

Effect of eye NGF administration on two animal models of retinal ganglion cells degeneration

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Summary. The aim of this study was to investigate the effect of nerve growth factor (NGF) administration on retinal ganglion cells (RGCs) in experimentally induced glaucoma (GL) and diabetic retinopathy (DR). GL was induced in adult rats by injection of hypertonic saline into the episcleral vein of the eye and diabetes (DT) was induced by administration of streptozotocin. Control and experimental rats were treated daily with either ocular application of NGF or vehicle solution. We found that both animal models present a progressive degeneration of RGCs and changing NGF and VEGF levels in the retina and optic nerve. We then proved that NGF eye drop administration exerts a protective effect on these models of retinal degeneration. In brief, our findings indicate that NGF can play a protective role against RGC degeneration occurring in GL and DR and suggest that ocular NGF administration might be an effective pharmacological approach.

Key words: glaucoma, diabetes, nerve growth factor, retinal ganglion cells, vascular endothelial growth factor.

Riassunto (*Effetti della somministrazione oculare di nerve growth factor in due modelli animali di degenerazione delle cellule ganglionari retiniche*). Lo scopo del presente studio è quello di studiare l'azione del NGF somministrato come collirio nella retinopatia diabetica (RD) e nel glaucoma (GL). Il GL è stato indotto in ratti adulti con la somministrazione di soluzione salina ipertonica nella vena episclerale del bulbo oculare. L'iperglicemia e la conseguente retinopatia è stata indotta mediante somministrazione di streptozotocina intraperitoneale. Gli animali con GL, RD e i controlli sani sono stati trattati con NGF o soluzione fisiologica per via topica (collirio). La retina e il nervo ottico sono stati in seguito isolati per l'analisi biochimica e strutturale. I risultati hanno evidenziato una progressiva degenerazione delle cellule ganglionari retiniche ed una ridotta espressione di recettori NGF sulle cellule del nervo ottico nei ratti con glaucoma e diabete. Queste alterazioni sono associate a significative alterazioni dei livelli di NGF e del VEGF. Infine, abbiamo dimostrato che il trattamento oculare con NGF in collirio esercita una marcata azione neuroprotettiva sulle cellule retiniche del roditore con glaucoma e con diabete.

Parole chiave: glaucoma, diabete, nerve growth factor, cellule ganglionari retiniche, vascular endothelial growth factor.

INTRODUCTION

Nerve growth factor (NGF) is the first discovered and best-characterized member of a neurotrophin family that it is produced by a number of different cells and [1, 2], including cells of the visual system [3]. The biological effect of NGF is mediated by two NGF-receptors, the high-affinity receptor TrkA and the low-affinity receptor p75, both receptors located on the surface of NGF-responsive cells [4]. Altered expression of these receptors and/or their ligands can cause degeneration of nerve cells within the peripheral and central nervous system leading to neuropathological manifestation [5]. It has been reported that retinal ganglion cell (RGC) degeneration can occur both in glaucoma (GL) and in diabetes [6, 7], though they have different developmental mechanisms. In fact, a key mechanism leading to

optic nerve injury and RGC loss is the elevated intraocular pressure [8], while in DR the degenerative event of these cells is associated to proliferation of new blood vessels, increased vascular permeability and vascular occlusion [9] and altered levels of vascular endothelial growth factor (VEGF) [10-14]. A number of recent studies have shown that NGF it is able to promote recovery of degenerating RGCs in animal model of ocular ischemia [11] and glaucoma [15-17], reduces retinal cell damage induced by intraocular hypertension [17] and to delays retinal cell degeneration in rodents with retinitis pigmentosa [19]. NGF has been shown also to protect retinal cell degeneration in human glaucoma [20] and maculopathy [21]. VEGF is a cytokine that is able to stimulate endothelial cell proliferation and vascular permeability [22, 23], and to affect retinal cell sur-

vival in certain ocular disorders [14, 22]. NGF and VEGF have reciprocal angiogenic and neurotrophic activity [24] and can activate common intracellular signalling cascade involved in cell proliferation and survival [25].

