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Decreased expression of glial-derived neurotrophic factor receptors in glaucomatous human retinas

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ABSTRACT

Purpose. The purpose of this study was to examine the expression of glial derived neurotrophic factor (GDNF), the GDNF receptors GFRα1 and GFRα2, ciliary neurotrophic factor (CNTF), and the CNTF receptor CNTFRα in normal and glaucomatous human tissue.

Methods. Human retinas were collected from 8 donors that had been clinically diagnosed and treated for glaucoma, and also from 9 healthy control donors. Immunohistochemical analysis for each trophic factor and receptor was performed. The percent of each retinal section labeled with each antibody was quantified for the total retinal thickness, and separately for the retinal ganglion cell (RGC) complex + retinal nerve fiber layer (RNFL). The expression of each protein was correlated with measures of the subject's ocular histories.

Results. The percentage area immunopositive for GFRα2 was significantly decreased in the total retinal thickness containing all retinal layers and in the combined RGC complex + RNFL in glaucomatous eyes in both the peripapillary region and more peripheral retinal locations. We also observed a decrease in GFRα1 expression in the peripapillary RGC Complex + RNFL in glaucoma patients compared to healthy control patients. We also observed a relationship between GDNF and its receptors with several outcomes obtained from the medical record. No differences in CNTF or CNTFR labeling were observed.

Conclusion. Decreases in GDNF receptor expression in glaucomatous tissue may limit the potential for neuroprotective therapy by supplementation with GDNF.

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INTRODUCTION

Glaucoma is an optic neuropathy that can cause progressive visual loss, and if left untreated can cause blindness. Although there are many sub-types of glaucoma, the result of this disease is an irreversible loss of retinal ganglion cells (RGCs) and their axons in the optic nerve. It is estimated that by 2040, 111.8 million people will suffer from glaucoma worldwide[1]. This prevalence has made glaucoma the second leading cause of vision loss in the world[2]. The processes leading to RGC death and dysfunction are multifactorial and depend on both the glaucoma sub-type and risk factors in individual patients. These risk factors can be environmental or hereditary, and include such factors as age, family history, high myopia, a history of diabetes or steroid use, hypertension and central corneal thickness[2]. One major risk factor that is closely tied to RGC death and progression of optic neuropathy is elevated intraocular pressure (IOP).

While there are multiple risk factors for glaucoma, nearly all treatments are designed to lower the IOP. Although the levels of increased IOP vary across individual and glaucoma subtypes, the expected overall effect of IOP lowering therapies is to inhibit the progression of glaucoma, and thus save visual function[3]. These therapies can include medications delivered orally, by eyedrop, or surgical intervention. The medical treatments typically encompass several classes of drugs, including: alpha adrenergic agonists, beta blockers, carbonic anhydrase inhibitors, and prostaglandin analogs. Beta blockers, alpha adrenergic agonists and carbonic anhydrase inhibitors predominantly function by decreasing the production of aqueous humor, while prostaglandin analogs primarily function by increasing the outflow of the eye.

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In addition to the ability to lower IOP, these medications may have off-target effects. We have recently evaluated the relationship of brain-derived neurotrophic factor (BDNF) and the BDNF receptor tropomyosin receptor kinase B (TrkB), with the use of glaucoma medications and outcomes derived from patient medical histories in control and glaucomatous eyes. This study identified a relationship between BDNF expression and use of prostaglandin analogs[4]. Additionally, the expression of TrkB was correlated with the use of carbonic anhydrase inhibitors, the use of beta blockers, and the total number of drugs used for the treatment of glaucoma. TrkB expression also correlated with the last measured intraocular pressure (IOP) from the medical records. In addition to BDNF, several other neurotrophic factors have been investigated for their ability to protect neurons from the effect of neurodegenerative disease. These include Ciliary Neurotrophic Factor (CNTF) and Glial Derived Neurotrophic Factor (GDNF).

CNTF is a member of the neuropoietic cytokine family[5], which also includes interleukin 6, IL-11, leukemia inhibitory factor, oncostatin M, cardiotropin 1, and cardiotropin-like cytokine[6]. CNTF signaling is accomplished by binding to a heterotrimeric receptor complex comprised of CNTF receptor-alpha(CNTFRα), gp130, and LIF receptor-beta[7]. GDNF is a neurotrophic factor, and a member of the GDNF-family of ligands (GFLs). All GLFs signal through transmembrane receptor tyrosine kinase (Ret), however, the complexes will only be activated when the GFL are first bound to GDNF-family receptor-alpha receptors (GFRα)[8].

CNTF and GDNF have been studied for their ability to protect neurons from death in a wide variety of animal models of disease, including those that affect the eye. Transplanted neural stem cells engineered to secrete CNTF were shown to protect

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RGCs following an intraorbital optic nerve lesion[9]. GDNF has also been shown to interact with cells derived from the eye. Exogenous application of GDNF to cultured lamina cribrosa cells from human donors increased the proliferation of the cells[10]. Intravitreal injection of both GDNF and CNTF have been shown to protect RGCs in a mouse model of optic nerve crush[11], and have been shown to work synergistically when co-administered[12].

