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Strategies for optic nerve rescue and regeneration in glaucoma and other optic neuropathies

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Glaucoma is the most common age-related optic nerve disease and also the most common neuropathy, affecting ~60 million people worldwide in its most common forms. This figure is expected to rise to 80 million by 2020. Glaucoma is a neurodegenerative disease in which various triggers induce cascades of secondary events, which ultimately lead to apoptotic retinal ganglion cell (RGC) death. The main risk factor for glaucomatous nerve damage is raised pressure in the eye. Understanding the cascades mediating optic nerve damage enables the development of new, neuroprotective treatment strategies that might not only target the initial insult but also prevent or delay secondary neurodegeneration. Furthermore, neuroregeneration and repopulation of the visual pathway by stem or neural precursor cells is becoming possible. Increasing understanding of the pathways involved in directed axon growth and manipulation of stem and progenitor cells towards an RGC fate have facilitated first successes in animal models of glaucoma.

Anatomy and terminology of optic nerve and visual pathway

In humans, the axons of 1.2 million retinal ganglion cells (RGCs) join to form the optic nerve. RGCs receive their input from the more than 100 million retinal photoreceptors via intermediate neurons (bipolar, horizontal and amacrine cells). After initial intraretinal processing, the optic nerve relays visual information to centres in the brain for further processing: to the lateral geniculate body (from which signals are conveyed to the visual cortex), the superior colliculus and the suprachiasmatic nucleus. These pathways are involved in visual perception, eye movements and circadian rhythms, respectively. The path from the eyeball to the lateral geniculate body is divided into three parts: the optic nerve, the chiasm (where axons from the nasal half of the retina cross to the contralateral side) and the optic tract. At the point where RGC axons leave the eyeball, they travel through a sieve of lamellar connective tissue pores lined with glial support cells, the lamina cribrosa. This region is called the optic nerve head (ONH). Myelination of the optic nerve begins after the axons have passed through the lamina cribrosa.

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Glaucoma: the most common optic neuropathy

RGC numbers decrease slightly with age, at a rate of ~0.5% per year [1]. Within the time frame of a normal life span, however, this reduction in numbers does not seem to cause symptoms of visual loss. Clinically far more relevant is a progressive neurodegenerative condition known as 'glaucoma', which is characterized by progressive loss of RGCs and characteristic excavation of the ONH.

Glaucoma currently affects 60 million people worldwide [2]. Second only to cataract, it is one of the leading causes of blindness [3]. Whereas cataract can be cured by an intraocular lens implant, optic nerve damage is at present irreversible, making glaucoma the leading cause of irreversible blindness worldwide [2]. In the USA, the prevalence of the most common type of glaucoma, primary open-angle glaucoma (POAG), in people over the age of 40 years is 1.86%; in people of African heritage, the prevalence is almost three times higher [4].

Age is a important risk factor for the development of the two most common types of glaucoma, POAG and angle-closure glaucoma. For POAG, the odds ratio per decade increase in age is 2.05 in white populations and 1.60 in black and Asian populations [5]. Prevalence in people over the age of 70 years has been estimated to

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be 6% in white populations, 16% in black populations and 3% in Asian populations [5].

Pathogenesis of glaucoma: mechanical theory and vascular hypothesis

The main risk factor for glaucomatous optic nerve damage is raised pressure inside the eye (intraocular pressure, or IOP), although in some individuals this damage occurs despite pressure within the normal range (sometimes termed 'normal tension glaucoma', or NTG). Vice versa, there are individuals with 'raised' IOP who do not develop optic neuropathy. This has led to two approaches to explaining the pathogenesis of this condition: the mechanical and the vascular theory of glaucoma.

The mechanical theory

RGCs can be damaged by lesions at any point of their trajectory: within the eye, at the lamina cribrosa or ONH, in the orbit, in the bony optic canal, at the base of the brain, at the chiasm or at the level of the optic tract. Although experimental models of optic nerve damage cause injury at any of these sites, injury in human glaucoma occurs in the eye and at the lamina cribrosa. Mechanical stress exerted by raised IOP is greatest at the lamina cribrosa or ONH and damage from hypoperfusion and subsequent reperfusion also occurs at this place.

The mechanical theory states that raised pressure causes direct and indirect damage to RGCs. Obstruction of axoplasmic flow prevents retrograde transport of neurotrophic factors to the RGC soma and accumulation of these factors at the lamina cribrosa [6–10]. In experimental models, raised pressure also directly damages RGC mitochondria [11]. Growth factor starvation and mitochondrial damage might shift the balance of RGC gene expression from cell maintenance to pro-apoptotic pathways. The deleterious effect of raised IOP on RGC numbers and electrophysiological function has been well documented in a rodent model [10,12].

Another important effect of raised pressure is the activation of glial cells in retina (Müller cells, astrocytes and microglia) and lamina cribrosa (astrocytes and microglia). *In vitro* raised pressure induces these cells to release neurotoxic substances such as nitric oxide (NO) and tumour necrosis factor α (TNF-α). Oxidative stress and free radicals as secondary insults can damage RGCs further [13,14]. In addition, ONH astrocytes *in vitro* secrete matrix metalloproteinases (MMP) and extracellular matrix molecules. *In vivo*, these processes might contribute to the characteristic remodelling of the lamina cribrosa in glaucoma and enhance structural and biochemical changes of the axon environment.

The vascular hypothesis

There is evidence of vascular dysregulation in individuals with some forms of glaucoma, which can cause chronic impairment of ONH blood flow [15]. Fluctuations in ONH perfusion caused by primary or secondary vascular dysregulation might induce ischaemia–reperfusion nerve injury. Primary vascular dysregulation was previously known as 'vasospastic syndrome' and includes Raynaud phenomenon and migraine. Secondary vascular dysregulation is seen in conditions with raised systemic levels of endothelin-1 (ET-1), such as rheumatoid arthritis and systemic lupus erythematosus [16]. Primary vascular dysregulation is associated with disturbances in autoregulation of ocular blood flow, leading to oxidative stress to RGCs, and is a risk factor for some forms of glaucoma. Secondary vascular dysregulation, on the other hand, does not seem to be a risk factor for glaucoma. Possible explanations include the fact that in these conditions blood flow is reduced globally or that vasoconstrictive peptides such as ET-1 are paracrine factors, with raised systemic levels not necessarily being pathogenic to the eye.

