OPEN ACCESS Freely available online

PLoS one

Ciliary Neurotrophic Factor Induces Genes Associated with Inflammation and Gliosis in the Retina: A Gene Profiling Study of Flow-Sorted, Müller Cells

Wei Xue[1☯], Radu I. Cojocaru[2☯], V. Joseph Dudley[1], Matthew Brooks[2], Anand Swaroop[2], Vijay P. Sarthy[1]*

1 Department of Ophthalmology, Northwestern University Feinberg Medical School, Chicago, Illinois, United States of America, **2** Neurobiology, Neurodegeneration and Repair Laboratory, National Eye Institute, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

Background: Ciliary neurotrophic factor (CNTF), a member of the interleukin-6 cytokine family, has been implicated in the development, differentiation and survival of retinal neurons. The mechanisms of CNTF action as well as its cellular targets in the retina are poorly understood. It has been postulated that some of the biological effects of CNTF are mediated through its action via retinal glial cells; however, molecular changes in retinal glia induced by CNTF have not been elucidated. We have, therefore, examined gene expression dynamics of purified Müller (glial) cells exposed to CNTF *in vivo*.

Methodology/Principal Findings: Müller cells were flow-sorted from *mgfap-egfp* transgenic mice one or three days after intravitreal injection of CNTF. Microarray analysis using RNA from purified Müller cells showed differential expression of almost 1,000 transcripts with two- to seventeen-fold change in response to CNTF. A comparison of transcriptional profiles from Müller cells at one or three days after CNTF treatment showed an increase in the number of transcribed genes as well as a change in the expression pattern. Ingenuity Pathway Analysis showed that the differentially regulated genes belong to distinct functional types such as cytokines, growth factors, G-protein coupled receptors, transporters and ion channels. Interestingly, many genes induced by CNTF were also highly expressed in reactive Müller cells from mice with inherited or experimentally induced retinal degeneration. Further analysis of gene profiles revealed 20–30% overlap in the transcription pattern among Müller cells, astrocytes and the RPE.

Conclusions/Significance: Our studies provide novel molecular insights into biological functions of Müller glial cells in mediating cytokine response. We suggest that CNTF remodels the gene expression profile of Müller cells leading to induction of networks associated with transcription, cell cycle regulation and inflammatory response. CNTF also appears to function as an inducer of gliosis in the retina.

Citation: Xue W, Cojocaru RI, Dudley VJ, Brooks M, Swaroop A, et al. (2011) Ciliary Neurotrophic Factor Induces Genes Associated with Inflammation and Gliosis in the Retina: A Gene Profiling Study of Flow-Sorted, Müller Cells. PLoS ONE 6(5): e20326. doi:10.1371/journal.pone.0020326

Editor: Paul Proost, University of Leuven, Rega Institute, Belgium

Received February 18, 2011; **Accepted** April 21, 2011; **Published** May 26, 2011

Funding: This work was supported by NIH grant EY019325 (VS), unrestricted funds from Research to Prevent Blindness and intramural program of the National Eye Institute. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: vjsarthy@northwestern.edu

☯ These authors contributed equally to this work.

Introduction

Cytokines are secretory proteins that were initially characterized as immune modulators, but have been subsequently found to promote proliferation and differentiation in the nervous system [1]. The cytokine, ciliary neurotrophic factor (CNTF: NM_170786.2), belongs to the interleukin 6 (IL-6: NM_031168.1) family of cytokines that share one or more of the receptor subunit, glycoprotein 130 (gp130: NM_010560.3) [2,3]. Activation by CNTF requires a heterotrimeric complex consisting of CNTF receptor α (CNTFRα: NM_001136056.2), leukemia inhibitory factor β (LIFRβ: NM_001113386.1) receptor and gp130 [2,3]. CNTF acts on cells primarily by stimulating the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway [3]. Additionally, CNTF may stimulate cell survival, through MEK [extracellular signal-regulated kinase (ERK) kinase]/MAPK (mitogen activated protein kinase), Phosphoinositide 3-kinase (PI3-K)/Akt, and Nuclear factor kB (NF-kB) pathways [4–12].

CNTF promotes the survival of a variety of neurons and oligodendrocytes, and induces neurite outgrowth and axon regeneration in both developing and mature nervous system [13–18]. In addition, it appears to be an effective neuroprotective agent in animal models of CNS neurodegenerative diseases [19]. CNTF has also been reported to activate leptin-like pathways in the brain and reduce body fat and stress in a leptin-independent manner [20].

In the vertebrate retina, CNTF exhibits numerous effects on the development, differentiation and survival of retinal neurons [21]. CNTF appears to play a critical role in progenitor commitment to the rod photoreceptor cell fate and in photoreceptor differentiation [22–24]. It is reported to increase the long-term survival of retinal ganglion cells after axotomy [25,26]. Furthermore, CNTF is capable of retarding retinal degeneration in several animal models

of retinitis pigmentosa [27–36]. CNTF appears to be the most effective and mutation-independent, neuroprotective agent known. A recent phase I clinical trial demonstrated the safety of chronic CNTF delivery in patients with retinitis pigmentosa [37], and phase II trials have been completed for patients with retinitis pigmentosa (RP) and age-related macular degeneration (AMD).

Molecular mechanisms proposed to explain the neuroprotective role of CNTF in the retina include (i) direct action on photoreceptors to prevent their apoptosis (ii) stimulation of Müller (glial) cells to produce photoreceptor survival factors [38] (iii) enhanced synthesis or distribution of glutamate transporters, thereby improving glutamate handling, resulting in less excitotoxic damage to retinal neurons [39] and (iv) induction of metabolic plasticity and increased resistance to metabolic damage [40]. Nevertheless, these mechanisms remain to be evaluated.

A primary target of CNTF action in the retina is the Müller cell, a predominant glial cell that is responsible for maintaining the health and activity of retinal neurons [41,42]. Müller cells contain CNTF receptors [19], and the JAK-STAT signaling pathway is rapidly activated in Müller cells in response to intravitreal CNTF injection [43–46]. Many of the biological effects of CNTF are proposed to be mediated through Müller cells [38]. Here, we have determined the global transcriptional response of Müller cells to CNTF *in vivo* with a goal to elucidate the molecular basis of its biological actions in the retina.

Results

Purification of Müller cells by flow-sorting

Müller (glial) cells constitute ~2% of the cells in the mouse retina [47]. A major hurdle in studying CNTF action on Müller cells has been the lack of a reliable method to separate these cells away from retinal neurons. To circumvent this problem, we recently used Fluorescence Activated Cell Sorter (FACS) to purify Müller cells from *mgfap-egfp* (mouseGlial fibrillary acidic protein (NM_001131020.1)-enhanced green fluorescent protein) transgenic mice in which only Müller cells express GFP [48] (Fig. 1A). Retinas were dissociated into a single cell suspension following treatment with papain, and GFP^+ (Müller) cells were enriched by FACS (Fig. 1B–D). During dissociation, Müller cells lose their radial processes and round up, but GFP remaining in the cell body is sufficient for flow sorting. We routinely obtained 30–50,000 viable cells per mouse retina. For microarray studies, we pooled retinas from 4–6 mice in each independent sample.

Transcriptional analysis of Müller cells

To study the transcriptional response of Müller cells to CNTF, *mgfap-egfp* transgenic mice were intravitreally injected with CNTF or PBS (control). One day later, GFP^+-Müller cells were flow-sorted, and total RNA was prepared for microarray analysis [49]. CNTF treatment resulted in differential expression of 923 transcripts that showed at least two-fold change (P-value <0.05). Of these, 691 transcripts exhibited 2-to 17-fold higher expression, whereas 232 transcripts revealed 2-to 5-fold reduction. We noticed, however, that genes such as *gfap* that are known to be induced by CNTF, were not detected. When the microarray data were analyzed with P-value<0.1, however, we found several genes of biological interest among 1261 differentially expressed transcripts. Of these, 939 transcripts were elevated 2- to 17-fold, and 322 transcripts were depressed 2- to 5-fold. The differentially expressed genes fell into several functional types (Table 1). A complete list of differentially regulated genes is presented in Table S1. A hierarchical cluster analysis also showed that genes with increased expression level outnumbered genes with decreased level, and that CNTF-induced genes fell into several clusters of co-expressed genes (Figure 2).

Validation of transcript level changes

Real time-PCR (qPCR) analysis of independent samples of FACS-purified Müller cells was carried out to validate the findings from the microarray data [23]. We performed primer design, one-step RT-PCR reaction, and qPCR analysis as previously described [49]. To determine the relative change in gene expression, we used the system software to compare the number of cycles (C_t) needed to reach the midpoint of the linear phase. All observations were normalized to the housekeeping gene *Gapdh* (NM_008084.2). We observed that among the 15 transcripts (from different gene families) tested, 12 genes demonstrated higher transcript levels, consistent with the microarray data (Figure 3). Two transcripts (NUD6: NM_008006.2, CKLF: NM_029295.2) showed a negative trend in the qPCR assay (Figure 3), whereas one transcript (MAPK8: NM_016700.4) that exhibited reduced expression in the microarray data also showed a downward trend in the qPCR analysis. These results establish that transcript changes observed in the microarray data represent the gene expression changes in Müller cells.

Pathways and biological processes associated with CNTF

Computational approaches were employed to identify biological pathways among the list of differentially expressed genes that

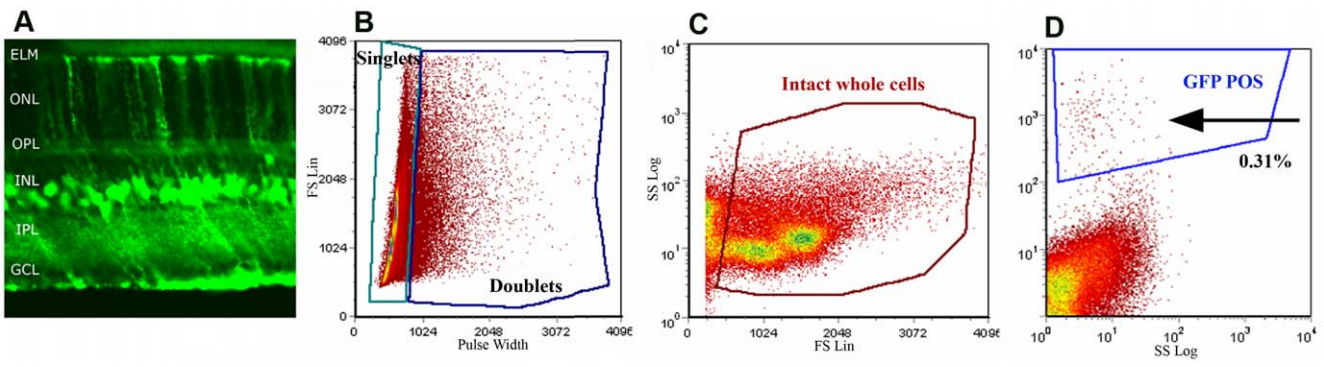

Figure 1. Flow-sorting Müller cells from mouse retina. (**a**) Transverse section of retina from mgfap-egfp transgenic mice. Note GFP expression in radially-oriented, Müller cell processes. (**b–c**) Scatter plot of flow-sorted, dissociated cells from mgfap-egfp transgenic mouse retina. The cells used for RNA preparation were collected from the enclosed area in F indicated by the arrow. Dissociated cells from non-transgenic mouse retina showed no GFP-fluorescence.
doi:10.1371/journal.pone.0020326.g001

Table 1. List of differentially regulated genes in Müller cells one day after CNTF treatment.