While GDNF and CNTF expression and function has been studied in animal models of retinal degeneration and glaucoma, there has been a paucity of studies to examine their expression in human tissue. Here we examine the expression of CNTF, GDNF, and their respective receptors in control and glaucomatous human retinas. We have examined the expression of each factor and receptor in the peripapillary and peripheral retina and correlated this expression with outcomes obtained from the medical history for each patient, including the IOP.

METHODS

Tissue

The collection of human tissue occurred in accordance with the World Medical Association Declaration of Helsinki and had the approval of the University of Iowa Institutional Review Board. Normal control retinas without glaucoma were collected postmortem from donors (male and female aged 64–93). Retinas from eyes with primary open-angle glaucoma (male and female age range 80–100) were collected post-mortem from patients diagnosed by a Board-certified ophthalmologist. A total of eight glaucoma patients and nine control patients were entered into this study. Human

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retinas were fixed within 8 hours of death, dissected from the globe, embedded in optimal temperature cutting compound, and frozen at −80°C, followed by sectioning with a cryostat (7 μm). Analysis of tissue was performed by investigators naïve to the disease status of the tissue. All data collected was used for analysis (Supplemental Table 1), with the following exclusion criteria: Images were only collected from tissue sections that were free of processing artifacts (such as torn or otherwise damaged tissue) in the region of interest. Images were also only collected from regions of tissue that could be positively identified as peripapillary retina and retina more peripheral to the optic nerve (at 500 microns and at 4 mm from the optic nerve border, respectively). Exclusion criteria were applied uniformly across all tissue sections by the individual performing quantification, and prior to unmasking the disease status of the tissue.

Immunohistochemical analysis

Frozen sections were rehydrated by incubation in potassium phosphate-buffered saline (KPBS) for 10 minutes. Endogenous peroxidase activity was blocked by incubation in 30% hydrogen peroxide. Following rinses in KPBS, tissues were incubated in blocking solution containing 5% normal donkey serum (NDS, 017–000-121, Jackson ImmunoResearch, West Grove, PA), 0.1% bovine serum albumin (BSA, A9647, Sigma), and 0.04% Triton X-100 for 2 hours at room temperature to eliminate non-specific antibody labeling. Tissue was then incubated in primary antibodies to GFRα2 (Abcam, ab8027), GFRα1 (Abcam ab8026), GDNF (Abcam, ab223347), CNTF (Abcam, ab190985), and CNTFRα (Abcam, ab89333) overnight at room temperature. Sections were then rinsed in KPBS and incubated with biotinylated conjugated secondary

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antibodies followed by incubation with Vectastain ABC complex kit (Vector Labs, Burlingame, CA) diluted in blocking solution. Antibodies were visualized by reaction with diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis MO) in nickel sulfate. Tissue was subsequently dehydrated by incubation with increasing amounts of ethanol followed by xylene. Tissue was mounted in Cytoseal permanent mounting medium (VWR, Radnor, PA). Control tissue from the same eyes was processed in parallel by the omission of the primary or secondary antibody (Supplemental Figure 1). The entire set of tissue used for quantification was processed at the same time to minimize processing artifacts.

Microscopy and antibody quantification

Four images along the vertical axis of the retina passing through the optic nerve were obtained 500 µm from the optic nerve border (peripapillary retina) and four images of more peripheral retina 4 mm from the optic nerve border were taken for each specimen using a Nikon Microphot Microscope (Nikon Inc. Garden City, NY) and a 40X oil immersion objective. In this study, we were interested in regions near the optic nerve (peripapillary retina), and those regions that are peripheral to the optic nerve (peripheral retina). The microscope settings of illumination during the acquisition of images from each tissue section labeled with either anti-GDNF, anti-GFRα2, anti-GFRα1, anti-CNTF, or anti-CNTFRα were left unchanged to eliminate variation in image intensity from one sample to the next. A blank image that did not contain any tissue from each slide was obtained and used for background correction of each slide to account for any variations of the optical properties of the slides. The intensity of each image from the region of the

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slide not containing tissue was used to set an intensity baseline threshold. This method was used to normalize light intensity, so that analysis of antibody staining was not influenced by changes in the optical properties of the slide. Metamorph image analysis software (Ver. 7 Molecular Devices, Sunnyvale CA) was used to quantify the percentage of retinal cross-sectional area that was immunoreactive for each antibody. A threshold intensity two standard deviations below the median staining intensity for each antibody was determined for the entire group as previously performed[4], the immunoreactivity was pseudocolored and the percentage area of the retina immunolabeled was calculated using Metamorph. The area of each retina that was immunolabeled by each antibody was quantified for the entire retinal thickness, including all of the retinal layers (extending from the retinal nerve fiber layer (RNFL) to, but not including, the retinal pigment epithelium) and separately for the retinal ganglion cell complex + RNFL (the ganglion cell layer + inner plexiform layer + RNFL).

Patient histories

Patient histories relevant to eye care were collected from the available medical records for individuals affected with glaucoma. These parameters included best-corrected visual acuity, refractive error, the IOP at the initial visit, the last recorded IOP, the average IOP, the maximum recorded IOP, the cup–disc ratio, and the age of the patient. The medical treatment of each patient was also collected and divided into three categories: prostaglandin analogs, carbonic anhydrase inhibitors, and beta blockers. No patients were treated with alpha-adrenergic agonists. Patients treated with more than one class of drugs during the course of the disease were analyzed for each treatment received. A

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correlation analysis between the percent retina labeled with each antibody and each parameter was performed to examine potential relationships.

