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# Pharmacological Neuroprotection for Glaucoma

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## Contents

## Abstract

Glaucoma represents a group of neurodegenerative diseases characterised by structural damage to the optic nerve and slow, progressive death of retinal ganglion cells (RGCs). Elevated intraocular pressure is traditionally considered to be the most important risk factor for glaucoma, and treatment options for the disease have hitherto been limited to its reduction. However, visual field loss and RGC death continue to occur in patients with well controlled intraocular pressures and, thus, a consensus has recently emerged that additional treatment strategies are needed.

One such strategy is pharmacological neuroprotection, which in the context of glaucoma, refers to the situation in which a drug is deployed to interact with neuronal or glial elements within the retina/optic nerve head and thereby facilitate the survival of RGCs. The advent of animal models of chronic glaucoma has enhanced our understanding of many of the pathological processes occurring in glaucoma and, in doing so, described logical targets for pharmacological intervention. Such targets, which have been manipulated with varying degrees of success in relevant animal paradigms include glutamate receptors, autoimmune elements, neurotrophin deprivation, nitric oxide synthesis, oxidative stress products, sodium and calcium channels, heat shock proteins and apoptotic pathways.

With exciting data now emerging from many research laboratories, it is obvious that pharmacological neuroprotection for glaucoma without doubt represents an exciting development in the search for a treatment modality for this debilitating disease.

## 1. Epidemiology and Definitions

Glaucomatous optic neuropathy (glaucoma) represents a group of neurodegenerative diseases characterised by structural damage to the optic nerve (ON) and slow, progressive death of retinal ganglion cells (RGCs). It is the second most common cause of blindness worldwide.[1] Risk factors for glaucoma include an elevated intraocular pressure (IOP), increasing age, family history, race and myopia. Historically, IOP was included in the definition of the disease; subtypes of glaucoma were classified according to whether the IOP was elevated or not, whether the aqueous outflow channels appeared to be open or closed, and whether or not a cause for an elevated IOP, if present, could be detected. Although IOP has now been removed from the definition and the pathogenesis of the disease is poorly understood, there is convincing evidence that glaucoma is an IOP-sensitive optic neuropathy,[2-5] and currently, the only clinically proven treatment is to reduce the IOP. This can be achieved either by

medical or surgical means.[3-5] IOP reduction halts the progression of the disease in the majority of patients, but there remain a recalcitrant group who continue to lose vision despite very low IOPs.[4] Furthermore, IOP reduction to the idealised levels can often not be achieved. Thus, a consensus has emerged proposing that additional treatment strategies are needed.

The term 'neuroprotection' is a relatively recent addition to the ophthalmological lexicon. It refers to physiological protection of undamaged, and rescue of damaged, RGCs and their axons embedded in a hostile environment. Most frequently the term is used in a pharmacological context, in which case a drug may be described as a neuroprotectant or as having neuroprotective properties. In ophthalmic research, the concept of neuroprotection has been regularly applied to diseases of the retina and ON, and in particular to glaucoma. Although any treatment strategy that preserves RGCs in glaucoma could be described as neuroprotective, most researchers limit use of this term to a drug that directly interacts with neuronal or glial elements within the retina/optic nerve head (ONH), thereby facilitating the survival of RGCs.[6-8] Many of the concepts *à propos* neuroprotection in glaucoma have been derived from the study of other neurodegenerative diseases. For example, there is some support for the idea that a proportion of RGCs in glaucoma may be lost via secondary degeneration.[9,10] Secondary degeneration is a phenomenon described in neurodegenerative diseases of the CNS in which neurons that are spared a primary insult are damaged by toxic products released from injured neighbouring neurons and glia. Thus, secondary degeneration is evident in a penumbra or zone that surrounds the ischaemic/traumatic lesion in affected brain tissue. A similar scenario is envisaged in glaucoma in which dying RGCs have the capacity to compromise the health of adjacent neurons.

