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Review Article

Understanding optic nerve degeneration: key to neuroprotection in glaucoma

Elisa Ferrer and Jordi Bozzo
Prous Science, P.O. Box 540, Barcelona 08080, Spain

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Abstract

Glaucoma is a group of neurodegenerative disorders characterized by degeneration of optic nerve axons and retinal ganglion cell death. While the most common strategy in glaucoma therapy is aimed at lowering elevated intraocular pressure (IOP), an emerging field of research is currently focused on protecting the optic nerve from degeneration. Neuroprotection is directed at preventing or slowing the death of retinal ganglion cells, thereby decreasing the progression of visual field loss. In all optic neuropathies including glaucoma, the initial site of injury are the axons of retinal ganglion cells. Axonal injury triggers apoptotic mechanisms that ultimately culminate in retinal ganglion cell death. Neuroprotective approaches are varied: 1) the prevention of apoptosis by inhibiting TNF-α and caspase activity; 2) blocking excessive Ca^{2+} overload due to overactivation of NMDA receptors; or 3) blocking nitric oxide toxicity. Axonal injury may cause the blockade of retrograde axoplasmic transport of neurotrophins essential for retinal ganglion cells. In this sense, neurotrophic factors such as brain-derived neurotrophic factor (BDNF) have been reported to prolong retinal ganglion cell survival, and α_2-adrenoceptor agonists such as brimonidine are thought to exert their neuroprotective effects partly by upregulating BDNF expression. Modulation of the immunological response has also proved effective in reducing retinal ganglion cell loss, suggesting a possible use of vaccines to prevent glaucoma progression.

Introduction

The major risk factor for the development of glaucoma is elevated intraocular pressure (IOP), which is thought to cause progressive optic nerve damage, leading to retinal ganglion cell (RGC) death and subsequent visual field loss. Traditional antiglaucoma therapy aimed at lowering elevated IOP has been reported to be effective for reducing disease progression. However, not all patients respond to IOP-lowering treatments and experiment further optic nerve degeneration in spite of reduced IOP, thereby creating the need for alternative therapeutic strategies.

As in other neurodegenerative diseases, the ultimate goal of neuroprotection in glaucoma treatment is to prevent or slow RGC death. The axons of RGCs represent the primary site of injury in glaucoma, as well as in other optic neuropathies. Axonal injury triggers a sequence of events that ultimately terminates in RGC death. Elevated IOP may result in an increased number of degenerated axons, as observed in optic nerve cross-sections (1). The mechanisms by which elevated IOP induces RGC death and optic nerve head changes (cupping) (Fig. 1) are still unclear, although focal damage due to compression of RGC axons at the lamina cribrosa (through which the RGC axons cross before condensing to form the optic nerve), or restricted local blood flow leading to optic nerve ischemia, appear to be plausible causes (1, 2) (Fig. 2).

The study of the cellular and molecular mechanisms of such processes in animal models of experimental glaucoma provides evidence that RGC death occurs by apoptosis (3, 4). Mechanisms of degeneration, such as deprivation of neurotrophic factors critical for RGC survival, glutamate-induced toxicity, intracellular Ca^{2+} overload and nitric oxide (NO) toxicity, can lead to activation of programmed cell death, or apoptosis. Therefore, successful neuroprotective strategies require an understanding of the predominant neurodegenerative mechanism to effectively tailor the treatment to each patient. The above-mentioned mechanisms together with the apoptotic program itself constitute attractive targets for potential neuroprotective therapies (see Table I).

Neurotrophin deprivation

One hypothesis to explain the activation of apoptosis in RGC suggests that elevated IOP can cause axonal

