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Original Article

Neuroprotective Mechanisms of Ciliary Neurotrophic Factor in Retinal Ganglion Cells: Insights from Microarray Analysis

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Purpose: This study investigated the changes in gene expression in retinal ganglion cells (RGCs) following ciliary neurotrophic factor (CNTF) treatment to elucidate the underlying mechanisms contributing to its neuroprotective effects.

Methods: RGCs isolated from Sprague-Dawley rat pups were treated with recombinant CNTF. Gene expression was analyzed via microarray. Differentially expressed genes (DEGs) were defined as those with a fold change greater than 2 or less than –2. The DEGs were further explored using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses.

Results: Our analysis identified 71 upregulated and 58 downregulated genes. A2m exhibited the highest increase, with a fold change of 4.97, whereas Rho displayed the most significant decrease in expression, with a fold change of –6.38. GO and KEGG pathway analyses revealed substantial involvement in sensory organ development and the phototransduction pathway.

Conclusions: This study provides new insights into the impact of CNTF on gene expression in RGCs, suggesting broader neuroprotective mechanisms that could inform future therapeutic strategies for retinal degenerative diseases. Our findings emphasize the importance of further investigation into the complex gene network responses to CNTF treatment.

Key Words: Ciliary neurotrophic factor, Microarray analysis, Retinal ganglion cells

Retinal ganglion cells (RGCs) are located in the inner retina, with axons that travel in the optic nerve and project to the brain. They are the final components of the neural network responsible for receiving visual stimuli in the eyes, facilitating the transmission of visual information to the brain. Hence, the loss of RGCs can lead to irreversible blindness, particularly in conditions such as glaucoma.

Neurotrophic factors are proteins that play a crucial role in the survival, growth, and function of neurons in the nervous system. Ciliary neurotrophic factor (CNTF) is one of the most extensively studied neurotrophic factors in the context of retinal degenerative disorders [1]. It is known to significantly protect photoreceptors, retinal pigment epithelium cells, and RGCs in experimental glaucoma [2,3]. CNTF improves RGC survival and enhances long-distance

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optic nerve regeneration [4]. The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways are reportedly involved in this process [3,5,6]. Based on various preclinical data supporting the potential of CNTF to safeguard RGCs, we hypothesized that treatment with CNTF would lead to significant changes in gene expression.

A comprehensive understanding of the overall mechanism and its effects at the genetic level remains insufficient. This knowledge gap may limit the capacity to utilize CNTF as a therapeutic option for treating retinal degenerative disorders. Therefore, in this study, we aimed to conduct a comprehensive analysis at the gene level using microarray technology to gain insights into the underlying mechanisms.

Materials and Methods

Ethics statement

Nine pregnant Sprague-Dawley rats were procured from Orientbio Inc. A total of 126 newborn pups were euthanized following protocols approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (No. 2019-0150). The handling and care of the animals adhered to the Association for Research in Vision and Ophthalmology guidelines for the use of animals in ophthalmic and vision research, with efforts to minimize animal use and suffering.

Isolation of RGCs

As detailed in prior studies, RGCs were isolated from 2- to 3-day-old rat pups using a two-step immunopanning method [7]. Three biological replicates were used in this study, each consisting of RGCs isolated from separate batches of pups. Retinal tissues were harvested from enucleated eyes, and the cells were suspended in a culture medium. The suspension was incubated for 5 minutes with rabbit anti-rat macrophage antibody (1:50, Fitzgerald Industries International), followed by a 30-minute incubation in Petri dishes pre-coated with goat anti-rabbit immunoglobulin G (1:200, Southern Biotechnology Associates). Nonadherent cells were then transferred to another Petri dish coated with mouse anti-rat Thy1.1 antibody (1:50, Bio-Rad) for 60 minutes and subjected to anti-biotin magnetic MicroBeads (Miltenyi Biotec) treatment. RGCs were magnetically separated, and all procedures were conducted at room temperature under sterile conditions. The purified RGCs were cultured on glass coverslips pre-coated with poly-L-ornithine and laminin (Sigma-Aldrich) in DMEM/F-12 (No. SH30023.01, HyClone Laboratories) supplemented with 10% fetal bovine serum (Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies) (Fig. 1A–1C). Cultures were maintained at 37 °C in an atmosphere containing 5% CO_2 and treated with recombinant rat CNTF (PeproTech) at a concentration of 50 ng/mL for 24 hours.