We have recently shown that NGF eye application can reach the posterior portion of eye and the brain, exerting protective effects on damaged RGCs induced by GL [15, 16], suggesting that this is a non-invasive route to deliver NGF into the posterior segment of the eye for preventing retinal cell damage. Whether eye NGF administration produces a similar or different effect on retinal cells in DR is not known.

To gain further insights into the role of NGF in the protective mechanism of RGC degeneration, we have investigated the effect of topical eye NGF administration on RGCs of rats with experimentally induced GL and DT. Using animal models of GL and DT, the present study investigate structural modification of RGCs and biochemical changes of NGF, NGF-receptors, and VEGF expression in the retina. Our findings showed that streptozotocin (STZ)-induced DT enhances the presence of NGF and reduces the expression of the high-affinity NGF receptor in the retina, particularly in the retinal ganglion layer.

MATERIALS AND METHODS

Animals and surgical procedures

Pathogen-free adult Sprague-Dawley rats (male weighing 300-350 g) were maintained on a 12-hrs light-dark cycle and provided with food and water ad libitum.

For induction of GL, we injected hypertonic saline into the episcleral vein of the adult rat, following the procedure described [26]. Briefly, rats were anesthetized by 1 ml/kg rat cocktail: 5:2,5:1 ketamine [100 mg/ml], xylazine [20 mg/ml], and acepromazine [10 mg/ml]. A volume of 50 μ l filtered hypertonic saline solution (1.75 M NaCl) was injected into the episcleral vein with a glass micro-needle. To measure the IOP, we used the TonoPen XL tonometer (Mentor, Norwell, MA) under topical anaesthesia. The IOP value was measured daily and determined as the mean of 10 valid readings as previously described [16].

DR was induced in adult rats by administration of STZ, a selective toxic compound for insulin-producing beta cells of the pancreas, by a single intraperitoneal injection of 65 mg/kg body weight of STZ dissolved in citrate buffer pH 4.5 [27]. The diabetic condition was checked using a blood glucose analyzer (Accutrend GC, Roche Diagnostic GmbH, Mannheim, Germany) eight weeks after STZ administration. Rats with blood glucose levels above 350 mg/dl were used for the STZ experimental group. Housing, care and experimental procedure of this study were carried out in conformity to the ARVO Statement for the Use of Animals in Ophthalmic

and Vision Research, the International laws (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987) and the Italian National Research Council's Ethical Commission on Animal Experimentation (1992).

Chemicals

The ELISA kits for NGF, Emax ImmunoAssay System, were purchased from Promega (Madison, WI, USA), and the ELISA kit for VEGF, DuoSet ELISA Development System, from R&D System, (Minneapolis, MN, USA). Anti-TrkA antibody was purchased from Upstate (Temecula, CA, USA), biotinylated anti-rabbit and anti-mouse IgG and avidin-conjugated horseradish peroxidase complex were purchased by Vector Laboratories (Burlingame, CA, USA).

2.5 S NGF was purified from adult male mouse submaxillary salivary gland following the described method [28]. Once purified, aliquots of NGF were dissolved in physiological solution (0.9% NaCl) at concentration 200 μ g/ml and stored at -70 °C until used for topical eye administration. NGF-antibodies were produced in goat and purified by affinity chromatography as previously reported [29].

NGF administration

Eight weeks after induction of diabetes and glaucoma, diabetic (no. = 12), glaucomatous (no. = 12) and normal (no. = 12) rats were treated twice a day with eye drops of 20 μ l of purified NGF (stock solution 200 μ g of NGF dissolved in 1 ml of 0.9% NaCl), for three consecutive weeks, in the right eye. As control, rats received equivalent amounts cytochrome C, a molecule with physiochemical properties similar to NGF, but lacking its biological activities into the fellow eye. Animals were killed with an overdose of Nembutal 21 days after NGF treatment.