Gene Symbol	Gene Name	Fold Change	Type
Ccl6	Chemokine ligand 6	13.5	Cytokine (19, 1)
Ccl5	Chemokine ligand 6	13.3	-
Spp1	Secreted phosphoprotein 1	10	-
Tnf	Tumor necrosis factor	7.8	-
Il1rn	Interleukin 1 receptor antagonist	7.5	-
Av3	Vav 3 guanine nucleotide exchange factor	-2.2	-
Hmox1	Heme oxygenase	15.9	Enzyme (115, 32)
Iigp1	Interferon inducible GTPase 1	8.8	-
Ptgs2	Prostaglandin-endoperoxide synthase 2	10.3	-
Oasl	2′-5′-oligoadenylate synthetase-like	6.9	-
XDH	Xanthine dehydrogenase	6.6	-
Pafah1b1	Platelet-activating factor acetylhydrolase 1b	-2.5	-
Rab2b	RAB2B, member RAS oncogene family	-2.5	-
Rab3c	RAB3C, member RAS oncogene family	-2.5	-
Rims1	Regulating synaptic membrane exocytosis 1	-2.6	-
Scd2	Stearoyl-Coenzyme A desaturase 2	-2.6	-
Ccrl2	Chemokine (C-C motif) receptor-like 2	12.5	G-protein (16, 9)
Gpr109a	G protein-coupled receptor 109A	9.4	-
Ccr1	Chemokine (C-C motif) receptor 1	8.9	-
C5ar1	Complement component 5a receptor 1	7.7	-
Emr1	Egf-like module, mucin-like chemokine-like recp 1	7.0	-
Tm2d1	TM2 domain containing 1	-2.0	-
Xpr1	Xenotropic and polytropic retrovirus receptor 1	-2.2	-
Ptger3	Prostaglandin E receptor 3 (subtype EP3)	-2.7	-
Lphn3	Latrophilin 3	-2.7	-
Gpr158	G protein-coupled receptor 158	-2.9	-
Edn2	Endothelin 2	6.7	Growth Factor (8, 2)
Gmfg	Glia maturation factor, gamma	5.3	-
Nudt6	Nudix-type motif 6	4.2	-
Tgfb1	Transforming growth factor, beta 1	3.8	-
Gmfg	Glia maturation factor, gamma	3.4	-
Angpt1	Angiopoietin 1	-2	-
Fgf9	Fibroblast growth factor 9 (glia-activating factor)	-2	-
Kcnk6	Potassium channel, subfamily K, member 6	4.5	Ion Channel (7, 11)
P2rx7	Purinergic receptor P2X, ligand-gated ion cha, 7	3.9	-
Kctd12	Potassium channel tetramerisation domain	3.2	-
Kcnn4	Potassium intermediate/small calcium-activated	2.7	-
Hvcn1	Hydrogen voltage-gated channel 1	2.4	-
Kcnj6	Potassium inwardly-rectifying channel	-2.3	-
Kcnip1	Kv channel interacting protein 1	-2.4	-
Scn2b	Sodium channel, voltage-gated, type II, beta	-2.4	-
Kcnma1	Potassium large conductance calcium-activated	-2.7	-
Stx1b	Syntaxin 1B	-3.1	-
Vav1	Vav 1 guanine nucleotide exchange factor	9.7	Transc. Regulator (78, 28)
Egr2	Early growth response 2	8.5	-
Atf3	Activating transcription factor 3	7.5	-
Klf4	Kruppel-like factor 4 (gut)	7.4	-
Ifi16	Interferon, gamma-inducible protein 16	7.3	-
Ldb2	LIM domain binding 2	-3.3	-
Nfib	Nuclear factor I/B	-3.3	-
Nfia	Nuclear factor I/A	-3.0	-
Ccnt2	Cyclin T2	-2.9	-
Pcgf6	Polycomb group ring finger 6	-2.5	-
Cd14	CD14 molecule	9.3	Transm. Receptor (50, 3)
Il7r	Interleukin 7 receptor	8.9	-
Msr1	Macrophage scavenger receptor 1	8.3	-
Tlr2	Toll-like receptor 2	8.0	-
Cd69	CD69 molecule	7.9	-
Cd247	CD247 molecule	-2.6	-
Gpc6	Glypican 6	-2.5	-
Ifnar1	Interferon (alpha, beta and omega) receptor 1	-2.1	-
Slc11a1	Solute carrier family 11	7.3	Transporters (33,15)
Slc15a3	Solute carrier family 15, member 3	5.6	-
Slc3a3	Solute carrier family 13	5.6	-
Tap1	Transporter 1, ATP-binding cassette, sub-family B	5.4	-
Mcl1	Myeloid cell leukemia sequence 1 (BCL2-related)	5.2	-
Atp6v1b2	ATPase, H+ transporting, lysosomal 56/58kDa	-2.7	-
Sec62	SEC62 homolog	-2.7	-
Rph3a	Rabphilin 3A homolog	-2.6	-
Vps41	Vacuolar protein sorting 41 homolog	-2.5	-
Slc6a1	Solute carrier family 6, member 1	-2.5	-

The table lists the top-five up- and down-regulated genes derived from IPA analysis. The total number of up- and down–regulated genes in each type are listed along with the functional types, in column 4.

doi:10.1371/journal.pone.0020326.t001

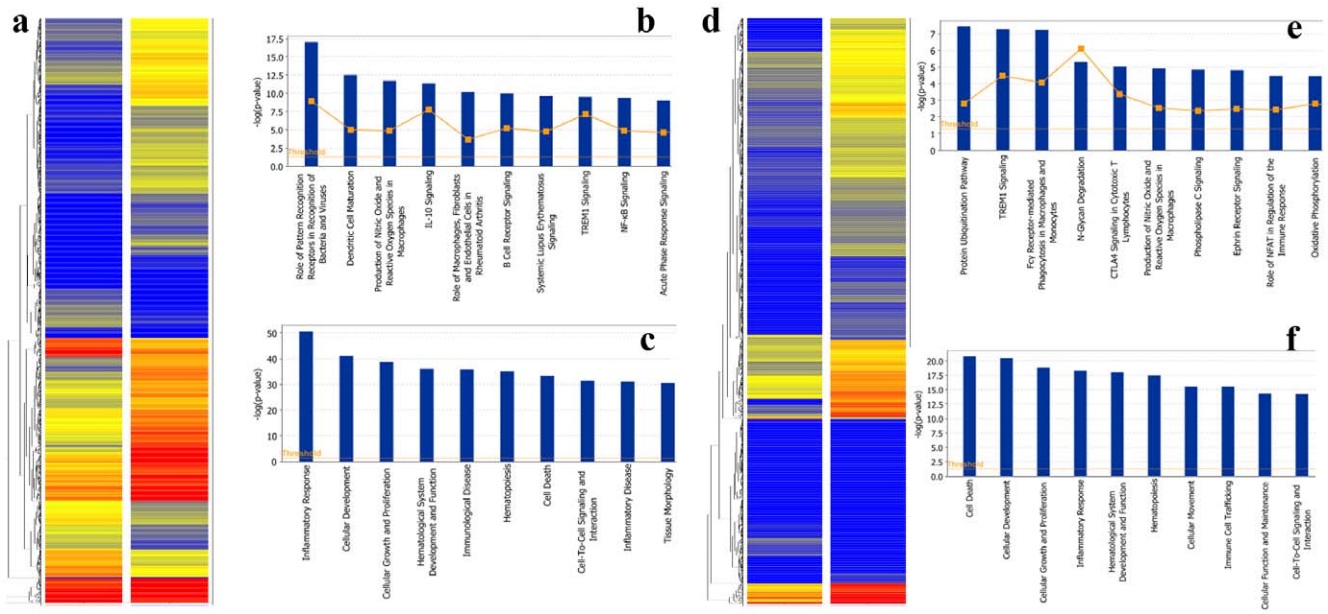

Figure 2. Microarray data analysis. (**a**) Hierarchical clustering showing 1261 probes that have a *P*-val ≤0.1 and a minimum 2-fold change between the CNTF and PBS samples at Day 1. Bright blue indicates lowest signal with increasing values indicated by yellow shading to bright red, representing peak signal. (**b**) Most significant 10 canonical pathways corresponding to Day 1. (**c**) Most significant biological functions for the same list of genes. (**d**) Hierarchical clustering showing 1541 probes that have a *P*-val ≤0.1 and a minimum 2-fold change between the CNTF and PBS samples at Day 3. (**e**) Most significant 10 canonical pathways corresponding to Day 3. (**f**) Most significant biological functions for the same list of genes.
doi:10.1371/journal.pone.0020326.g002

respond to CNTF. We used the Ingenuity® Pathway Analysis (IPA) program (Ingenuity Systems, Red Wood City, CA), which sorts genes into canonical pathways based on the scientific literature and indicates significantly overrepresented pathways [www.ingenuity.com] (Figure 2). The top canonical pathways were concerned with the role of pattern recognition receptors, production of reactive oxygen species, IL10 signaling and role of macrophages in immune disease, consistent with the known roles of IL-6 family of cytokines (Table 2). The top biological functions were cellular development, cell death and cell- to -cell signaling (Fig. 2), which are related to immune response, immunological disease and inflammatory disease.

Genes induced by CNTF

Cytokines represented one of the highly induced sets of genes in Müller cells after exposure to CNTF (Table 1,S1). Furthermore, many cytokines types including interleukins, chemokines and chemokine-like factors, tumor necrosis factor and colony stimulating factor were elevated 2 to 14-fold. Among chemokines, both C-C and CXC family members were detected. Intriguingly, multiple proinflammatory chemokines were induced. Several enzymes involved in intermediary, nucleic acid and lipid metabolism were also elevated. Heme oxygenase-1 (NM_010442.2) was induced at a high level, pointing to a role for CNTF in neuroprotection against oxidative damage. Interestingly, transcript levels for several chemokine receptors, CCRL2 (NM_017466.4), CCR1 (NM_009912.4), CXCR4 (NM_009911.3) and CCR5 (NM_009917.5) were increased in CNTF-treated Müller cells. The induction of chemokines and chemokine receptors suggests existence of paracrine loops that could further modify CNTF-induced gene expression pattern.

The well-known growth factor and anti-inflammatory agent, transforming growth factor ß (TGFß: NM_011577.1) was induced by CNTF. Expression of both TGFß and proinflammatory chemokines suggests that CNTF has complex effects on Müller cells. Surprisingly, endothelin 2 (NM_007902.2), a signaling molecule released by degenerating photoreceptors that initiates Müller cell gliosis [50], was strongly induced by CNTF; however, no change in endothelin receptor B (*endrB*: NM_001136061.1) expression was detected.

