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Review

Neurotrophic Factors in Glaucoma and Innovative Delivery Systems

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Abstract: Glaucoma is a neurodegenerative disease and a worldwide leading cause of irreversible vision loss. In the last decades, high efforts have been made to develop novel treatments effective in inducing protection and/or recovery of neural function in glaucoma, including neurotrophic factors (NTFs). These approaches have shown encouraging data in preclinical setting; however, the challenge of sustained, targeted delivery to the retina and optic nerve still prevents the clinical translation. In this paper, the authors review and discuss the most recent advances for the use of NTFs treatment in glaucoma, including intraocular delivery. Novel strategies in drug and gene delivery technology for NTFs are proving effective in promoting long-term retinal ganglion cells (RGCs) survival and related functional improvements. Results of experimental and clinical studies evaluating the efficacy and safety of biodegradable slow-release NTF-loaded microparticle devices, encapsulated NTF-secreting cells implants, mimetic ligands for NTF receptors, and viral and non-viral NTF gene vehicles are discussed. NTFs are able to prevent and even reverse apoptotic ganglion cell death. Nevertheless, neuroprotection in glaucoma remains an open issue due to the unmet need of sustained delivery to the posterior segment of the eye. The recent advances in intraocular delivery systems pave the way for possible future use of NTFs in clinical practice for the treatment of glaucoma.

Keywords: glaucoma; neurotrophic factors (NTFs); neurotrophins (NTs); neuroprotection; drug delivery systems; microspheres; gene therapy; polymers; nanoparticles; implants

1. Introduction

Glaucoma is a chronic and progressive optic neuropathy, characterized by degeneration of retinal ganglion cells (RGCs) and axons. It affects more than 60 million people worldwide, causing legal blindness in more than 10% of cases [1]. The only available treatments for glaucoma are effective in lowering intraocular pressure (IOP) and therefore in halting or slowing the disease progression [2]. Currently, treatments able to recover retinal and neural function are not available for clinical use. In the last decades, encouraging perspectives in glaucoma treatment have emerged, and ongoing research is focusing on the study of novel molecules with neuroprotective and/or neuroregenerative activity [2]. To date, only glutamate N-methyl-D-aspartate (NMDA) receptor antagonist memantine and α_2- agonist brimonidine have reached large scale randomized controlled trials (RCTs), although results on their potential neuroprotective effects have not proven decisive [3,4].

Several preclinical studies have demonstrated that topical or intravitreal neurotrophic factors (NTFs) are able to prevent, slow, or reverse RGC death in animal models of experimental glaucoma [5–9]. In fact, it has been demonstrated that the deprivation of intrinsic growth factors promotes apoptotic ganglion cell death in chronic course, and that the administration of exogenous neurotrophic agents

has the potential to establish survival permissive conditions [10,11]. Neurotrophins (NTs) are key modulators of multiple signaling pathways essential for neuronal survival and differentiation of central and peripheral nervous systems, as well as for synaptic plasticity and axonal regeneration [12–14]. The family of NTs consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BNDF), neurotrophin 3 (NT3), and neurotrophin 4/5 (NT4/5). The glial cell-derived neurotrophic factor (GDNF) family and the neuropoietic cytokines have also been shown to regulate development and maintenance of neuronal cells [15].

Currently, clinical use of NTFs in glaucoma has been hindered by the difficulty to provide enough evidence of a safe, steady, controlled in vivo delivery to RGCs. Therefore, the potential of neuronal survivals might be further enhanced from advancement in intraocular delivery devices, enabling sustained drug release to the retina and optic nerve and improved safety profile.

In this paper, we review available knowledge on the use of NTFs for the treatment of glaucoma and related innovative delivery methods, as promising research strategy for preventing RGCs degeneration.

2. Neuroprotection: Insights on Biochemical Pathways and Treatment Opportunities

Neuroprotection for glaucoma refers to any intervention aiming at preserving retinal ganglion cells and related signal transduction pathways. Mean increase of IOP is still considered as the most common risk factor for glaucoma progression, and it is well known that its reduction allows slowing or halting the disease progression in most cases [16]. However, up to 20% of patients with glaucoma show disease progression despite optimal IOP control [17]. In addition, 30–90% of patients showing glaucomatous optic disc damage and visual field loss have normal values of IOP, suggesting that multiple factors may contribute to the pathogenesis of RGCs death in glaucoma [16,18–21]. Several studies demonstrated that neuronal death represents the ultimate process in the pathophysiology of glaucoma damage, and, in this scenario, neuroprotective therapeutical approaches appear as crucial to prolong RGC life and possibly improve visual function [22,23].

The rationale for neuroprotectants is to act against the main pathways involved in the apoptotic ganglion cell death process, including: (i) the deprivation of NTs resulting from the blockage of the retrograde axonal transport from the lateral geniculate nucleus of the thalamus; (ii) the abnormal increase of excitatory neurotransmitters and reactive oxygen species; (iii) the deregulation of ion channel activities; and (iv) the loss of intracellular self-repair processes. All of these different cellular mechanisms invariably lead to RCG loss and glial cells activation.

Several classes of neuroprotective agents have been studied in glaucoma, most notably NTFs, glutamate N-methyl-D-aspartate (NMDA) receptor antagonists, α_2-adrenergic agonists, antioxidant and free radical scavengers, and calcium channel blockers. Among them, only memantine, a non-competitive NMDA receptor antagonist, and brimonidine, a topical α_2- agonist, were evaluated in large randomized controlled trials (RCTs). Specifically, the efficacy of memantine in reducing glaucoma progression was evaluated through two long-term, phase III RCTs conducted by Allergan (Irvine, CA). The results of these studies show that daily treatment with 10 or 20 mg of memantine for 48 months was not proven to significantly delay glaucomatous damage progression, when compared with placebo [3]. Neuroprotective properties of brimonidine were investigated in a multi-center, phase II RCT and compared with topical timolol in patients with low-pressure glaucoma (Low Pressure Glaucoma Treatment Study, LoGTS) [4]. The potential mechanisms of neuroprotective effects of brimonidine include upregulation of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) in RGCs, activation of cell survival pathways and antiapoptotic genes, and modulation of NMDA receptor.