Although many studies have reported reduced ONH blood flow in glaucoma patients, the causal relationship with RGC damage has not yet been established, although evidence from animal experiments might support this hypothesis [17,18].

NO is a potent vasodilator synthesized by three isoforms of the enzyme nitric oxide synthase (NOS), neuronal, endothelial and inducible NOS (nNOS, eNOS and iNOS) [19]. Whereas NO formed by nNOS and eNOS is involved in multiple cardiovascular and nervous regulatory mechanisms, activation of iNOS by immunological and inflammatory stimuli results in the production of excessive amounts of NO, which can cause cytotoxicity, neurodegeneration, apoptotic cell death and circulatory failure [20–23]. NO mediates glutamate excitotoxicity [24]. Conversely, *N*-methyl-D-aspartic acid (NMDA) glutamate receptor activation can trigger NO production by nNOS, and NO can stimulate further release of glutamate [25,26]. Confusingly, NO also has antioxidative and neuroprotective effects, owing to its ability to interact with reactive free radicals [27].

Continuous production of NO by nNOS and eNOS might maintain the basal tone of the posterior ocular vessels [28,29]. Reduction of nNOS or eNOS activity can result in vasoconstriction, but impairment of NO production by endothelial cells in glaucomatous individuals has not been proven so far [23]. Expression of all three isoforms of NOS in the prelaminar ONH region might be increased in glaucomatous eyes, indicating either neurotoxic levels of NO or neuroprotective vasodilation and increased blood flow or both [30–32]. However, not all studies found a difference in iNOS expression in glaucomatous and non-glaucomatous ONH in humans or in experimental models [33].

Endogenous vasoconstrictors might cause ischaemic insult to RGCs at the ONH [34,35]. Endothelin, a potent vasoactive peptide involved in blood flow control, is such a vasoconstrictor. In animal models, intravitreal injection of ET-1 reduces ONH blood flow [36], causes disruption of axoplasmic transport at the ONH [37,38] and induces RGC loss [39–41]. ET-1 levels in the aqueous are raised in human glaucomatous eyes [42,43], and glaucoma patients react to cold-induced stress with an increase in plasma ET-1 levels not observed in non-glaucomatous individuals [44]. In addition to its vasoconstrictive effect, ET also has a potent effect on glial cells and might induce ONH astrocyte activation [45,46] with subsequent neurotoxic changes in the ONH microenvironment and ONH matrix remodelling.

Regardless of the type of initial insult, axonal damage triggers death of RGCs by apoptosis [47,48]. Initial signs of RGC damage are a shrinking of the dendritic tree, followed by a reduction in size of cell body and axons [49].

The events leading to RGC death can be divided broadly into damage to RGC-survival pathways and triggers of RGC apoptosis pathways.

RGC maintenance and survival pathways

RGC damage occurs in two steps: initial insult and subsequent degeneration through apoptosis. Current treatments target the first step; however, the insight that continuous degeneration occurs even when the initial insult is controlled will have great significance for the future management of glaucoma and ischaemic optic neuropathies.

In the healthy eye, RGCs receive continuous supplies of neurotrophic factors from the brain via retrograde axonal transport and from the retinal Müller glia. These factors include the neurotrophin (NT) family of growth factors – brain-derived growth factor (BDNF), nerve growth factor (NGF) and NT-3 and NT-4/5 – and receptors they bind to, the tropomyosin-receptor kinases (Trk) and the p75-neurotrophin receptor (p75NTR) [50,51]. Other growth factors relevant for RGC maintenance are ciliary- and glial-derived neurotrophic factors (CNTF and GDNF) and basic fibroblast growth factor (bFGF or FGF-2) [52]. BDNF, CNTF, NT-4 and GDNF, but not NT-3, reduce RGCs after axotomy and optic nerve crush in rodents [53–55].

Amongst these, BDNF and its receptor TrkB in particular have a prominent role in RGC development and survival. Downstream of the TrkB receptor, AKT and mitogen-activated protein kinase (MAPK, also known as 'extracellular signal-regulated kinase', or ERK1/2) pathways induce the phosphorylation of cyclic AMP response element binding protein (p-CREB) and transcription of survival-promoting genes, such as *bcl-2* (originally described in B-cell lymphomas) and, in the retina, *bcl-x*.

In glaucoma, disruption of axoplasmic transport might prevent neurotrophic factors and receptors crucial for the survival of RGCs from reaching the cells [7,8]. The point of disruption seems to be the ONH, the area mechanically most susceptible to raised IOP. In addition, recent work indicates that not only absolute IOP but also the pressure gradient across the lamina cribrosa is relevant to axonal damage. The retrobulbar optic nerve is surrounded by a cuff of cerebrospinal fluid. In several individuals with glaucomatous visual field deficits, abnormally low CSF pressure has been described, resulting in a high translaminar pressure gradient [56].

According to the 'neurotrophin hypothesis', NT starvation as a result of impairment of axoplasmic transport at the lamina cribrosa might impair RGC survival and promote RGC apoptosis. Evidence from experimental glaucoma supports this hypothesis, showing a decrease in BDNF and NT-4/5 in the inner retina [57]. However, contradictory findings demonstrating a lack of change or even upregulation of BDNF, NT-4/5 and TrkB and C expression might indicate a compensatory retinal response to injury or a more complex interplay of these and other growth factors [51].

Signalling pathways downstream of the p75 NTR differ from those downstream of NT receptors and modulate growth factor signalling, for example by modulating TrkB activity. Pathways downstream of the p75 NTR are involved in both apoptosis and cell survival [58,59].