There were substantial changes in the expression of ion channels including the potassium channels, KCNK6 (NM_0010-33525.3) and KCNN4 (NM_001163510.1). We did not, however, detect transcript level changes for two potassium channels, Kir4.1 (NM_001039484.1) and Kir2.1 (NM_008425.4), which are known to be involved in potassium ion-homeostasis in Müller cells [42]. As CNTF treatment induces a large number of genes, it was not surprising that several transcriptional regulators such as CCAAT/enhancer binding proteins, early growth response proteins, and forkhead box proteins were elevated. In comparison, far fewer translational regulators were altered.

We also identified higher expression of several transmembrane receptors including cell adhesion molecules such as CD14 (NM_009841.3) and CD86 (NM_019388.3) although there was no change in CD44 (NM_009851.2), a hyaluronic acid receptor, expressed in Müller cells [41]. In addition, a large number of interleukin receptors were induced, suggesting the existence of feed back loops in CNTF-induced networks. Expression of Toll-like receptors 1 and 2 (TLR1: NM_030682.1 and TLR2: NM_011905.3) was increased following CNTF treatment (Table S1, Figure 3), suggesting that Müller cells are part of the innate immune response in the retina. One of the roles of Müller cells involves transport and export of nutrients and related small molecules in the retina [41,4]. Induction of genes for a variety of transporters belonging to ABC and SLC superfamilies is in accord with the metabolic functions of Müller cells [41]. Interestingly, SLC11A1 (NM_013612.2) has been proposed as an autoimmunity susceptibility gene.

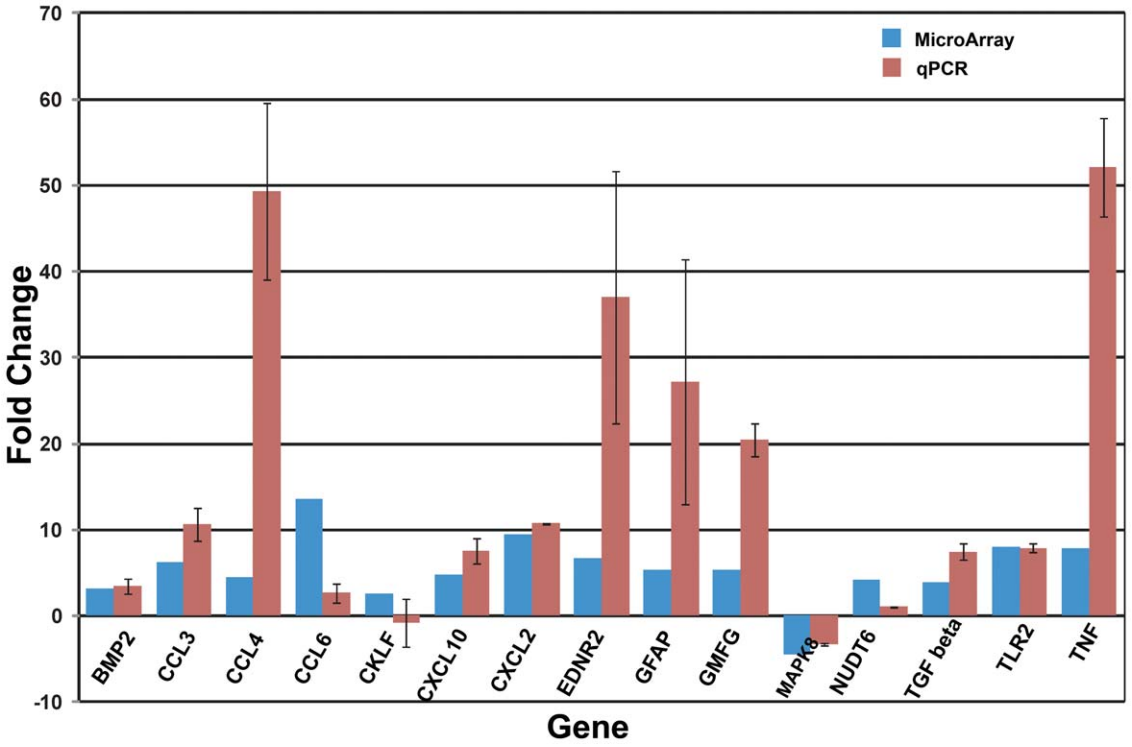

Figure 3. qRT-PCR validation of selected genes using independent biological samples. Predicted fold changes from microarray analysis and relative gene expression fold change from qRT-PCR for 3–6 independent biological replicates of flow-sorted Müller cells. Error bars indicate ±SEM.
doi:10.1371/journal.pone.0020326.g003

Table 2. Canonical Pathway analysis of CNTF-induced genes at Day 1 and Day 3.

Canonical Pathways- Day 1	**CNTF–associated genes**	**p-value**
Role of Pattern Recognition Receptors of Bacteria and Viruses	*Tlr1, Pik3r5, Oas1b, Ccl5, C1qb, Ifih1, C5ar1, Ticam1, Pik3cg, Creb1, Tlr7, Casp1, Oas1, C3, Nlrp3, Oas2, Nfkb2, Oas3 (includes EG:4940), Tlr2, Irf7, Clec7a, Syk, Ddx58, Tlr6, Pik3r6, Pik3cd, Elf2ak2, C3ar1, Tnf*	9.59E
Dendritic Cell Maturation	*B2m, Rac2, Il1a, Icam1, Nfkbie, Pik3r5, Hla-drb1, Hla-dmb, Fcgr2b, Fcgr1a, Col1a2, Nfkbia, Pik3cg, Creb1, Hla-b, Stat1, Tnfrsf1b, Fcgr3a, Hla-c, Tyrobp, Tnfrsf1a, Fcgr2a, Mapk8, Ikbke, Nfkb2, Tlr2, Col5a3, Il1rn, Pik3r6, Fcer1g, Cd86, Pik3cd, Irf8, Tnf, Ifnar1*	3.06E
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	*Rap1b, Rac2, Nfkbie, Pik3r5, Rhoh, Ppp1r14b, Spii1, Map3k10, Rhog, Nfkbia, Pik3cg, Cyba, Ppm1l, Cybb, Stat1, Tnfrsf1b, Map3k2, Ptpn6, Tnfrsf1A, Mapk8, Ikbke, Nfkb2, Ncf4, Irf1, Tlr2, Prkcd, Plcg2, Ncf2, Pik3r6, Map3k8, Pik3cd, Irf8, Jak3, Tnf*	1.93E
IL-10 Signaling	*Ccr1, Il18rap, Socs3, Ccr5, Il4r, Il1a, Il1rl1, Fcgr2a, Nfkbie, Mapk8, Ikbke, Nfkb2, Stat3, Fcgr2b, Il1r2, Hmox1, Nfkb, Il1rn, Il10ra, Il10rb, Cd14, Tnf*	4.67E
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatiod Arthritis	*Il18rap, Rac2, Socs3, Tlr1, Fcgr1a, Il1r2, Tgfb1, Pik3cg, Osm, Wnt5b, Tnfsf13b, C1s, Il6r, Stat3, Apc, Tlr2, Il1rn, Cebpd, Plcg2, Prkcd, Pik3r6, Pik3cd, Tnf, Il1a, Fn1, Icam1, Il1rl1, Nfkbie, Pik3r5, Ccl5, Il17ra, C1r, Nfkbia, C5ar1, Ppp3cb, Creb1, Tlr7, Cebpa, Tnfrsf1b, Fcgr3a, Traf1, Tnfrsf1a, Ikbke, Cebpb, Irak3, Ripk1, Csf1, Tlr6, Tlr13*	6.21E
Canonical Pathways- Day 3	**CNTF–associated genes**	**p-value**
Protein Ubiquitination Pathway	*Usp14, Psmd7, Psma7, Ube2l3, Ube2v2, Dnajc10, Psmb8, Hspa5, Psmc5, Usp48, Uchl1, Uso1, Dnajc28, Hsp90b1, Hsp90ab1, Psmd10, Uchl5, Psmd14, Psma2, Psmc2, Birc3, Hspa4l, Hla-c, Psmb4, Psmb9, Psma6, Hsph1, Usp9x, Psmd6, Psma1, Dnajb9, Psmd8, Psmb7, Psmc1, Cul2, Psmb2, Psmd12, Hspa13, Psmb1, Dnajc5b, Smurf2, Uba1, Usp25, Dnajc7, Tceb1*	3.8E-08
TREM 1 Signaling	*Itgb1, Rac2, Tyrobp, Tlr8 (includes EG:51311), Cd83, Jak2, Stat3, Fcgr2b, Tlr4, Ccl2, Plcg2, Tlr6, Casp1, Tlr7, Cd86, Tlr13, Il1b, Itgax*	5.48E-08
Fcg Receptor-mediated Phagocytosis in Macrophages and Monocytes	*Actr2, Rac2, Gab2, Fcgr2a, Actb, Arpc5, Fcgr1a, Inpp5d, Pld4, Myo5a, Hmox1, Rab11b, Pik3cg, Prkcd, Syk, Arpc4, Vamp3, Lyn, Rab11a, Arpc3, Vav1, Fcgr3a, Lcp2, Prkcb*	6.07E-08
N-Glycan Degradation	*Chi3l4, Fuca2, Edem1, Gm2a, Man1a1, Hexb, Lct, Hexa, Man2b1, Man2a1, Chi3l3*	4.99E-06
CTLA4 Signaling in Cytotoxic T Lymphocyte	*Hla-dma, Ap2b1, Rac2, Ptpn6, Ppp2r2a, Ap1s2, Cltc, Hla-dqa11, Hla-drb1, Jak2, Hla-dqb1, Ppp2r1a, Syk, Pik3cg, Clta, Fcer1g, Cd86, Pik3cb, Lcp2, Ap1g1*	9.22E-06

The top-five pathways and associated genes are presented.
doi:10.1371/journal.pone.0020326.t002

In general, only a few genes showed a decrease in transcript levels (Table 1, S1), with most showing a modest change of about two-fold.

Long-time effects of CNTF

Cytokines are known for their rapid and transient action [1]. To examine whether transcripts induced initially by CNTF continue to be expressed or the gene expression pattern is further altered, we investigated transcriptional changes in Müller cells three days following CNTF treatment. The biological activity of CNTF appears to last 2–3 days, due perhaps both to degradation of the cytokine [43] and activation of suppressor of cytokine signaling systems [51]. We found that 1192 transcripts were increased 2- to 26-fold, and 349 transcripts were reduced 2- to 10-fold. (Table 3; see Table S2 for a complete list). A comparison of transcriptome data from Müller cells at one and three days after CNTF exposure showed that (i) there is an increase in the number of genes transcribed from 1261 to 1541; and (ii) the pattern of transcribed genes is changed, only 273 overlapping transcripts were detected at these two time-points.