Statistical analysis

Statistical analysis was performed using Graphpad Prism using statistical tests as described in the manual (ver. 9.0 for Macintosh). Summary statistics are expressed as mean ± SD. Differences between groups were considered statistically significant for $P < 0.05$. Groups were analyzed using the unpaired t-test with assumptions for equal variances of groups and also nonparametric statistics using rank-sums. Data were normally distributed and had equal variances. Scatter plots of GDNF, GFRα2, GFRα1, CNTF, and CNTFRα expression against medical patient characteristics were reviewed. The strength of the association was assessed through the Pearson correlation coefficient (r) and its probability value. The probability value uses the fact that under normality and under the null hypothesis of no correlation, $(r\sqrt{(n-2)})/\sqrt{(1-r^2)}$ follows a t-distribution with n-2 degrees of freedom; n denotes the number of data pairs. Scatter plots shown in the supplementary materials confirm the linearity of the association and the absence of unusual observations that could affect the fitted regression slope and the associated correlation coefficient. Subject's measures on prostaglandin analogs, carbonic anhydrase inhibitors, and beta blockers use yes/no indicators; in these situations, the p-value for the correlation expresses the significance of the difference of the group averages.

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RESULTS

Total Retinal Expression of GDNF, GFRα1 and GFRα2

The expression of GDNF, CNTF and their respective receptors was analyzed in control and glaucomatous retinas in all retinal layers, and separately for the RGC Complex + RNFL in both the peripapillary and peripheral retina (Fig. 1, Table 1). GDNF expression (Fig. 2 A, B) was observed in the retinal nerve fiber layer (RNFL), the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL). Less staining was observed in the inner plexiform layer (IPL). The percentage of GDNF labeling across all retinal layers combined was 9.4 ± 9.9% (control) and 8.3 ± 8.0% (glaucoma) in the peripapillary region, which was not significantly different between groups (P = 0.86, Unpaired t-test). GDNF expression across all retinal layers in the peripheral retina was 11.8 ± 14.5% (control) and 20.5 ± 21.3% (glaucoma), which was not statistically significant (P = 0.44, Unpaired t-test). GFRα1 expression was observed in the ONL, INL, IPL, GCL, and the RNFL (Fig. 2 C, D). Expression of GFRα1 was lower in the peripapillary region of glaucomatous retinas (15.1 ± 13.2%) compared to control retinas (22 ± 16.3%), but was not significantly different (P = 0.45, Unpaired t-test). GFRα1 expression in the peripheral retina across all retinal layers showed a slight decrease in glaucomatous retinas (10.1 ± 19.8%) compared to control retinas (19.8 ± 9.7%) but was also not significantly different (P = 0.13, Unpaired t-test). GFRα2 expression was observed in the RNFL, GCL, and INL (Fig. 2, E, F). However, analysis of GFRα2 expression across all retinal layers was significantly decreased in glaucoma retinas in the peripapillary region (24.4 ± 16.3%) compared to control retinas (49.6 ± 19.5%, P=0.04, Unpaired t-test; also significant with rank-sum analysis). Likewise,

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GFRα2 expression across all retinal layers was significantly decreased in glaucoma retinas in the peripheral region (26.3 ± 9.8%) compared to healthy control retinas (40.9 ± 15.3%, P=0.03, Unpaired t-test; also significant with rank-sum analysis).

Retinal Ganglion Cell Complex + RNFL Expression of GDNF, GFRα1 and GFRα2

The percentage of GDNF labeling in the RGC Complex + RNFL was 8.3 ± 11.3% (control) and 11.3 ± 9.8% (glaucoma) in the peripapillary region, which was not significantly different (P = 0.64, Unpaired t-test). GDNF expression across all retinal layers in the peripheral retina was 9.0 ± 13.4% (control) and 12.02 ± 12.2% (glaucoma), which was not significantly different (P = 0.70, Unpaired t-test). Analysis of GFRα1 peripapillary expression in the RCG complex + RNFL showed a significant decrease in retinas with glaucoma (12.4 ± 6.1%) compared to control retinas (21.3 ± 15.4%, P=0.02, Unpaired t-test; also significant with rank-sum analysis). However, the expression in the peripheral retina in glaucomatous (24.4 ± 14.5) and control (18.87 ± 10.32) retinas was not significantly different (P = 0.40). GFRα2 expression in the peripapillary RGC Complex + RNFL was significantly reduced in glaucomatous (22.6 ± 14.1) retinas compared to control retinas (45.5 ± 15.5%, P=0.01, Unpaired t-test). This trend was also observed in the peripheral RGC Complex + RNFL when comparing retinas from patients with glaucoma (28.8 ± 7.2%) to healthy control retinas (42.5 ± 14.5%, P=0.03, Unpaired t-test) in analysis of GFRα2.