Microarray data analysis

Immediately following the 24-hour CNTF treatment, total RNA was extracted (using the RNeasy Micro Kit, Qia-

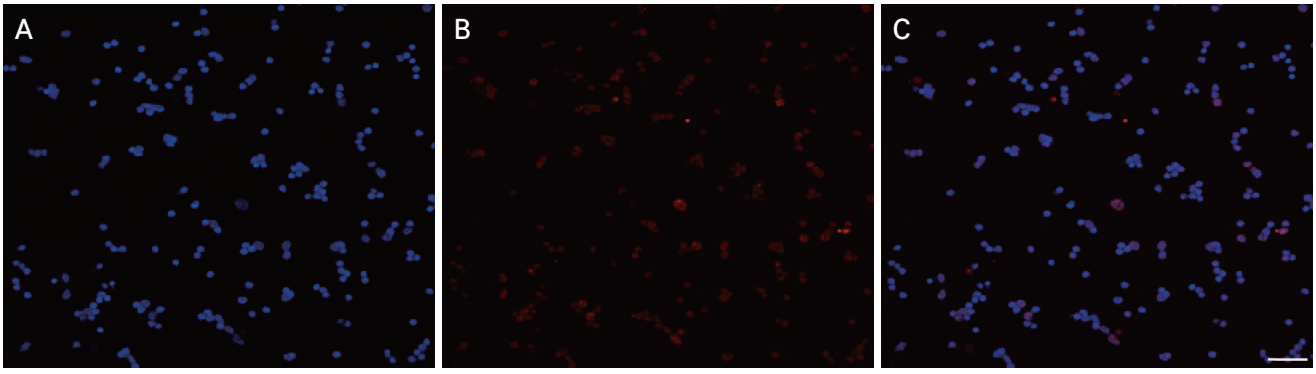

Fig. 1. Immunofluorescence staining of primary retinal ganglion cells at day 2 of culture (×400; scale bar, 50 μm). (A) DAPI nuclear staining. (B) Positive expression of Brn-3a is shown. (C) Merged image.

Table 1. Upregulated and downregulated genes

Gene description	Gene symbol	mRNA accession	Fold change
Alpha-2-macroglobulin	*A2m*	NM_012488	4.97
Signal peptide, CUB domain, EGF-like 1	*Scube1*	NM_001134884	4.01
Cysteine dioxygenase type 1	*Cdo1*	NM_052809	3.56
B-cell CLL/lymphoma 3	*Bcl3*	NM_001109422	3.32
Glutamate receptor, metabotropic 6	*Grm6*	NM_022920	3.28
Myeloid leukemia factor 1	*Mlf1*	NM_001107680	3.27
GRAM domain containing 3	*Gramd3*	NM_001014011	3.19
Biglycan	*Bgn*	NM_017087	3.16
Leucine rich repeat containing G protein coupled receptor 5	*Lgr5*	NM_001106784	3.06
Solute carrier family 39 (zinc transporter), member 8	*Slc39a8*	NM_001011952	2.77
Proprotein convertase subtilisin/kexin type 2	*Pcsk2*	NM_012746	2.71
Shisa family member 5	*Shisa5*	NM_001006989	2.67
Potassium channel, inwardly rectifying subfamily J, member 15	*Kcnj15*	NM_133321	2.63
ATP/GTP binding protein 1	*Agtpbp1*	NM_001106100	2.52
Myosin X	*Myo10*	NM_001107657	2.51
Procollagen C-endopeptidase enhancer	*Pcolce*	NM_019237	2.50
Lipopolysaccharide-induced TNF factor	*Litaf*	NM_001105735	2.47
Neurotrophin 3	*Ntf3*	NM_001270868	2.45
Mortality factor 4 like 1	*Morf4l1*	XM_008766445	2.44
Prohibitin, pseudogene 1	*Phb-ps1*	XR_594831	2.44
Rho GDP dissociation inhibitor (GDI) gamma	*Arhgdig*	NM_001108269	2.41
SLIT and NTRK-like family, member 6	*Slitrk6*	NM_001106057	2.33
Acyl-CoA synthetase short-chain family member 3	*Acss3*	NM_001108091	2.29
Secreted phosphoprotein 1	*Spp1*	NM_012881	2.27
Leucine-rich, glioma inactivated 1	*Lgi1*	NM_145769	2.26
Glycine dehydrogenase (decarboxylating)	*Gldc*	NM_001107583	2.25
Cd80 molecule	*Cd80*	NM_012926	2.22
MicroRNA 181a-1	*Mir181a-1*	NR_031926	2.21
Aminoadipate-semialdehyde synthase	*Aass*	NM_001100963	2.19
Similar to histone 1, H2ai	*LOC682330*	XM_001061048	2.17
Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucos-aminyltransferase, isozyme A	*Mgat4a*	NM_001012225	2.14
Glutamate cysteine ligase, modifier subunit	*Gclm*	NM_017305	2.14
Vomeronasal 1 receptor 104	*Vom1r104*	NM_001166761	2.13
Growth arrest and DNA-damage-inducible, gamma	*Gadd45g*	NM_001077640	2.12
Platelet derived growth factor D	*Pdgfd*	NM_023962	2.10
Calcium regulated heat stable protein 1	*Carhsp1*	NM_152790	2.07
Guanylate cyclase activator 1B	*Guca1b*	NM_001108198	2.06
Methionine sulfoxide reductase B2	*Msrb2*	NM_001031660	2.06
Protogenin	*Prtg*	NM_001037651	2.05
Stomatin	*Stom*	NM_001011965	2.05