IPA analysis was used to categorize genes according to canonical pathways and functions (Table 2). At day 3, the networks involved in CNTF signaling had changed and new canonical pathways and functional groups were operative (Figure 4). The top canonical pathways were now associated with protein ubiquitination, TREM1 signaling and receptor mediated phagocytosis. The molecular and cellular functions involved cell death, and cell growth and proliferation (Table 2). These network changes are likely to represent the altered role of Müller cells in CNTF signaling in the retina.

cThe differentially regulated transcripts could be classified into distinct functional types using IPA (Table 3). At three days, we found the induction of chemokine, CCl2 (NM_011333.3), which is known to be involved in the activation and chemoattraction of circulating macrophages into tissues [52]. Relatively few growth factors were induced at day three unlike at day one. We also observed a high induction of the glycoprotein, GPNMB (NM_053110.4), a melanosome-associated protein implicated in cancer metastasis [53]. The chemokine receptor, CCR5, continued to be expressed at 3 days. In support of the continuing role of Müller cells in innate immunity, we found a 14-fold increase in TLR13 (NM_205820.1) and 7-fold increase in TLR7 (NM_133211.3) levels in Müller cells.

Networks induced by CNTF

Next, we examined network(s) that contained genes relevant to CNTF action. Several networks were generated using Metacore (www.genego.com/metacore.php), and genes associated with one of the networks together with their direct and indirect interactions are presented in Fig. 5a. The following gene products emerged as hubs (at day one): STAT3 (NM_213659.2), a transcription factor involved in cytokine signaling; CDKN1A (NM_007669.4), cyclin-dependent kinase inhibitor 1, which inhibits cyclin-CDK2 (NM_016756.4) or CDK4 (NM_009870.3) and acts as a regulator of cell cycle; CEBPA (NM_007678.3), a bZIP transcription factor involved in cytokine (leptin) signaling; Insulin, a well known hormone associated with carbohydrate and fat metabolism; AP-1, a transcription factor stimulated by cytokines and growth factors; Ras homolog, which is involved in cell contraction and cytoskeleton rearrangement; and MIR124, a microRNA involved in brain-specific pre-mRNA splicing. Overall, genes associated with transcription, cell cycle regulation and inflammatory response appear to occupy prominent positions (hubs) in the network topology.

In accord with the changes in gene expression pattern, there were substantial changes in the CNTF-induced networks three days after CNTF treatment (Fig. 5b). The following gene products were found as hubs: CCL3 (NM_011337.2) and CCL4 (NM_013652.2), chemokines involved in the activation and recruitment of polymorphonuclear leukocytes; CCL13, a chemoattractant induced by IL1b (NM_008361.3) and TNF-a (NM_013693.2), and a ligand for CCR5 (NM_009917.5); CCR5 and CXCR4 (NM_009911.3), chemokine receptors that act as co-receptors for HIV entry into cells; TyroBP (NM_011662.2), a transmembrane signaling receptor with a role in inflammation; PLCG2 (NM_172285.1) and PLC gamma (NM_021280.3), phospholipases associated with cell signaling; SYK (NM_001198977.1), a non-receptor tyrosine kinase involved in B cell signaling; and TGFBR1 (NM_009370.2), transforming growth ß receptor 1.

CNTF is an inducer of gliosis in Müller cells

CNTF can also act as an inducer of reactive gliosis in the CNS [41,54]. Intravitreal injection of CNTF in rodents rapidly induces GFAP, a hallmark of gliosis in the nervous system [43,46]. Virtually every retinal disease is associated with 'activation' of Müller cells and reactive gliosis [41,42]; it is possible that CNTF might be one of the endogenous inducers. Moreover, CNTF is upregulated in response to retinal degeneration and injury [55,56].

To further establish CNTF as a candidate inducer of gliosis in the retina, we compared transcripts with increased expression in CNTF-stimulated Müller cells with genes expressed in 'reactive' Müller cells. Rattner and Nathans [50] previously reported retinal transcriptional profile changes in two mouse models of retinal degeneration, the procadherin-knockout (proCAD) strain and mice with light damage [50]. Using *in situ* hybridization to localize some of the upregulated transcripts, they found that many of the upregulated genes were localized to Müller cells. A comparison of our microarray data with those of Rattner and Nathans [50] is presented in Table 4. All ten genes upregulated in light-damaged retina and six genes upregulated in proCAD mutant retina also showed increases in transcript levels, although the fold-changes differed. For example, ceruloplasmin (NM_001042611.1) increased ~ 8-fold in light damaged retinas compared to 2-fold in CNTF-treated retina. These data show that CNTF-activated Müller cells express several genes, some of which are also induced in reactive Müller cells from proCAD mice or mice with light damage; thus, suggesting that CNTF is a likely to be an inducer of gliosis in the retina.

Comparison of gene profile of Müller cells with astrocytes and RPE

Müller (glial) cells perform metabolic functions similar to astrocytes and RPE [41,57]. To determine whether this similarity is also reflected in the gene expression profiles of these cell types, we compared the top 2000 highly expressed transcripts from Müller cells and astrocytes (Table S1), using previously published astrocyte transcriptome data from postnatal day-17 mice [58]. As shown in Figure 6, 642 genes were common to Müller cells and astrocytes yielding a 32% overlap in transcriptional pattern. In comparison, Müller cells and retinal pigment epithelial cells (RPE) have 565 genes in common. The RPE transcriptome data were obtained from a recent publication [59]. There is a 14% overlap (381 genes) in highly expressed genes among the three different cell types (Fig. 6a). When compared with transcriptional profiles of neurons, there was an overlap of 27% (Fig. 6b, 58).

IPA of the 381 genes (common to Müller cells, astrocytes and RPE) showed that the top canonical pathways were concerned

Table 3. List of differentially regulated genes in Müller cells three days after CNTF treatment.

Gene Symbol	Gene Name	Fold Change	Type
Spp1	Secreted phosphoprotein 1	15.8	Cytokines (16, 4)
Ccl3l3	Chemokine (C-C motif) ligand 3-like 3	14.8	-
Pf4	Platelet factor 4	12.4	-
Nampt	Nicotinamide phosphoribosyltransferase	4.0	-
Ccl13	Chemokine (C-C motif) ligand 13	3.9	-
Thpo	Thrombopoietin	-2.0	-
Prl	Prolactin	-2.1	-
Ifnk	Interferon, kappa	-2.1	-
Il1f8	Interleukin 1 family, member 8 (eta)	-2.3	-
Cybb	Cytochrome b-245, beta polypeptide	14.9	Enzymes (271, 28)
Gpnmb	Glycoprotein (transmembrane) nmb	14.0	-
Gatm	Glycine amidinotransferase	11.2	-
Ppib	Peptidylprolyl isomerase B (cyclophilin B)	10.0	-
Hpgds	Hematopoietic prostaglandin D synthase	10.0	-
Fut1	Fucosyltransferase 1	-2.5	-
Bche	Butyrylcholinesterase	-2.5	-
B3gat2	Beta-1,3-glucuronyltransferase 2	-2.6	-
Gng13	Guanine nucleotide binding protein, gamma 13	-3.6	-
Gcsh	Glycine cleavage system protein H	-4.7	-
C3ar1	Complement component 3a receptor 1	20.4	G-proteins (14, 82)
Emr1	Egf-like module containing, mucin-like	9.2	-
Cxcr4	Chemokine (C-X-C motif) receptor 4	8.1	-
Ccr5	Chemokine (C-C motif) receptor 5	6.5	-
Gpr65	G protein-coupled receptor 65	5.8	-
Olfr703	Olfactory receptor 703	-3.4	-
Olfr619	Olfactory receptor 619	-3.5	-
Olfr921	Olfactory receptor 921	-3.9	-
Olfr777	Olfactory receptor 777	-4.2	-
Olfr153	Olfactory receptor 153	-4.8	-
Grn	Granulin	5.2	Growth Factors (3, 0)
Gmfb	Glia maturation factor, beta	3.2	-
Hgf	Hepatocyte growth factor (hepapoietin A)	2.8	-
Clic1	Chloride intracellular channel 1	2.7	Ion Channels (6, 0)
Clic4	Chloride intracellular channel 4	2.6	-
Fxyd5	FXYD domain containing ion transport regul 5	2.6	-
P2rx4	Purinergic receptor P2X, ligand-gated ion chan, 4	2.2	-
P2rx7	Purinergic receptor P2X, ligand-gated ion chan, 7	2.0	-
Taf9	TAF9 RNA polymerase II, TATA box binding protein	8.1	Transc. Reg. (80, 20)
Sp100	Protein SP100 nuclear antigen	7.3	-
Tcea1	Transcription elongation factor A (SII), 1	7.1	-
Supt4h1	Suppressor of Ty 4 homolog 1 (S. cerevisiae)	5.7	-
Rel	V-rel reticuloendotheliosis viral oncogene homolog	5.6	-
Phb	Prohibitin	-2.3	-
Gli3	GLI family zinc finger 3	-2.3	-
Erg	V-ets erythroblastosis virus E26 oncogene homolog	-2.4	-
Abra	Actin-binding Rho activating protein	-2.4	-
Ankz1	Ankyrin repeat and zinc finger domain	-2.5	-
Hla-c	Major histocompatibility complex, class I, C	17.0	Transm. Receptors (48, 1)
Msr1	Macrophage scavenger receptor 1	12.9	-
Clec7a	C-type lectin domain family 7, member A	10.2	-
Fcrl2	Fc receptor-like 2	7.1	-
Tlr7	Toll-like receptor 7	6.9	-
Gfra2	GDNF family receptor alpha 2	-2.0	-
Saa1	Serum amyloid A1	15.4	Transporters (101, 11)
M6pr	Mannose-6-phosphate receptor (cation dependent)	6.3	-
Gabarap	GABA(A) receptor-associated protein	5.9	-
Mfsd1	Major facilitator superfamily domain containing 1	5.7	-
Atp6v1e1	ATPase, H+ transporting, lysosomal 31kDa, V1, E1	5.4	-
Timm9	Translocase of inner mitochondrial membrane 9	-2.3	-
Atp6ap2	ATPase, H+ transporting, lysosomal protein 2	2.3	-
Igfbp7	Insulin-like growth factor binding protein 7	-2.5	-
Apod	Apolipoprotein D	-2.9	-
Disp1	Dispatched homolog	-3.9	-

The table lists the top-five up- and down-regulated genes derived from IPA analysis. The total number of up- and down –regulated genes in each type is listed along with the functional types, in column 4.
doi:10.1371/journal.pone.0020326.t003

with oxidative phosphorylation, mitochondrial function, glycolysis/gluconeogenesis, hypoxia signaling and ubiquinone biosynthesis. This finding is in accord with the known supportive roles of the three cell types in energy metabolism in the retina [41,57]. The top biological functions involve protein synthesis, RNA post-transcriptional modification, and energy production (Fig. 7).