NGF and VEGF determination

Tissues were homogenized by ultrasonication in extraction buffer (10 mM tris-HCl, pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 10% glycerol; 0.1% SDS; 2 mM Na_3VO_4 ; 20 mM $\text{Na}_4\text{P}_2\text{O}_7$; 1 mM NaF; 2 μ g/ml aprotinin; 1 mM PMSF; 1 μ g/ml leupeptin), centrifuged at 4 °C for 20 min at 13 000 rpm, then supernatant was recovered and used for NGF and VEGF protein determination. The tissue concentration of NGF and VEGF was measured using an ELISA kit, DuoSet ELISA assay (R&D Systems, MN, USA), following the instructions provided by the manufacturer. All assays were performed in duplicate and the data are expressed as concentration of growth factors pg/g of total proteins.

Histological and immunohistochemical analyses

For these studies, all rats were anesthetized and transcardially perfused with 4% paraformaldehyde in phosphate buffer 0.1 M, pH 7.4; eyecup and ON were removed and post-fixed overnight with the

same fixative and placed in a buffered solution containing 20% sucrose for 24 hrs. Coded sections of the eyes were cut at 20 μm with a cryostat (Leica CM 1850 UV, Germany) at $-20\text{ }^{\circ}\text{C}$, taking care that the cross sections of each retina were performed in the same orientation. Sections were then stained with haematoxylin-eosin. The number of RGCs was counted under a Zeiss microscope equipped with a 40X objective.

For immunohistochemistry, sections of the retinal and ON were first exposed to 0.03% of hydrogen peroxide (H_2O_2) and 10% of methanol W/V for 20 minutes, followed by exposure to 0.1M PBS containing 10% of horse or goat serum for 1 hrs, then incubated overnight at $4\text{ }^{\circ}\text{C}$ with antibodies against TrkA at concentration 2 $\mu\text{g}/\text{ml}$ in PBS, containing 2% of serum for 2 hrs at room temperature. Sections were then exposed to biotinylated anti-mouse or anti-rabbit IgG (1:300), with 2% of goat or horse serum, depending on the animal in which the secondary antibody was produced, for 2 hrs at room temperature, and then to avidin-conjugated horseradish peroxidase complex in PBS 0.1% Triton for other 2 hrs at room temperature and for 15 minutes with a solution of 3,3'-diaminobenzidine. All the sections studied passed through all procedures simultaneously to minimize any difference from immunohistochemical staining itself. Staining specificity was assessed by omission of the primary antibodies. The number of immunostained cells in retinal ganglion layer and in the ON were counted under a Zeiss mi-

croscope equipped with a 40X objective in random areas (no. = 10) of 6 different sections of each retina and ON. All counts were performed in a masked manner and the results were averaged as a percentage of immunostained RGCs.

Statistical analysis

All statistical evaluations were performed using the StatView package for Windows and the data were expressed as mean \pm SEM. A *post-hoc* comparison within logical sets of means was performed using Tukey's test. A p-value less than 0.05 was considered significant.

RESULTS

Eight weeks after administration of STZ, the level of glucose in the blood was significantly elevated compared to control rats and the body weight markedly reduced (data not shown).

Figure 1A shows the eye globe of a rat and the administration of hypertonic saline in the episcleral vein to induce EIOP. Eight weeks after the injection the intraocular pressure was 35.7 ± 0.5 mm Hg, as compared to 26.9 ± 0.6 mm Hg of control (Figure 1B). NGF eye drops application is unable to modify the IOP of glaucomatous eye (data not shown).

Figure 1C reports the level of NGF before and after NGF administration in glaucomatous and diabetic retinas and the level of VEGF in both animal models before NGF administration. NGF is reduced