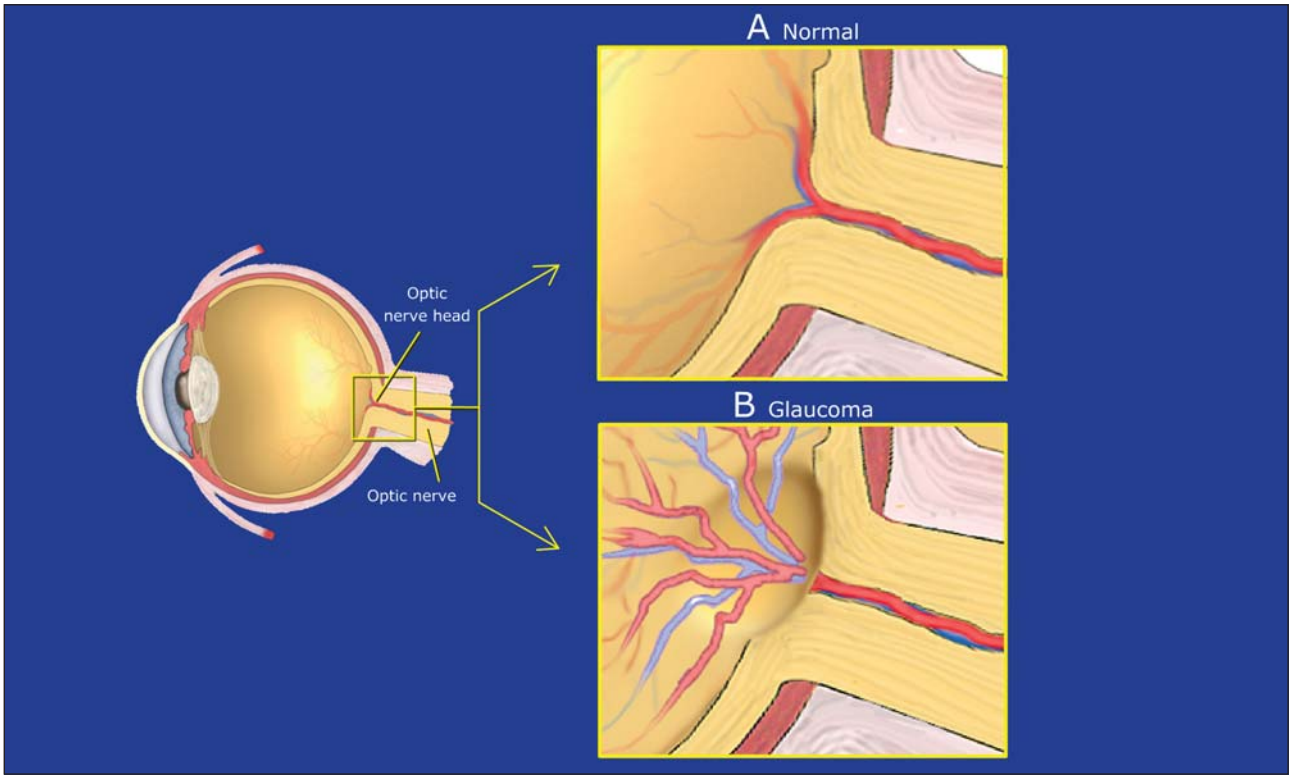

Fig. 1. **A**: Axons of retinal ganglion cells converge on the optic nerve head (or optic disk) and form the optic nerve. The normal optic nerve head has a small central depression known as the cup. The lamina cribrosa is located below the optic nerve head. **B**: In the glaucomatous eye, the cup is enlarged and deepened due to loss of retinal ganglion cell axons.