Total Retinal Expression of CNTF and CNTFRα

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CNTF expression was observed in the ONL, INL, GCL, and RNFL (Fig. 3. A, B). Across all retinal layers, CNTF expression in retinas with glaucoma (22.9 ± 32.4) was not significantly different than the expression in control retinas (15.8 ± 18.7%) in the peripapillary region (P=0.6, Unpaired t-test). Likewise, there was not a significant difference in the peripheral retina (Control=25.0 ± 28.5%, glaucoma=16.6 ± 25.4%, P=0.7, Unpaired t-test). CNTFRα expression was observed in the RNFL, GCL, IPL, INL and ONL (Fig. 3 C, D). CNTFRα expression in the peripapillary region did not vary between the glaucoma group (13.1 ± 16.2%) and the control group (8.6 ± 11.4%, P = 0.57, Unpaired t-test). The expression of CNTFRα across all retinal layers in the peripheral region in the glaucoma group (9.9 ± 13.1%) was not significantly different than the control group (9.1 ± 9.9%, P=0.89, Unpaired t-test).

Retinal Ganglion Cell Complex + RNFL Expression of CNTF and CNTFRα

Evaluation of CNTF expression in the RCG complex + RNFL did not show a significant difference in either the peripapillary region (control = 23.8 ± 23.6%, glaucoma = 17.1 ± 23.9%, P=0.57, Unpaired t-test) or the peripheral region (control = 24.2 ±18.9%, glaucoma = 18.7 ± 22.43%, P=0.61, Unpaired t-test). CNTFRα expression in the peripapillary RGC complex + RNFL was 21.6 ± 21.5% (control) and 19.9 ± 27.3% (glaucoma), which was not significantly different between groups (P=0.83, Unpaired t-test). There was not a difference in CNTFRα expression in the peripheral retina RGC complex + RNFL (control = 21.6 ± 21.6%, glaucoma = 19.2 ± 26.1%, P=0.85, Unpaired t-test).

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Correlation of antibody labeling with ocular history parameters

The percentage expression of each antibody was correlated with parameters obtained from the patients' medical history. We analyzed the correlation of medical history parameters with: GDNF expression in the total retina (Table 2) and in the RGC Complex + RNFL (Table 3); GFRα1 expression in the total retina (Table 4) and in the RGC Complex + RNFL (Supplemental Table 2); GFRα2 expression in the total retina (Table 5) and the RGC Complex + RNFL (Table 6); CNTF expression in the total retina (Supplemental Table 3) and the RGC Complex + RNFL (Supplemental Table 4); and CNTFRα expression in the total retina (Supplemental Table 5) and the RGC Complex + RNFL (Supplemental Table 6). For medical treatments: two subjects received treatment with prostaglandin analogs; one subject with was treated with beta blockers only; one subject received carbonic anhydrase inhibitors and beta blockers; one subject received both prostaglandin analogs and beta blockers; two subjects were treated with prostaglandin analogs, beta blockers, and carbonic anhydrase inhibitors; one subject did not receive medical treatment.

We did not note any correlation of CNTF, or CNTFRα with any parameter obtained from the medical records. The GDNF expression across all retinal layers correlated with the cup to disc ratio in the peripheral retina (Supplemental Fig. 2 A, r = -0.9, P = 0.04) and in the peripheral RCG complex + RNFL (Supplemental Fig. 2 B, r = -0.91, P = 0.03). Additionally, GDNF expression was correlated to the maximum IOP in the peripapillary retina in the RCG complex + RNFL (Supplemental Fig. 2 C, r = - 0.90, P = 0.04). The GFRα1 expression across all retinal layers in the peripheral retina was

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also correlated to the use of carbonic inhibitors (Supplemental Fig. 3 A, r = - 0.81, P = 0.02). The retinal expression of GFRα2 across all retinal layers was correlated with the use of prostaglandin analogs (Supplemental Fig. 4 A, r = - 0.76, P = 0.047) in the peripapillary region. We also identified a correlation of GFRα2 expression in the RCG complex + RNFL with the use of carbonic anhydrase inhibitors (Supplemental Fig. 4 B, r = - 0.81, P = 0.02) in the peripheral region.

DISCUSSION

In this study we have systematically examined the distribution of GDNF, GFRα1, GFRα2, CNTF, and CNTFRα in healthy and glaucomatous human retinas using immunohistochemical staining. A main finding from this analysis is that within the eyes sampled, the area immunopositive for GFRα2 was significantly decreased across all retinal layers as well as in the RGC complex + RNFL in glaucomatous eyes in both the peripheral and peripapillary regions. We also observed a decrease in GFRα1 expression in the peripapillary RGC Complex + RNFL in glaucoma patients compared to healthy control patients. We did not detect any significant differences in the expression of GDNF, CNTF, and CNTFRα in glaucomatous retinas compared to healthy control retinas.

In the eyes examined, GDNF, GFRα1, and GFRα2 expression was significantly correlated to several clinical parameters obtained from the medical record. We observed a negative correlation of GDNF expression in the RGC Complex + RNFL with an increasing cup-disc ratio in the peripapillary and peripheral retina. We also observed a negative correlation of GDNF expression in the peripapillary RGC Complex + RNFL

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with the maximum recorded IOP. Analysis of GDNF receptors further showed that GFRα1 expression was negatively correlated to the use of carbonic anhydrase inhibitors in all retinal layers of the peripheral retina. GFRα2 expression was negatively correlated to the use of carbonic anhydrase inhibitors in the RNFL Complex in the peripheral retina, and to prostaglandin analog use in the peripapillary region.