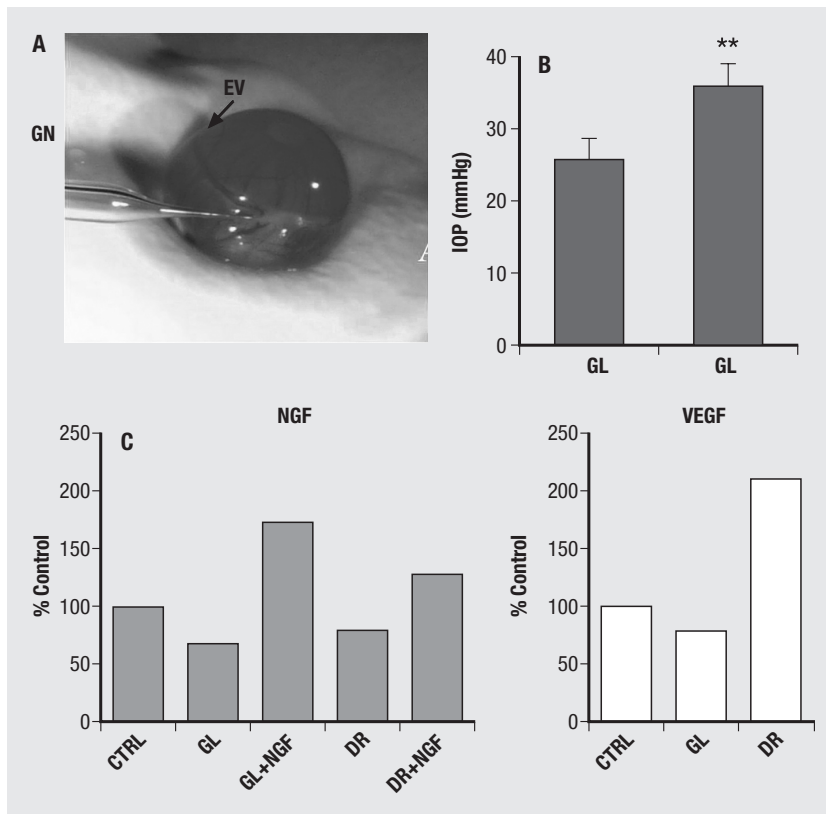


Fig. 1 | (A) A representative image of the rat eye globe, the injection of hypertonic saline with a glass needle (GN) in the episcleral vein (EV). (B) The hypertonic saline in the EV causes an IOP that is significantly higher compared to the untreated control eye ($p < 0.01$). (C) Levels of NGF and VEGF in the retina of rats with GL and DR. The concentration of NGF, both in glaucomatous and diabetic rat retinas are significantly reduced compared to their respective controls ($*p < 0.01$). The level of VEGF is elevated in both the diabetic rat retinas.