In LoGTS, twice-daily treatment with topical 0.2% brimonidine tartrate for four years appeared to have beneficial effect on visual function independently of IOP lowering when compared with 0.5% timolol maleate eye drops. Nevertheless, problems related with the study design, patient adherence to treatment, the occurrence of adverse events leading to significant drop-out, the different profiles and diurnal effects of used drugs, and the missing data on visual acuity and vertical cup-disc

ratio questioned the reliability of the study. A recent Cochrane systematic review concluded that results were not decisive, and that additional clinical trials are strongly recommended to demonstrate whether neuroprotective agents, including brimonidine eye drops, may be beneficial for patients with glaucoma [24].

2.1. Neurotrophic Factors Rationale for Use in Glaucoma Treatment and Preliminary Results

NTFs are secreted peptides regulating neuronal survival and function, which include the family of NTs, the glial cell-derived neurotrophic factor (GDNF) family, and the neuropoietic cytokine family.

NTs belong to a small family of pleiotropic molecules that are indispensable for regulating neuronal development, survival, and differentiation and promoting synaptic plasticity and axonal regeneration [12]. Currently, four NTs have been isolated: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). NTs and their receptors modulate multiple signaling pathways through the activation of two types of transmembrane glycoproteins: the tropomyosin receptor kinases (TrkA, TrkB, and TrkC) and the low-affinity neurotrophin receptor $p75^{NTR}$. Specifically, NGF and BDNF bind with high affinity to TrkA and TrkB, respectively. NT-3 preferentially binds to TrkC, but it may bind with low affinity to both TrkA and TrkB depending on cellular context. NT-4/5 predominantly acts through TrkB. The Trk activation induces signaling cascades including the Ras/ERK (extracellular signal-regulated kinase) protein kinase pathway with stimulation of mitogen-activated protein (MAP) kinases, the phosphatidylinositol-3-kinase (PI-3 kinase)/Akt kinase pathway, and phospholipase C (PLC)-γ1. On the other hand, immature precursor forms of NTs (proNTs) are able to bind and activate $p75^{NTR}$ with different functional outcomes in terms of apoptosis or cell survival in dependence on the concurrent expression of Trk receptors. Therefore, a delicate balance between the relative percentage of pro- and mature NTs and/or the interaction between Trk and $p75^{NTR}$ receptor availability determines cellular homeostasis in the nervous system [13,14].

The role of NTs in the maintenance and survival of retinal cells during several degenerative diseases including glaucoma has been clearly demonstrated [23–26]. Specifically, various evidence showed that the blockage of axoplasmic flow at the lamina cribrosa in glaucoma would markedly compromise axonal long-range retrograde transportation via endosome neurotrophic signaling from the lateral geniculate nucleus in the CNS to ganglion cell bodies [10,11]. The decrease in neuronal trophic support, in turn, triggers programmed cell death in RGCs, as shown after experimental axotomy of the optic nerve in animals [27]. Furthermore, as a result of degenerative changes in the RGCs and optic nerve head, local production of NTs in the retina is markedly reduced, contributing to disease progression (Figure 1) [28–30].

In recent decades, scientific efforts have been strongly directed at investigating the potential use of neurotrophic agents in promoting RGCs survival in glaucoma.

2.1.1. Nerve Growth Factor

Nerve growth factor (NGF) is the first discovered and best-characterized member of NTs family. Several studies demonstrated that NGF promotes survival and differentiation of neurons in the peripheral and central nervous system, including the optic nerve and visual pathway [31–39]. In fact, both NGF and its receptors TrkA and $p75^{NTR}$ are widely expressed in the retina, optic nerve, lateral geniculate nucleus, and visual cortex.

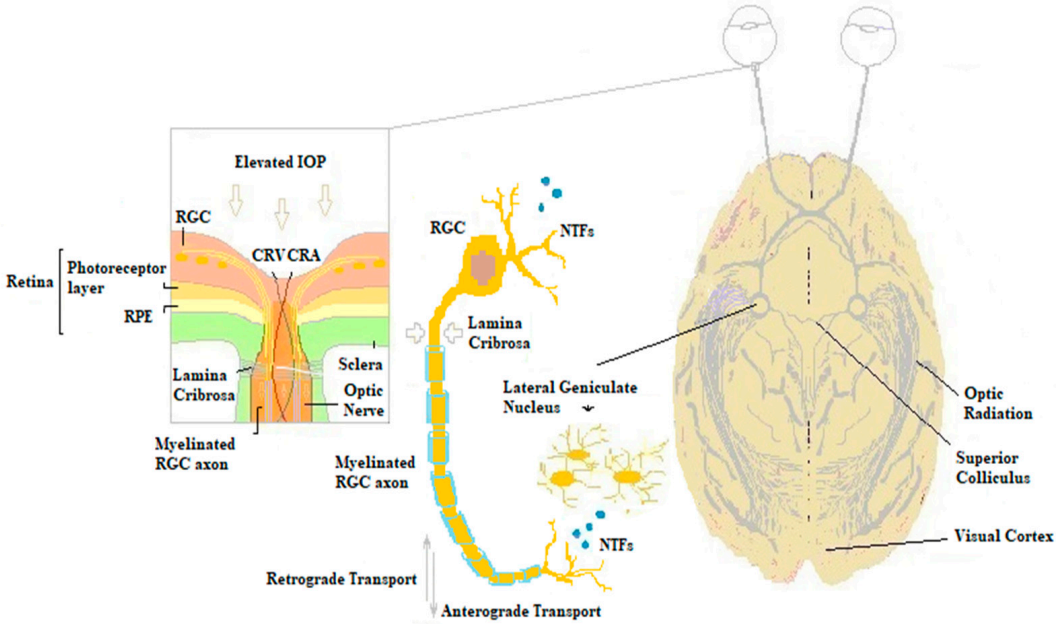

Figure 1. Biochemical mechanisms of glaucomatous damage. The elevated intraocular pressure (IOP) at the lamina cribrosa is responsible for the blockage of the retrograde axonal transport of NTs from the lateral geniculate nucleus of the thalamus to retinal ganglion cells. The deprivation of neuronal trophism in turn triggers apoptotic ganglion cells death, resulting in decreased local NTFs processing and impaired anterograde transportation along RGCs axons. RGC, retinal ganglion cell; CRV, central retinal vein; CRA, central retinal artery; NTFs, neurotrophic factors; RPE, retinal pigment epithelium.