A related concept to neuroprotection, and one that is sometimes referred to as 'vasoprotection',[11] is the capacity of drugs to improve blood flow to the ONH. Numerous studies, utilising a variety of techniques, have demonstrated that, on average, perfusion to the ONH is decreased in glaucoma patients.[12] Administration of drugs, such as calcium channel antagonists, that counteract vascular insufficiency at the ONH via direct vasodilatory effects on blood vessels represents a potential therapeutic strategy for glaucoma; furthermore, some drugs may improve blood flow to the ONH and also have neuroprotective properties. Detailed discussion of the effect of drugs on ONH perfusion is, however, largely beyond the scope of this review and readers are directed to the comprehensive review by Costa and colleagues.[13]

All of the drugs that are currently used for the treatment of glaucoma lower IOP. Some of these agents also have laboratory-proven neuroprotective properties;[14,15] in some cases there is tantalising clinical evidence to support the laboratory findings. A large-scale, prospective, randomised study is currently evaluating the neuroprotective potential of one topical agent, brimonidine, for the treatment of normal tension glaucoma.[16] Furthermore, there is evidence that drugs which have no effect on IOP may be neuroprotective in glaucoma. A 5-year, prospective, phase III clinical trial is currently underway investigating the effectiveness of the NMDA receptor antagonist memantine in open-angle glaucoma, while the vaccine glatiramer acetate, which displays neuroprotective properties in animal models of glaucoma, is expected to enter phase II trials in the near future.

Effective neuroprotection for glaucoma would offer a new treatment strategy and have a profound clinical impact. This review identifies potential targets for neuroprotection in glaucoma, discusses the evidence in favour of each target being a valid point for intervention, and highlights pharmacological classes of compounds that might prove useful as therapeutic agents. The search engine PubMed was primarily used to identify relevant references using numerous key words, such as neuroprotection, glaucoma, ganglion cell, retina, optic nerve and ocular hypertension in combination with keywords relevant to each individual section of the article. Papers for those references, as well as pertinent review articles and original publications cited by others, were evaluated for inclusion in this article. The final search data for inclusion of references within the article was 31 December 2006.

## 2. Outline of Relevant Pathology

The most striking clinical feature of glaucoma is the characteristic changes at the ONH, and there is a consensus that the primary site of injury in glaucoma is at this site. There are several different patterns of glaucoma, as distinguished by the ophthalmoscopic appearance; however, they all share a common feature: loss of RGC axons at the ONH. There are a number of different types of RGC; in particular, the parvo and magnocellular RGCs, subserving different visual functions. Evidence had accrued suggesting that the magnocellular RGCs with their larger somas and axons were preferentially affected in glaucoma;[17,18] however, there is now considerable evidence against this concept.[19,20]

The gradual loss of axons at the ONH produces characteristic changes to vision. Initially there is a generalised depression of retinal sensitivity followed by localised scotomata in a nerve-fibre bundle type distribution. Contrast sensitivity and motion detection are also affected early. There is evidence that one-third of RGCs can be lost before detectable field loss occurs,[21] indicating considerable redundancy in the system; however, as axons continue to degenerate, the field loss continues: unchecked, blindness ensues.

An understanding of the early pathological changes that precede obvious axonal loss at the ONH has been impeded by the lack of available human tissue for study; however, some progress has been made; particularly as animal models have improved. In early glaucoma, there is a compression of the laminar sheets, followed by a backward bowing of the lamina cribrosa superiorly and inferiorly.[22] Microvascular changes in the ONH have been described as an early feature,[23] but other studies have indicated that capillaries are lost in proportion to the neural tissue.[24] Axons at the superior and inferior poles are usually preferentially lost prior to generalised thinning of the neuroretinal rim (cupping of the disc).[18,22] This has been attributed to anatomical differences in the lamina pores, with larger pores at the poles and less intervening supportive tissue.[18,22] Changes to elastin and extracellular matrix have been reported,[25,26] suggesting that loss of lamina cribrosa integrity contributes to the axonopathy. Activated astrocytes and production of matrix metalloproteinases have also been reported,[27,28] and loss of glial tissue is a late feature.[22]

In addition to the loss of axons at the ONH, there is a loss of RGC somata in the retina. This is a characteristic pathological feature. In fact, retinal pathology appears to be limited to this feature.[29-31] There is no convincing evidence that other retinal cells are affected. Moreover, at least some of the RGC somata undergo apoptotic death;[30,32] however, the precise cause for the apoptosis remains obscure. Furthermore, there is evidence that RGCs in glaucomatous eyes are on a pathological continuum. A number of RGCs are in a transitory 'sick' phase with altered morphology, including shrinkage of their dendritic tree.[33,34] This pathological feature has very important clinical ramifications, because it offers a target population for potential neuroprotectants. Hence, current neuroprotective strategies are aimed at healthy and/or sick RGCs. Neuroregeneration of RGCs is generally considered to be a separate concept.