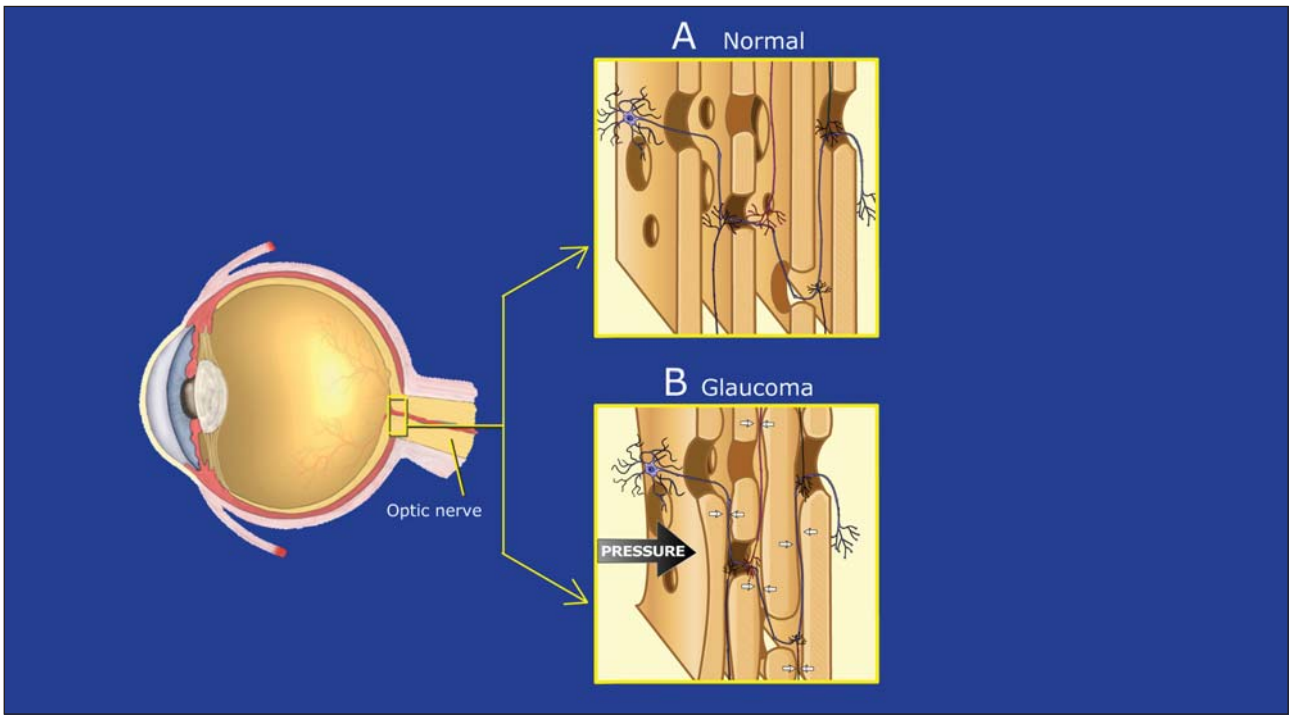

Fig. 2. **A**: Optic nerve fibers cross a series of porous connective tissue sheets of scleral origin below the optic nerve head, known as the lamina cribrosa. **B**: Elevated intraocular pressure (IOP) causes a pressure gradient on the lamina cribrosa that can cause focal injury of the retinal ganglion cell axons passing through the pores. Axonal compression could lead to restricted blood flow (ischemia) or impaired axonal transport.

Table I: Neuroprotective drugs under active development for glaucoma.

Drug name	Mechanism(s) of action	Neuroprotective effect
Memantine*	Non-competitive NMDA receptor antagonist (16)	Blockade of glutamate-induced excitotoxicity
Lomerizine**	Ca^{2+} channel antagonist that prevents Ca^{2+} entry through voltage-dependent Ca^{2+} channels or glutamate receptors (30)	Prevention of Ca^{2+} overload
PN-277	Immunomodulator	Modulation of the immune response
TV-5010	Immunomodulator	

* Phase III clinical studies under way. ** Phase II clinical studies under way.

compression at the lamina cribrosa that would result in impaired retrograde axonal transport of neurotrophic factors secreted by the major RGC brain targets (lateral geniculate nucleus or superior colliculus). This hypothesis is supported by the finding that the transport of brain-derived neurotrophic factor (BDNF) injected into the rat superior colliculus was slower in eyes with elevated IOP (5). Additionally, BDNF appears to be essential for RGC survival (6) by activating phosphatidylinositol 3-kinase (PI3-kinase) and mitogen-activated protein kinase (MAPK) survival pathways (7, 8), and also by suppressing caspase-3 activation (7). According to this hypothesis, BDNF would bind to high-affinity neurotrophin receptors in the RGC axon terminals (tyrosine kinase Trk receptors) to form a complex that would be retrogradely transported to the RGC somata. Evidence supporting this theory has been found in a model of chronic glaucoma in monkeys (9). Furthermore, in a rat model of experimental glaucoma, overexpression of the BDNF gene by RGCs transfected using an adeno-associated viral (AAV) vector increased RGC survival by at least 4 weeks, suggesting that sustained overexpression of BDNF can have a therapeutic effect (10). In this sense, the α_2-adrenoceptor agonist brimonidine, used in glaucoma treatment due to its IOP-lowering effect, has been reported to upregulate BDNF expression in rat RGCs (11), thus partially explaining its neuroprotective effect, although further mechanisms of action have also been proposed (12).