Experimental studies demonstrated that NGF promotes development, differentiation, and survival of retinal ganglion cells (RGCs) by acting as local paracrine/autocrine mediator. In addition, NGF is provided by retrograde/anterograde transport along the axons of the RGCs (optic nerve) [16,29,40–46]. Specifically, intravitreal (IVT) injection of NGF was effective in increasing the survival of injured RGCs in animal models of optic nerve transection and ocular ischemia [47,48]. Moreover, it was reported that exogenous retro-bulbar or intraocular NGF administration significantly inhibited retinal degeneration in animal models of retinitis pigmentosa [49]. Preclinical studies in animal models of glaucoma showed that NGF exerts a protective role after retinal damage induced by increased IOP [8,9] Specifically, it was demonstrated that local NGF levels transiently increased in the aqueous humor after four days of ocular hypertension, with progressive decrease after 10 days and again after 15 days of high IOP [8]. Moreover, it was reported that retro-ocular injections of murine NGF at concentration of 10 μg/100 μL administered three times a week were effective in promoting a significant increase in the density of injured RGCs in glaucoma animal models [8]. An additional experimental study showed that NGF increased RGC cell density in an animal model of glaucoma also when topically administered [9]. In this study, the beneficial effect of NGF on promoting RGCs survival was directly related to the inhibition of apoptosis, as shown by decrease of TUNEL RGC immunostaining and increase of retinal Bcl-2/Bax ratio [9]. Furthermore, it was demonstrated that NGF binding with TrkA on RGCs upregulated Bcl-2 protein and prevented caspase activation and consequently rescued cells from apoptosis and glutamate excitotoxicity [50]. Moreover, the neuroprotective effects of topical NGF treatment were associated with an increase in BDNF protein and mRNA levels in the rat retina [51]. Results from a preclinical pharmacokinetic study support the topical use of NGF for the treatment of retinal degenerative diseases, by demonstrating that topically applied NGF was able to reach the retina and optic nerve, suggesting a direct passage of this high molecular-weight protein through the conjunctiva and sclera [41]. Based on this evidence, an open, uncontrolled clinical study was performed in three patients with progressive glaucoma showing that topical treatment with murine NGF at concentration of 200 μg/mL four times a day for seven weeks was effective in improving

electrofunctional parameters, visual field, contrast sensitivity, and visual acuity, and that improvement lasted up to 90 days after the end of treatment [9]. Recently, a recombinant human nerve growth factor (rhNGF) molecule named Cenegermin, has been developed for clinical use and it is currently available for topical treatment of patients with neurotrophic keratopathy (NK) [52–57]. This compound has been tested in preclinical setting in animal models of retinitis pigmentosa and of optic nerve crush showing efficacy in improvement of retinal cells survival [57–59].

As possible further applications, the efficacy and safety of Cenegermin eye drops solution are currently being tested in a large, controlled, randomized phase I clinical trial in patients with primary open-angle glaucoma despite IOP control, but results are not yet available [59].

2.1.2. Brain Derived Neurotrophic Factor

Among the other NTs, BDNF is of interest for its potential neuroprotective effect in several neuronal populations involved in glaucoma, peripheral sensory neuropathies, amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD) [60,61].

BDNF synthesis occurs locally by RGCs and astrocytes in the retina, as well as in the superior colliculus and the lateral geniculate nucleus in higher mammals from where it is retrogradely transported [62].

Experimental studies showed that hampered axonal transport of BDNF has been observed in glaucoma animal models, and that BDNF injection into the superior colliculus of neonatal hamster led to a 13–15-fold reduction in RGCs pyknosis, suggesting that BDNF plays a key role in promoting RGC survival [6]. Moreover, clinical and experimental studies demonstrated that BDNF/TrkB complex was downregulated in inner retina and optic nerve head in glaucoma [11,63]. Similarly, TrkB was shown to be gradually downregulated in response to neuronal injury, with potential decreased responsiveness to existing levels of BDNF [64]. In addition, BDNF levels in the serum of primary open-angle glaucoma patients and in tears of normal tension glaucoma patients were significantly lower when compared with control group [30,65,66].

On this a basis, previous research on experimental animals demonstrated that exogenous, topical, or intravitreal BDNF was effective in activating RGCs pro-survival signaling pathways after ocular hypertensive damage [5,67].

Specifically, results from experimental study on animal models of chronic ocular hypertension show that recombinant human BDNF eye drop treatment was effective in inducing recovery of pattern electroretinogram (P-ERG) and visual cortical evoked potentials (VEP) damage. Moreover, it was associated with an increase of RGCs survival, as assessed by Brn3 immunopositive cell density in the RGC layer using retinal immunocytochemistry [67]. Furthermore, three repeated intraocular injections of BDNF at concentration of 1.0 µg/µL in moderately chronic hypertensive eyes of rats, resulted in two weeks of lasting increase in RGC survival with no cumulative effect [5]. However, high-doses BDNF as exposed on cultured rat hippocampal neurons and intravitreally injected in animal models of optic nerve injury, caused a rapid and significant downregulation of TrkB expression which reduced BDNF effectiveness [68,69]. In addition, studies showed that upregulated expression of BDNF/TrkB can be detrimental for neuronal homeostasis as it enhances glutamate excitotoxicity [70,71]. In this respect, elevated BDNF levels were found to be expressed in muscle from ALS patients, suggesting possible negative action, and TrkB activation was observed to accelerate glutamate-induced death in rat neuroblastoma cells [70,71]. In addition, no significant reduction in the number of RGCs was demonstrated in BDNF knock-out mice [72,73].

Clinical trials using high-dose subcutaneous or intrathecal rhBDNF for neuroprotective intent in ALS did not provide favorable results [74,75]. Several efforts have been consequently directed at selectively targeting TrkB with low molecular weight exogenous agents. Specifically, a flavonoid-based TrkB agonist 7,8-dihydroxyflavone (7,8 DHF) has been developed. This molecule was effective in activating TrkB downstream signaling and exerting neuroprotective effects in an animal model of PD and in excitotoxic and oxidative stress-induced RGC apoptosis in vitro [76,77]. In addition, it has been demonstrated that highly specific TrkB agonist antibodies promoted RGC survival in vitro and in vivo in acute and chronic models of glaucoma [78,79]. As an option to directly activate survival signaling downstream of TrkB, a cell permeable phosphine–borane complex was demonstrated to promote RGC protection in rat model of optic nerve injury by activating the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway [80]. However, further studies are required to assess pros and cons of activated BDNF/TrkB signaling pathway in neurodegenerative conditions.