Network analysis of the genes as well as their direct and indirect interactions (Fig. 7) revealed the following gene products emerged as hubs: ATP5I (NM_007507.2), mitochondrial ATP synthase; H^+-transporting, two-sector ATPase; vacuolar H^+-ATPase; LDHA (NM_001136069.2), lactate dehydrogenase; YWHAZ (NM_011740.3), an adapter protein implicated in several signal transduction pathways; and NFkB complex, which controls transcription and is involved in cellular response to cytokines. Overall, genes associated with carbohydrate metabo-

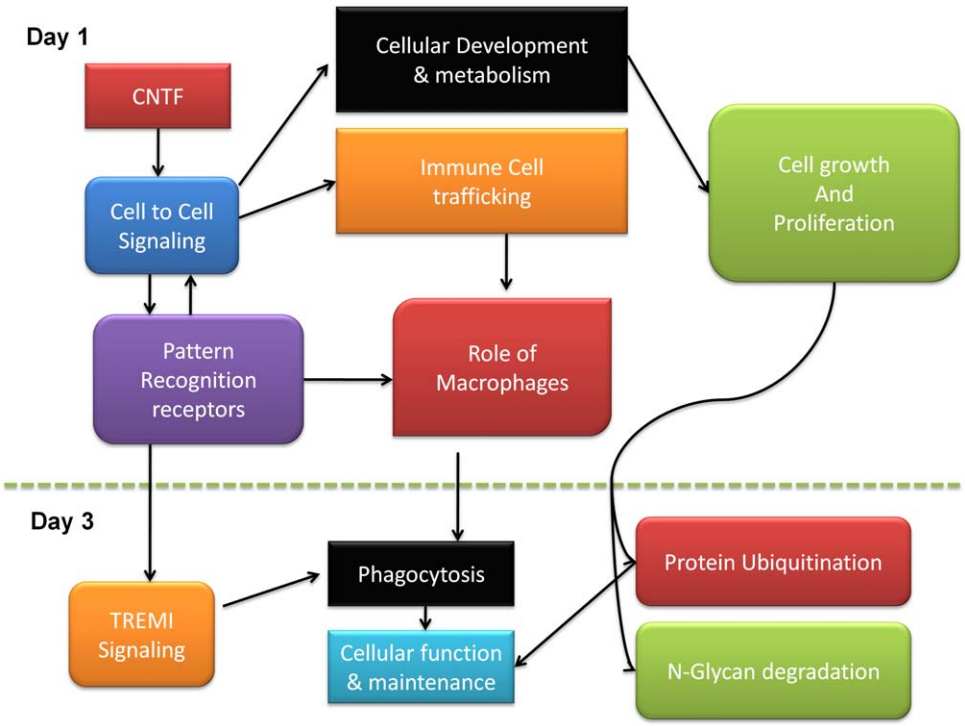

Figure 4. Changes in pathways and processes induced by CNTF. The diagram indicates potential biological processes and their inter-relationships in Müller cells at day 1 and day 3 after CNTF treatment. The processes and pathways were generated from IPA analysis.
doi:10.1371/journal.pone.0020326.g004

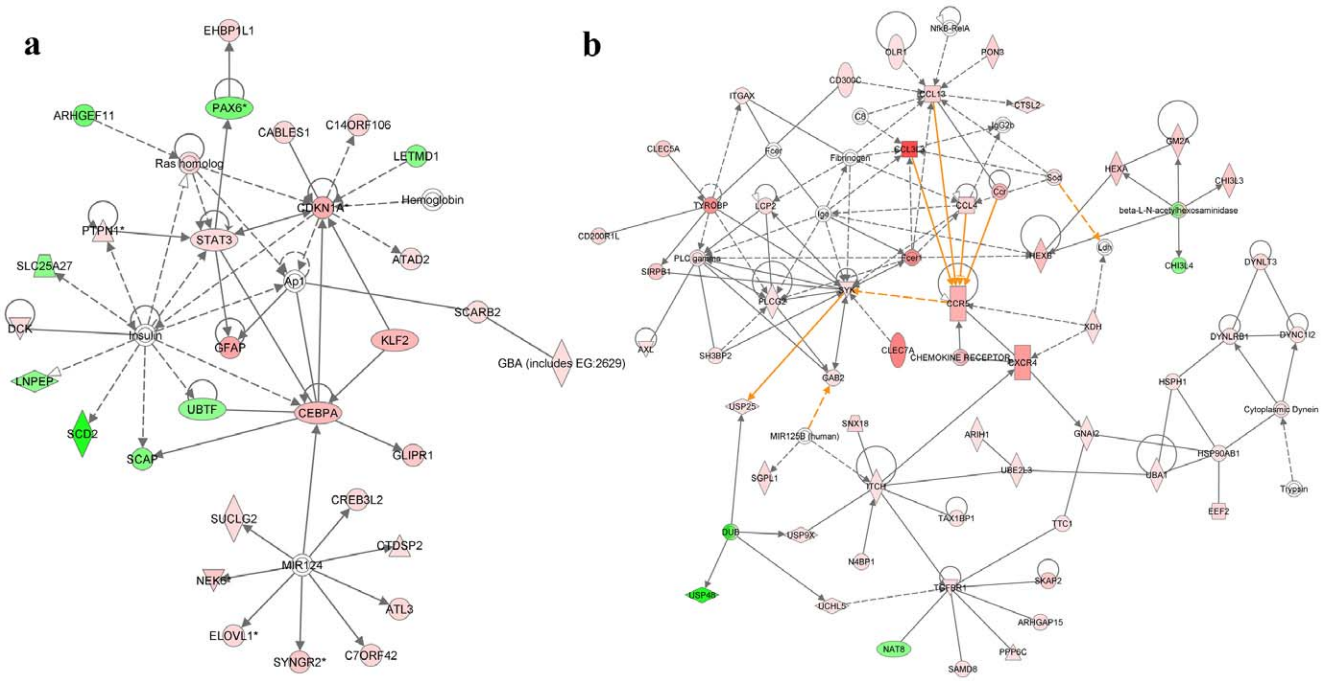

Figure 5. The most prominently affected gene networks generated by Ingenuity Pathway Analysis. (**a**) Day 1- Genes in this network are responsible for cellular development, connective tissue disorder and metabolic disease. (**b**) Day 3- Genes in this network are responsible for cellular function and maintenance, molecular transport and inflammatory response. Red color indicates induction, while green represents repression; color intensity correlates with fold change.
doi:10.1371/journal.pone.0020326.g005

Table 4. Comparison of transcript level changes in retinas from procadherin-knockout (proCAD) and light damaged mice with Müller cells from CNTF-treated mouse retinas.

		Fold Change		
Gene name	**Gene symbol**	**proCAD mutant**	**Light damage**	**CNTF-treated**
CCAT enhancer binding protein	*Cebpd*	5.7	19.3	4.26
Ceruloplasmin	*Cp*	0	8.2	2.43
Endothelin 2	*Edn2*	11.4	12.9	6.71
GFAP	*Gfap*	5	3.8	5.41
Lipocalin 2	*Lcn2*	0	17.9	2.63
Oncostatin M receptor	*Osmr*	3.4	8.2	3.5
S100 protein	*S100a6*	0	5.5	3.51
Serpin a3n	*Serpin a3n*	2.1	5.7	5.02
SOCS-3	*Socs3*	2.9	13.5	3.6
Soluble galactose binding protein	*Lgals3*	0	5.9	6.04

The pro-CAD and light damage data are from ref. 50.
doi:10.1371/journal.pone.0020326.t004

lism, energy production, protein synthesis and nucleic acid metabolism appear to occupy prominent positions (hubs) in the network topology.

Discussion

We report that CNTF induces rapid and extensive changes in the transcriptional profile of Müller (glial) cells *in vivo*. The activated genes involve growth factors, cytokines, ion channels, kinases, phosphatases, and G-protein coupled receptors that are components of cellular pathways concerned with cell- to -cell signaling, cell cycle and inflammation. The widespread genomic response of Müller cells is likely the basis for the numerous effects of CNTF on the development, differentiation and survival of retinal neurons.

Genes induced by CNTF are reported to carry a CNTF-response element, 5′-TTCC (N_{3-5}) AA-3′ (CNTF-RE) in their 5′-flanking sequence [60]. We observed that a majority of upregulated genes had one or more (up to 6) CNTF-RE sites in their 5′-flanking sequence (~1 kb). For example, among genes that are upregulated at least 6-fold (n = 51), 45 had the CNTF-RE on the direct or coding strand, one gene (Tnfaip2: NM_009396.2) did not have CNTF-RE site and four genes had a CNTF-RE site on the non-coding strand. Genes that carry the CNTF-RE are likely to be activated by the direct action of CNTF while genes without CNTF-RE element could be induced by secondary mechanisms.

CNTF-mediated photoreceptor neuroprotection

CNTF acts as a neuroprotective agent in many animal models of retinal degeneration [27–36,61]. One hypothesis postulates that CNTF acts directly on photoreceptors to promote their survival. The absence of CNTF receptors on rodent photoreceptors and the lack of STAT3 phosphorylation in photoreceptors following CNTF treatment have been advanced as evidence against this suggestion [38]. Recent studies with LIF (NM_001039537.1), a close relative of CNTF, show that this cytokine might directly act on photoreceptors [62]. In transgenic mice with conditional ablation of the gp130 gene in photoreceptors, LIF was unable to rescue light-induced photoreceptor degeneration whereas LIF was able to promote photoreceptor survival in mice with conditional inactivation of gp130 gene in Müller cells [62]. It could be argued that CNTF might act in a manner analogous to LIF.