The neurotrophin deprivation hypothesis may not be totally accurate, as neurotrophin (BDNF and others) synthesis does not appear to be restricted exclusively to brain targets, but also occurs in the human lamina cribrosa, where the expression of neurotrophins, including BDNF, nerve growth factor (NGF), neurotrophin-3/4 (NT-3/4) and Trk receptors, has been reported (13). Furthermore, ischemic insults such as oxygen/glucose deprivation have been related to increased neurotrophin (BDNF, NGF, NT-3) expression in human lamina cribrosa cells and optic nerve head astrocytes (14), which may represent a compensatory mechanism to protect RGCs against ischemia. In contrast, decreased retinal expression of TrkB receptors and absence of retinal neurotrophin upregulation have been reported in response to chronically elevated IOP (15), which may compromise the compensatory mechanism. Phosphorylated (*i.e.*, activated) TrkB receptors were also found in the cells of lamina cribrosa and optic nerve head astrocytes in the absence of exogenous BDNF, suggesting a paracrine/autocrine regulation via endogenous neurotrophins (16). This paracrine/autocrine regulation due to the release of neurotrophic factors may occur in response to ischemic insults.

Glutamate-induced excitotoxicity

Glutamate is the major excitatory neurotransmitter in the brain. Excitotoxicity due to excessive glutamate levels has been reported to contribute to neurodegeneration in a variety of neurological disorders, such as Alzheimer's disease and Parkinson's disease (17). RGCs receive synaptic input from bipolar and amacrine cells and translate it into action potentials that propagate down their axons to their major targets in the brain. These synapses use glutamate as the major excitatory neurotransmitter (18). Although RGCs express both NMDA and non-NMDA (AMPA) ionotropic receptors, glutamate-induced excitotoxicity in the retina appears to be mostly mediated by the NMDA receptor subtype (18). The NMDA receptor (Fig. 3) has two relevant modulatory sites: a pore binding site blocked by Mg^{2+} in resting conditions and an extracellular cysteine sulfhydryl group susceptible to nitrosylation by NO, a reaction that downregulates NMDA receptor activity. High glutamate levels, such as those present during retinal ischemia (19), or excessive cell membrane depolarization (as occurs after axonal injury) (2) would overactivate the receptor, causing increased Ca^{2+} (and other cations) influx and initiating Ca^{2+}-dependent signaling cascades, which may lead to cell apoptosis (18, 19).

It has been suggested that glutamate neurotoxicity may contribute to the pathogenesis of glaucoma, as elevated glutamate levels have been encountered in the vitreous humor of glaucoma patients (18, 20). Furthermore, an elevation of glutamate levels accompanying a reduction in RGC number has been reported after acute IOP elevation in rats (21). In this sense, NMDA receptor antagonists have been reported to increase RGC survival in animal models of glaucoma (20-22). A promising experiment showed that memantine, a noncompetitive antagonist of the NMDA receptor, conferred protection to RGCs in rats exposed to chronic small increases in glutamate for 3 months (20). Memantine binds close to the intracellular Mg^{2+} site within the NMDA receptor, and will therefore be pharmacologically active only if the receptor is open for a sufficient length of time, as occurs under excitotoxic conditions (17).