Continued on the next page

Table 1. (Continued)

Gene description	Gene symbol	mRNA accession	Fold change
Keratin 15, type I	*Krt15*	NM_001004022	2.04
Similar to Zinc finger protein 267 (Zinc finger protein HZF2)	*LOC691257*	XR_601214	2.01
Low-density lipoprotein receptor–related protein 2	*Lrp2*	NM_030827	2.00
GULONOLACTONE (L-) oxidase	*Gulo*	NM_022220	–2.02
NEURAL retina leucine zipper	*Nrl*	NM_001106036	–2.05
FRUCTOSAMINE 3 kinase	*Fn3k*	NM_001109051	–2.06
VOMERONASAL 1 receptor 78	*Vom1r78*	NM_001008924	–2.07
MicroRNA let-7f-1	*Mirlet7f-1*	NR_031806	–2.09
Calcium binding protein 5	*Cabp5*	NM_001108907	–2.17
Olfactory receptor 32	*Olr32*	NM_001000690	–2.18
Recoverin	*Rcvrn*	ENSRNOT00000004880	–2.22
Double C2-like domains, beta	*Doc2b*	NM_031142	–2.30
Solute carrier family 1 (glutamate transporter), member 7	*Slc1a7*	NM_001108973	–2.38
Similar to glyceraldehyde-3-phosphate dehydrogenase	*LOC291543*	ENSRNOT00000039569	–2.40
MicroRNA 6323	*Mir6323*	NR_106695	–2.48
Cyclic nucleotide gated channel alpha 1	*Cnga1*	NM_053497	–2.56
BPI fold containing family B, member 6	*Bpifb6*	NM_001107791	–2.62
Heat shock factor binding protein 1-like 1	*Hsbp1l1*	NM_001136183	–2.63
Retinitis pigmentosa 1	*Rp1*	NM_001195676	–2.65
Phosphodiesterase 6B, cGMP-specific, rod, beta	*Pde6b*	NM_001106024	–2.73
Spermatogenesis associated multipass transmembrane protein 2	*Samt2*	XM_001065393	–2.74
MicroRNA 3584	*Mir3584*	NR_037378	–2.76
Retinoschisin 1	*Rs1*	NM_001104643	–3.00
Phosducin	*Pdc*	NM_012872	–3.29
Fatty acid binding protein 7, brain	*Fabp7*	NM_030832	–3.84
Sterile alpha motif domain containing 7	*Samd7*	NM_001191703	–4.65
Rhodopsin	*Rho*	NM_033441	–6.38

mRNA = messenger RNA.