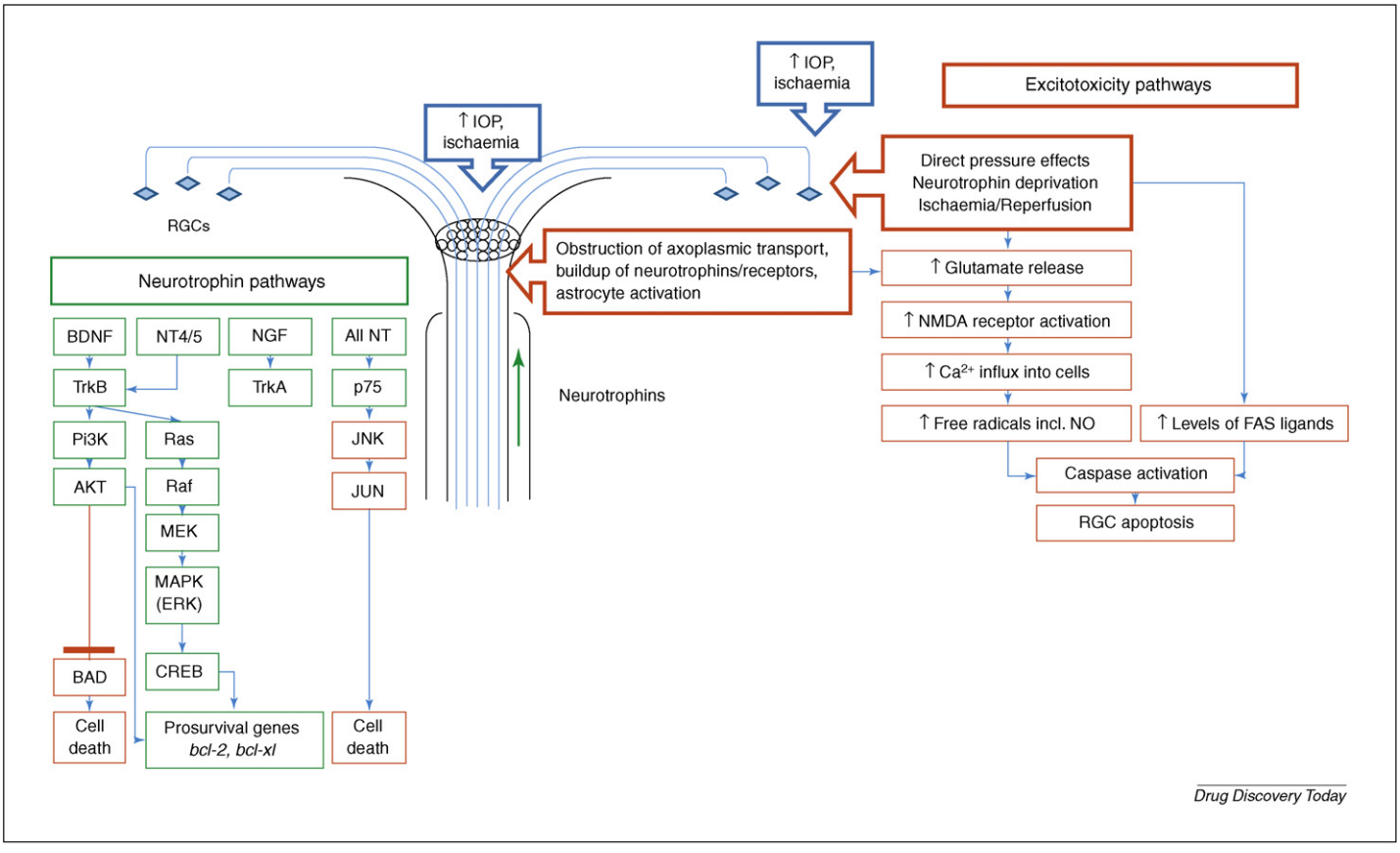

FIGURE 1

RGC maintenance in the healthy eye and pathways involved in RGC survival and apoptosis after injury. Green: RGC maintenance in the healthy eye. Blue: primary insults – raised IOP and ischaemia. Red: injury events and pathways. Abbreviation: CREB, cyclic AMP response element binding protein.

RGC apoptosis pathways

The major excitatory neurotransmitter in the retina, mediating the transmission of light signals from photoreceptor to bipolar cells and on to RGCs, is glutamate. Excessive glutamate levels, however, are toxic to cells and trigger a cascade resulting in apoptosis. Glutamate excitotoxicity plays a part in several neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, Huntingdon's disease, amyotrophic lateral sclerosis and cell death after stroke [60].

In neuronal tissues in CNS and retina, increased levels of glutamate induce excessive NMDA receptor activation and subsequent excessive calcium ion influx into cells. This results in an increase in free radicals, including NO, and activation of caspases involved in apoptosis [61] (Fig. 1). Other activators of caspases downstream of raised IOP are FAS ligands, such as Fas-Associated protein with Death Domain [62]. After experimental optic nerve transection or optic nerve crush, cell-death-related genes are upregulated in a sustained manner, particularly those encoding proteins involved in apoptosis, such as caspase-3, TNF receptors and c-fos [63]. The expression of the corresponding proteins is also increased [64].

TNF-α signalling pathways activate caspases and lead to the generation of reactive oxygen species. *In vitro*, microglial cells exposed to raised pressure release TNF-α and NO, which induce RGC apoptosis. TNF-α is also a key component of local immune responses, and disturbances in the immunoregulatory function of ONH and retinal glia might contribute to secondary neuronal damage in glaucoma [14,65]. Indications that immune dysregulation might contribute to glaucomatous damage include the presence of antibodies against ONH glycosaminoglycans, heat-shock proteins (HSPs), gamma-enolase and glutathione-*S*-transferase in the serum of glaucoma patients. In addition, antibodies against HSPs (HSP 70 and αB-crystallin) and vimentin have been be found in aqueous humour from patients with NTG [65]. *In vitro,* these antibodies induce apoptosis of retinal neurons.

The causal link between glutamate levels and human glaucomatous optic nerve damage is not established, however, and other studies present arguments against this mechanism. *In vitro* retinal preparations of glaucomatous rat eyes indicate the presence of a retinal glutamate clearance mechanism that might reduce toxicity *in situ* [66]. A non-human primate model of glaucoma did not find a difference in vitreous levels of glutamate between glaucomatous as compared with fellow eyes or a correlation between glutamate levels and RGC loss [67].

One study reported characteristic ONH damage and visual field deficits in a non-human primate model of glaucoma but no difference in vitreal glutamate levels between glaucomatous and control eyes [68]. A study on human glaucomatous eyes reported similar levels of glutamate in glaucomatous and healthy eyes [69].

Inhibition of TNF-α reduces RGC death [70,71]. TNF-α might be elevated not only in experimental models of raised IOP but also in retina and ONH of glaucoma patients [72]; however, TNF-α receptor binding can also activate cell survival signals and anti-apoptotic pathways that involve the transcription factor NF-κB. The balance between several TNF-α activation pathways determines the eventual RGC fate [65].