2.1.3. Glial Cell-Derived Neurotrophic Factor and Ciliary Neurotrophic Factor

In the recent years, members of other neurotrophic factor families including glial cell-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) have been shown to regulate survival, development, and function in the nervous system by activating tyrosine kinases. GDNF is a neuroprotective agent related to the TGF-β family of cytokines, involved in the growth, differentiation, maintenance, and regeneration of different neuronal populations, including mesencephalic dopaminergic neurons and locus coeruleus noradrenergic neurons [81,82].

Both the GDNF family receptor-α (GFRα) and transmembrane Ret receptor tyrosine kinase are expressed on embryonic chick RGCs as well as on amacrine and horizontal cells [83]. Intravitreal injection of GDNF demonstrated to promote RGC survival after transection of the optic nerve in rats [84,85]. In addition, in vitro and in vivo studies have shown that GDNF may represent an effective therapeutic approach for neurodegenerative diseases of the retina including retinitis pigmentosa and diabetic retinopathy [86,87].

CNTF is a member of neuropoietic cytokine family which was demonstrated to be effective in promoting cell survival and differentiation of multiple types of peripheral and central neurons and glial cell populations [88]. Nevertheless, phase I clinical trials in ALS showed that systemic delivery of CNTF induced severe side effects, which limited the dosage and ultimately the efficacy of the drug [89,90]. CNTF and related tripartite receptor complex were shown to be expressed at the level of the RPE, photoreceptors, outer plexiform layer (OPL), internal nuclear layer (INL), internal plexiform layer (IPL), ganglion cell layer (GCL), and nerve fiber layer (NFL), as well as in the optic nerve head in humans and animals, enabling paracrine as well as autocrine signaling [91,92]. The expression of CNTF, as evaluated after optic nerve section in animals, was observed to increase one week after injury, peak at two weeks, and drop to control levels at four weeks [93]. Conversely, retinal expression of CNTF declined in advanced stages of experimental IOP elevation in animals [94]. Previous research demonstrated that intravitreally injected CNTF enhanced RGCs survival after nerve transection [95,96]. Intravitreal administration of a single dose of CNTF at concentration of 2 μg/2 μL reduced RGC loss from approximately 22% to 5% after four weeks in animal model of glaucoma [7]. Neuroprotective effects of CNTF were proposed to be exerted through an increase in the phosphorylated STAT3 protein in the ganglion cell and inner nuclear layer of the retina, with activation of JAK-STAT pathway (Figure 2) [7]. In addition, CNTF levels were found to be reduced than expected in the aqueous humor and tears of patients with glaucoma [97].

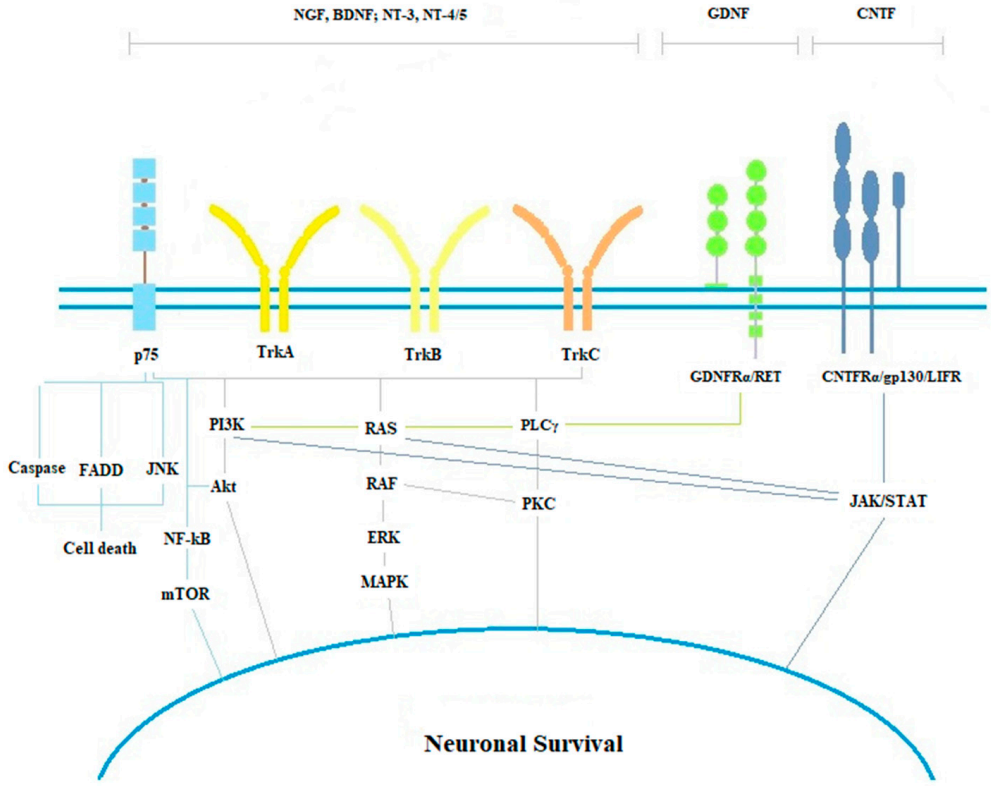

Figure 2. Neurotrophic factors (NTFs) multiple signaling pathways. The tropomyosin receptor kinases (TrkA, TrkB, and TrkC) promote neuronal survival through the activation of the Ras/ERK/mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, and the phospholipase C (PLC)-γ pathway. The low-affinity neurotrophin receptor $p75^{NTR}$ induces cell death through the activation of caspases, c-JUN N-terminal kinase (JNK), and fas-associated protein with death domain (FADD) pathways, but it is able to induce cell survival through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)/mTOR pathway and AKT in dependence of Trk receptors concurrent expression. The GDNFα receptor and tyrosine kinase RET receptor activate PLC-γ, Ras/ERK/ MAPK and PI3K pathways. The CNTFRα receptor and the two subunits gp130 and leukemia inhibitory factor (LIFR) receptor activate the JAK/STAT, Ras/ERK/MAPK, and PI3K pathways.

3. Advances in Drug and Gene Delivery Systems for Neuroprotection in Glaucoma

3.1. Routes of Administration and Devices

Among neuroprotective molecules, NTFs represent promising candidates for their recognized effectiveness in promoting neuronal recovery in human subjects and in experimental models of glaucoma. However, their use for treatment of retinal and/or optic nerve diseases are limited by short half-life in vivo and low bioavailability. Therefore, these compounds require multiple topical or intravitreal administrations to achieve therapeutic effects in the posterior ocular segment. Sustained delivery to the back of the eye probably represents the major difficulty that has most seriously hindered clinical application of NTFs for treatment of glaucoma. In recent years, increasing interest has been addressed to the development of intraocular drug delivery systems in order to provide therapeutic levels of neuroprotective agents in a targeted, local, and sustained way to the retina and optic nerve.