A second hypothesis, termed the Müller cell hypothesis of neuroprotection, states that the CNTF effect on photoreceptors is indirect, and that CNTF mediates its neuroprotective effect by stimulating Müller cells [40–42]. Many lines of evidence support this hypothesis. CNTF receptors are found on Müller cells, and CNTF treatment rapidly stimulates the JAK-STAT pathway in Müller cells [43–46,63]. Our experimental data clearly show that several growth factors and cytokines are induced by CNTF in Müller cells. However, none of the previously-tested, photoreceptor neuroprotective agents— Basic Fibroblast growth factor, bFGF (NM_008006.2); Brain-derived neurotrophic factor, BDNF (NM_001048139.1); Glia-derived neurotrophic factor, GDNF (NM_010275.2); Pigment epithelium-derived factor, PEDF (NM_011340.3); Lens epithelium-derived growth factor, LEDGF (NM_133948.4); Fibroblast growth factor-5, FGF-5 (NM_

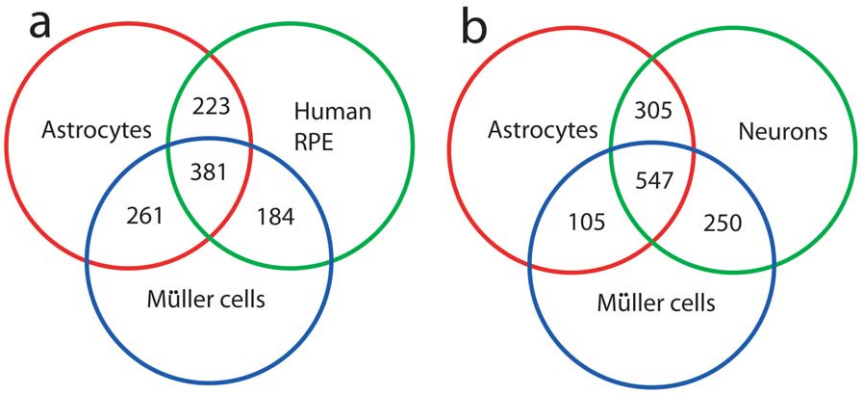

Figure 6. Overlapping genes in transcription profiles from Müller cells, astrocytes and retinal pigment epithelium (RPE). Intensity signals for the top 2000 probes were used to generate sets of overlapping genes. The astrocyte and RPE transcriptome data are derived from published studies (56,57). Müller cell data represent signals from cells not exposed to CNTF. (**a**) The diagram shows that 381 transcripts were common to the three cell types, while 642 were shared between Müller cells and astrocytes, 565 between Müller cells and the RPE, and 604 between astrocytes and RPE. (**b**) Diagram showing overlap of transcripts among astrocytes, Muller cells and neurons. The neuron transcriptome data was obtained from a published study (56).
doi:10.1371/journal.pone.0020326.g006

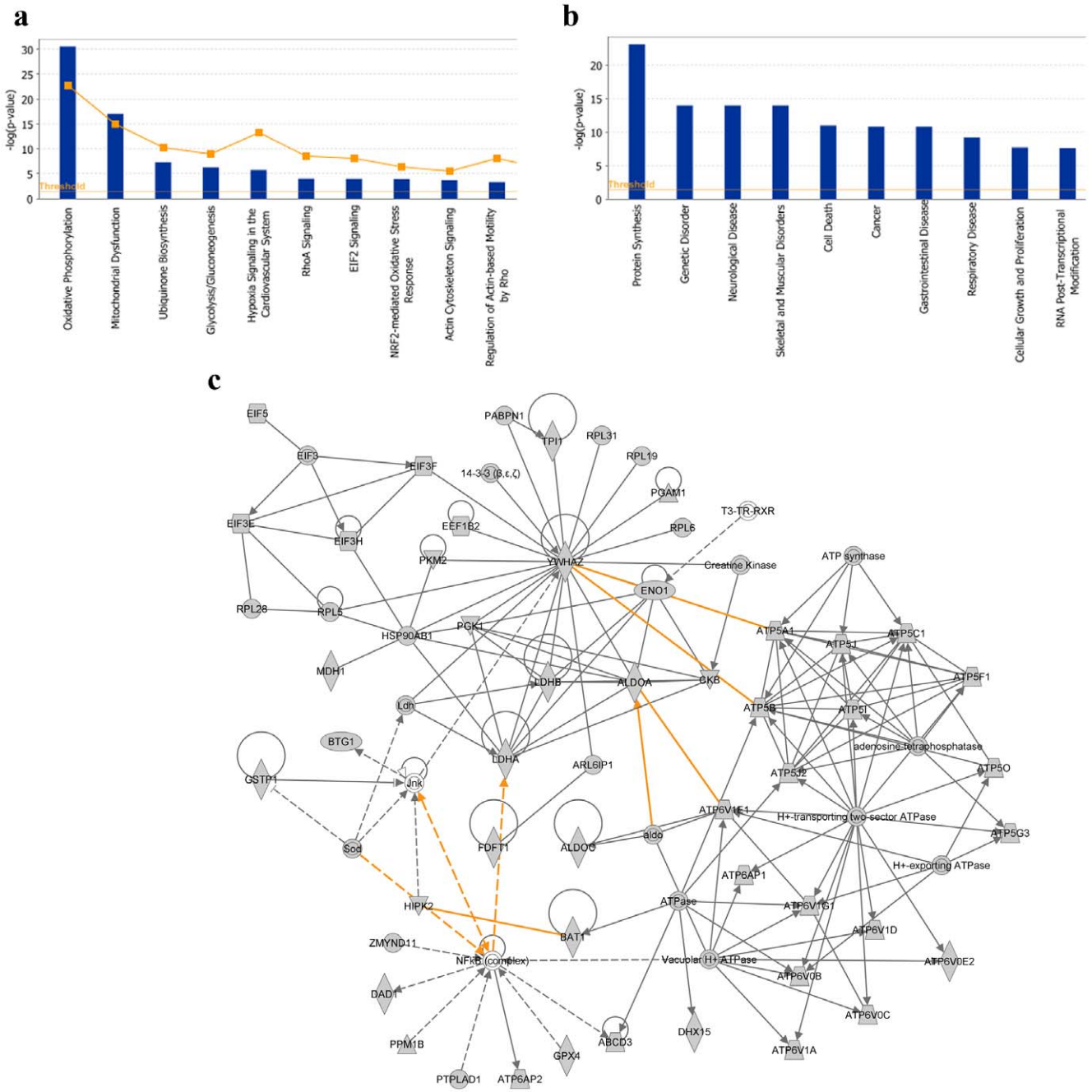

Figure 7. Gene networks common to Müller cells, astrocytes and RPE. (**a**) Most significant canonical pathways; (**b**) Most significant biological functions for the same list of genes; and (**c**) Genes in this network are responsible mitochondrial function and metabolism.
doi:10.1371/journal.pone.0020326.g007

010203.4); Fibroblast growth factor-18, FGF-18 (NM_008005.1); Interleukin 1ß, IL1ß; Rod-derived cone survival factor, RDCVF (NM_145598.2); Insulin-like growth factor II, IGFII (NM_0011 22736.1); Colony stimulating factor, CSF-1 (NM_001113529.1); and LIF are induced in Müller cells by CNTF [64–72]. This observation may argue against the Müller cell hypothesis of neuroprotection. It is, nevertheless, possible that other growth factors or cytokines induced by CNTF (Tables S1, S2) are involved in photoreceptor neuroprotection.

Two other mechanisms have been implicated in CNTF-mediated neuronal survival. One mechanism states that CNTF might enhance synthesis or distribution of glutamate transporters, which could improve glutamate handling resulting in less excitotoxic damage to neurons. In the rat striatum, CNTF has been reported to increase glutamate uptake by redistribution of glutamate transporters [39]. Although we have not examined glutamate transporter distribution, our data does not reveal an increase in glutamate transporter transcripts in response to CNTF (Table S1). Another mechanism involves CNTF-induced metabolic changes that enhance resistance to severe metabolic insults [40]. In CNTF-treated Müller cells, there were no changes in the expression glucose or lactate transporters (Table S1, S2).

CNTF has been reported to increase the long-term survival of retinal ganglion cells after axotomy [25,26]. The mechanism underlying this neuroprotective effect is not known. CNTF could directly act on retinal ganglion cells (RGCs) or it could act on Müller cells to trigger production on RGC survival factors [73–82]. Alternatively, CNTF injection could lead to production of Oncomodulin or another RGC survival factor from retinal microglia/macrophages [83].

One limitation of the present study is that gene expression profiling could be potentially affected by post-mortem changes in gene transcription during tissue removal, cell sorting and RNA preparation. Independent in vivo measurements of gene expression will be needed to understand in situ CNTF response. Another concern is that we cannot completely rule out the possibility that there might be a few GFP-positive astrocytes among flow-sorted cells from GFAP-GFP transgenic retinas. But the majority of cells in the flow-sorted population are likely to be Müller cells with $<1\%$ contribution from astrocytes. The density of Müller cells in the mouse retina is $\sim$12,000 per mm^2 [84]. The density of astrocytes in the mammalian retina is $\sim$600 per mm^2 [85], which is 5% of Müller cells. If 10% GFP-positive astrocytes remain in one-month old, GFAP-EGFP mouse retina, astrocyte contamination in flow-sorted cells would amount to $<1\%$. Therefore, the gene expression profile we have studied is largely representative of transcription profile changes in Müller cells rather than astrocytes.

CNTF is an inducer of reactive gliosis in Müller cells

A variety of agents including growth factors, cytokines, glutamate and purines have been suggested as inducers of reactive gliosis in the CNS [86]. In the retina, cytokines belonging to the IL-6 family as well as bFGF have been shown to induce gliosis (as determined by GFAP induction) in Müller cells [43,46,87]. We found that several genes that are induced by CNTF in quiescent Müller cells are also upregulated in gliotic Müller cells from inherited and experimentally-induced retinal degenerations [49]. These data strongly suggest that CNTF can act as an inducer of gliosis in the retina. This inference is supported by the finding that CNTF is upregulated in response to retinal degeneration and injury that results in Müller cell gliosis [55,56].

Müller cells, astrocytes and RPE serve support functions

It is generally accepted that Müller cells are functionally similar to astrocytes [42], although they are generated through different lineages [41]. Whereas Müller cells are derived from a retinal progenitor common to rods, bipolar cells and Müller cells [88], retinal astrocytes are generated from an oligodendrocyte/astrocyte precursor [89]. A comparison of transcript profiles of Müller cells and astrocytes shows that of the 2000 highly expressed genes, 642 genes (32%) were common to Müller cells and astrocytes. A closer examination shows that for many genes, the level of expression, however, is different between the two cell types, consistent with the previous transcriptome data on Müller cells [90]. In addition, there is a significant overlap ($\sim$14%) among genes expressed by Müller cells, astrocytes and RPE. Muller cells and astrocytes are believed to have analogous functions in the CNS [41]. The RPE has been considered a separate entity. The present study, however, suggests that the RPE shares some metabolic pathways with Muller cells and astrocytes; it appears that Müller cells, astrocytes and RPE have evolved to support the metabolic needs of the neural retina.

In summary, the present study shows that (i) CNTF induces rapid and extensive changes in the transcriptional profile of Müller (glial) cells; (ii) that several genes induced by CNTF in normal Müller cells are also upregulated in gliotic Müller cells from inherited and experimentally-induced retinal degenerations, which suggests that CNTF is an inducer of gliosis in the retina; and (iii) the transcript profiles of Müller cells and astrocytes are similar although they are derived from distinct cell lineages. Finally, CNTF induces networks in which genes associated with transcription, cell cycle regulation and inflammatory response appear to occupy prominent positions (hubs) in the network topology.

Materials and Methods

Mice

All mice were used in accordance with the approved Northwestern University Institutional Animal Care and Use Committee (IACUC) protocol, 2008-1398 that specifically approved this study. Northwestern University Animal Welfare assurance is on file with the Office of Laboratory Animal Welfare (A3283-01).

Purifying GFP$^+$-Müller cells by FACS

We previously described a transgenic mouse line, *mgfap-egfp*, in which GFP is expressed only in retinal Müller cells [48]. We used the transgenic mice to purify Müller cells based on GFP-expression. CNTF or PBS was intravitreally injected into anesthetized, transgenic mice (one month old) as described earlier [42]. We used one eye for CNTF treatment and the fellow eye as PBS control. In order to ensure CNTF action, we routinely selected a few injected animals and examined retinal sections for GFAP expression in Müller cells [48]. Similarly, we also examined PBS injected eyes. We always found GFAP expression in Müller cells in the CNTF- but not PBS-injected eyes

Retinas from *mgfap-egfp* transgenic mice were treated with papain for 30 min, and dissociated into a single cell suspension. The cells were filtered through a 70 μm nytex mesh to get rid of cell clumps. GFP$^+$-cells were enriched by FACS (MoFlo; Dako, Carpintaria, CA) at Northwestern Flow Cytometry Core facility.