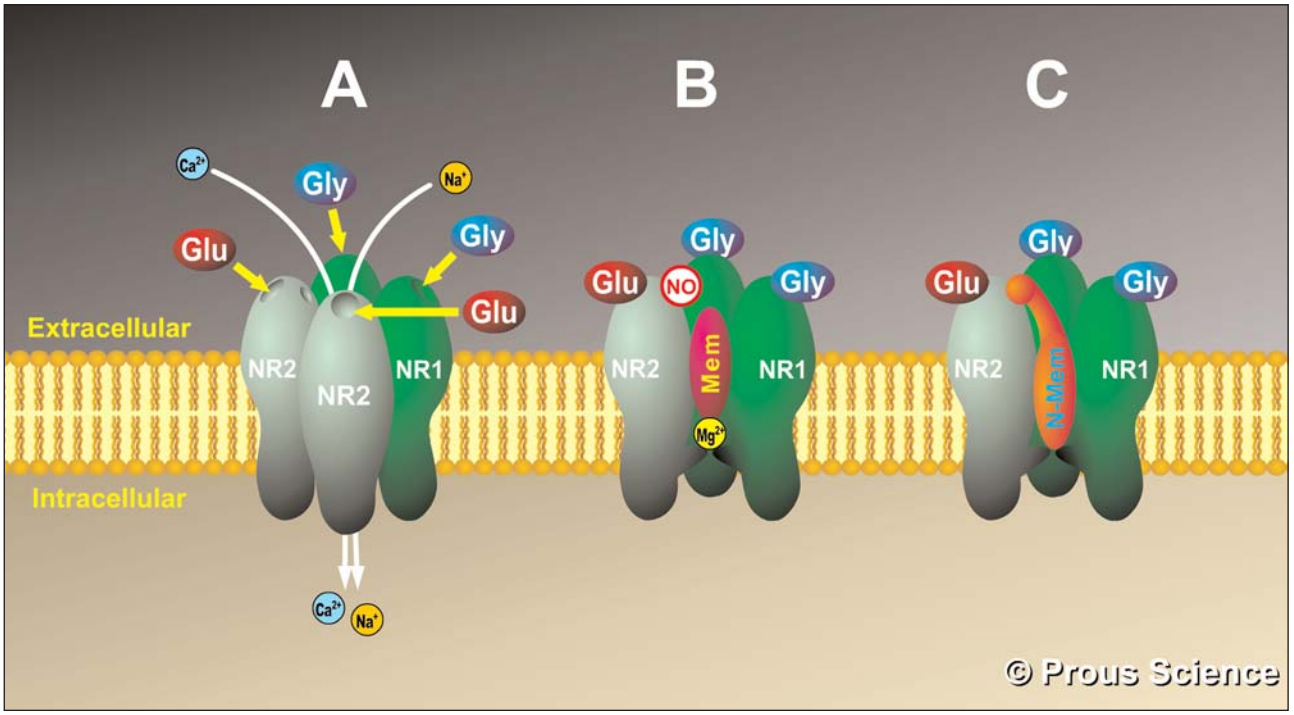

Fig. 3. **A**: Schematic structure of the NMDA ionotropic glutamate receptor showing the NMDA receptor subunits, NR1 and NR2, and important binding sites: glutamate or NMDA binding site (Glu), glycine binding site (Gly) (co-agonist for receptor activation). Activation of the NMDA receptor results in the opening of a selective cation channel within the receptor complex, allowing Na^+ and Ca^{2+} entry, thus depolarizing the membrane. Overactivation of the NMDA receptor causes excessive Ca^{2+} influx that ultimately leads to cell apoptosis. **B**: Illustration of the intracellular region of the channel pore blocked by Mg^{2+} in resting conditions. Upon receptor activation by glutamate, the open channel blocker memantine enters the channel and binds near the Mg^{2+} site, blocking Ca^{2+} entry. The diagram also shows a nitrosylation site (NO) located at the extracellular domain of the receptor. **C**: Nitric oxide reacts with the sulfhydryl group of a cysteine residue (*S*-nitrosylation), downregulating receptor activity. Nitromemantines interact with both the intracellular memantine site and the *S*-nitrosylation site.

Memantine, which was recently approved for the treatment of Alzheimer's-type dementia (23), is currently undergoing phase III clinical studies to evaluate its effects on the progression of glaucoma (24, 25). Furthermore, new derivatives of this drug named nitromemantines are currently being developed. As discussed earlier, *S*-nitrosylation of an extracellular modulatory site of the NMDA receptor results in a decrease in channel opening. These memantine derivatives interact with both the memantine binding site on the NMDA receptor and the *S*-nitrosylation site, thus showing enhanced neuroprotective effects compared to memantine (17).

Other NMDA receptor antagonists, such as MK-801, cannot be used in therapy due to systemic side effects. MK-801 has high affinity for the Mg^{2+} site, where it accumulates and interferes with the normal function of the NMDA receptor (17).

The signaling pathway underlying NMDA-mediated glutamate-induced toxicity in the retina has not been clearly elucidated, although p38 mitogen-activated protein kinase (p38 MAPK) has been implicated in glutamate-induced neurotoxicity (26). In rats, optic nerve axotomy induced apoptosis of RGCs mediated by p38 MAPK (27). In this model, intravitreal administration of MK-801 prior to axotomy inhibited p38 MAPK phosphorylation, suggesting that after axotomy activation of p38 MAPK is mediated by NMDA receptors. Further development of p38 MAPK inhibitors as neuroprotectants is encouraged by these findings.

Pretreatment with α_2-adrenoceptor agonists, in particular brimonidine, has been shown to be neuroprotective in several models of optic neuropathy. Besides upregulating BDNF expression, brimonidine is known to prevent the accumulation of vitreal glutamate and aspartate in RGCs after transient ischemia, by a mechanism yet to be clarified (28). Brimonidine also protected RGCs in a rat model of laser-induced chronic ocular hypertension (29) and in a model of partial optic nerve crush (30).