Novel drug delivery systems consist of technologies and materials allowing for considerable benefits over conventional formulations such as improvement in bioavailability and therapeutic efficacy, sustained and controlled release, reduction of dosing schedule frequency with consequent better tolerability, and patient compliance [98].

The ocular barrier represents substantial constraints for topically applied ophthalmic drugs in terms of rapid tear fluid clearance and drainage, relative impermeability of the corneal epithelium, and potential systemic toxicity related to conjunctival and nasolacrimal absorption [99,100]. Alternatively, intravitreal administration offers the advantage of direct access to the vitreous cavity, but it represents an invasive treatment option and increases the risk of ocular complications (endophthalmitis, IOP increase, uveitis, cataract formation, vitreous hemorrhage, and retinal detachment) [99,100]. In addition, it has been demonstrated that, after intravitreal injection, NTFs in the vitreous humor are rapidly degraded by resident microglia in the retina or by free or degenerating RGC-induced extracellular proteases [101]. The trans-scleral route is less invasive and safer than IVT, but it requires drugs to cross the sclera, choroid blood flow, and RPE, resulting in low bioavailability, especially for large molecules such as NTs [99,100]. Similarly, the suprachoroidal route is at lower risk of side effects if compared with IVT, but it is not a valid choice for large molecules since it is associated with slow diffusion from suprachoroidal space to choroid and retina, and rapid clearance of drug via adjacent choroidal blood vessels [100,102]. Conversely, intravenous route is associated with invasive procedure, systemic side effects, and is subjected to blood–retinal barrier (BRB) crossing [103].

As advances in drug sustained delivery to the posterior segment of the eye, various vehicles amenable for intravitreal use have been proposed, including nanoparticles (1–1000 nm), microparticles (1–1000 μm), and implants (>1 mm). Carriers are designed to encapsulate the small active fraction and provide a slow, controlled release. Therefore, low levels of drug achieve therapeutical relevance if constantly administered in the target microenvironment.

Microparticles and implants provide delivery of the active substances for longer periods of time compared with nanoparticles [104]. In addition, microparticles can be easily administered through conventional 25–34-gauge needles. Two structurally distinct variants are described among microparticles: microcapsules and microspheres (MSs). Microcapsules are composed of a polymer or a mixture of several polymers creating a reservoir for the inclusion of the active substance, while in the MSs the active substance is dispersed into a polymeric matrix [104].

Different implants from non-biodegradable polymers have been approved for intravitreal delivery, with the relevant limitation of another surgical procedure for treatment replacement. These include the static implants Vitrasert™ for cytomegalovirus retinitis and Retisert™ for chronic non-infectious uveitis, and the suture-free implant Iluvien™ for diabetic macular edema (DME), respectively, lasting 6, 30, and 36 months [105,106].

However, scientific efforts are now focusing on the development of biodegradable intravitreal devices for long-lasting benefit, rather than non-biodegradable implants.

The synthetic and biocompatible poly-DL-lactide-co-glycolide (PLGA) polyester is the most commonly used for biodegradable MSs and implants manufacturing, with one currently approved product for ophthalmic use containing corticosteroid dispersed in a PLGA matrix system (Ozurdex®) [107]. PLGA is combined with bioactive molecules while modeling, and subsequent gradual degradation of the material which is converted into CO_2 and water in vivo results in slow, sustained release from one to approximately four months. Biodegradable PLGA MSs are relatively inert in the vitreous cavity, with reported minimal inflammatory response, and are able to encapsulate molecules of practically any size with high reproducibility [104].

However, the release of complex large molecules such as NTs from micro-scale devices remains challenging since biological activity is strongly related to three-dimensional structure of these molecules.

In accordance, distinct technological strategies have been proposed to increase protein stability after microencapsulation and allow for sustained delivery of neuroprotective agents in neurodegenerative diseases.

3.2. Neurotrophic Factors Delivery in Glaucoma

The use of NTF-loaded biodegradable microspheres has been tested in preclinical setting for the treatment of ocular diseases including glaucoma [108–111].

Ward et al. demonstrated a significant increase in long-term RGC survival in a glaucoma animal model (DBA/2J mice) by intravitreally transplanting GDNF- loaded PLGA microspheres. In this study, they demonstrated that the release kinetics was nonlinear, with an initial burst of 59% registered within the first two days followed by a 30-day plateau stage in which approximately 1–2 ng/mg was released. Subsequently, a sustained delivery of 15 ng/mg was observed over the final 40 days, until a cumulative release 35.4 ng/mg was reached at 71 days. The average diameter of microspheres was approximately 10 µm. Different injection time points were established in order to design four treatment protocols, while maintaining an interval of two months between each injection. Mice receiving early treatment with microsphere-delivered GDNF showed up to 3.5 times greater RGC density than untreated mice at 15 months survival [112].

The same authors performed a dosage analysis of microsphere-released NTs in the vitreous over eight weeks of IOP elevation in a rat model of glaucoma, demonstrating that 10% GDNF microspheres promoted a significant higher percentage of survival of RGCs and axons (61.58%) when compared with 2% GDNF microspheres (38.56%), blank microspheres (35.25%), and phosphate buffered saline (PBS) alone (33.12%). Similar significant results were achieved in terms of retinal inner plexiform layer (IPL) thickness preservation and glial cell inhibition in the retina and optic nerve. Moreover, an associated reduction in optic nerve head excavation was reported [111].

In view of the above, Kyhn et al. evaluated the neuroprotective effect of GDNF-loaded PLGA microspheres in a pig model of acute retinal ischemia induced by controlled low ocular perfusion pressure. Treatment was intravitreally administered at post-ischemic Day 3 and resulted in significant increase in RGC survival at 42 days following ischemic insult with associated functional improvement, as evaluated by multifocal electroretinography (mfERG) studies [113].