## 3. Pathogenesis of Glaucoma

The exact nature and cause of the demise of RGCs in glaucoma is still essentially unknown, although a multitude of cellular and tissue events, pathways and mediators that could play important roles in the process have been hypothesised.[12,19,30,35-46] Moreover, although glaucoma has traditionally been associated with ocular mechanical strain arising from an elevation of IOP, this is now known not to be exclusively the case, with vascular, autoimmune and excitotoxic components among those influences also having been hypothesised to play roles.

### 3.1 The Mechanical Theory

It is believed that the major destructive effect of elevated IOP is a deformation in the lamina cribrosa leading to a misalignment of inter-lamellae pores and, thus, kinking and distortion of axon bundles.[25,26,30,44,47-49] Morphologically this presents as retrograde depression of the lamina cribrosa, disorganisation of its structure and a decreased density of connective tissue in both inferior and superior poles of the ONH.[50] This explains why different individuals exhibit damage at a range of IOP levels: varia-

tions in mechanical or elastic properties of lamina cribrosa components determine the susceptibility of the ONH to deviations in IOP. Damage to RGCs can also occur indirectly through mechanical compression of sensitive microvessels (hypoxia) or via the toxic influence of substances released from stretched (stressed) local glia including cytokines or trophic factors such as tumour necrosis factor-α (TNFα), nitric oxide (NO), glutamate or aspartate, D-serine or potassium ions.[40,51-54] It is also possible that the compromised ONH area becomes infiltrated with blood-borne peptides, antibodies or other potentially harmful factors from the relatively permeable choroidal circulation.[38]

One of the major consequences of mechanical compression to RGC axons is thought to be a reduction or blockade of target-derived trophic support, which is provided under physiological conditions by intra-axonal retrograde transport of brain-derived neurotrophic factor (BDNF) or other trophic factors from the lateral geniculate body.[30,55-57] In this situation, the affected RGC experiences a similar fate to those RGCs that die off during retinal development: lack of appropriate target-derived trophic support causes cells to die in an apoptotic manner. The appearance of RGC apoptosis after both experimental glaucoma[57-60] and ON transection,[58,61,62] along with the demonstrable protective effect of exogenous BDNF or other neurotrophins in paradigms of axonal injury,[63-69] lends good support to this theory.

### 3.2 The Vascular Hypothesis

There is considerable evidence for blood flow incompetence at the ONH in glaucoma patients (see review by Flammer et al.[12]), with a clear correlation between retinal damage and vascular risk factors such as systemic hypotension, low perfusion pressure, increased local resistance and vasospasm. It has been proposed that the ONH and/or retina undergo chronic hypoglycaemic, hypoxic or ischaemic injury, as a result of compromised local blood flow.[11,12,41,43,55] Vascular disturbances have long been known to have profound effects on brain neurons (see reviews by Nagahiro et al.[70] and Siesjo[71,72]) and a large body of evidence now exists also linking ischaemia or hypoxia with RGC death in experimental models.[73]

The major consequence of blood flow compromise to a tissue and to its component cells stems from a reduction of nutrients and substrates for energy production.[70,72] When a cell is deprived of glucose or oxygen a fall in cellular adenosine triphosphate (ATP) production will result. Without ATP, there will be a failure in many vital cellular systems, such as membrane pumps and channels, biosynthetic pathways, intracellular signalling pathways and maintenance of cellular and organellular (e.g. mitochondrial) integrity.[73]