The molecular mechanisms by which these drugs exert their neuroprotective effect are not completely understood, although α_2-adrenergic stimulation may activate the antiapoptotic PI3-kinase/Akt (or protein kinase B, PKB) pathway (discussed later), preventing proapoptotic mitochondrial signaling (31).

It has also been suggested that part of the toxic effects of glutamate on RGC survival could be mediated by activation of AMPA/kainate receptors after optic nerve crush, since blockade of AMPA receptors with DQNX increased RGC survival (32).

Intracellular Ca^{2+} overload leads to RGC toxicity

As discussed above, overactivation of NMDA or non-NMDA receptors would depolarize the membrane, causing voltage-dependent calcium channels (VDCC) to open and hence excessively increasing the intracellular Ca^{2+} concentration, which would activate Ca^{2+}-dependent processes leading to cell death. Therefore, preventing Ca^{2+} overload would likely contribute to increasing the RGC survival.

The calcium channel antagonist lomerizine has been reported to prevent retinal damage in an *in vivo* model of retinal ischemia in rats. Further studies *in vitro* showed that glutamate-induced RGC toxicity mediated by both NMDA and AMPA/kainate was blocked by preincubating retinal cell cultures with lomerizine (33). However, the mechanism of action of lomerizine remains unknown and it still needs to be clarified whether it prevents Ca^{2+} entry by blocking glutamate receptors or VDCCs. Nonetheless, it is currently undergoing phase II clinical studies to evaluate its effect on visual field loss progression in glaucoma. Lomerizine has also been reported to improve optic nerve head circulation in rabbit and human studies, an effect that would also be beneficial in glaucoma (34).

Additionally, neuronal degeneration has been related to persistent activation of voltage-gated sodium channels. Also, ischemia in the optic nerve triggers a series of events including failure of the Na^+/K^+-ATPase pump, accumulation of intra-axonal Na^+ which leads to inversion of the Na^+/Ca^{2+}exchanger and subsequent abnormal intra-axonal Ca^{2+} accumulation. Sodium channel blockade with phenytoin protected RGCs and their axons from IOP-induced neuronal injury in a rat model of experimental glaucoma (35). According to this paradigm, β-adrenoceptor antagonists, widely used as hypotensive agents, are thought to exert neuroprotective effects on RGCs by reducing Na^+ and Ca^{2+} influx through voltage-sensitive sodium and calcium channels, and not through an interaction with β-adrenoceptors (36).

Nitric oxide toxicity

It has been postulated that an excessive amount of NO causes neurodegeneration of RGC axons in glaucoma. In fact, all isoforms of the enzyme nitric oxide synthase (NOS), NOS-1, -2 and -3, appear to be increased in optic nerve head astrocytes of patients with open-angle glaucoma (37). NO released by astrocytes in these conditions would react with superoxide anions generated by the mitochondria to form peroxynitrite, a free radical that is highly reactive with protein tyrosine residues, hence forming nitrotyrosine. Nitrotyrosine staining is used as a marker of peroxynitrite-mediated damage in glaucoma.

Inhibition of NO synthesis with aminoguanidine, an NOS-2 inhibitor, has been reported to be protective against RGC loss in experimental glaucoma in rats (38, 39). However, a recent report has shown that expression of the inducible isoform NOS-2 did not increase in response to chronic elevated IOP in experimental glaucoma in monkeys (40). Furthermore, the severity of pressure-induced optic nerve damage was not modified by aminoguanidine, contradicting the above-mentioned study (38). Nevertheless, there is evidence that NO plays a role in neurodegeneration, although the mechanisms have not yet been fully elucidated (37).

Interestingly, nitrotyrosine staining has been found in neural and vascular tissue of the lateral geniculate nucleus in monkeys with experimental glaucoma, confirming that glaucomatous damage is not limited to the RGCs, and encouraging further research to limit the extension of the neural degeneration in central brain targets (41).

It has been reported that glutamate-induced neurotoxicity is partly mediated by excess levels of NO (37). An interesting study showed that NMDA-induced RGC death in a rat model was due to increased formation of peroxynitrite, which was blocked by the addition of NOS inhibitors or superoxide scavengers, thus increasing cell survival (42). This study also showed that two cannabinoids, Δ^9-tetrahydrocannabinol and cannabidiol, prevented NMDA-induced retinal neurotoxicity by decreasing peroxynitrite formation. In addition to their neuroprotective effect, cannabinoids are also known to lower IOP in different animal models (43), indicating their potential as antiglaucoma drugs.