gen). The messenger RNA was reverse transcribed into complementary DNA (cDNA) using the GeneChip Whole Transcript (WT) Amplification Kit (Affymetrix). The cDNA was then fragmented and biotin-labeled with the GeneChip WT Terminal Labeling Kit (Thermo Scientific). A 1.0-μg sample of the labeled DNA target was hybridized for 16 hours at 45 °C on the GeneChip Rat Gene 2.0 ST Array (Affymetrix). The arrays were subsequently washed and stained in the Fluidics Station 450 and scanned using the GeneChip Scanner 3000 (Affymetrix). Fragmentation, labeling, hybridization, washing, staining, and scanning steps were performed by Macrogen Inc, following the manufacturer's protocols. Probe cell intensity data and CEL file generation were conducted using the Affymetrix GeneChip Command Console Software (Affymetrix). Data summarization and normalization were performed using the robust multi-array average (RMA) method within the Affymetrix Power Tools (Affymetrix). Gene-level RMA analysis results were exported, and differentially expressed genes (DEGs) were identified based on fold changes, setting thresholds at ≥2-fold increase for upregulation and ≤–2-fold decrease for downregulation compared to untreated control samples. Enrichment and functional annotation analyses of the DEGs were conducted using

Gene Ontology (GO; https://biit.cs.ut.ee/gprofiler/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/), with gene network analyses performed via STRING (https://string-db.org). All data handling, analyses, and visualization of DEGs were executed using R ver. 4.2.3 (R Foundation for Statistical Computing).

Results

This analysis identified 129 genes exhibiting significant expression changes, with 71 genes upregulated and 58 downregulated due to treatment. Table 1 summarizes the diverse biological functions and processes of these genes. The most prominently upregulated genes are alpha-2-mac-