The initial outcome of ATP synthesis failure in neuronal tissue pathology is a failure of the plasma membrane-based, ATP-driven sodium-potassium pump.[70,72] The resultant slow elevation in membrane potential increases the likelihood of depolarisation and also release of the voltage-dependent $Mg^{2+}$ block on the NMDA-type glutamate receptor channel.[74,75] This leads to membrane destabilisation and opening of multiple ligand- and voltage-gated ion channels with a net elevation in intracellular levels of sodium, calcium and chloride ions, an uncontrolled osmotic influx of associated water molecules causing cell swelling and necrotic lysis, action potential generation, intracellular acidosis and extracellular accumulation of neurotransmitters, either by synaptic release or by reversal of transmitter carriers. An excessive influx of ions, particularly calcium in the cell body and sodium in the axon region, leads to a loss of control for many cellular regulatory systems and an activation of enzymes that may contribute to cell death or degradation, e.g. lipases, NO synthases, kinases, endonucleases, proteases, phosphatases and caspases.[70,76] The net result of the energy production failure in a cell is, therefore, an irreversible commitment to death via a series of self-reinforcing events. Secondary death, often by apoptosis, is also a natural consequence of tissue energy failure.[70,76] In neuronal tissues it is believed that secondary death processes, involving cells initially unaffected by an energy production deficit, arise because of the increased extracellular levels of neurotransmitters (e.g. glutamate) and other factors such as TNFα, NO, endothelin, D-serine or potassium ions. Elevated neurotransmitter levels will necessarily activate appropriate local receptors and in the absence of energy-dependent transmitter removal, this process will continue in an uncon-

trolled manner. In such an instance, the receptor profile of neighbouring neurons becomes extremely important; if a cell expresses a preponderance of inhibitory receptors (e.g. for transmitters such as GABA or glycine), then it may not be unduly affected by this process. However, if expression of excitatory receptors predominates (particularly those for glutamate), then the cell can effectively be 'excited to death' in the process of excitotoxicity.[7,40]

Excitotoxic neuronal injury involves a self-reinforcing cascade of events stemming from persistent activation of ionotropic glutamate receptors.[77] It leads to a loss in cellular ionic homeostasis, membrane depolarisation, build-up in intracellular calcium and sodium levels and, ultimately, cell death, in a manner similar to that detailed for ischaemia. Calcium-induced cellular demise is triggered more efficiently when influx occurs through certain channels rather than others: influx through the NMDA-type glutamate receptor is believed to be particularly efficient at causing cell death.[77] Excitotoxicity during glaucoma could theoretically arise from both mechanical and vascular insults, but it is mainly considered to be of consequence during hypoxia/ischaemia.[37,39] Hence, if hypoxia/ischaemia plays a role in the pathogenesis of glaucoma, as is widely believed, then excitotoxicity will be a major component. Nevertheless, the precise molecular mechanisms by which excitotoxicity may act as a destructive component of 'optic nerve head' rather than 'retinal' ischaemia remain unclear (see section 6.1.3).

A pathological set of events that is considered central to the vascular hypothesis, but that is also implicated in the mechanical theorem, is damage caused by oxidative stress. All cells maintain a defence against oxidative damage. This comprises a series of enzymatic (e.g. catalase, superoxide dismutase) and non-enzymatic (e.g. ascorbate, α-tocopherol) antioxidants to detoxify oxidative free radical species arising as a result of normal cellular respiration (e.g. superoxide, hydroxyl, singlet oxygen). When energy levels are depleted or stressful events numerous, as is predicted to occur in glaucoma, this system will not operate at full capacity, meaning that there will be an intracellular build-up of damaging radical species. Such species have detrimental consequences for a cell by attacking macromolecular constituents and causing both structural and functional damage. Furthermore, in the event of circulatory restoration following an ischaemic episode, oxygen will flood the tissue, further overloading the capacity of a cell to detoxify radicals. This phenomenon, termed 'reperfusion injury', is a well known consequence of re-establishing a compromised blood supply,[78] and has been described in the retina following restoration of the blood supply after experimental ischaemia. Oxidative free radicals are not only produced in the retina after ischaemia-reperfusion but as a consequence of other injurious situations, for example, excitotoxic glutamate receptor stimulation, and oxidative stress may well contribute to tissue damage and cellular death in both the retina and lamina cribrosa during glaucoma (see section 6.7).