Certain β-adrenoceptor antagonists (carvedilol and propranolol) have been reported to act as free radical scavengers (36). However, the antioxidant properties of these antiglaucoma drugs need to be further investigated.

Apoptosis activation in RGCs

Apoptosis is clearly associated with glaucomatous neurodegeneration and one of the proposed mechanisms is based on the activation of caspase cascades (Fig. 4; online publication only). CPP32, or caspase-3, one of the key executors of apoptosis, was found to be activated in RGCs cultured under different apoptotic stimuli (hypoxia, excitotoxicity), together with poly(ADP-ribose) polymerase (PARP; NAD^+ ADP-ribosyltransferase) cleavage (44). Activation of caspase-3 was also found in RGCs from rats with ocular hypertension and after optic nerve transection. Furthermore, intraocular injection of several CPP32-like caspase inhibitors enhanced RGC survival after optic nerve transection in rats (45), suggesting that CPP32 and CPP32-like proteases are primarily mediating axotomy-induced RGC apoptosis. Surprisingly, caspase-3 has been reported to be expressed in astrocytes and Müller cells of experimental glaucomatous rats, but is absent in RGCs, suggesting that glial cells may be involved in the apoptotic process in the retina (46).

In addition to caspase-3 activation, cleavage of the amyloid precursor protein (APP) and β-amyloid (Aβ) peptide have also been encountered in retinas of ocular hypertensive rats. These interesting findings suggest that glaucoma may share similar pathogenic features with Alzheimer's disease, where abnormal APP processing with subsequent accumulation of Aβ peptide are pathognomonic signs.

Transcription of both caspase-8 and caspase-9, initiators of the extrinsic and intrinsic caspase cascades, respectively, was found to be upregulated in a model of experimental glaucoma in rats where IOP was elevated for 10 days (47). Interestingly, activation of cell survival regulators, such as phosphorylated Akt and BAD (Bcl-2-associated death domain), occurs concomitantly with caspase activation in experimental glaucoma (48), suggesting that cell survival mechanisms are activated in response to injury to maintain cellular homeostasis, providing a further site for therapeutic intervention. Caspase-8 is the initiator of the extrinsic apoptotic pathway, which is mainly induced by binding of tumor necrosis factor-α (TNF-α) with TNF-α receptor 1 (TNFR1). Stress factors such as ischemia or elevated hydrostatic pressure, simulating glaucoma conditions, have been reported to increase the production of TNF-α by glial cells, causing apoptosis in co-cultured RGCs (49). Supporting these findings, TNF-α has been found to be predominantly expressed in glial cells, whereas TNFR1 is mainly localized in RGCs from glaucomatous human eyes (50, 51). These results are similar to those reported for increased expression of caspase-3 in glial cells of rat retina in experimental glaucoma (46). In contrast, TNF-α has also been reported to promote RGC survival after optic nerve transection in rats by inducing Akt phosphorylation and reducing outward potassium currents (52). Moreover, RGCs have been shown to be insensitive to TNF-α-mediated cell death in mixed retinal cell cultures, via activation of NF-κB, probably induced by a factor released by other retinal cells (51).

Another interesting mediator of RGC death is calcineurin. Experimentally elevated IOP in rats induced cleavage of this Ca^{2+}/calmodulin-dependent protein phosphatase, yielding a truncated form of the protein that was not present in control eyes. Calcineurin inhibition with FK-506 (tacrolimus) not only increased RGC survival but also blocked BAD dephosphorylation and cytochrome *c* release, all caused by elevated IOP (53). Confirmation of these observations using different calcineurin inhibitors would be desirable, since FK-506-mediated neuroprotection may be due to mechanisms other than calcineurin inhibition.

A recent study proposed the eukaryotic translation initiation factor 5A (eIF5A) as a new therapeutic target, since its inhibition with small inhibitory RNAs strongly blocked TNF-α-induced apoptosis of human lamina cribrosa cells (54). eIF5A is a nucleocytoplasmic shuttle protein required for cell division that was recently implicated in the expression of proteins essential for apoptosis. However, its potential efficacy to protect RGCs against apoptosis has not yet been tested.

