in PBS containing a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, MO), and, after sonication, the protein level was determined by the Bradford method (Bio-Rad protein assay; Bio-Rad, Hercules, CA). Samples consisting of dog and chicken retinal protein lysates and recombinant rat CNTFRα (amino acid residues 1-346; R&D Systems, Minneapolis, MN) were placed in the sample buffer containing 4% glycerol, 0.4% sodium dodecyl sulfate, 1% β-mercaptoethanol, 0.005% bromo-phenol blue in 12.5 mM Tris-HCl buffer (pH 6.8), and heated at 100°C for 5 minutes. Samples and molecular weight standards were separated by SDS-PAGE (4% stacking gel, 10% separating gel). Transfer of proteins from gels to polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Bedford, MA) was performed in prechilled transfer buffer by SDS-PAGE (4% stacking gel, 10% separating gel). Transfer of proteins from gels to polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Bedford, MA) was performed in prechilled transfer buffer by goat anti-rabbit secondary antibody conjugated with horseradish peroxidase. Sections were then treated with 0.25% Triton X-100 in PBS containing 0.5% Tween-20 overnight at 4°C. The membrane was then blocked with 10% skim milk in Tris-buffered saline containing 0.5% Tween-20 overnight at 4°C. The membrane was incubated for 1.5 hour with a protein A-purified rabbit anti chick CNTFRα antibody (1:100,000; developed by one of the authors [HR]), followed by goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10,000; Zymed, San Francisco, CA). The blots were developed with the enhanced chemiluminescence (ECL) method, according to the manufacturer's recommendations (Amersham Biosciences, Piscataway, NJ).

Immunocytochemistry

Tissue sections (7–10 μm thick) 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 minutes, followed by 10% normal goat serum (NGS) with 0.25% Triton X-100 and 0.05% sodium azide in PBS for 20 minutes. They were then incubated with primary antibodies diluted in PBS with 0.25% Triton X-100 and 0.05% sodium azide overnight at 4°C. Primary antibodies used in this study were: an affinity-purified polyclonal rabbit anti-chick CNTFRα (1:1000 dilution) and a protein A-purified polyclonal rabbit anti-chick CNTFRα (1:2000 dilution). These two antibod-
higher between the rat and the human sequences and lower between the rat and the chicken sequences. Hallmarks of cytokine receptors, such as clusters of cysteine residues, putative N-glycosylation sites, and the cytokine receptor consensus motif (WSXWS box), were conserved in the canine CNTFRα amino acid sequence (Fig. 1A).

**RH Mapping**

Radiation hybrid mapping using the RH083000 canine-hamster panel placed CNTFRα on canine chromosome 11 (CFA 11) in a position approximately 70.41 cr3000 telomeric to the microsatellite REN275MO5 and 56.19 cr3000 centromeric from the gene markers IFNA3/IFNA1 (Fig. 2). Marker order was supported with a lod score of 3.5 and was similar to that obtained with a different radiation hybrid panel made with rosatellite REN275MO5 and 56.19 cR3000 centromeric from the cytogenetic p13 region of human chromosome 9 (HSA 9p13). Thus our results are in agreement with human mapping data, which localizes CNTFRα to HSA 9p13.

**Transcription of CNTFRα in Different Tissues**

RT-PCR was used to examine the expression of CNTFRα in various tissues. The transcript was detected in brain, retina, spleen, lung, liver, and kidney tissues (Fig. 3). The specificity of the RT product was based on the product size that was obtained with control retina treated by DNase I digestion, as well as on the absence of any product amplification in tissues processed by omitting the reverse transcriptase enzyme (data not shown).

**Localization of CNTFRα Expression in the Adult Canine Retina**

In situ hybridization was used to determine which retinal cells transcribe canine CNTFRα. With the antisense probe, the CNTFRα message was found in the retinal pigment epithelium (RPE), photoreceptors, inner nuclear layer (INL), and ganglion cells; no labeling was observed with the sense probe, although a very weak background was noticed in some sections (Fig. 4). The labeling was most intense in the central retina and decreased toward the periphery. In the visual cells, labeling was present at the level of the external limiting membrane (ELM), and in the proximal region of the inner segments (IS; myoid region). Labeling was present throughout the INL but was most intense in the cells located in the vitreal and scleral borders.

To complement the in situ hybridization results, we performed immunocytochemical studies using the polyclonal antibodies directed against chick CNTFRα. Both antibodies (affinity-purified, and protein A-purified) produced a similar and consistent pattern of labeling that was absent in control sections (Fig. 5C). There was robust staining of all the inner segments in the photoreceptor layer (Figs. 5D–F) and also distinct labeling of the outer and inner plexiform layers (OPL, IPL, respectively), cells of the INL, and ganglion cells and their axons (nerve fiber layer, NFL; Figs. 5A, 5D, 5F). With the affinity-purified antibody, different populations of cells, predominantly located at the scleral and vitreal borders of the INL were identified, but they were not distinct with the protein A-purified antibody (compare Figs. 5A and 5D with 5F). Labeling of the RPE was present but variable in intensity (Figs. 5B, 5F). Staining of the photoreceptor inner segments was observed, and, in addition, distinct labeling was present throughout a subclass of photoreceptor cells with nuclei that were located at the outermost border of the ONL (Figs. 5E, 5F). The position and cytologic characteristics of these cells, as well as the similar pattern of cone labeling obtained with two antibodies that label only cones (anti-human cone arrestin) or cones and rods (anti-PDEγ; Figs. 5G, 5H), suggest that, in addition to its localization at the rod inner segment, CNTFRα is present throughout most of the cone photoreceptor cell.