One free radical that has received much attention is NO. NO is a gaseous free radical species that acts as a potent physiological vasodilator as well as a messenger molecule within the CNS. It is synthesised by nitric oxide synthase (NOS) from arginine and oxygen. Three distinct isoforms of NOS have been identified: neuronal NOS (NOS-1; nNOS) and endothelial NOS (NOS-3; eNOS) are constitutively expressed by a variety of neurons and by endothelial cells of blood vessels, respectively, while inducible NOS (NOS-2; iNOS) is not typically found under normal physiological conditions, but is induced by certain, usually toxic, stimuli. NO has the advantage of not requiring synapses to pass from one neuron to another, and it is this ease of passage that underlies its detrimental influence: a rapid intracellular and intercellular diffusion combined with the ready combination with superoxide radicals to produce peroxynitrite lends this compound a potentially lethal character. Increased levels of NO production in the retina have been described as a direct consequence of pathological situations such as ischaemia-reperfusion,[79,80] inflammation[81] and excitotoxicity.[82] For example, stimulation of NMDA receptors activates NOS-1 and causes transcriptional upregulation of NOS-2, leading to large increases in the cellular NO level.[83] Importantly, NOS-1 and NOS-3 have been shown to be upregulated, and NOS-2 to be expressed, in astrocytes and microglia in the ONH of primary open-angle glaucoma (POAG) patients and experimental glaucomatous

rats, and these findings in particular have implicated NO in the pathogenesis of glaucoma (see section 6.4).[84-89]

### 3.3 Autoimmunity and Heat Shock Proteins

Recent studies have suggested that the immune system may play one of two roles in glaucoma pathogenesis.[38] The first is to actually cause RGC damage by stimulation of an autoimmune response subsequent to expression of autoantibodies or by a mimicked response to a sensitising antigen (e.g. the immunodominant heat shock proteins [HSPs]). The second is to provide a means of surveillance for RGC damage stimulated by other stresses (e.g. hypoxia, elevated pressure, raised glutamate levels, excessive TNFα or NO release). In the latter scenario, stressed RGCs produce antigens that will stimulate an immune response at the site of damage, thus protecting other RGCs from a similar destruction.[38]

Evidence for a link between HSP reactivity and glaucoma pathogenesis has arisen from several studies that show that HSPs have an increased expression in the eyes of glaucoma patients,[90,91] in animals subjected to chronic hypertension,[92,93] and in stressed neurons and astrocytes in culture.[94] Some glaucoma patients additionally possess an elevated titre of serum antibodies to HSP27.[90] Furthermore, addition of HSP27 antibody itself can induce widespread apoptotic death of cultured retinal cells including RGCs.[94] Other candidate antibodies for involvement that are elevated in the serum of glaucoma patients include those to glycosaminoglycan and glutathione *S*-transferase (see Tezel et al.[38] for a review).

Such data are convincing, but it is not clear at present whether HSP expression exists as cause or effect in RGC axon injury. Caprioli,[95] for example, has suggested that these proteins may just be expressed as part of an endogenous retinal protective response to stress (see section 6.8). Careful temporal studies may detail whether the increased HSP or HSP-antibody expression precedes all other signs of injury, thus indicating that these proteins may play a central role in stimulating an autoimmune attack on RGCs.

## 4. Laboratory Models of Glaucoma

The use of animal models to investigate glaucoma has been the subject of some excellent recent reviews.[44,96-99] As a consequence, the topic is covered relatively briefly in this review. A number of different *in vitro* and *in vivo* models of RGC death have been used to study neuroprotection for glaucoma. Given that the aetiology and pathogenesis of glaucoma remain ill-defined, it seems reasonable to suggest that any potential neuroprotectant should be tested in multiple experimental models first. It follows that the more paradigms in which the drug shows activity, the more likely it is that the compound will be useful in the clinical setting. Nevertheless, the goal of a model is to resemble the disease as closely as possible, thus making extrapolation from bench to bedside more credible.

Tissue cultures and single cell type cultures provide a direct and rapid means of comparing different compounds. They are ideal for determining the potency and efficacy of a drug, and allow direct cellular accessibility and microenvironmental control, as well as efficient comparison between many experimental conditions or potential therapeutic compounds; however, they generally have less relevance to clinical glaucoma than *in vivo* models because of the unique complexity of the retina, the importance of the intact ON for RGC survival, the need to measure physiological parameters, the interaction between the anterior and posterior chambers of the eye, and the differing intra- and extra-cellular milieu of particular ocular tissues and cell layers, which cannot be accurately replicated *in vitro*.

A variety of insults can cause RGC loss *in vivo*, including models of retinal ischaemia-reperfusion, ON transection/crush and excitotoxic injury. There is evidence that ischaemia, mechanical injury and excitotoxicity all play a role in glaucoma pathogenesis; hence, successful neuroprotectants using these models can to some extent be extrapolated to glaucoma; however, the models do not closely mimic the clinical situation. Conversely, primate laser models of glaucoma probably exhibit the closest comparison to human glaucoma,[100] but the high monetary and welfare costs associated often limit their use to what is essentially an immediately preclinical setting.