Disruption of mitochondrial function, production of caspases and degradation of DNA eventually result in apoptotic cell death via activation of the p53 pathway, resulting in activation of pro-apoptotic bcl2-associated X protein (*bax*) genes and inhibition of expression of anti-apoptotic *bcl-2* and *bcl-x* genes.

Treatment strategies for glaucomatous optic neuropathy – current and future

Current treatments mainly target raised IOP, the predominant trigger of glaucomatous optic neuropathy (Fig. 2). Some treatments aim to improve vascular perfusion of the ONH, although the evidence for efficacy via this mechanism is poor.

Pharmacological reduction of IOP is achieved by topical β blockers, prostaglandin analogues, α_2-adrenergic receptor agonists, parasympathomimetics and topical or systemic carbonic anhydrase inhibitors (Table 1). All these either reduce production of aqueous fluid by the ciliary body or increase its outflow from the

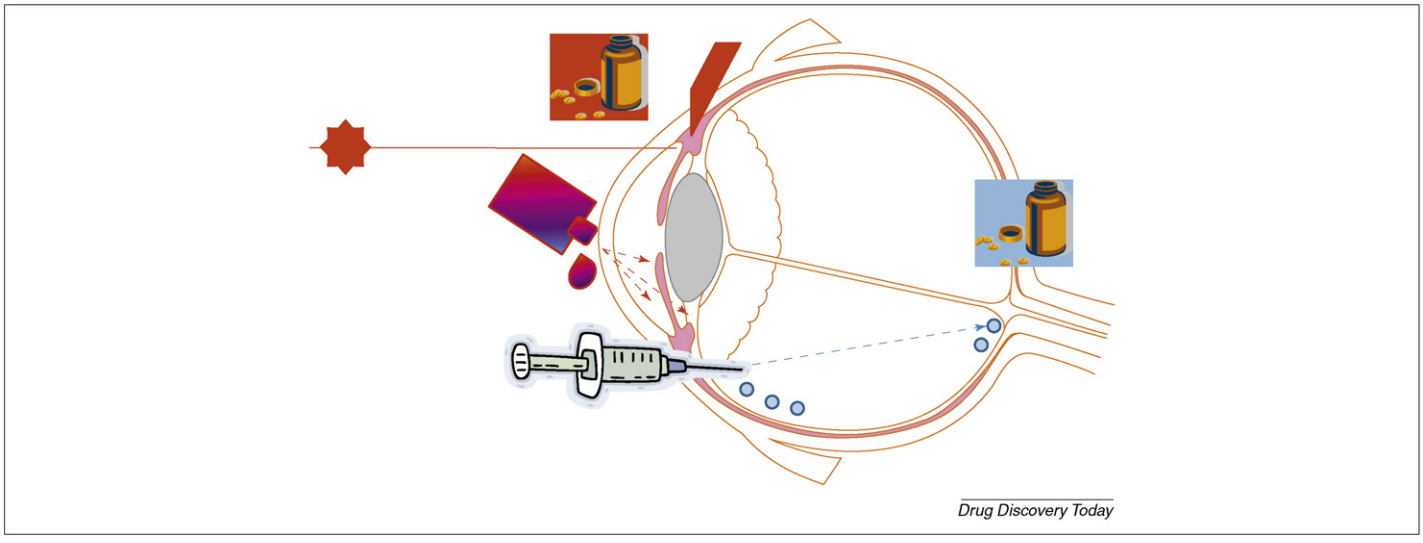

FIGURE 2

Current (red) and potential future (blue) management strategies in glaucoma. Red: surgical or laser treatment of iridocorneal drainage angle, topical or systemic pharmacological enhancement of aqueous production and drainage and/or optic nerve head blood flow. Blue: injection or implantation of neuroprotective and/or neuroregenerative agents into the vitreous cavity and/or in front of the lamina cribrosa.

TABLE 1

Current treatment strategies in the management of glaucoma

Pharmacological treatment	Examples	Mechanism
Topical beta-blocker	Timolol, carteolol, betaxolol, nipradilol	Reduction of aqueous fluid production
Topical carbonic anhydrase inhibitors	Dorzolamide, brinzolamide	Reduction of aqueous fluid production in ciliary body
Topical prostaglandin analogue	Latanoprost, bimatoprost, travoprost	Increase in uveoscleral outflow of aqueous
Topical alpha$_2$ receptor agonist	Brimonidine, apraclonidine	Reduction of aqueous fluid production in ciliary body
Systemic carbonic anhydrase inhibitor	Acetazolamide	Reduction of aqueous fluid production in ciliary body
Parasympathetic muscarinic receptor agonist	Pilocarpine	Miotic, opens trabecular meshwork and increases aqueous outflow
Surgical interventions	Trabeculectomy	Increase in aqueous outflow by bypassing trabecular meshwork
±Augmentation by anti-fibrosing agents	Mitomycin C, 5-fluorouracil, beta-irradiation	Prevention of scarring at drainage site
Laser interventions	Trabeculoplasty Iridotomy Ciliary body ablation	Increase in aqueous outflow Increase of aqueous passage into anterior chamber in some types of angle-closure glaucoma Reduction of aqueous fluid production in ciliary body

eye via the trabecular meshwork in the iridocorneal angle or via uveoscleral tissues.

Carbonic anhydrase inhibitors might also improve ocular blood flow at the ONH, thereby potentially reducing damage from reperfusion injury [73]. In addition to their IOP-lowering effect, brimonidine, betaxolol and latanoprost are claimed to offer neuroprotection. Betaxolol might act as a calcium-channel blocker, and the neuroprotective mechanism of brimonidine might be mediated by upregulation of endogenous BDNF production [74]. In rodent models of transient retinal ischaemia, a single systemic or topical application of brimonidine reduces RGC loss for several months [75–77].