Previous studies of GDNF and CNTF have shown that these neurotrophic factors are capable of protecting neurons from a variety of insults[12-17]. In this study GDNF and CNTF were not differentially expressed in glaucomatous and healthy control retina. A previous study by Shpak et al. identified a reduction of CNTF in the aqueous humor, and in the lacrimal fluid[18] of patients with POAG. This effect was magnified as the disease status progressed. However, the CNTF in the aqueous humor may not be comparable to these studies, as it was likely not produced by retinal cells. Less work has been done examining the expression of GDNF. Wordinger et al. found that cells of the human optic nerve head expressed mRNA for GDNF but did not examine glaucomatous tissue[10]. However, the absence of expression change of neurotrophins in glaucomatous vs control tissue is in harmony with our previous study that did not observe changes in BDNF in glaucomatous patients.

We did identify a decrease in the GDNF receptor GFRα2 in glaucomatous eyes. Little is known about the regulation of GDNF receptors in the retina, although one study has implicated GDNF receptors in the formation of epiretinal membranes in diabetic retinopathy[19].

There has been a significant amount of research invested in evaluating the potential therapeutic benefit of GDNF and CNTF to protect retinal neurons from

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neurodegeneration. Previous studies using mice have shown that GDNF and CNTF have synergistic effects in protecting RGCs following axotomy[12]. Application of exogenous GDNF in combination with BDNF has also been shown to be effective in preventing apoptosis of isolated RGCs[13]. GDNF has also been shown to increase levels of glutamate transporter and excitatory amino acid transporter when injected into rat eyes with glaucoma[14], and protect RGCs from cell death when released by microspheres in a rat model of chronic ocular hypertension[15]. CNTF delivered alone via an adeno-associated viral vector was shown to preserve axons in rat glaucomatous eyes[16]. Transient delivery of CNTF via intraocular injection has been shown to protect RGCs for up to four weeks in hypertensive rat eyes[17]. This approach has also been examined using CNTF-producing neural stem cells injected into the eyes of axotomized mice, which was shown to protect RGCs[12]. However, it is likely that supplementation of CNTF and GDNF results in expression levels in the tissue that are above those observed naturally and it is conceivable that this level is needed for effective neuroprotection.

Our study has shown a negative correlation of GDNF receptors with carbonic anhydrase inhibitors and prostaglandin analogs. We believe this to be the first description linking these drugs with the GDNF receptors. While we can only speculate the mechanism, we have previously shown that prostaglandin analogs corrlelate with the expression of BDNF, and that carbonic anhydrase inhibitors correlate with the expression of the BDNF receptor, TrkB[4]. Another study has shown that Betaxolol, a beta-blocker, has been shown to upregulate CNTF mRNA in a model of retinopathy[20]. These data suggest that treatments may be associated with off-target effects of

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expression of neurotrophic growth factors and their receptors. This may lead to increased neuroprotection or may inhibit intrinsic neuroprotective pathways that are upregulated in response to stress. Further study is needed to clearly elucidate these pathways.

Our current study had caveats that are important to note. This study was conducted with a limited number of samples. Future studies to confirm the findings presented here will be important to conduct on larger cohorts, and also in different forms of glaucoma. We used semi-quantitative immunohistochemistry, which may miss global changes that could be detected using protein analysis of the entire retina. This study also examined the mature proteins but did not examine the expression of pro-GDNF. In the case of the GDNF and CNTF receptors we analyzed the major subunits, but did not analyze other important receptor components, such as gp130, LIF receptor-beta, and Ret. Also, the relationship of protein expression may be affected by the number of RGCs present in the tissue. Future studies using retinal whole mounts will be important to examine the relationship between protein expression normalized to the number of surviving RGCs. Additionally, this study did not evaluate the co-localization of proteins to specific cells in the retina. Future studies will be needed to determine if individual cells express multiple neurotrophins, neurotrophic growth factors, and their receptors. The relationships that we examined were restricted to eyes with glaucoma, and to those parameters that could be obtained from the medical record. This data did not include functional tests of vision. Studies that examine the relationship of protein expression to changes in visual field will be important, as decreased in the visual field will represent a more severe form of the disease.

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In summary, we have demonstrated that the percentage of the retina immunopositive for GDNF, CNTF, and CNTFRα in tissue sections does not vary between control and glaucomatous tissue. We did observe a decrease in GFRα1 and GFRα2 in glaucomatous patients that appear to be correlated to several clinically important parameters. We have shown that GDNF expression correlated with the cup to disc ratio and the max IOP. GFRα1 and GFRα2 expression were both correlated to the use of carbonic inhibitors and GFRα2 expression was further correlated with carbonic anhydrase inhibitor use. Our findings suggest that expression of neurotrophic growth factor receptors may be influenced by choice of drug, which may become a consideration in the treatment of the disease.

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TABLES

	All Retinal Layers				RGC Complex + RNFL			
	Peripapillary Retina		Peripheral Retina		Peripapillary Retina		Peripheral Retina	
Protein	Control	Glaucoma	Control	Glaucoma	Control	Glaucoma	Control	Glaucoma
GDNF	9.4 (9.9)	8.3 (8.0)	11.8 (14.5)	20.5 (21.3)	8.3 (11.3)	11.3 (9.8)	9 (13.4)	12.0 (12.2)
GFR1	22.0 (16.3)	15.1 (13.2)	19.8 (9.7)	10.1 (19.8)	21.3 (15.4)*	12.4 (6.1)*	18.8 (10.3)	24.4 (14.5)
GFR2	49.6 (19.5)*	24.4 (16.3)*	40.9 (15.3)*	26.3 (9.8)*	45.4 (15.5)*	22.6 (14.1)*	42.5 (14.5)*	28.8 (7.2)*
CNTF	15.8 (18.7)	22.9 (32.4)	25.0 (28.2)	16.6 (25.4)	23.8 (23.6)	17.1 (23.9)	24.2 (18.9)	18.7 (22.4)
CNTFR	8.6 (11.4)	13.1 (16.2)	9.1 (9.9)	9.9 (13.1)	21.6 (21.5)	19.9 (27.3)	21.6 (21.6)	19.2 (26.1)

Table 1. The mean (standard deviation) percentage of retinal area labeled with antibodies to each protein. * = denotes a significant difference between control and glaucoma.