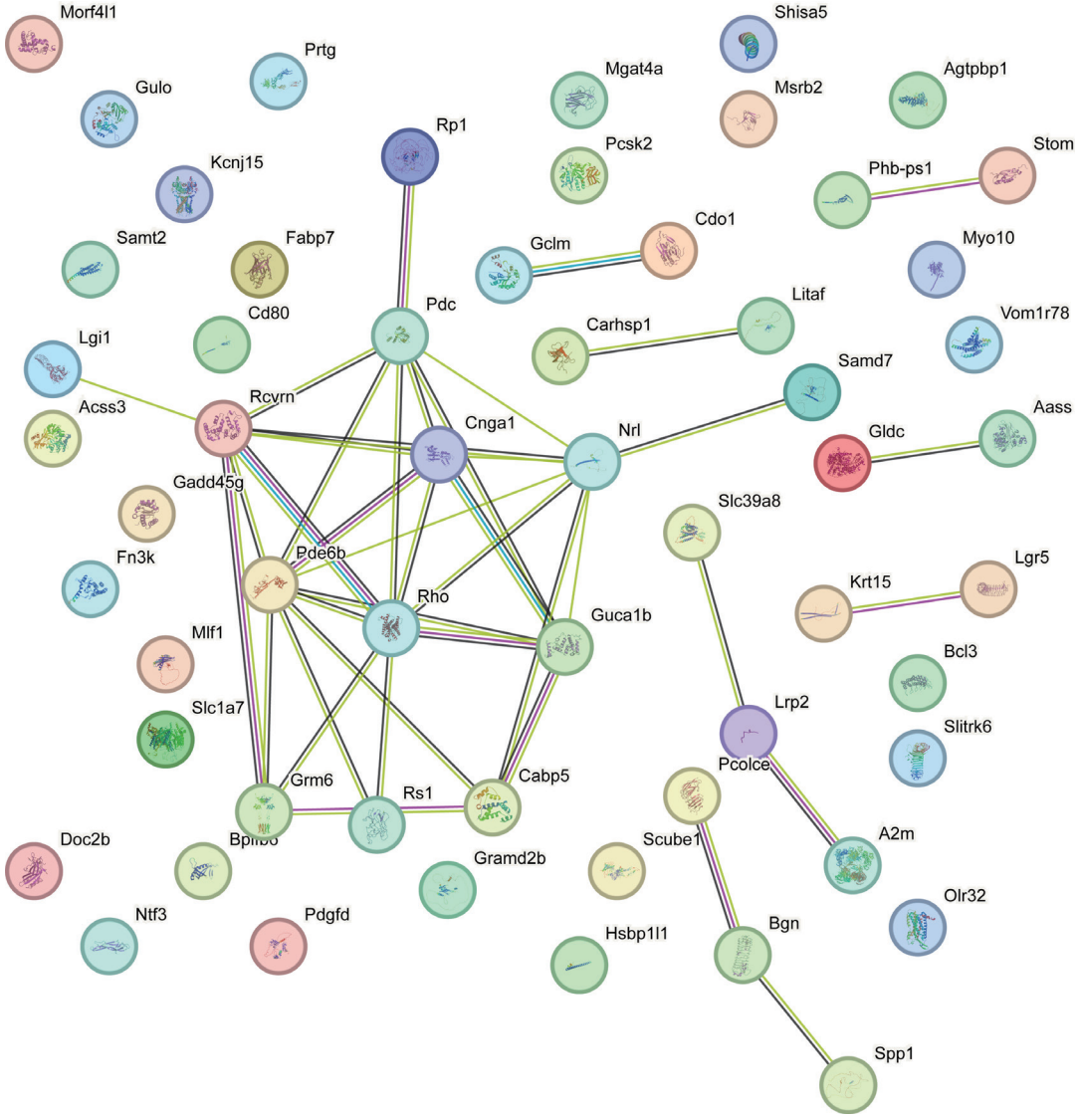

Fig. 2. Interactive gene network in ciliary neurotrophic factor (CNTF)-treated retinal ganglion cells. This diagram visualizes the protein-protein interaction network among differentially expressed genes after treatment with CNTF. Nodes represent individual proteins, with each node corresponding to proteins produced by a single gene locus. Colored nodes indicate query proteins and their direct interactors, while white nodes represent secondary interactors. Filled nodes denote proteins with a known three-dimensional structure, and empty nodes indicate those without a known structure. The edges connecting the nodes depict protein-protein associations, color-coded based on the type of interaction evidence: turquoise for curated database interactions, purple for experimentally determined interactions, green for predicted interactions within gene neighborhoods, red for gene fusion predictions, blue for gene co-occurrence predictions, yellow for text-mining–derived interactions, black for co-expression evidence, and violet for protein homology. This network provides a comprehensive overview of the molecular interactions potentially affected by CNTF treatment in retinal ganglion cells.

roglobulin (*A2m*), signal peptide CUB domain EGF-like 1 (*Scube1*), and cysteine dioxygenase type 1 (*Cdo1*), with fold changes of 4.97, 4.01, and 3.56, respectively. Conversely, among the downregulated genes, rhodopsin (*Rho*) exhibited the most significant decrease, with a fold change of –6.38, followed by sterile alpha motif domain-containing 7 (*Samd7*) and fatty acid binding protein 7, brain (*Fabp7*). Gene network analysis revealed extensive interconnections among the DEGs, indicating complex regulatory mechanisms activated in response to treatment (Fig. 2).

GO analysis classified DEGs in response to CNTF treatment into biological processes and cellular component categories. The biological process analysis revealed pronounced enrichment in processes related to visual function, including sensory organ development, visual system development, and sensory perception of light stimuli (Fig. 3A). Cellular component categorization highlighted numerous genes associated with neuron projection (Fig. 3B). Conversely, molecular function analysis in GO did not yield any significant findings. Additionally, KEGG pathway analysis identified the phototransduction pathway as significantly enriched (adjusted *p*-value, 3.95e-06), with *Guca1b, Rcvrn, Cnga1, Pde6b,* and *Rho* playing pivotal roles (Table 2).

Discussion

In this study, the effects of CNTF on gene expression in RGCs through microarray analysis, including GO and KEGG pathway assessments were analyzed. A total of 129 DEGs were identified, with 71 upregulated and 58 downregulated. Notable upregulated genes included *A2m, Scube1*, *Grm6*, and *Cdo1*, while the most downregulated genes were *Rho, Samd7*, and *Fabp7*. GO analysis revealed significant enrichment in processes related to visual function and components associated with neuron projection. Additionally, KEGG pathway analysis highlighted the phototransduction pathway as significantly enriched.