In recent years, a number of rodent models of ocular hypertension and glaucoma have been developed (see Morrison et al.[44]). These rat models produce elevated IOP for several weeks, which results in glaucomatous-like changes at the ONH and a loss of RGCs. The models differ primarily in the means by which the IOP is raised. Elevated IOP can be achieved by cauterising two or three of the episcleral vessels,[101,102] by scarring Schlemm's canal with hypertonic saline,[60] or by lasering the trabecular meshwork.[103,104] They are thought to represent a significant improvement upon ischaemia-reperfusion and mechanical injury models, and studies performed using these paradigms form the core of this review; however, more work is needed to determine their true similarity to glaucoma and whether any one model is superior to the others. In addition to rat models, mutant mice, such as the DBA/2J variant, have been developed, which spontaneously develop a form of secondary glaucoma.[105,106] Mice offer the distinct advantage that their genetic background can be altered.

## 5. Criteria for Evaluating Neuroprotection

In order to evaluate whether any specific pharmacological agent has potential for use in the treatment of glaucoma, Wheeler et al.[107] have set out four criteria that should be met by the drug in question. A prospective neuroprotective agent should (i) have (a) specific target(s) in the retina/optic nerve; (ii) increase RGC survival in laboratory models of glaucoma; (iii) reach the target at a neuroprotective concentration after clinical dosing; and (iv) display neuroprotective activity in human trials.

In the following sections, potential targets for neuroprotectants in glaucoma are identified. For each target, the evidence in favour of it being a valid point for intervention is discussed. Focus is then on pharmacological agents that have been tested in animal models of glaucoma or in the clinic, using the criteria listed above as a general framework for discussion (table I).

**Table I.** Selected studies detailing drugs tested in animal models of chronic ocular hypertension which promote survival of retinal ganglion cells (RGCs) via mechanisms that are independent of intraocular pressure

| Drug | Potential Strategy | Species | Model | Reference |
|---|---|---|---|---|
| MK-801 | NMDA receptor antagonism | Rat | Cauterisation | 108 |
| | | Rat | Hypertonic saline | 109 |
| Memantine | NMDA receptor antagonism | Rat | Lasered | 110 |
| | | Mouse | DBA/2J | 111 |
| | | Primate | Lasered | 112-114 |
| Glatiramer acetate | Boosting autoimmunity | Rat | Lasered | 115-117 |
| BDNF | Neurotrophin deprivation | Rat | Lasered | 67 |
| | | | Cauterisation | 63,118 |
| CNTF | Neurotrophin deprivation | Rat | Lasered | 119 |
| Aminoguanidine | Nitric oxide inhibition | Rat | Cauterisation | 120 |
| | | Rat | Hypertonic saline[a] | 86 |
| Phenytoin | Sodium channel blockade | Rat | Cauterisation | 121 |
| *Ginkgo biloba* | Reducing oxidative stress | Rat | Cauterisation | 122 |
| S-PBN | Reducing oxidative stress | Rat | Cauterisation[b] | 63 |
| Geranylgeranyl-acetone | Induce heat shock protein 70 | Rat | Lasered | 123 |
| FK506 | Calcineurin inhibition | Rat | Hypertonic saline | 124 |
| BIRC4 | Caspase-3 inhibition | Rat | Hypertonic saline | 125,126 |
| Brimonidine | Activation of $\alpha_2$-adrenoceptors | Rat | Lasered cauterisation | 104,127 |

a No protection of RGCs was observed using this model.

b Enhanced protection afforded by BDNF, failed to improve RGC survival when administered alone.

**BDNF** = brain-derived neurotrophic factor; **BIRC4** = baculoviral; **CNTF** = ciliary neurotrophic factor; **IAP** =repeat-containing protein 4; **S-PBN** = N-*tert*-butyl-(2-sulfophenyl)-nitrone.