Latanoprost reduces the expression of apoptosis marker caspase-3 and glutamate-induced calcium influx in RGC *in vitro* [78]; however, evidence for clinical neuroprotection is lacking. NO donors such as nitroglycerine, isosorbide mononitrate or isosorbide dinitrate have potent vasodilator effects. They increase posterior ocular blood flow, although the individual response is variable [79,80]. In case of progression of glaucomatous optic nerve damage despite a combination of non-invasive pharmacological treatments, laser and conventional surgical treatment enhance aqueous circulation and drainage or reduce its production. In some individuals, visual loss progresses despite successful IOP reduction. Neural degeneration and the apoptotic cascade can proceed after the initial insult has been removed. This indicates the need for additional treatment modalities targeting injured neurons and promoting their survival. However, neuroprotective strategies alone are unlikely to succeed if the initial insult persists. In the future, combination treatments targeting both IOP and neuronal injury will offer the best potential for halting the progression of RGC loss.

Present research in optic neuropathy management focuses on the exploration of three approaches: the development of new strategies or agents to lower IOP or improve ONH perfusion, the administration of agents that can rescue damaged RGCs (neuroprotection) and the ambitious long-time goal of regenerating optic nerve depleted of RGCs (optic nerve regeneration).

New agents to lower IOP or improve ONH perfusion

Two new classes of agents lower IOP in experimental glaucoma: inhibitors of Rho-associated kinase (ROCK) and synthetic agonists of the cannabinoid receptors CB1 and CB2. In addition, one new calcium-channel blocker (flunarizine) also has a pressure-lowering effect, and two others (nimodipine and lomerizine) improve ocular blood flow.

ROCK inhibitors HA-1077 and Y-27632, applied topically or via injection into the anterior chamber or the vitreous cavity of rabbits, reduce IOP and increase aqueous outflow [81–83]. The synthetic CB1/CB2 agonist WIN-55-212-2 lowers IOP after topical application in a rat glaucoma model [84]. Although this effect has been known for some time, topical administration has been difficult because of ocular irritation. With Tocrisolve as carrier, prolonged application does not induce local side effects [85].

A human trial using cannabis extracts delta-9-THC and cannabidiol demonstrated temporary IOP reduction with good tolerability [86].

Systemic calcium antagonists are often prescribed to reduce blood pressure; these agents work by relaxing the vascular smooth muscle cells, reducing blood vessel tone. In the eye, they increase ocular blood flow. In animal studies, calcium antagonists increase ONH blood flow, and some increase RGC survival in rodent models of acute retinal ischaemia [87–90]. In humans, they might improve posterior ocular blood flow and visual fields without lowering and compromising systemic blood pressure, although reported results are variable [88,91–97]. There is some evidence that centrally acting calcium-channel blockers such as nimodipine and lomerizine increase ocular blood flow, whereas peripherally acting agents such as nifedipine do not have this effect [98].

In addition to its neuroprotective role by inhibiting glutamate or NMDA toxicity, flunarizine also has an IOP-lowering and aqueous-outflow-enhancing effect in a primate model of glaucoma [99].

Rescuing damaged RGCs – neuroprotection

Potential neuroprotective agents aim to target known players of RCG maintenance and apoptosis pathways.

Strategies promoting RGC survival

Strategies promoting RGC survival can be divided into approaches that supply RGCs with exogenous neurotrophic factors, induce increased endogenous expression of neurotrophic factors, upregulate Trk or inactivate p75 receptors, increase *bcl-2*/*blc-x* expression or use pharmacological agents whose neuroprotective mechanisms of action are not clear. In addition to efforts to protect RGC cell bodies, the protection of axons might be another distinct approach [11,100,101].

Exogenous neurotrophic factors

Application of exogenous neurotrophic factors prolongs RGC-survival *in vitro*, and their direct injection into the vitreous or optic nerve lesion sites prolongs RGC-survival *in vivo*, as demonstrated for BDNF, CNTF, NT-4, FGF-2 and neurturin [55]. CNTF mediates its neurotrophic effect via the Janus kinase/signal transducer and activator of transcription 3 and phosphoinositide-3 kinase (PI3K)/AKT pathways [102,103], whereas neurturin enhances glutamate uptake by upregulating glutamate transporter GLAST-1, involving PI3K and the Sarcoma family of tyrosine kinases [104]. The effect of FGF-2 is signalled through FGF receptor 1, upregulating expression of endogenous BDNF via the ERK pathway and expression of TrkB via the protein kinase A (PKA) pathway. Both pathways end with CREBs, increasing the expression of survival-promoting *bcl-2* and *bcl-x* genes whilst reducing the expression of the pro-apoptotic *bax* gene [105–107]. A novel approach tested *in vitro* is the use of short peptides derived from activity-dependent neurotrophic factor and activity-dependent neurotrophic protein. Both promote RGC survival and axon outgrowth [108]. Successful strategies may require a combination of neurotrophic factors such as bFGF, NT-3 and BDNF, mediating dysinhibition of axon regeneration, as well as cell survival [109].

Sustained increased endogenous expression of neurotrophic factors

Any neuroprotective effect after direct injection of growth factors into the eye is transient. Strategies used to achieve sustained increased endogenous expression of neurotrophic factors include transfection of growth factor genes into RGCs using different viral vectors, cell-based delivery approaches and physical methods. Intravitreal injection of adeno-associated virus (AAV) or lentiviral vectors encoding BDNF, CNTF or GDNF prolongs the growth factor effect RGC survival in glaucoma and axotomy models [110–116]. Physical methods, such as electroporation after intravitreal injection of BDNF or GDNF, also enhance the RGC-protective effect [117,118]. Increased expression of endogenous CNTF by astrocytes and Müller cells is observed after retinal injury, after inflammatory stimuli and even after intravitreal injection of exogenous CNTF or viral transfection [103,119–122].

Other methods that successfully prolong RGC survival include combined intravitreal application of Ad-GDNF and application of the caspase inhibitor X-chromosome-linked inhibitor of apoptosis, Ad.XIAP, to the optic nerve stump in a transection model [123] and the intravitreal injection of slow-release poly(DL-lactide-co-glycolide) microspheres in a glaucoma model [124,125].

Finally, transcorneal electrical stimulation enhances RGC survival by increasing calcium influx into retinal Müller cells, which, in turn, increases production of insulin-like growth factor IGF-1 by these cells [126,127].

Another way of providing sustained secretion of neurotrophic growth factors in the retina is the transplantation of stem or progenitor cells. Cells transplanted into and onto the retina in experimental glaucoma models include mesenchymal stem cells (MSCs), oligodendrocyte precursor cells (OPCs), retinal progenitor cells (such as Müller cells), neural stem and precursor cells and olfactory ensheathing cells (OECs). Of these, MSCs, OPCs and neural stem and precursor cells promote RGC survival in experimental glaucoma [128–130]. OPCs require 'activation' by interaction with inflammatory cells [129].