	GDNF All Retinal Layers (Peripapillary Region)		GDNF All Retinal Layers (Peripheral Region)	
Covariate	**Correlation**	**P Value**	**Correlation**	**P Value**
IOP (initial)	- 0.24	0.76	- 0.03	0.96
IOP (last recorded)	- 0.51	0.49	0.39	0.51
IOP (Average)	- 0.59	0.41	0.67	0.21
IOP (Max)	- 0.85	0.15	0.45	0.45
Cup to disc ratio	0.15	0.85	**- 0.90**	**0.04**
Use of prostaglandin analogs	**X**	**X**	0.10	0.87
Use of carbonic anhydrase inhibitors	0.31	0.69	- 0.76	0.13
Use of beta blockers	0.31	0.69	- 0.14	0.82

Table 2. The relationship of GDNF in all retinal layers and parameters from the medical record. The strength of the association was assessed through the Pearson correlation coefficient (r) and its probability value. X = all samples used prostaglandin analogs.

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	GDNF RCG complex + RNFL (Peripapillary Region)		GDNF RCG complex + RNFL (Peripheral Region)	
Covariate	**Correlation**	**P Value**	**Correlation**	**P Value**
IOP (initial)	- 0.62	0.26	- 0.04	0.94
IOP (last recorded)	0.48	0.41	0.41	0.49
IOP (Average)	- 0.58	0.31	0.70	0.18
IOP (Max)	**- 0.90**	**0.04**	0.47	0.42
Cup to disc ratio	- 0.27	0.66	**- 0.91**	**0.03**
Use of prostaglandin analogs	- 0.87	0.06	0.11	0.86
Use of carbonic anhydrase inhibitors	- 0.28	0.65	- 0.77	0.12
Use of beta blockers	0.43	0.47	- 0.20	0.74

Table 3. The relationship of GDNF in the RCG complex + RNFL and parameters from the medical record. The strength of the association was assessed through the Pearson correlation coefficient (r) and its probability value.

	GFRα1 All Retinal Layers (Peripapillary Region)		GFRα1 All Retinal Layers (Peripheral Region)	
Covariate	**Correlation**	**P Value**	**Correlation**	**P Value**
IOP (initial)	- 0.25	0.69	- 0.70	0.06
IOP (last recorded)	- 0.33	0.59	0.23	0.48
IOP (Average)	- 0.07	0.91	- 0.13	0.75
IOP (Max)	0.26	0.68	0.08	0.85
Cup to disc ratio	0.08	0.90	- 0.39	0.33
Use of prostaglandin analogs	**X**	**X**	- 0.40	0.33
Use of carbonic anhydrase inhibitors	- 0.50	0.39	**- 0.81**	**0.02**
Use of beta blockers	- 0.13	0.84	- 0.27	0.53

Table 4. The relationship of GFRα1 expression in all retinal layers and parameters from the medical record. The strength of the association was assessed through the Pearson correlation coefficient (r) and its probability value. X = all samples used prostaglandin analogs.

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	GFRα2 All Retinal Layers (Peripapillary Region)		GFRα2 All Retinal Layers (Peripheral Region)	
Covariate	**Correlation**	**P Value**	**Correlation**	**P Value**
IOP (initial)	- 0.62	0.14	- 0.35	0.39
IOP (last recorded)	0.40	0.37	0.41	0.31
IOP (Average)	- 0.40	0.37	0.02	0.96
IOP (Max)	- 0.38	0.40	- 0.06	0.90
Cup to disc ratio	- 0.07	0.88	- 0.15	0.72
Use of prostaglandin analogs	**- 0.76**	**0.04**	- 0.70	0.05
Use of carbonic anhydrase inhibitors	- 0.20	0.66	- 0.35	0.39
Use of beta blockers	- 0.10	0.83	- 0.03	0.94

Table 5. The relationship of GFRα2 expression in all retinal layers and parameters from the medical record. The strength of the association was assessed through the Pearson correlation coefficient (r) and its probability value.

	GFRα2 RCG complex + RNFL (Peripapillary Region)		GFRα2 RCG complex + RNFL (Peripheral Region)	
Covariate	**Correlation**	**P Value**	**Correlation**	**P Value**
IOP (initial)	- 0.23	0.58	- 0.23	0.58
IOP (last recorded)	0.50	0.21	0.64	0.09
IOP (Average)	- 0.11	0.80	0.31	0.46
IOP (Max)	- 0.49	0.21	0.0018	0.99
Cup to disc ratio	- 0.30	0.48	- 0.61	0.11
Use of prostaglandin analogs	- 0.31	0.45	- 0.08	0.85
Use of carbonic anhydrase inhibitors	- 0.15	0.72	**- 0.81**	**0.02**
Use of beta blockers	0.03	0.94	- 0.05	0.90

Table 6. The relationship of GFRα2 expression in the RCG complex + RNFL and parameters from the medical record. The strength of the association was assessed through the Pearson correlation coefficient (r) and its probability value.