Checa-Casalengua et al. described the effects of gamma-irradiation on proteins microencapsulated according to a novel method based on S/O/W (solid-in-oil-in-water) emulsion process using PLGA microsphere combined with antioxidants [114,115]. Recombinant human GDNF was encapsulated conforming to three technological approaches aimed at preserving protein biological activity after microencapsulation and sterilization: water-in-oil-in-water (W/O/W), solid-in-oil-in-water included in PLGA microspheres (S/O/W) without oil additive (S/O/W 1), and S/O/W in PLGA in combination with Vitamin E in the inner phase (S/O/W 2) [114]. The microspheres prepared (mean particle size 19 µm; 25.4 ng GDNF/mg MSs) were able to slowly release bioactive GDNF in vitro for at least 19 weeks. The results demonstrate that the protein released from the S/O/W 2 microspheres was the most biologically active, with significant increased survival in RGCs and axons than those released from either W/O/W or S/O/W 1 [115]. The introduced changes included: (i) increased protein stability as GDNF was not pre-treated before encapsulation and unfolding-refolding phenomena at the organic, i.e., aqueous interface were avoided; (ii) the inclusion of antioxidant vitamin E in the internal phase to promote additional protein protection; and (iii) release modulation [114,115].

The same group later evaluated the intravitreal pharmacokinetics of GDNF after a single GDNF/vitamin E-loaded microspheres (4%*w*/*v*; 74.85 ng GDNF) IVT injection (50 µL) in rabbits over an extended period of 24 weeks. Rabbits were selected due to their bigger vitreous cavity in comparison to rats (1.27 mL vs. 20 µL). The intravitreal GDNF levels were quantified in the vitreous at each time point: 24 h and 1, 4, 6, 8, 12, 18, and 24 weeks after injection. The results demonstrate that intravitreal GDNF concentrations reached levels of 717.1 ± 145.1 pg/mL 24 h after the injection, followed by maintained levels of 745.3 ± 25.5 pg/mL for the following four weeks. At six weeks post-injection, the intravitreal GDNF concentration was 5.9 ± 0.6 pg/mL. After that, a second plateau occurred, with constant levels of 17.4 ± 3.7 pg/mL from the eighth week to the end of the assay. Thus, GDNF/vitamin E-loaded PLGA MSs prepared using S/O/W2 technique resulted in providing a sustained controlled release of the neurotrophic factor for up to six months, with significantly higher concentration than basal level [116].

As additional intriguing application of NTF-loaded microspheres, subretinal administration of PLGA microspheres delivering BDNF, has been shown to improve the functional efficacy of transplanted retinal progenitor cells in a rat model of retinal degeneration. Interestingly, the improvement of functional outcomes as evaluated by behavioral and electrophysiology data was not significant with BDNF alone, suggesting a synergistic effect between BDNF and the graft [117].

In an animal model of acute IOP elevation, intravitreally injected PLGA microspheres loaded with BDNF and GDNF were demonstrated to be effective over CNTF microspheres for retinal functional recovery, as evidenced by pupil light reflex (PLR) parameters and electroretinograms (ERG). The authors speculated that intrinsic production of CNTF may have occupied the majority of available receptors, thus preventing any additional therapeutic effect of exogenous CNTF [118].

Although there are no available in vitro or in vivo studies on rhNGF-loaded PLGA microspheres for ocular delivery, encouraging in vitro and in vivo results have been described on the use of rhNGF-loaded PLGA microspheres for targeted delivery to the basal forebrain (BF) in AD. Specifically, rhNGF release kinetics from microspheres after implantation in the BF was sustained for 4–5 weeks, with low initial burst (11.4%) related to the low amount of rhNGF added (1/2000 *w*/*w*). The encapsulated rhNGF was biologically active and significatively effective at rescuing p75NTR- and ChAT-positive neurons in the medial septum (MS) and vertical diagonal branch (VDB) [119,120].

The carriage options thus far described, however, do not avoid the need for repeated injections in the context of life-long treatment, and, therefore, delivery of transfected cells permanently expressing NTFs would firmly represent a promising prospect.

3.3. Gene Delivery for Neurotrophic Factors in Glaucoma

Gene therapy for neuroprotection in glaucoma is aimed at increasing endogenous retinal production of selected NTFs without recurring to the in vivo delivery of proteins. It represents an attractive technology in the context of sustained delivery to the posterior segment of the eye, as RGCs are highly accessible target structures [121–123]. Consequently, several efforts have been made to develop gene therapy approaches for neurotrophic agents in retinal neurodegenerative disorders including glaucoma. The most studied are encapsulated cell technology (ECT) systems, based on delivery of cells ex vivo transfected with human NTF genes, and NTF gene therapy using viral and non-viral carriers such as polymeric micelles and dendrimers. Intravitreal ECT implants containing immortalized pigment epithelial cells transfected with human CNTF (NT-501) gene have been developed to release therapeutic factors directly into the vitreous over an extended time of 1–1.5 years.

NT-501 was evaluated in a non-randomized phase I safety clinical trial over a six-month period on patients with retinitis pigmentosa (RP) and in two randomized, phase II safety clinical one-year trials in patients with early and more advanced RP, with overall positive results in terms of visual outcomes and tolerability [124–127].

A randomized phase II, one-year clinical trial on NT-501 implant has been conducted in patients with atrophic macular degeneration, showing similar good safety profile and benefit in preserving vision [128]. Moreover, from a study evaluating the pharmacokinetics of NT-501 implants over a period of up to two years in patients with retinitis pigmentosa (RP) and geographic atrophy (GA), it was demonstrated that CNTF was continuously produced for the whole implant period with a calculated half-life in the vitreous of 51 months [129].

The NT-501 encapsulated cell system is currently under evaluation in phase II clinical trials for the treatment early-stage retinitis pigmentosa and Usher syndrome [130] and macular telangiectasia [131], as well as in a phase I clinical trial for ischemic optic neuropathy [132].

Randomized phase I and II clinical trials are currently ongoing to evaluate the safety and efficacy of intravitreal implantation of NT-501 CNTF cell devices in patients with primary open-angle glaucoma (POAG) [133,134]. More in detail, CNTF-secreting capsules, 1 mm in diameter, are characterized by internal poly (ethylene terephthalate) scaffold supporting human RPE cells and present a semipermeable polymer outer membrane with 15-nm pores to release therapeutic factors directly into the vitreous over an extended time of 1–1.5 years. The encapsulated retinal cells expressing CNTF need to be surgically implanted into the vitreous cavity and then surgically removed at the end of the study [124].

In addition to cell-mediated gene therapy, gene delivery to the retina achieved through viral vectors has also provided encouraging preclinical results in the context of retinal neurodegenerative diseases including glaucoma [135].