Microarray data procurement and analysis

Microarray analysis was carried out as described previously [49]. We performed total RNA extraction (TRIZOL; Invitrogen, Carlsbad, CA) from ($\sim$50–100,000) flow-sorted cells, and confirmed the integrity of RNA with a bioanalyzer (model 2100; Agilent Technologies, Palo Alto, CA). All RNA samples had average RIN of 8.66 ± 0.79 (12 samples). We synthesized, labeled, and hybridized cRNA onto arrays at Genome Explorations (Memphis, TN) according to standard Affymetrix methods, previously described [49]. We performed transcriptional profiling using mouse Affymetrix chips (mouse Expression Array 230 2.0). The experiments were done in biological triplicates and microarray data were analyzed as reported previously [49].

For Day 1, four biological replicates were used on Affymetrix GeneChip Mouse Genome MOE430 2.0. For Day 3 we also used 4 biological replicates on Affymetrix Exon Chip MoEx ST1.v1. Raw data were normalized and analyzed using GeneSpring GX 11.0.2 software (Silicon Genetics, Redwood City, CA). For normalization we used Robust Multichip Average (RMA) method. We performed background correction followed by quantile normalization and used the average mean for summarization. We performed a one-way ANOVA (a simple t-test for this simple situation) for comparison between CNTF and PBS at Day 1 and Day 3. We used an un-corrected p-value ≤ 0.1 which led to a total of 5336 probes for Day 1 and 6777 for Day 3. These two sets were subjected to a 2-fold change filter value between CNTF and PBS at Day 1 and Day 3. This resulted in a set of 1261 for Day 1 and

1541 probes for Day 3, respectively. Moreover, for Day 1 we obtained 1063 independent genes while for Day 3 the list contained 1530 genes.

RNA preparation and Real-Time PCR Analysis (qPCR)

qPCR analysis of FACS-purified Müller cells was carried out to validate the findings from the microarray data [23]. We performed primer design, one-step RT-PCR reaction, and qPCR analysis as previously described [49]. Primers were designed using the PrimerQuest^{SM} IDT-DNA site. To eliminate genomic contamination, we treated total RNA with RQ1 RNase-free DNase (Promega, Madison, WI). We performed qPCR reaction in a thermocycler (iCycler; Bio-Rad, Richmond, CA), using the reagents in the SYBR Green iQ Real-time PCR kit; (Bio-Rad, Richmond, CA). To determine the relative change in gene expression, we used the system software to compare the number of cycles (C_t) needed to reach the midpoint of the linear phase. All observations were normalized to the housekeeping gene *Gapdh*. We used actin, *hsp90* or *hprt1* as additional controls along with *Gapdh*. The qPCR data are from 3-6 biological replicates.

Supporting Information

Table S1 Supporting table.
(DOC)

Table S2 Supporting table.
(DOC

Acknowledgments

We thank members of the Sarthy and Swaroop labs for assistance and discussions. The authors have no commercial interest in the subject of the manuscript or in entities discussed in the manuscript.

Author Contributions

Conceived and designed the experiments: WX VPS AS. Performed the experiments: WX VJD MB. Analyzed the data: RIC VPS. Contributed reagents/materials/analysis tools: WX RIC VJD MB AS VPS. Wrote the paper: VPS AS.

References

1. Nicola NA (1994) Guidebook to cytokines and their receptors. Oxford, UK: Oxford University Press. 284 p.
2. Ip N, Yancopoulos GD (1996) The neurotrophins and CNTF: Two families of collaborative neurotrophic factors. Ann Rev Neurosci 19: 491–515.
3. Levy DE, Darnell JE (2002) STATS: Transcriptional control and biological impact. Nature Rev Mol Cell Biol 3: 651–662.
4. Nishimune H, Vasseur S, Wiese S, Birling MC, Holtmann B, et al. (2000) Reg-2 is a motoneuron neurotrophic factor and a signalling intermediate in the CNTF survival pathway. Nature Cell Biol 2: 906–914.
5. Rane SG, Reddy EP (2000) Janus kinases: components of multiple signaling pathways. Oncogene 19: 5662–5679.
6. Servidei T, Aoki Y, Lewis SE, Symes A, Fink JS, et al. (1998) Coordinate regulation of STAT signaling and c-fos expression by the tyrosine phosphatase SHP-2. J Biol Chem 273: 6233–6241.
7. Yokogami KM, Wakisaka S, Avruch J, Reeves SA (2000) Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR. Current Biology 10: 47–50.
8. Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UAK, et al. (2001) Role of STAT3 and Pl 3-Kinase/Akt in mediating the survival actions of cytokines on sensory neurons. Mol Cell Neurosci 18: 270–282.
9. Boulton TG, Stahl N, Yancopoulos GD (1994) Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. J Biol Chem 269: 11648–11655.
10. Oh H, Fujio Y, Kunisada K, Hirota H, Matsui H, et al. (1998) Activation of phosphatidylinositol 3-kinase through glycoprotein 130 induces protein kinase B and p70 S6 kinase phosphorylation in cardiac myocytes. J Biol Chem 273: 9703–9710.
11. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, et al. (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 374: 1–20.
12. Gallagher D, Gutierrez H, Gavalda N, O'Keefe G, Hay R, et al. (2007) Nuclear factor-kB activation via tyrosine phosphorylation of inhibitor kB-a is crucial for ciliary neurotrophic factor-promoted neurite growth from developing neurons. J Neurosci 27: 9664–966.
13. Hagg T, Varon S (1993) Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons in vivo. Proc Natl Acad Sci U S A 90: 6315–631.
14. Lo AC, Li L, Oppenheim RW, Prevette D, Houenou LJ (1995) Ciliary neurotrophic factor promotes the survival of spinal sensory neurons following axotomy but not during the period of programmed cell death. Exp Neurol 134: 49–55.
15. MacLennan AJ, Vinson EN, Marks L, McLaurin DL, Pfeifer M, et al. (1996) Immunohistochemical localization of ciliary neurotrophic factor receptor alpha expression in the rat nervous system. J Neurosci 16: 621–630.
16. Sendtner M, Kreutzberg GW, Thoenen H (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 345: 440–441.
17. Sendtner M, Schmalbruch H, Stockli KA, Carroll P, Kreutzberg GW, et al. (1992) Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. Nature 358: 502–504.
18. Deverman BJ, Patterson PH (2009) Cytokines and CNS development. Neuron 64: 61–78.
19. Linker RA, Mäurer M, Gaupp S, Martini R, Holtmann B, et al. (2002) CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. Nature Med 8: 620–624.
20. Lambert PD, Anderson KD, Sleeman MW, Wong V, Tan J, et al. (2001) Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat, without cachexia or rebound weight gain, even in leptin-resistant obesity. Proc Natl Acad Sci U S A 98: 4652–4657.
21. Rhee KD, Yang XJ (2010) Function and mechanism of CNTF/LIF signaling in retinogenesis. Adv Exp Med Biol 664: 647–654.
22. Kirsch M, Lee MY, Meyer V, Wiese A, Hofmann HD (1997) Evidence for multiple, local functions of ciliary neurotrophic factor (CNTF) in retinal development: expression of CNTF and its receptors and in vitro effects on targets. J Neurochem 68: 979–990.
23. Ezzedine ZD, Yang X, DeChiara T, Yancopoulos G, Cepko CL (1997) Postmitotic cells fated to become rod photoreceptors can be respecified by CNTF treatment. Development 124: 1055–67.
24. Rhee KD, Goureau O, Chen S, Yang XJ (2004) Cytokine-induced activation of signal transducer and activator of transcription in photoreceptor precursors regulates rod differentiation in the developing mouse retina. J Neurochem 224: 9779–88.
25. Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA (1995) Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron 15: 805–819.
26. Cui Q, Lu Q, So KF, Yip HK (1999) CNTF, not other trophic factors, promotes axonal regeneration of axotomized retinal ganglion cells in adult hamsters. Invest Ophthalmol Vis Sci 40: 760–766.
27. LaVail MM, Unoki K, Yasumura D, Matthes MT, Yancopoulos GD, et al. (1992) Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. Proc Natl Acad Sci U S A 89: 11249–11253.
28. Cayouette M, Gravel C (1997) Adenovirus-mediated gene transfer of ciliary neurotrophic factor can prevent photoreceptor degeneration in the retinal degeneration (*rd*) mouse. Human Gene Ther 8: 423–430.
29. LaVail MM, Yasumura D, Matthes MT, Lau-Villacorta C, Unoki K, et al. (1998) Protection of mouse photoreceptors by survival factors in retinal degenerations. Invest Ophthalmol Vis Sci 39: 592–602.
30. Liang FQ, Dejneka NS, Cohen DR, Krasnoperova NV, Lem J, et al. (2001) AAV-mediated delivery of ciliary neurotrophic factor prolongs photoreceptor survival in the rhodopsin knockout mouse. Mol Ther 3: 241–248.
31. Bok D, Yasumura D, Matthes MT, Duncan JL, Chappelow AV, et al. (2002) Effects of adeno-associated virus-vectored ciliary neurotrophic factor on retinal structure and function in mice with a P216L rds/peripherin mutation. Exp Eye Res 74: 719–735.
32. Liang FQ, Aleman TS, Dejneka NS, Dudus L, Fisher KJ, et al. (2001) Long-term protection of retinal structure but not function using RAAV CNTF in animal models of retinitis pigmentosa. Mol Ther 4: 461–472.
33. Wen R, Song Y, Kjellstrom S, Tanikawa A, Liu Y, et al. (2006) Regulation of rod phototransduction machinery by ciliary neurotrophic factor. J Neurosci 26: 13523–13530.
34. Tao W, Wen R, Goddard MB, Sherman SD, O'Rourke PJ, et al. (2002) Encapsulated cell-based delivery of CNTF reduces photoreceptor degeneration in animal models of retinitis pigmentosa. Invest Ophthalmol Vis Sci 43: 3292–3298.