## 6. Targets for Neuroprotection

### 6.1 Glutamate Receptors

The rationale behind the excitotoxic hypothesis of glaucoma is simple: during glaucoma, there is an increase in the level of the excitatory neurotransmitter glutamate in the retina that is toxic to RGCs. While there is substantive evidence that high doses of, or prolonged exposure to, glutamate kills RGCs via overactivation of ionotropic glutamate receptors,[128-131] no consensus has been reached in answering the question: is the level of glutamate elevated during glaucoma?

#### *6.1.1 Is There Evidence for Increased Glutamate in Glaucoma?*

There has been much speculation as to the pathological processes that could result in an elevated glutamate level, but conclusive data supporting an elevated level have proved tantalisingly elusive. Essentially, at the cellular level, two mechanisms are most likely to account for a rise in glutamate: (i) an increased release of glutamate from dying RGCs or metabolically compromised glial cells; and (ii) a reduced clearance of glutamate as a result of inefficient uptake.

Initial studies suggested that the level of glutamate was elevated in glaucomatous eyes. Dreyer et al.[132] reported that in patients with glaucoma and in monkeys with experimentally elevated IOP the concentration of glutamate in the vitreous body was at least twice as high as in control eyes. This finding was replicated in studies that analysed the vitreous humours of dogs with breed-related primary glaucoma[133] and quail with a glaucoma-like disorder.[134] However, recent examinations of the glutamate content of vitreous bodies from human patients[135] and from monkeys[112,136,137] and rats[138] with experimental glaucoma have failed to repeat these findings; in all of these studies the level in glaucomatous eyes was normal. Despite these negative findings, the excitotoxicity theory has not been markedly weakened. A number of authors have pointed out that the vitreal glutamate level may not correspond to the concentration present at the synapses of the RGCs. It is also possible that the glutamate concentration is only sporadically elevated, for example during ONH ischaemic episodes.

Lately, researchers have turned their attentions to investigating whether retinal glutamate clearance mechanisms are compromised in experimental glaucoma. Using the lasered model of chronic hypertension, Martin et al.[139] found a significant reduction in the level of the glutamate transporters GLAST (EAAT1) and GLT-1 (EAAT2) in the retina when analysed by Western blot. They speculated that if the observed reductions were associated with a concurrent decrease in glutamate uptake capability, there would be an increased likelihood of glutamate-mediated injury. Conversely, WoldeMussie et al.,[140] using the same model of chronic hypertension as Martin et al.,[139] showed a sustained increase in the level of GLAST expression in the retina, when analysed by immunohistochemistry and Western blotting. In this case, the authors suggested that the increase in GLAST was most likely to be a compensatory mechanism in response to an increase in the extracellular glutamate concentration. The underlying reason for the contradictory results of the two studies is unclear and neither result is supported by recent work from the laboratory of Pow,[141] who showed no change in the level of GLAST in glaucomatous rats. Interestingly, however, they showed a vastly altered expression and distribution of a splice variant of GLT-1 in glaucomatous rats and humans, which they suggest may be indicative of an anomaly in glutamate homeostasis. Nevertheless, none of these studies determined whether alterations in glutamate transporter expression in experimental glaucoma correlate with a change in functional capacity.

This issue was recently addressed in a study performed by Hartwick et al.[142] They reasoned that if experimental glaucoma causes a decrease in the functional capacity of retinal glutamate clearance mechanisms, then exogenously applied glutamate would stimulate RGC glutamate receptors at lower concentrations. They tested the hypothesis by assessing the effect of glutamate on RGC calcium dynamics in living retinal wholemounts prepared from rats with experimental glaucoma and from controls. To validate the technique, they initially confirmed the presence of glutamate clearance mechanisms in the intact retinal preparation. They achieved this by demonstrating that in retinal wholemounts prepared from normal eyes, very high

concentrations of glutamate were necessary to affect RGC calcium dynamics, but pharmacological inhibition of glutamate transporters resulted in an increased sensitivity to glutamate. Subsequently, they investigated the responses in rats with experimental glaucoma (the hypertonic saline model). The results showed no difference between glaucomatous or control retinas in their sensitivity to exogenously applied glutamate. The authors concluded that there was no evidence for a global defect in retinal glutamate clearance mechanisms in the hypertonic saline rat model of glaucoma.

The inevitable weakness in the study of Hartwick et al.[142] is that the functional capacity of the retinal glutamate transporters *in vitro* may not be identical to their capacity *in vivo*. If, for example, the energy requirements of the Müller cells are compromised during experimental glaucoma then the transporters may not be operating efficiently. This would not be evident in the retinal wholemount when the tissue is superfused with balanced salt solution and oxygen.