Sufficiently high concentrations of CNTF effectively promote neurite growth in cultured adult RGCs via the JAK/STAT3 and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathways. Reducing MAPK/ERK activity also increases neurite outgrowth in mature

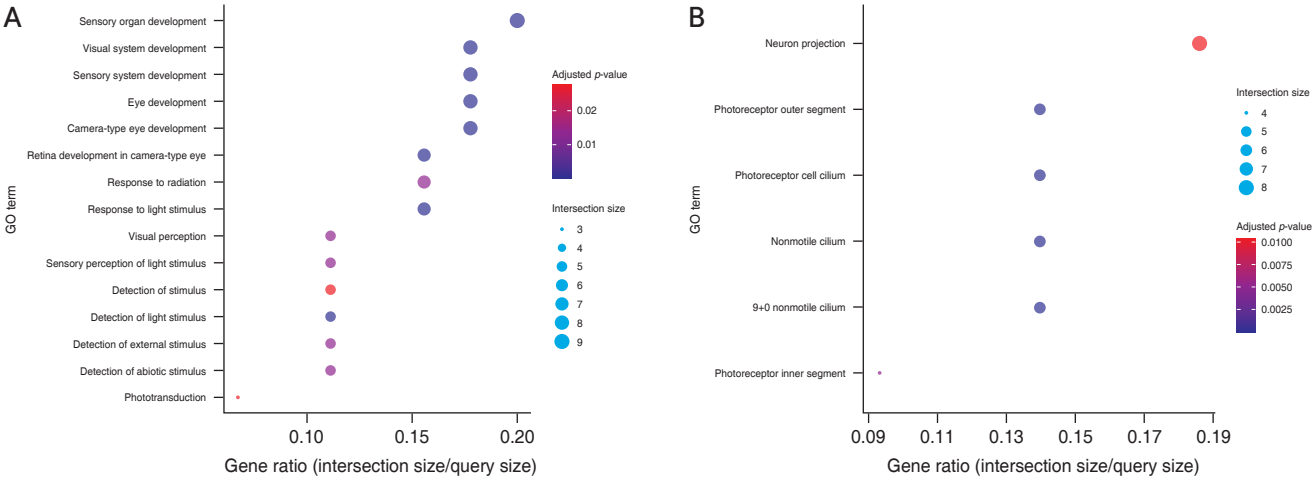

Fig. 3. Gene Ontology (GO) term enrichment analysis for retinal ganglion cells following ciliary neurotrophic factor treatment. Dot size indicates the number of associated genes (intersection size), and color denotes the significance level of the adjusted *p*-value. (A) Enrichment of biological processes illustrates pathways predominantly related to visual system development affected by differentially expressed genes. (B) Cellular component enrichment highlights involvement in neuronal projection, with the most significant terms associated with photoreceptor segments.

Table 2. Result of the KEGG pathway analysis

Pathway ID	Pathway name	Adjusted *p*-value	Associated gene
Rno04744	Phototransduction	3.95e-06	*Guca1b, Rcvrn, Cnga1, Pde6b, Rho*

KEGG = Kyoto Encyclopedia of Genes and Genomes.

RGCs in culture. Moreover, intravitreal administration of CNTF *in vivo* directly activates the JAK/STAT3 signaling pathway in RGCs [8]. In a murine model of nonarteritic anterior ischemic optic neuropathy, intravitreal administration of CNTF significantly improved the survival rate of RGCs. [9].

One upregulated gene, *Grm6*, encodes glutamate metabotropic receptor 6 (GRM6 or mGluR6), which is involved in the visual information processing signaling cascade. When light stimulates photoreceptor cells, it reduces glutamate release, activating mGluR6 on the dendrites of ON-bipolar cells, ultimately transmitting visual signals to the brain through RGCs [10]. Conversely, CNTF downregulated several neurotoxic genes. *Cnga1*, which encodes the alpha subunit of the rod CNG channel, exhibits neurotoxic effects, and mutations in this gene cause autosomal recessive retinitis pigmentosa [11]. *Samd7*, a gene encoding a Crx-regulated transcriptional repressor in the retina [12], also exhibits neurotoxic effects and is downregulated by CNTF treatment.