Mitochondrial dysfunction

Apoptosis can be induced in response to external stimuli such as the binding of TNF-α to the TNFR (extrinsic pathway) or to stimuli generated within the cell (intrinsic pathway). Both pathways merge into activation of caspase-3. Mitochondrial dysfunction characterized by loss of mitochondrial membrane potential and formation of a permeability transition pore, with subsequent release of cell death activators (cytochrome *c* and apoptosis-inducing factor [AIF]), has been reported to mediate RGC death (55). In this study, caspase inhibition did not completely protect RGCs against TNF-α and hypoxia, suggesting that other mechanisms that are caspase-independent may also play a role in RGC death. It appears that different steps of the apoptotic pathway will need to be targeted in order to achieve complete RGC survival.

Cell survival signals

The balance between cell death and cell survival signals ultimately determines the fate of a cell (Fig. 5; online publication only). The hematopoietic factor erythropoietin (EPO) and its receptor were recently identified in the central nervous system and have shown neuroprotective effects in different conditions of neuronal damage, such as mechanical trauma, neuroinflammation and retinal and cerebral ischemia. The neuroprotective effects of EPO on RGCs were recently studied in a rat model of optic nerve transection *in vivo*, where intravitreal injection of EPO enhanced RGC survival, possibly mediated by activation of the PI3-kinase/Akt pathway, reducing caspase-3 cleavage (56) (Fig. 6; online publication only).

A similar model has been proposed to explain the neuroprotective action of brimonidine, a selective α_2-adrenoceptor agonist extensively used in glaucoma. Activation of the antiapoptotic PI3-kinase/Akt pathway leads to BAD phosphorylation. In this sense, BAD is able to shift the equilibrium between the proapoptotic BAX (Bcl-2-associated X protein) and the antiapoptotic Bcl-X (57) towards cell survival (31).

Apoptosis-independent mechanisms of optic nerve degeneration

An emerging field of research focuses on discovering mechanisms other than apoptosis to explain the death of RGCs that occurs in glaucoma. Neuronal apoptosis is restricted to the cell body, whereas evidence supports the existence of different death programs in other cellular compartments, such as axons, dendrites and synapses. Since the initial site of injury of the RGC in glaucoma is the axon, it seems logical to suggest that localized axonal degeneration may be an early factor contributing to the pathogenesis of glaucoma (2).

To support this hypothesis, researchers examined apoptosis-dependent RGC death in a model of inherited glaucoma in mice. These mice were subjected to deletion of the proapoptotic *BAX* gene. Homozygous *BAX*-deficient mice showed important axon degeneration (axon loss > 95%), with no modification of RGC counts (58). Thus, *BAX* deletion prevents RGC death but not axonal degeneration, supporting the existence of different soma and axon degeneration pathways and suggesting that the axonal pathway is independent of apoptosis induction.

Furthermore, the *BAX* gene dose appears to determine disease progression, which provides another potential target for therapeutic intervention.

Role of immune response: a vaccine for glaucoma?

The immune system has been proposed to play a role in the pathogenesis of neurodegenerative diseases including glaucoma. Several studies pointed out that the accumulation of T-cells at the site of lesion after traumatic injury to the optic nerve has a beneficial rather than a destructive function. Furthermore, injection of T-cells specifically directed to myelin-associated antigens was reported to be neuroprotective (59). In this sense, vaccines activating T-cells specific against immunodominant antigens localized at the site of injury may slow optic nerve degeneration. In fact, vaccination with a synthetic peptide copolymer (Cop-1, or glatiramer acetate) provided significant protection to RGCs against glutamate toxicity and ocular hypertension (60). Glatiramer (Copaxone®) is a safe vaccine currently used for the treatment of multiple sclerosis, and TV-5010 (Teva), a copolymer comprised of the same amino acids, is in preclinical development for glaucoma and other neurodegenerative disorders. Also, PN-277 (Proneuron), a polymer that induces a strong immune response in mice, is currently being investigated in preclinical studies.

Conclusions

Glaucoma is a multifactorial disease that undoubtedly will benefit from novel therapeutic strategies. Understanding the mechanisms underlying neuroprotection in glaucoma is crucial to develop innovative treatments, which, combined with already existing IOP-lowering drugs, will prevent loss of visual function. These advances, together with new tools for early diagnosis, will lead to tailored therapies before irreversible optic nerve degeneration occurs.

Acknowledgements

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Online links

Subscribers to Drugs of the Future can access online animations of Figures 4-6.