In their native environment in the nasal mucosa, OECs organize the continuously regenerating axons of the olfactory nerve into fascicles and guide them from the nose to their target, the olfactory bulb at the base of the brain. In transplantation studies, these cells support regenerating axons, offering hope in the treatment of spinal cord injury [131–134]. The axon-ensheathing and neuroprotective effects of OECs involve several mechanisms, including secretion of neurotrophic and membrane adhesion factors [135–137]. After transretinal injection into healthy rat eyes, OECs migrate within the retina and along the RGC axon layer into the ONH, ensheathing RGCs with their cytoplasm [138].

Upregulating Trk or inactivating p75 receptors

Different strategies have been used in experimental models to upregulate Trk or inactivate p75 receptors in an attempt to enhance the effect of exogenous growth factor application and to promote RGC survival. Upregulation of the receptor TrkB via gene transfer enhances BDNF-induced RGC survival after axotomy [139]. TrkA activation by a selective partial agonist, the peptidomimetic D3, combined with administration of the IOP-lowering β blocker betaxolol is highly effective in reducing RGC death [140]. Combination of selective TrkA agonists with p75 NTR antagonists enhances RGC survival in a rodent axotomy model [141]. In this model, retinal expression of the proform of NGF (proNGF) was found to be upregulated after axotomy. It has since been demonstrated that proNGF induces RGC death via activation of the p75 NTR and subsequent TNF-α expression by Müller cells [142]. Blocking the function of a p75NTR signalling partner, LINGO-1, by intravitreal injection of soluble LINGO-1 protein or an anti-LINGO-antibody reduces RGC death in axotomy and glaucoma models. LINGO-1 protein blocks the RhoA–JNK pathway and promotes Akt activation [143].

Overexpression of the retinal anti-apoptotic protein bcl-x

RGC death after axotomy is reduced in transgenic mice that overexpress *bcl-2* [144,145]. The effect of *bcl-2* is mediated through an increase in intracellular calcium signalling and activation of CREB and ERK [146]. In these animals, additional AAV-mediated expression of CNTF increases RGC survival further [115]. Overexpression of the retinal anti-apoptotic protein bcl-x can be induced by TAT-mediated protein transduction. In this approach, the HIV–TAT protein carries proteins across cell membranes. After intraocular injection, this construct reduces RGC death in a mouse optic nerve lesion model [147].

Various other agents that activate the *bcl-2* pathway promote RGC survival, such as *N*-β-alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine (5-*S*-GAD, a substance isolated from flesh fly), cilostazol, citicoline, lithium and ROCK inhibitors. Low doses of 5-*S*-GAD promote RGC survival after application of NMDA and optic nerve crush [148]. Cilostazol is a selective inhibitor of cyclic nucleotide phosphodiesterase 3 used for the treatment of intermittent claudication and is known to reduce neuron death after cerebral infarct. It upregulates p-CREB and *bcl* gene expression through the PKA pathway [149]. The choline precursor citicoline, freely available for cognitive dysfunction and transient cerebral ischaemia, enhances RGC survival after optic nerve crush [150]. Citicoline serves as choline donor in the biosynthesis of neuronal phospholipids. In humans, both oral and intramuscular administration of citicoline lead to long-term stabilization or even improvement of retinal and visual pathway function, as assessed by electroretinogram and visually evoked potentials as objective outcome parameters [151].

In vitro, lithium promotes RGC survival and axon sprouting via a *bcl-2*-dependent mechanism [152]. *In vivo*, it increases RGC survival after optic nerve crush [150]. ROCK plays an essential part in myelin-derived inhibition of axon regrowth. *In vitro* and *in vivo* ROCK inhibitors such as Y-27632 and C3 transferase (C3–11) promote RGC survival via MAPK signalling [153,154].

Other pharmacological agents

Other pharmacological agents that enhance RGC survival include erythropoietin (EPO) and nipradilol. In a rat model of raised IOP, retinal EPO and its receptor proteins are upregulated in RGCs and intermediate neurons. EPO enhances RGC axon sprouting *in vitro* [155] and intravitreal injection of EPO promotes RGC survival [156,157]. Nipradilol, a nonselective β-adrenergic receptor and selective α_1-adrenergic receptor blocking agent with NO-donor action that *in vivo* lowers IOP and increases OHN perfusion [158] also promotes RGC survival and regeneration in an axotomy model [159].

Several pharmacological agents reduce RGC apoptosis by interfering with the glutamate excitotoxic cascade: glutamate release inhibitors, antagonists of the NMDA receptor or its polyamine site, calcium-channel blockers, NO donors and free radical scavengers and antioxidants [60]. Recent advances have been made in the understanding of the mechanism of action of all these compounds and their potential use in the treatment of glaucoma. In this line of research, the glutamate release inhibitor riluzole has been shown to be neuroprotective in a rat retinal ischaemia model [160], although inhibition of iNOS by aminoguanidine has delivered variable results [21,33].

NMDA receptor antagonists and polyamine site blockers

NMDA receptor antagonists and polyamine site blockers are neuroprotective in experimental glaucoma models. However, complete or prolonged blocking of NMDA receptors by competitive antagonists can cause serious adverse events such as seizures, psychosis, coma and death [13]. Memantine, a low-affinity, noncompetitive NMDA antagonist, is safe and already in use in the treatment of Parkinson's disease [161]. After showing great promise in experimental glaucoma, including in primate models [162,163], memantine reached the stage of a phase III clinical trial. The trial failed to demonstrate a beneficial effect over placebo, although disease progression was lower in patients receiving a higher dose of memantine than in those on a lower dose [164]. This result might be due to memantine acting only on a small number of RGCs with a particular density of NMDA receptors, whilst apoptosis might be triggered by different mechanisms in other RGCs [164]. A similar discrepancy between very positive effects in experimental models and negative clinical effects has been observed in several clinical trials of neuroprotection after stroke. Taken together, the negative results in glaucoma and stroke trials (despite very good results in experimental models) have had a large negative impact on the prospects for clinical neuroprotective drugs in glaucoma. Positive results, at least for neuroprotection, in animal models have to be approached with caution when making decisions to embark on large clinical trials.