35. Chong NH, Alexander RA, Waters L, Barnett KC, Bird AC, et al. (1999) Repeated injections of a ciliary neurotrophic factor analogue leading to long-term photoreceptor survival in hereditary retinal degeneration. Invest Ophthalmol Vis Sci 40: 1298–1305.
36. Schlichtenbrede FC, MacNeil A, Bainbridge JW, Tschemutter M, Thrasher AJ, et al. (2003) Intraocular gene delivery of ciliary neurotrophic factor results in significant loss of retinal function in normal mice and in the Prph2Rd2/Rd2 model of retinal degeneration. Gene Ther 10: 523–527.
37. Sieving PA, Caruso RC, Tao W, Coleman HR, Thompson DJ, et al. (2006) Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. Proc Natl Acad Sci U S A 103: 3896–3901.
38. Zack DJ (2000) Neurotrophic rescue of photoreceptors: Are Müller cells the mediators of survival? Neuron 26: 285–286.
39. Escartin C, Brouillet E, Gubbellini P, Trioulier Y, Jacquard C, et al. (2006) Ciliary neurotrophic factor activates astrocytes, redistributes their glutamate transporters GLAST and GLT-1 to raft microdomains, and improves glutamate handling in vivo. J Neurosci 26: 5978–5989.
40. Escartin C, Pierre K, Colin A, Brouillet E, Delzescaux T, et al. (2007) Activation of astrocytes by CNTF induces metabolic plasticity and increases resistance to metabolic insults. J Neurosci 27: 7094–7104.
41. Sarthy V, Ripps H (2001) The Retinal Müller Cell. New York, NY: Structure and Function. Kluwer Academic/Plenum Press. 278 p.
42. Bringmann A, Pannicke T, Grosche J, Francke M, Wiedemann P, et al. (2007) Müller cells in the healthy and diseased retina. Prog Ret Eye Res 25: 397–424.
43. Peterson WM, Wang Q, Tzekova R, Wiegand SJ (2000) Ciliary neurotrophic factor and stress stimuli activate the Jak-STAT pathway in retinal neurons and glia. J Neurosci 20: 4081–4090.
44. Wahlin KJ, Campochiaro PA, Zack DJ, Adler R (2000) Neurotrophic factors cause activation of intracellular signaling pathways in Müller cells and other cells of the inner retina, but not photoreceptors. Invest Ophthalmol Vis Sci 41: 927–936.
45. Wahlin KJ, Adler R, Zack DJ, Campochiaro PA (2001) Neurotrophic signaling in normal and degenerating rodent retinas. Exp Eye Res 73: 693–701.
46. Wang Y, Smith SB, Ogilvie JM, McCool DJ, Sarthy V (2002) Ciliary neurotrophic factor induces glial fibrillary acidic protein in retinal Müller cells through the JAK/STAT signal transduction pathway. Current Eye Res 24: 305–312.
47. Jeon C-J, Strettoi E, Masland RH (1998) The major cell populations of the mouse retina. J Neurosci 18: 8936–8946.
48. Kuzmanovic M, Dudley VJ, Sarthy VP (2003) GFAP promoter drives Müller cell-specific expression in transgenic mice. Invest Ophthalmol Vis Sci 44: 3606–3613.
49. Akimoto M, Cheng H, Zhu D, Brzezninski JA, Khanna R, et al. (2006) Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. Proc Natl Acad Sci U S A 103: 3890–3895.
50. Rattner A, Nathans J (2005) The genomic response to retinal disease and injury: Evidence for endothelin signaling from photoreceptors to glia. J Neurosci 25: 4540–4549.
51. Park KK, Hu Y, Muhling J, Pollett MA, Dallimore EJ, et al. (2009) Cytokine-induced SOCS expression is inhibited by cAMP analogue: Impact on regeneration in injured retina. Mol Cell Neurosci 41: 313–324.
52. Biber K, de Jong EK, van Weering HRJ, Boddeke HWGM (2006) Chemokines and their receptors in central nervous system disease. Curr Drug Targets 7: 29–46.
53. Rose AA, Grosset A-A, Dong Z, Russo C, Macdonald PA, et al. (2010) Glycoprotein Nonmetastatic B Is an Independent Prognostic Indicator of Recurrence and a Novel Therapeutic Target in Breast Cancer. Clinical Cancer Research 16: 2147–2156.
54. Winter CG, Saotome Y, Levison SW, Hirsh D (1995) A role for ciliary neurotrophic factor as an inducer of reactive gliosis, the glial response to central nervous system injury. Proc Natl Acad Sci U S A 92: 5865–5869.
55. Wen R, Song Y, Cheng T, Matthes D, Yasumura D, et al. (1995) Injury induced upregulation of bFGF and CNTF mRNAs in the rat retina. J Neurosci 15: 7377–7485.
56. Walsh N, Valter K, Stone J (2001) Cellular and subcellular patterns of expression of bFGF and CNTF in the normal and light stressed adult rat retina. Exp Eye Res 72: 495–501.
57. Strauss O (2005) The retinal pigment epithelium in visual function. Physiol Rev 85: 845–881.
58. Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, et al. (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci 28: 264–278.
59. Strunnikova NV, Maminishkis A, Barb JJ, Wang F, Zhi C, et al. (2010) Transcriptome analysis and molecular signature of human retinal pigment epithelium. Human Mol Genet 19: 2468–2486.
60. Segal RA, Greenberg ME (1996) Intracellular signaling pathways activated by neurotrophic factors. Ann Rev Neurosci 19: 463–89.
61. Bramall AN, Wright AF, Jacobson SG, McInnes RR (2010) The genomic, biochemical, and cellular responses of the retina in inherited photoreceptor degenerations and prospects for the treatment of these disorders. Ann Rev Neurosci 33: 441–72.
62. Ueki Y, Le Y-Z, Chollangi S, Muller W, Ash JD (2009) Preconditioning-induced protection of photoreceptors requires activation of the signal-transducing receptor gp130 in photoreceptors. Proc Natl Acad Sci U S A 106: 21389–21394.
63. Walsh N, Valter K, Stone J (2001) Cellular and subcellular patterns of expression of bFGF and CNTF in the normal and light stressed adult rat retina. Exp Eye Res 72: 495–501.
64. Faktorovich EG, Steinberg RH, Yasumura D, Matthes MT, LaVail MM (1990) Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. Nature 347: 83–86.
65. Frasson M, Picaud S, Leveillard T, Simonutti M, Mohand-Said S, et al. (1999) Glial cell line-derived neurotrophic factor induces histologic and functional protection of rod photoreceptors in the rd/rd mouse. Invest Ophthalmol Vis Sci 40: 2724–2734.
66. Cayouette M, Smith SB, Becerra SP, Gravel C (1999) Pigment epithelium-derived factor delays the death of photoreceptors in mouse models of inherited retinal degenerations. Neurobiol Dis 6: 523–532.
67. Lau D, McGee LH, Zhou S, Rendahl KG, Manning WC, et al. (2000) Retinal degeneration is slowed in transgenic rats by AAV-mediated delivery of FGF-2. Invest Ophthalmol Vis Sci 41: 3622–3633.
68. Ogilvie JM, Speck JD, Lett JM (2000) Growth factors in combination, but not individually, rescue rd1 mouse photoreceptors in organ culture. Exp Neurol 161: 676–685.
69. Machida S, Chaudhry P, Shinohara T, Singh DP, Reddy VN, et al. (2001) Lens epithelium-derived growth factor promotes photoreceptor survival in light-damaged and RCS rats. Invest Ophthalmol Vis Sci 42: 1087–1095.
70. Green ES, Rendahl KG, Zhou S, Ladner M, Coyne M, et al. (2001) Two animal models of retinal degeneration are rescued by recombinant adeno-associated virus-mediated production of FGF-5 and FGF-18. Mol Ther 3: 507–515.
71. Whiteley SJ, Klassen H, Coffey PJ, Young MJ (2001) Photoreceptor rescue after low-dose intravitreal IL-1beta injection in the RCS rat. Exp Eye Res 73: 557–568.
72. Leveillard T, Mohand-Said S, Lorentz O, Hicks D, Fintz A, et al. (2004) Identification and characterization of rod-derived cone viability factor. Nat Genet 36: 755–759.
73. Mey J, Thanos S (1993) Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. Brain Res 602: 304–317.
74. Di Polo A, Aigner LJ, Dunn RJ, Bray GM, Aguayo AJ (1998) Prolonged delivery of brain-derived neurotrophic factor by adenovirus-infected Müller cells temporarily rescues injured retinal ganglion cells. Proc Natl Acad Sci U S A 95: 3978–3983.
75. Chun M-H, Ju W-K, Kim K-Y, Lee M-Y, Hofmann H-D, et al. (2000) Upregulation of ciliary neurotrophic factor in reactive Müller cells in the rat retina following optic nerve transection. Brain Res 868: 358–362.
76. Cui Qi, Harvey AR (2000) CNTF promotes the regrowth of retinal ganglion cell axons into murine peripheral nerve grafts. Neuroreport 11: 3999–4002.
77. Martin KR, Quigley HA, Zack DJ, Levkovitch-Verbin H, Kielczewski J, et al. (2003) Gene therapy with brain-derived neurotrophic factor as a protection: retinal ganglion cells in rat glaucoma model. Invest Ophthalmol Vis Sci 44: 4357–4365.
78. Leaver SG, Cui Q, Plant GW, Arulpragasam A, Hisheh S, et al. (2006) AAV-mediated expression of CNTF promotes long-term survival and regeneration of adult rat retinal ganglion cells. Gene Ther 13: 1328–1341.
79. Pease ME, Zack DJ, Berlinicke C, Bloom K, Cone F, et al. (2009) Effect of CNTF on retinal ganglion cell survival in experimental glaucoma. Invest Ophthalmol Vis Sci 50: 2194–2200.
80. Leibinger MA, Müller A, Andreadaki A, Hauk TJ, Kirsch M, et al. (2009) Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on Ciliary neurotrophic factor and Leukemia inhibitory factor. J Neurosci 29: 14334–14341.
81. Müller A, Hauk TG, Leibinger M, Marienfeld R, Fischer D (2009) Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF. Mol Cell Neurosci 41: 233–246.
82. Bai Y, Shi Z, Zhuo Y, Liu J, Malakhov A, et al. (2010) In Glaucoma the upregulated truncated TrkC.T1 receptor isoform in glia causes increased TNF-a production, leading to retinal ganglion cell death. Invest Ophthalmol Vis Sci 51: 6639–6651.
83. Yi Y, Henzl MT, Lorber B, Nakazawa T, Thomas TT, et al. (2006) Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. Nature Neurosci 9: 843–852.
84. Drager U, Edwards DL, Barnstable CJ (1984) Antibodies against filamentous components in discrete cell types of the mouse retina. J Neurosci 8: 2025–2042.
85. Schnitzer J (1988) Astrocytes in mammalian retina. Prog Ret Res 7: 209–231.
86. Sofroniew MV (2009) Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci 32: 638–647.

87. Lewis GP, Erickson PA, Guerin CJ, Anderson DH, Fisher SK (1992) Basic fibroblast growth factor: a potential regulator of proliferation and intermediate filament expression in the retina. J Neurosci 12: 3968–3978.
88. Turner DL, Cepko CL (1987) A common progenitor for neurons and glia persists in rat retina late in development. Nature 328: 131–136.
89. Rowitch DH, Kriegstein AR (2010) Developmental genetics of vertebrate glial-cell specification. Nature 468: 214–222.
90. Roesch K, Jadhav AP, Trimarchi JM, et al. (2008) The transcriptome of retinal Müller glial cells. J Comp Neurol 509: 225–238.