However, vectors based on adenovirus showed limitations due to transient transgene expression and their tendency to trigger significant inflammatory reaction. Conversely, adeno-associated virus (AAV) and lentivirus (LV) vectors are capable of long-term efficient transgene expression in the retina and cause minimal ocular inflammation.

Martin et al. developed a modified adeno-associated virus (AAV) incorporating cDNA for BDNF to transfect retinal ganglion cells (RGCs), reporting a reduction in RGC axon loss from 52.3% to 32.3% in four weeks of high IOP model in rats. The novel AAV-BDNF vector included hybrid cytomegalovirus (CMV) immediate early enhancer, chicken β-actin (CBA) promoter, and the woodchuck hepatitis post-transcriptional regulatory element (WPRE) to promote efficient transfection in the rat RGC layer within two weeks of a single 2-µL intravitreal virus injection [136].

Intraocular injection of lentiviral (LV) or AAV vectors delivering human CNTF gene to the retina proved effective for rescuing injured RGCs and photoreceptors in animal models of retinal degeneration and optic nerve transection for an extended period [137–141]. Interestingly, CNTF released by transduced RGCs facilitated the survival of neighboring non-transduced RGCs, plausibly via the stimulation of other neuronal and glial populations in the retina [138]. However, discordance between RGCs increased survival and decline in electroretinographic potentials was observed by Liang et al., likely to be ascribed to a prevalent effect by CNTF on inner retinal neurons and Muller cells masking the positive action on photoreceptors [139].

An additional gene therapy approach, consisting of a combination of exogenous BDNF and AAV-mediated TrkB gene transfer into RGCs, was proposed to upregulate the TrkB receptor after optic nerve injury, and it was demonstrated to transiently increase neuronal survival [142].

A single intravitreal injection of adenoviral vector expressing the rat GDNF gene from a cytomegalovirus promoter (AdCMV-GDNF) increased the number of surviving RGCs by 67% 14 days after optic nerve transection as compared with axotomized untreated control animals [143].

Furthermore, adenoviral transmission of GDNF combined with adenoviral X-chromosome-linked inhibitor of apoptosis (ad.CMV-XIAP) was more effective at promoting RGCs survival (47.3%) than the two treatments given separately (37.4%), as described in an animal model of optic nerve transection [144].

XIAP, also known as human baculoviral IAP repeat-containing protein-4 (BIRC-4), represents a promising neuroprotectant for glaucoma therapy, and it acts by directly inhibiting at least three downstream cell death proteases, caspase 3, 7, and 9.

Adeno-associated viral vector containing chicken-b-actin (AAV-CBA) coding for human BIRC-4 intravitreally injected in a rat model of glaucoma promoted and sustained significant axon survival, as evaluated after 12 weeks [145]. To date, clinical trials using similar vectors to treat Leber's congenital amaurosis (LCA) have provided encouraging results, with translational prospective for other neurodegenerative ocular diseases [146,147].

Advances in recombinant AAV technology led to introduction of new viral vectors with novel serotypes showing optimal tropism towards specific target cells, less susceptibility to ubiquitin-mediated damage, increased stability, and longer lasting effects with the need of lower doses [147].

The AAV vectors showing ability to efficiently transduce RGC in rodents following intravitreal and subretinal injection have proven to be those including serotypes 2 and 8 [148,149]. In a recent study, adeno-associated virus serotype 2 (AAV2) delivering BDNF was neuroprotective against NMDA toxicity in a mouse model of inner retinal injury in comparison with control vector group [150].

Intravitreal injection of AAV2-vectored ciliary-derived neurotrophin factor (CNTF) gene at the low dosage of 2 µg was effective in reducing RGC axon loss from 53.9% to 39.7% in a four-week study in experimental glaucoma. Intriguingly, combined CNTF and BDNF overexpression did not improve RGC axon survival; the authors hypothesized that this finding may be related with the reduced transduction efficiency of CNTF [151].

Gene therapy approaches using AAV2 vectors encoding NGF (CERE-110) were investigated in a few clinical trials on AD patients, showing good safety profile and long-term NGF expression. Nevertheless, AAV2-NGF demonstrated limited spread and inaccurate stereotactic targeting, and functional results were not satisfactory [152–155]. Specifically, intraparenchymal AAV2-NGF delivery by stereotactic injection in the nucleus basalis of Meynert proved safe and showed prolonged expression of biologically active human NGF in a dose-escalating phase I clinical trial for AD [152,153]. However, from a multicenter, sham placebo-controlled phase II clinical trial for AD, AAV2-NGF was not able to improve cognition [155]. A recent post-mortem analysis conducted on three patients from the phase I dose-escalation trial revealed that NGF gene expression had persisted for at least seven years at sites of AAV2-NGF injection; however, the mean distance of AAV2-NGF spread had been only 0.96 ± 0.34 mm. Particularly, NGF had not directly reached cholinergic neurons at any of the 15 injection sites [154].

Thus, gene transfer could overcome the obstacles associated with delivering NTFs to the brain, and even possibly to the posterior segment of the eye, but further insight is recommended as available evidence is limited and inconclusive.

The eventuality of insertional mutagenesis and the immunogenicity concerns associated with the use of viral vectors led to the development of non-viral gene delivery techniques for NTFs in the eye.

Interestingly, eye drops of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO-PPO-PEO) polymeric micelles demonstrated effective potential for noninvasive, topical ocular gene delivery in in vivo studies [156]. In the same direction, polyamidoamine (PAMAM) dendrimer-driven expression of BDNF and NT-3 was achieved in vitro in rodent and human stem cells [157].

In addition, a cationic cell-penetrating peptide was studied for ocular delivery (POD) conjugated with polyethylene glycol (PEG-POD). PEG-POD nanoparticles, containing glial cell line-derived neurotrophic factor transgene (PEG–POD-GDNF), were injected into the subretinal space of adult murine retina, showing a significant reduction in light-induced photoreceptor apoptosis at 14 days post exposure compared to control-injected animals [86].

Direct intravitreal injection of BDNF cDNA was studied in mice models of optic nerve transection, followed by in vivo electroporation, and it resulted in increased survival of axotomized RGCs [158].

Another interesting application of NTs delivery involves the use of artificial substrates for promoting axonal repair.

A graft made from a silicone tube enriched with cultured Schwann cell, extracellular matrix, NGF, BDNF, and NT-4 was tested in murine models subjected to axotomy, resulting in regeneration of blood vessels, RGC, and related axons [159] (Table 1).