Other noncompetitive NMDA receptor antagonists, such as amantadine, or substances blocking the polyamine site of the NMDA receptor, such as eliprodil and ifenfrodil, also reduce RGC apoptosis in culture and in experimental retinal ischaemia [165–167] but have not been used in clinical trials. L-Kynurenine, a precursor to the endogenous NMDA receptor antagonist kynurenic acid, is another agent that protects against NMDA-mediated toxicity in animal models [168]. A metabotropic glutamate receptor inhibitor, LY354740, promotes RCG survival in a rat model of ocular hypertension [167].

Several calcium-channel blockers show neuroprotective effects by inhibiting NMDA toxicity *in vivo* – for example, flunarizine, nivaldipine, lomerizine and cilnidipine [90,169–174]. Neuroprotective effects in humans have not yet been demonstrated.

NO donor NOC-18 has an RGC-survival-promoting effect in an *in vivo* model using NMDA injection [175]. A different agent, LA-419, is currently in a phase II clinical trial for cardiovascular disease and might have a beneficial effect on glaucoma [176].

An antioxidant enzyme, peroxiredoxin 6, reduces TNF-α- and glutamate-induced RGC death in a rat model by reducing levels of reactive oxygen species, NF-κB activation and intracellular calcium influx [177].

NOS inhibitors such as the NOS-2 inhibitor animoguanidine or the nonspecific NOS inhibitor *N*(G)-nitro-L-arginine-methyl-ester reduce RGC loss in models of ocular hypertension [21,178,179].

Flavonoids are naturally occurring substances that act as free radical scavengers and have antioxidant properties. Black and green tea, coffee, dark chocolate and red wine all contain flavonoids. One flavonoid richly present in green tea, epigallocatechin gallate, reduces retinal degeneration when injected into the vitreous in a model of oxidative stress [180]. A commercial oral preparation is available under the name Epinerve [181]. Ginkgo biloba extract also contains polyphenolic flavonoids that protect mitochondria from oxidative stress. Ginkgo extract improves visual fields in some patients with NTG, as shown in a clinical trial [182].

Resvetrol, another polyphenol found in grapes and wine, reduces expression of reactive oxygen species and markers of cellular ageing and has an anti-apoptotic effect when applied to trabecular meshwork cells *in vitro* [183]. Another natural antioxidant, ubiquinone (coenzyme Q10), protects mitochondria and

has a neuroprotective effect in an animal model of retinal ischaemia/reperfusion [184]. Whereas most antioxidants have only been tested *in vitro* and in animal models, one clinical trial in glaucoma patients demonstrated a beneficial effect of α tocopherol (vitamin E) on visual field loss progression [185].

Optic nerve regeneration

At present, loss of RGCs is irreversible, and uncontrolled glaucoma can lead to blindness. For many individuals in whom current treatment is insufficient, regeneration of the optic nerve offers the only hope of restoring vision; however, optic nerve regeneration is fraught with difficulties. To replace RGCs, a source of stem cells capable of differentiating into RGCs needs to be identified and manipulated towards an RGC fate. These cells need to be transplanted into the eye and integrate into the retina without being rejected. The environment needs to be made permissive to axon regrowth. In the CNS, glial scars play a major part in preventing neural repair. In glaucoma, activated astrocytes at the lamina cribrosa and Müller cells in the retina alter the biochemical microenvironment [186]. Although different from the glial scar created by optic nerve transection or crush, these changes still need to be addressed to promote axon regrowth through the ONH. Finally, transplanted and successfully integrated cells need to form functional synapses with retinal interneurons and their target neurons in the brain, preserving the retinotopic organization of the visual pathway.

Peripheral nerve grafts

In rodent optic nerve transection models, RGC axons grow through a peripheral nerve graft bridging the lesion and form synapses at their targets in the superior colliculus and re-establish the pupillary light reflex [187–190]. Peripheral nerve grafts inserted into the vitreous also promote RGC regeneration beyond the lesion, via modulation of the NgR/p75NTR/EGFR axis by Schwann-cell-derived factors, disinhibiting axon growth through the otherwise inhibitory milieu of CNS myelin [191].

Differentiation of stem or precursor cells into RGC-like cells and integration of transplanted cells into the retina

From a clinical point of view, optic nerve regeneration seems a distant target, but initial steps have been taken successfully in experimental models. Both embryonic and adult-derived stem and precursor cells have been applied to experimental glaucoma models. Ethical concerns surround the use of embryonic stem cells, and there is fear that after transplantation, embryonic stem-cell-derived neural precursors can form tumours in the eye [192].

As discussed above, most studies to date conclude that stem-cell treatment prolongs RGC survival by providing neurotrophic factors. In the following, we summarize stem and precursor cell studies that investigate whether transplanted cells differentiate into RGC-like phenotypes. This type of study requires specific histological and biochemical RGC markers and ideally would also investigate improvement of vision as functional outcome. Several proteins serve as RGC differentiation markers, such as β-3 tubulin, gamma-synuclein and brain-specific homeobox/POU domain protein 3 (Brn3) [193–196]. The latter plays a key part in the differentiation of RGCs. In particular, Brn3b expression is considered as a marker of the initial differentiation of progenitor cells transplanted into retina [193,196]. Functional outcome measures to demonstrate RGC differentiation include standard and multifocal electroretinograms and visually evoked potentials. Behavioural visual tests in laboratory animals can assess the effect of transplanted cells or drugs on visual function. The tests include detection of orientation, startle and tracking of moving objects. Frequently used in rodents, the head-tracking test is based on the principle that animals with good vision will use head movements to track a moving object [197].

Few stem and precursor cell types injected into the vitreous of eyes with experimental glaucoma or depleted of RGCs demonstrate signs of neural or RGC differentiation: cells from an embryonic stem-cell-derived eye-like structure, hippocampus-derived neural stem cells, OPCs, a cell line of spontaneously immortalized Müller glial cells and neural progenitor cells.

Cells from an embryonic stem-cell-derived eye-like structure, injected into the vitreous of mouse eyes depleted of RGCs by NMDA injection, spread on the inner retina and frequently differentiate into cells expressing RGC markers but fail to form functional connections, as indicated by negative visually evoked potentials [198].

Hippocampus-derived neural stem cells integrate into retina that has undergone mechanical or ischaemic injury, spontaneous dystrophy or selective RGC depletion and undergo partial differentiation to retinal neuronal lineage [199–202].