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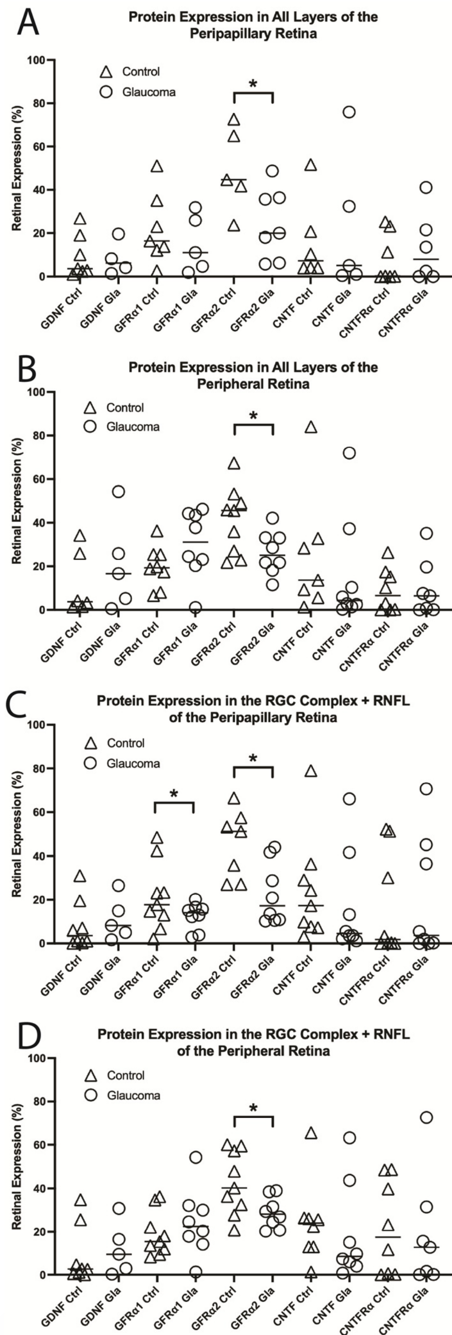

Figure 1. Scatterplots of protein expression from all samples collected in this study in all retinal layers of the peripapillary retina (A), all retinal layers of the peripheral retina (B), the RGC complex + RNFL in the peripapillary retina (C), and the RGC complex + RNLF in the peripheral retina (D). * = P < 0.05, unpaired t-test.