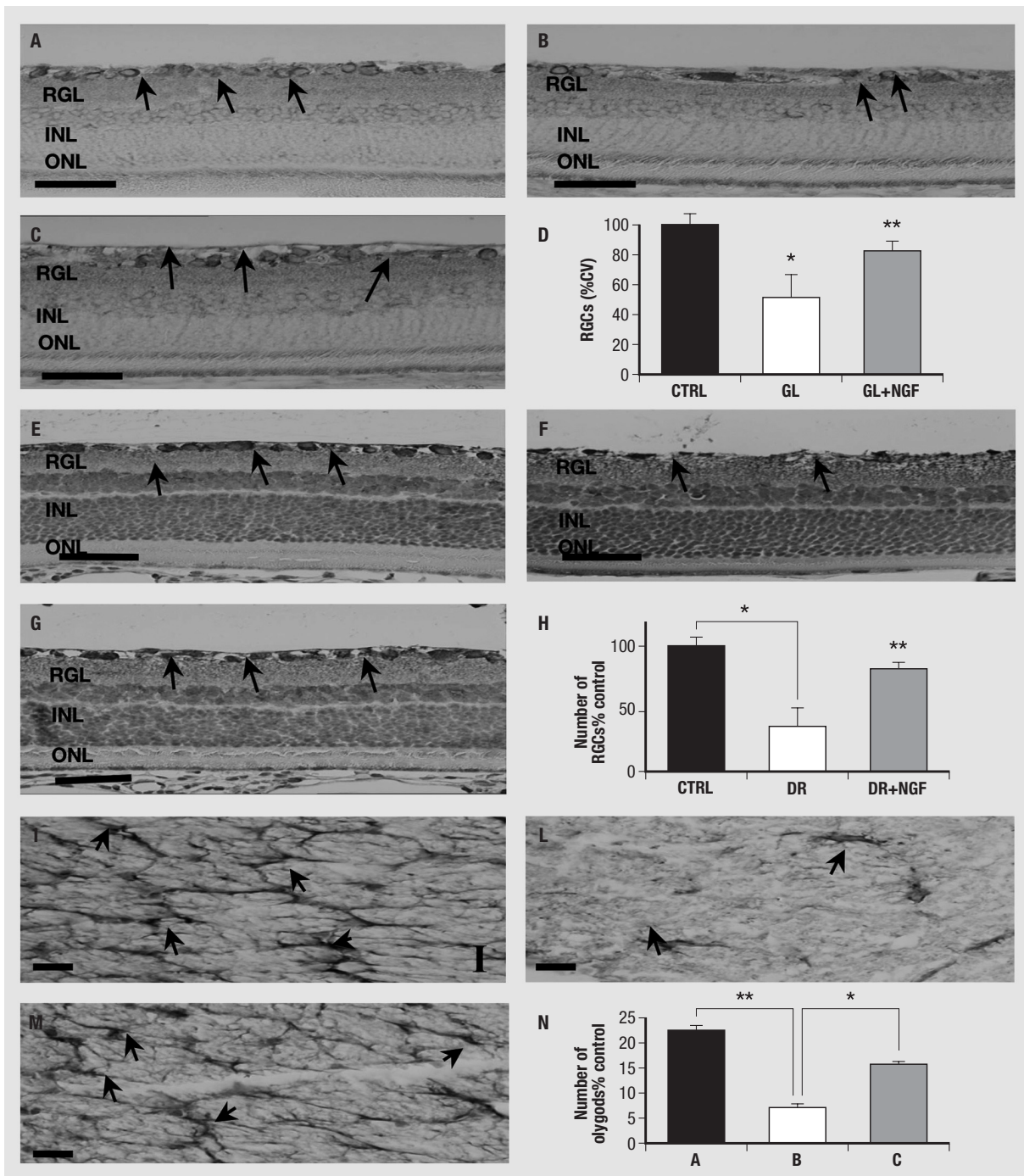


Fig. 2 | A-N. Representative microscopic fields of *TrkA*-immunostained retina sections of control (A), untreated glaucomatous (B) and glaucomatous NGF-treated eyes (C). Eleven weeks after hypertonic saline injection, the number of *TrkA*-positive RGCs decrease, compared to control retina. Quantitative evaluation indicated that eye NGF administration, largely prevented this decrease (D). (* $p < 0.01$; ** $p < 0.001$). Retinal layers, the retinal ganglion layer (RGL), inner retinal layer (INL) and the outer nuclear layer (ONL). Scale bars: 60 μm . (* $p < 0.01$; ** $p < 0.001$).

Representative section of rat retina of control (E), untreated diabetic (F) and diabetic NGF-treated eye (G), immunostained with *TrkA*. Note the reduced presence of *TrkA* immunostained cells (arrows) in the RGC of diabetic retina, compared to control retina and the protective action of eye NGF administration. Quantitative evaluation indicated that eye NGF administration, largely prevented this decrease (H).

Representative sections of the ON of control (I), untreated glaucomatous (L) and glaucomatous NGF-treated rat eye (M). Cells (oligodendrocytes) of the ON express the *TrkA* receptor (I) and its presence is markedly reduced in the ON of glaucomatous eye (L) compared to control eye (I) and is nearly normally expressed in the NGF-treated eye (M). NGF administration prevented the loss of *TrkA*-receptor, with a difference that was statistically significant (N; * $p < 0.01$). Scale bars: 90 μm .

No significant differences in the expression of *TrkA* receptor expression was found in the ON of diabetic rats (data not shown).

in the retina of rats with GL by 32% and in diabetic rats by 22% when compared to controls. The level of VEGF in the glaucomatous retina is also reduced compared to control retina while it is increased in the diabetic retina. As reported in *Figure 1C*, eye NGF administration enhances the presence of NGF in the retina of rats with GL by 72% and with DR by 28%.

Effect of EIOP and diabetes on RGCs

Histological analysis retina of rats sacrificed eleven weeks after induction of GL and DT shows a decreased number of retinal ganglion cells (RGCs), compared to control retina. Daily ocular NGF administration for 3 consecutive weeks reduces the number of RGC loss and prevents also the loss of the high-affinity NGF-receptor, TrkA in the retinal ganglion cell layer (RGL).