Transplanted into eyes with acute retinal ischaemia induced by raised IOP, neural progenitor cells demonstrate limited differentiation into neuronal cell types but no functional improvement [203,204].

The eye itself contains progenitor cells, which can be manipulated towards an RGC fate. These cells reside in the ciliary body and within the retina, the latter being a subpopulation of Müller cells. In lower vertebrates, optic nerve regeneration occurs even in adult animals, and Müller cells provide the newly formed RGCs. In adult mice, optic nerve injury by transection or crush upregulates cell proliferation and nestine expression in ciliary body and retina; again, it is Müller cells and reactive astrocytes that proliferate, indicating that these cells might hold a regenerative potential for optic nerve lesions even in mammals [205]. Indeed, a spontaneously immortalized Müller cell line shows stem-cell characteristics and, under appropriate conditions, can migrate and differentiate into various retinal cell types [206,207]. When transplanted into eyes with experimental glaucoma, the migration of these cells into the RGC layer can be promoted by modulating extracellular matrix with chondroitinase ABC and by controlling microglial reactivity with anti-inflammatory therapy [208,209].

Other cell types show integration into the retina but do not spontaneously differentiate into an RGC-like phenotype. These include neural precursor-like cells transplanted into eyes selectively depleted of RGCs by axotomy or superior colliculus ablation and human cortical neural progenitor cells [210,211]. Human cortical neural progenitor cells transplanted into the subretinal space of healthy monkeys and rats form a single cell layer in the nuclear layer of the retina and survive for up to five weeks without disturbing retinal function but have not yet been investigated for neuronal or RGC differentiation [211].

Of interest for their availability, human umbilical cord blood mesenchymal stromal cells rescue axotomized RGCs when injected into a lesion site in the optic tract but have not been evaluated in a glaucoma model [212].

Creation of an environment permissive to axon regrowth – the role of glia and extracellular matrix

Raised pressure activates glial cells in the retina and the lamina cribrosa, including Müller cells and astrocytes. After a rise in IOP, astrocyte morphology changes and, at the same time, changes occur in RGC axons [213,214]. Lamina cribrosa astrocytes initiate remodelling of the extracellular matrix via secretion of MMP and matrix proteins. *In vivo*, these effects can be observed by raising IOP; *in vitro*, raising hydrostatic pressure on cell cultures is used as a model [186,215,216].

In experimental models of optic nerve regeneration, application of substances toxic to astrocytes enhances axon regrowth. The capacity of RGC axons to sprout from the optic nerve stump into an experimentally attached peripheral nerve graft is increased when a microglial inhibitor such as the immunoglobulin-derived tripeptide Thr-Lys-Pro is applied [217].

Inhibition of the epidermal growth factor receptor (EGFR), involved in transduction of mechanical signals on glial cells, by an EGFR tyrosine kinase inhibitor also prevents astrocyte activation and subsequent RGC loss in experimental glaucoma [218].

A different approach is the overexpression of the *bcl*-2 gene. In very young mice whose astrocytes are still immature, overexpression of *bcl-2* is sufficient to facilitate optic nerve regeneration. In older animals, additional suppression of astrocytes by α-aminoadipate, a glutamate analogue toxic to astrocytes (but not neurons), is required to achieve similar results [219]. Instead of gene overexpression, lithium can be used to induce *bcl-2* expression and stimulate RGC axon outgrowth *in vitro* and *in vivo*. Administration of lithium combined with α-aminoadipate enables optic nerve regeneration in adult mice [220].

Activated ONH astrocytes secrete MMPs, which might cause breakdown of the extracellular matrix necessary for anchorage of RGC, thereby creating a biochemical environment hostile to RGCs. The broad-spectrum MMP inhibitor GM6001 prevents RGC loss in an animal model of glutamate toxicity [221].

Promotion of axon regrowth and pathfinding

Future clinical approaches will need to include neurotrophic treatment to promote axon outgrowth from both surviving and transplanted RGCs or precursors. Several of the strategies discussed under neuroprotection will be applicable at this step. In addition to their RGC-survival-enhancing effect, several growth factors also promote axonal outgrowth: intravitreal injection of CNTF promotes RGC axon outgrowth directly and indirectly via increased expression of endogenous CNTF by astrocytes [103].

Intravitreal injection of an AAV to deliver FGF-2 to RGCs leads to increased axon regrowth after axotomy [222]. The ERK1/2 pathway is essential to this process [223]. Conditioned medium from Müller cell cultures rich in NTF, but not BDNF nor CTNF, enhances RGC survival and axon regrowth *in vitro* [224]. Activated macrophages also secrete proteins that promote RGC axon extension [225]. Retinoic acid agonists promote neural regrowth in a spinal cord lesion model [226].

Neural-tube-derived embryonic stem cells from chicken transplanted into the site of optic nerve transection promote regrowth of RGC axons across the lesion to targets in the brain, involving expression of MMP-2 and -14 by ONH astrocytes and digestion of extracellular matrix proteins such as chondroitin sulfate proteoglycans, along with the secretion of trophic factors [227].

Another cell-based approach involves transplantation of olfactory ensheathing cells. These cells have the potential to penetrate glial scars, possibly by secreting MMPs or by altering the biochemical microenvironment for sprouting axons. In an optic nerve transection model, OECs applied to the lesion site promote the growth of axons across the lesion but do not restore normal optic nerve structure [228].

Channel formation and creation of a neurotrophic environment can also be accomplished by nanotechnology strategies. Amphiphilic carbon nanofibres promote the differentiation of progenitor cells into neurons rather than astrocytes by delivering neurite growth factors whilst growing around the cells. Self-assembling nanofibres can provide a higher density of neurite growth factor around the cells than conventional cell-culture techniques or *in vivo* situations [229].

Concluding remarks and future perspectives

Glaucoma research has unveiled numerous complex and interacting pathways that lead from initial insult to eventual apoptotic RGC death. These insights are being translated into new approaches to glaucoma management, adding neuroprotective strategies to the treatment repertoire. Neuroregeneration will incorporate these strategies to create a biochemical environment permissive to axon regrowth. In the future, various stem and progenitor cells might enable us to repopulate the optic nerve and visual pathway, restoring sight in individuals for whom at present no treatment is available.

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