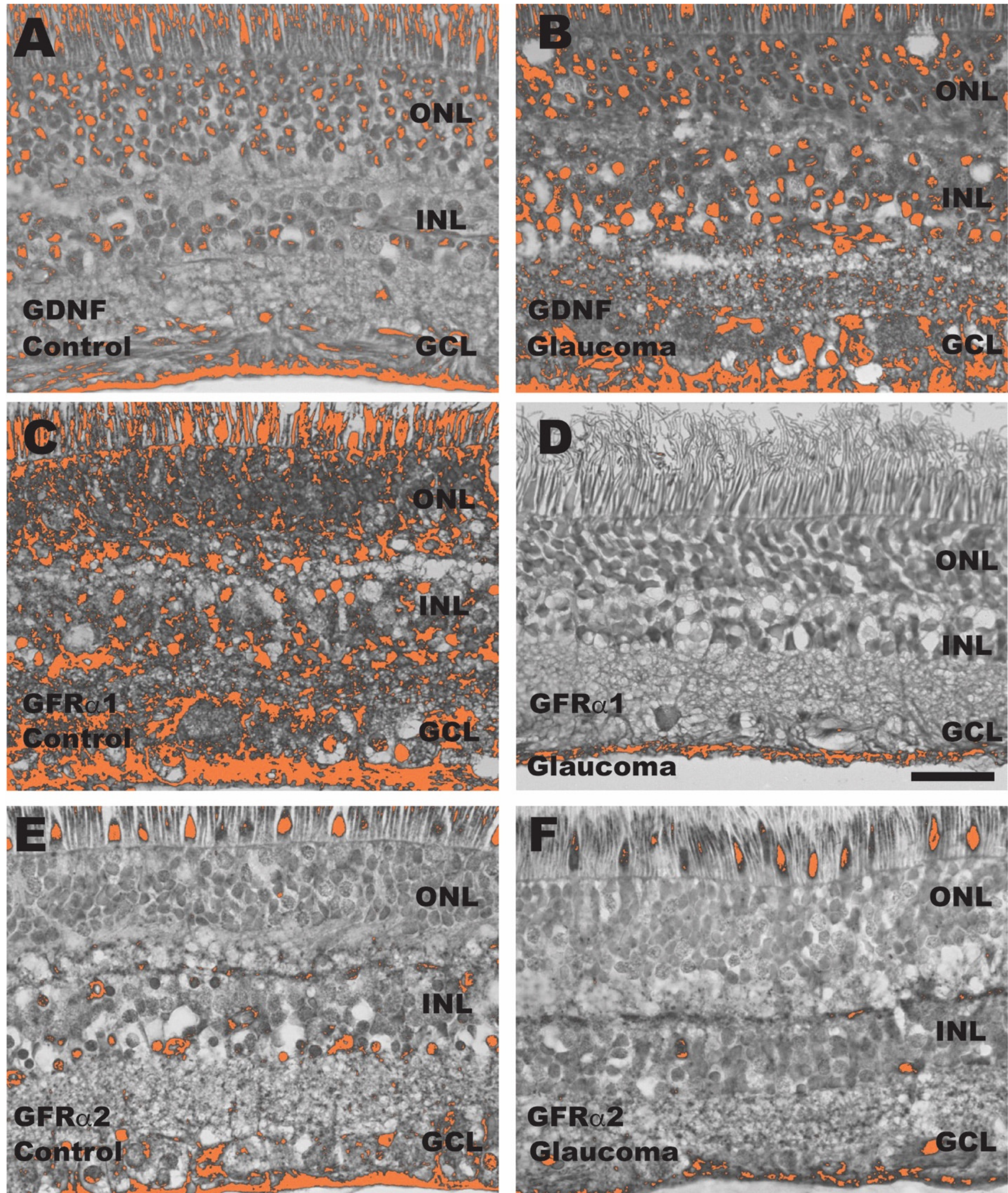

Figure 2. Expression GDNF and GDNF receptors in control and glaucomatous tissue. Representative antibody labeling of GDNF in control (A) and glaucomatous retinas (B); GFRα1 in control (C) and glaucomatous (D) retinas; and GFRα2 in control (E) and glaucomatous (F) retinas. Scale bar in D for all images, 70μm.

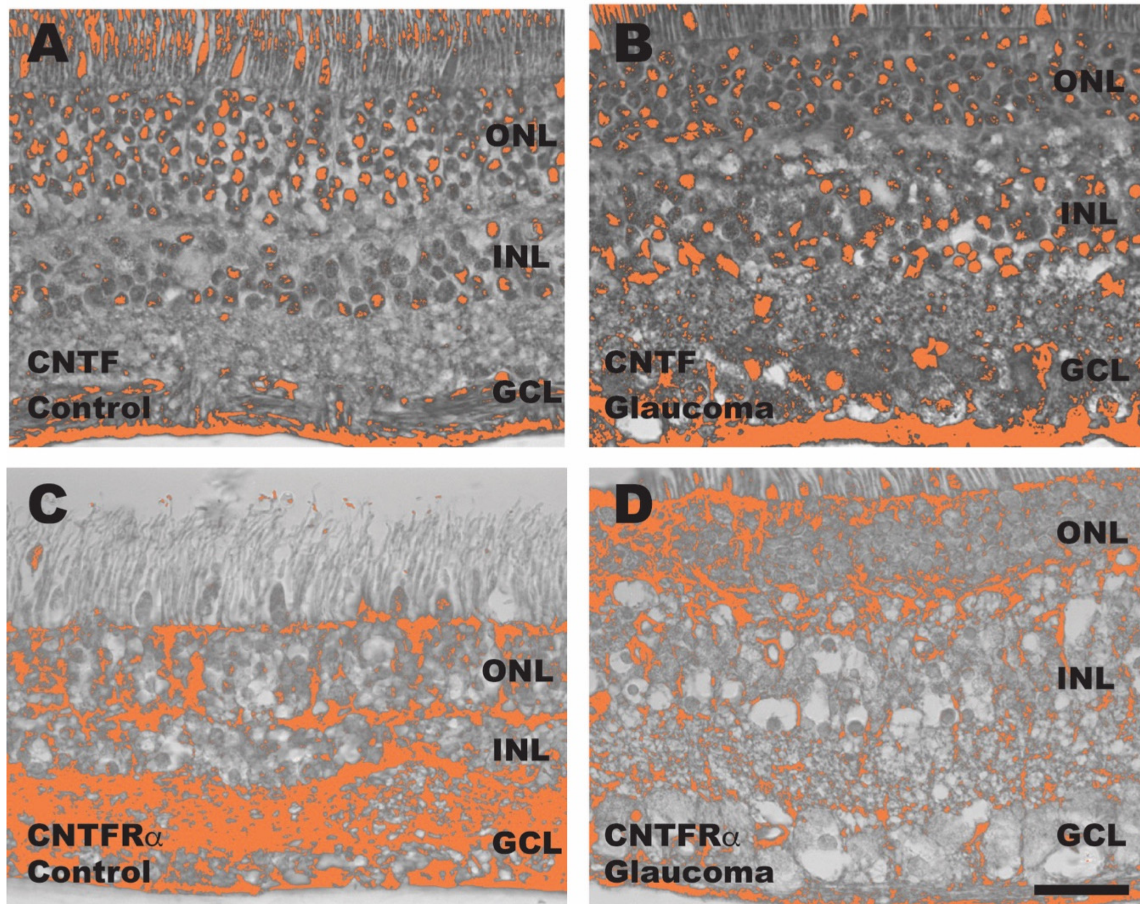

Figure 3. Expression CNTF and CNTF receptors in control and glaucomatous tissue. Representative antibody labeling of CNTF in control (A) and glaucomatous retinas (B); CNTFRα in control (C) and glaucomatous (D) retinas. Scale bar in D for all images, 70μm.

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