Indeed, under normal conditions, RGCs express the TrkA receptor (*Figure 2A*), in glaucomatous retina these cells and TrkA are markedly reduced (*2B*), and in the retina of glaucomatous retina treated with NGF are comparable to control retina (*2C*) see also arrows. As shown in *Figure 2E-G*, the retina of diabetic rats is also characterized by a reduction of RGC number and TrkA receptor (*E*), compared to control retina (*2F*), while eye topical NGF administration prevent this reduction (*2G*), see arrows. As reported in *Figures 2D* and *2H*, in glaucomatous and diabetic retina the protective effect of NGF is statistically significant, (* $p < 0.01$; ** $p < 0.001$).

As shown in *Figure 2I*, the receptor TrkA is also expressed by cells of the ON of normal rats. The number of these cells expressing this receptor is significant less compared to control ON (*2L*), while the administration of NGF protects, though not completely, but significantly the deficits of these cells (*2M*), see arrows. (* $p < 0.01$; ** $p < 0.001$). No similar differences were found in the ON of diabetic rats (data not shown).

DISCUSSION

GL is widely accepted as a neurodegenerative disease in which RGC loss is initiated by a primary insult to the optic nerve head caused by EIOP [8, 26, 27], while DR is characterized by retinal neovascularization, endothelial cell proliferation, and up-regulation of VEGF [12-14]. GL and DT are also recognized as belonging to a group of neurodegenerative diseases, that can be affected by progressive degeneration of RGCs [6, 7]. In the present study, we have investigated the role of NGF in RGC damages of adult rats with experimentally induced GL and DT and focused our attention on NGF role, because recent studies indicated that the reduced presence of NGF can lead to loss of RGCs through a mechanism involving apoptotic cell death [15, 31, 32]. The result of the present study showed that the level of NGF in the retina of rats with GL and diabetes decreased significantly compared to the level of NGF in the retina of normal rats and that the expression of NGF-receptor, TrkA,

by RGCs is markedly reduced. We also found that eye topical NGF administration can protect from RGCs degeneration, indicating that NGF delivered as eye drops can prevent the deleterious events that can lead to progressive RGC death, both in GL and diabetes. However, the possibility that eye NGF administration might not only have a direct effect, but also act indirectly by means of other growth factors, as suggested by other studies [10, 12] cannot be excluded. Moreover, as RGCs are known to need multiple factors for survival and neuritis outgrowth, the hypothesis that the responsiveness of RGCs to NGF may have distinct temporal dependence to these factors should be also taken into consideration.

One critical question raised by our findings is how a high-molecular-weight protein, such as NGF, when administered as eye drops, can be transported from the anterior ocular surface to the posterior segment of the eye and to cells of the retina and optic nerve and protect them. Based on the available data, a reasonable hypothesis is that the NGF reach the retina via two possible routes; directly by travelling through the cornea, conjunctiva, sclera, choroid and retinal pigment epithelium; or indirectly, throughout the suprachoroidal and the retrobulbar space [33, 34]. This hypothesis is supported by recent studies in humans showing that eye NGF application can reach the posterior segment of the eye exerting a protective action on damaged RGCs induced by either GL [20] or by maculopathy [21]. Of note is the observation that the IOP remains elevated. It has been reported that certain patients with low IOP can experience visual loss and others with RGC and ON degeneration can have normal IOP [35, 36]. The critical mechanism(s) involved in these two different conditions is not clear and need to be investigated. The fact that eye NGF administration is able to protect RGC from further degenerative events suggests that that environmental factors can have an important protective role in RGC damages occurring in GL and DR. Our working hypothesis is that NGF either alone or cooperatively with other growth factors can be part of this protective mechanism. Our data here, obtained from two animal models of RGC degeneration, extend previous and recent findings regarding the protective action of eye NGF administration on RGCs and suggest a possible potential benefit of NGF-based therapy for ocular disorders such as GL and DR.

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Conflict of interest statement

None of the Authors has competing or proprietary interests.

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