Table 1. Current status of delivery systems for Neurotrophic factors (NTFs).

Neurotrophic Factors (NTFs)	Delivery Systems	Main Preclinical Studies	Main Clinical Studies	Clinical Trials Identifier
NGF	IVT PLGA microspheres-NGF IVT AAV2-NGF (CERE-110) Artificial graft transplantation-NGF	*Gu et al. 2007, in vitro and in vivo (AD)* *Gu et al. 2009, in vitro and in vivo (AD)* *Bishop et al. 2008, in vivo (AD)* *Negishi et al. 2001, in vivo (ON transection)*	*Rafii et al.2014 (AD)* *Rafii et al.2018 (AD)*	
BDNF	Mimetic ligands binding TrkB - 7,8-dihydroxyflavone (7,8 DHF) - IVT TrkB selective antibodies - IVT phosphine-borane complex Subretinal/IVT PLGA microspheres-BDNF IVT AAV-BDNF IVT AAV-TrkB+BDNF IVT AAV2-BDNF IVT AAV2-BDNF Polyamidoamine dendrimer-BDNF IVT BDNF+electroporation Artificial graft transplantation-BDNF	*Jang et al. 2010, in vivo (PD)* *Gupta et al. 2013, in vitro (excitotoxic and oxidative stress induced RGC apoptosis)* *Hu et al. 2010, in vitro and in vivo (ON transection)* *Bai et al. 2010, in vivo (ON transection and POAG)* *Almasieh et al. 2011, in vivo (ON transection and POAG)* *Seiler et al. 2008, in vivo (photoreceptor degeneration)* *Grozdanic et al. 2010, in vivo (retinal ischemia)* *Martin et al. 2003, in vivo (POAG)* *Pease et al. 2009, in vivo (POAG)* *Cheng et al. 2002, in vivo (ON transection)* *Leaver et al. 2006, in vivo (ON transection)* *Shiozawa et al.2020 in vivo (inner retinal injury model)* *Shakhbazau et al. 2012, in vitro (rodent and human stem cells)* *Mo et al. 2002, in vivo (ON transection)* *Negishi et al. 2001, in vivo (ON transection)*		
GDNF	IVT PLGA microspheres-GDNF IVT Ad. GDNF IVT Ad. GDNF+Ad.XIAP IVT AAV-GDNF Subretinal PEG-POD-GDNF	*Ward et al. 2007, in vivo (POAG)* *Jiang et al. 2007, in vivo (POAG)* *Kylhn et al. 2009, in vivo (retinal ischemia)* *Checa-Casalengua et al. 2011, in vitro and in vivo (POAG)* *Checa-Casalengua et al. 2012, in vitro (retinal cells)* *Garcia-Caballero et al. 2017, in vitro and in vivo (wild-type)* *Grozdanic et al. 2010, in vivo (retinal ischemia)* *Schmeer et al. 2002, in vivo (ON transection)* *Straten et al. 2002, in vivo (ON transection)* *Pease et al. 2009, in vivo (POAG)* *Read et al. 2010, in vitro and in vivo (photoreceptor degeneration)*		
CNTF	IVT PLGA microspheres-CNTF IVT NT 501 CET-based implants IVT AAV-CNTF Subretinal AAV-CNTF IVT LV-CNTF IVT AAV2-CNTF	*Grozdanic et al. 2010, in vivo (retinal ischemia)* *Thanos et al. 2004, in vitro and in vivo (wild-type)* *Liang et al., 2001, in vivo (RP)* *Bok et al.2002, in vivo (photoreceptor degeneration)* *Adamus et al.2003, in vivo (photoreceptor degeneration)* *van Adel et al., 2003 in vivo (ON transection)* *Leaver et al., 2006, in vivo (ON transection)* *Pease et al. 2009, in vivo (POAG)*	*Sieving et al. 2006, (RP)* *Birch et al. 2013, (RP)* *Zhang et al. 2011 (AMD)* *Kauper et al. 2012 (RP/AMD)*	*Phase I NCT01408472 (POAG)* *Phase II NCT02862938 (POAG)* *Phase II NCT01530659 (RP/Usher synd.)* *Phase II NCT03071965 (MacTel)* *Phase I NCT01411657(ION)*
NT-3	Polyamidoamine dendrimer-NT-3	*Shakhbazau et al. 2012, in vitro (rodent and human stem cells)*		
NT-4/5	Artificial graft transplantation-NT-4/5	*Negishi et al. 2001, in vivo (ON transection)*		

IVT, Intravitreal; PLGA, Poly-DL-lactide-co-glycolide; AD, Alzheimer's disease; AAV, Adeno-associated virus; ON, optic nerve; PD, Parkinson's disease; POAG, Primary open-angle glaucoma; Ad., adenoviral; XIAP, X-linked inhibitor of apoptosis protein; RP, Retinitis Pigmentosa; AMD, Atrophic macular degeneration; CET, cell encapsulation technology; LV, Lentivirus; MacTel, Macular Telangiectasia; ION, ischemic optic neuropathy.

4. Conclusions

Glaucoma is a primary optic neuropathy characterized by irreversible retinal ganglion cells loss. Neuroprotective treatments act directly on the pathogenetic mechanism of glaucomatous damage and have the potential to reverse the progressive degeneration of RGCs.

The deprivation of NTFs has been shown to play a crucial role in the loss of RGCs and related axonal damage in glaucoma, and numerous pre-clinical studies have demonstrated that exogenous topical or intravitreal NTFs efficiently promote RGC recovery.

Although NTFs represent an innovative therapeutical approach in clinical management of glaucoma, the medical need of a continuous delivery system to the retina and optic nerve is still open.

In recent decades, considerable progress has been made in drug and gene delivery technology for neurotrophic active substances. Delivery systems allow for steady release of factors at the required

physiological site of action, prolonged interval between treatments, fewer adverse events, and improved patient adherence.

The results emerging from clinical trials in several neurodegenerative diseases support the possible effectiveness of NTFs treatment in glaucoma. Various evidence shows that different delivery strategies for the use of NTFs in the treatment of glaucoma are proving effective for achieving long-term RGC survival and functional improvements, associated with a good safety profile. Specifically, the most promising technologies include cell-mediated gene therapy, currently being evaluated in phase II clinical trials for the treatment of glaucoma, and gene delivery approaches via viral and non-viral vectors, which showed encouraging preclinical results in glaucoma models and need prompt clinical investigation.

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