To verify the specificity of the protein A-purified anti-chick CNTFRα antibody, we performed immunoblot analysis. When recombinant rat CNTFRα (amino acid residues 1-346) was resolved by SDS-PAGE, the anti-chick CNTFRα antibody identified it at an apparent size of approximately 50 kDa, consistent with the molecular mass indicated by the manufacturer. In protein lysates from dog retina, the antibody recognized a single protein migrating with a molecular mass of approximately 62 kDa (Fig. 6). A similar result was observed with protein lysates from retinas of a 2-week-old chick resolved by SDS-PAGE under reducing conditions (data not shown). This molecular mass was slightly smaller than that reported for the chick under nonreducing conditions, and could be explained by differences in the electrophoresis conditions. Our immunoblot results with the chicken and dog retinal lysates are similar to those reported for rat central nervous system and retina using a different polyclonal antibody. A band with a molecular mass of approximately 50 kDa could also be observed when the film was exposed to the blot for longer periods of time. Specificity of the anti-chick CNTFRα antibody has been previously demonstrated both on immunoblots and immunohistochemical sections of chick dorsal root ganglia. The specific labeling was inhibited by preincubating the antibody with the recombinant chick CNTFRα used to generate the antibody. Because the source of the recombinant chick CNTFRα used to block the anti-chick CNTFRα antibody is no longer available, we could not repeat these experiments with the dog tissues.

**Discussion**

In this study, we cloned the canine CNTFRα cDNA and showed that there was a high level of homology, both at the nucleotide and amino acid levels, when comparing it with that of the...
The degree of amino acid homology between dog and chicken was lower, but still sufficient to allow us to use in the dog retina polyclonal antibodies that were raised against chicken CNTFRα/H9251. Such a level of conservation, at least for some domains of the protein, suggests that the CNTFRα/H9251 signaling pathway is conserved across evolution.

In our results in the normal adult canine retina, the RPE, photoreceptors, and cells in the INL and ganglion cell layer (GCL) transcribed the CNTFRα/H9251 gene. We observed a strict concordance between the retinal cells labeled by in situ hybridization and those stained by immunocytochemistry, particularly with the affinity-purified antibody. This suggests that all cells that transcribe the CNTFRα gene also express its protein. However, we cannot exclude the possibility that some of these cells also may bind the soluble form of CNTFRα. Such a soluble form could be released by neighboring cells after cleavage of the glycosyl-phosphatidylinositol link by PI-PLC. Labeling of the INL, both by in situ hybridization and immunocytochemistry, was more intense in cells located at the outer and innermost part of this layer, suggesting that horizontal, amacrine, and Müller cells express CNTFRα. This was consistent with the location of CNTFRα transcript in the rat INL. However, the use of cell-specific markers, coupled with CNTFRα immunolocalization, is necessary for specific identification of the cells in the INL that express this neurotrophic receptor.

The antibodies directed against CNTFRα that were used in our study showed an intense labeling pattern at the level of the RPE, photoreceptors, OPL, INL, IPL, GCL, and NFL. In addition to the photoreceptor IS labeling, the perinuclear cytoplasm, axon, and synaptic terminal of cones also were intensively labeled with the antibodies raised against chick CNTFR. Although we did not use any antibodies specific to rods, the labeling pattern of the entire IS layer was very different from that obtained with antibodies that solely stain cone photoreceptor cells (Fig. 5H), confirming that CNTFRα is present in both cone and rod inner segments.
tion that tissue fixation is critical in these procedures, at least in the canine retina. Our findings are based on using a very mild fixation protocol; however, we have observed (data not shown) a reduction or absence of labeling of the inner retina, but not of the photoreceptor IS, with more prolonged fixation (24 hours in 4% PAF). In an attempt to evaluate whether the expression of CNTFRα by photoreceptors is specific to the dog or is common to a wide variety of species, we are currently undertaking similar studies in several other mammalian species.

Determining the localization of the expression of the receptor for CNTF is critical, because this survival factor is being considered as a potential treatment for retinal degenerations. Its sustained delivery is currently in late preclinical development and clinical trials are expected to begin in patients with retinitis pigmentosa (www.neurotech.fr/presse/index.htm). If CNTFRα is also found in human photoreceptors, then evaluating its level of expression during the course of retinal degeneration may be a valid approach for evaluating the potential therapeutic role of CNTF.

In conclusion, we have shown the expression of CNTFRs in photoreceptor cells of the normal adult canine retina. These results suggest that, at least in the dog, CNTF may act through a direct mechanism to rescue photoreceptors in the rd1 model of retinal degeneration. If such a site of action is also present in the human retina, it may lead to novel therapeutic approaches for RP.

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