Interestingly, *A2m* was the most upregulated gene, recognized for its neurotoxic rather than neuroprotective properties. Previous studies indicate that an early increase in *A2m* expression occurs in response to ocular hypertension and persists long after intraocular pressure normalizes [12]. Neutralizing *A2m* delays the loss of RGC in the presence of ocular hypertension, while the administration of *A2m* to normal eyes induces progressive apoptosis of RGCs, mimicking glaucoma conditions [12,13]. This contradictory result, namely the increase in *A2m* expression when RGCs are treated with CNTF, challenges the neuroprotective effects of CNTF. We hypothesize that this discrepancy may reflect the vulnerability of isolated RGCs or apoptotic processes occurring during culture. While oxidative stress models could clarify this role, limitations of such experiments precluded their inclusion in this study. Another neuroprotective gene, *Fabp7*, plays a functional role in the development and regeneration of RGC axons [14]. However, it exhibited a negative fold change contrary to our expectations.

Genes such as *Scube1*, which encodes signal peptide, CUB domain, EGF-like 1, and *Cdo1*, which encodes cysteine dioxygenase type 1, have not previously been associated with RGCs. Their fold changes are significant; however, gene network analysis indicates that they do not belong to the hub genes. Nonetheless, based on the current findings, it can be inferred that their association with CNTF-treated RGCs is plausible. Subsequent studies should investigate this mechanism further.

Our results demonstrate that CNTF plays a critical role in phototransduction and visual system development. This finding aligns with previous studies showing that CNTF exerts a neuroprotective effect on RGCs by stimulating regeneration, and its administration can significantly increase the survival of RGCs [15,16]. CNTF has been shown to regulate gene expression and stimulate neuron regeneration in mice and zebrafish [17,18]. Additionally, CNTF can stimulate axonal regeneration, potentially mediated by astrocytes [6,19].

Overall, since CNTF enhances RGC survival and axon growth via multiple pathways, direct injection of CNTF or modulation of specific genes could aid in treating various glaucoma conditions. A phase 1 trial of the former has been reported, conducted on patients with primary open-angle glaucoma to assess the safety, neuroprotection, and neuroenhancement of a high-dose CNTF-secreting NT-501 implant. The trial revealed structural and functional improvements, indicating biological activity in patients with primary open-angle glaucoma [20]. While the direct application of a CNTF implant presents a potent approach, our study aims to establish the basis for gene therapy by identifying gene alterations in RGCs induced by CNTF. For instance, therapeutic genome editing or gene surgery using neuroprotective genes such as *GRM6*, *GUCA1B*, and *PDE6B*, implicated in phototransduction, could be explored [21]. Next-generation gene therapy, involving *in vivo* gene editing of neurotoxic genes such as *CNGA1* and *A2M* via CRISPR/Cas9, might also be feasible [22]. This study is significant in demonstrating the potential of gene therapy for glaucoma through the analysis of gene expression in RGCs after CNTF treatment. Furthermore, if such gene therapy promotes RGC survival and enables regeneration, it could facilitate the treatment of conditions like ischemic optic neuropathy by rebuilding connections to the brain via axon regeneration.

This study presents several limitations. First, neonatal rat RGCs were utilized instead of adult RGCs, potentially leading to differences in vulnerability to apoptotic stimuli. This susceptibility may stem from inherent differences in the expression of pro-apoptotic machinery at neonatal and adult stages [23]. Nevertheless, neonatal RGCs share numerous characteristics with adult RGCs, offering valuable

insights into RGC survival and development [24]. Second, the study was conducted *in vitro*, raising the possibility of disparate results compared to *in vivo* conditions, where RGCs interact with various cell types, including Müller cells and astrocytes. Third, RGCs were incubated for a brief 2-day period, limiting the assessment of neurite growth; therefore, further investigations monitoring RGC neurite growth over an extended duration are warranted. Fourth, experiments under oxidative stress conditions were not included in this study. In our preliminary studies, RGCs were highly susceptible to oxidative damage under the conditions tested, resulting in inconsistent results, which were not included. Evaluating CNTF's effects under oxidative stress or other pathological conditions would greatly strengthen our understanding of its neuroprotective capacity, and further study performed under the optimized experimental conditions is needed. Despite these limitations, the study underscores the influence of CNTF on RGCs, even during short exposures, and demonstrates gene expression changes in RGCs.

This study provides novel insights into the impact of CNTF on gene expression in RGCs. The findings lay the groundwork for developing potential gene therapy approaches to treat glaucoma and other optic neuropathies by identifying DEGs and their associated pathways. Future research should focus on validating these findings *in vivo* and exploring the therapeutic potential of modulating these genes to enhance RGC survival and regeneration.

Conflicts of Interest: None.
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