#### 6.1.2 NMDA Receptor Antagonists

RGCs express multiple subtypes of ionotropic and metabotropic glutamate receptors;[143-145] yet excitotoxic loss of RGCs is thought to result primarily from glutamate interacting with the NMDA receptor subtype. Administration of NMDA receptor antagonists is an effective method of preventing RGC loss in various models of RGC death in which excitotoxicity is implicated, such as injection of glutamate or NMDA,[131] in models of retinal and ON ischaemia,[146-148] and after ON crush.[110,149,150] Therefore, if NMDA receptor antagonists similarly reduce RGC loss in models of chronic hypertension, then a role for excitotoxicity in glaucoma could be considered. The first study to address this issue was by Chaudhary et al.,[108] who elevated IOP in rats by cauterising the episcleral veins, and administered the prototypical, non-competitive NMDA receptor antagonist MK-801. They found a marked preservation of RGC numbers in animals treated with the drug. A similar effect was documented by Guo et al.,[109] who used the hypertonic saline model of RGC injury. However, MK-801 is unsuitable for use clinically because it blocks normal glutamatergic neurotransmission and would, therefore, give rise to potentially severe adverse effects. Accordingly, other studies have made use of the uncompetitive NMDA receptor antagonist memantine.

##### Memantine

Memantine (1-amino-3,5-dimethyl-adamantane) is an NMDA receptor antagonist that has recently been shown to be clinically useful in the treatment of moderate to severe Alzheimer's disease.[151]

*Activity*: Memantine is a highly effective neuroprotective agent in acute animal models of RGC death.[110,131,146-150] In the lasered rat model of chronic hypertension, daily infusion of memantine (5–10 mg/kg) via a subcutaneous osmotic pump increased the number of surviving RGCs and preserved the compound action potential of the ON.[110] In the DBA/2J mouse model of glaucoma, twice daily intraperitoneal injections of memantine (5 mg/kg) protected against loss of RGCs to a degree similar to that achieved by the IOP-lowering drug timolol.[111] Most significantly, in monkeys with experimental glaucoma, daily oral administration with memantine (4 mg/kg) afforded protection against structural changes and functional loss that occurred in vehicle-treated animals.[112-114]

*Mode of action*: Memantine is classified as an uncompetitive NMDA receptor antagonist. An uncompetitive antagonist is defined as an inhibitor the action of which is contingent upon prior activation of the receptor by the agonist: the degree of blockade for a given concentration of antagonist increases as the concentration of the agonist increases. Thus, memantine is inactive during normal glutamatergic synaptic activity, but is an increasingly effective blocker under prolonged/excessive activation of the receptors, which occurs in pathological situations.[152]

*Penetration*: The oral administration regimen used in the primate study resulted in a vitreous memantine concentration of approximately 1 μmol/L at the time the animals were killed.[112] Memantine is known to block NMDA-mediated death of cultured RGCs at this concentration.[152]

*Clinical trials*: A large-scale, randomised, prospective, phase III clinical trial is currently in progress to determine the effect of memantine on visual field loss in glaucoma patients. The results are not expected until 2007.

#### 6.1.3 How are NMDA Receptor Antagonists Neuroprotective in Experimental Glaucoma?

The ability of NMDA receptor antagonists to attenuate injury is easy to explain when the injury involves ischaemia/trauma to a neuronal cell body that expresses NMDA receptors; however, the mechanism by which NMDA receptor blockade could attenuate experimental glaucoma remains unclear. If glaucomatous pathology occurs principally at the level of the unmyelinated axons in the ONH, which are unresponsive to NMDA,[153] how could NMDA receptor blockade protect RGCs? Several explanations for the success of NMDA receptor antagonists in this situation exist.

- NMDA receptor antagonists prevent secondary degeneration of RGCs. In this context, secondary degeneration is hypothesised to occur when uninjured RGCs are exposed to the released toxic cellular contents (i.e. glutamate) of neighbouring RGCs that have died as a result of the primary insult.
- NMDA receptor blockade reduces glutamatergic injury to ONH astrocytes and this prevents subsequent injury to axons.
- The models inadvertently produce a primary injury to the retina and it is this injury that is being attenuated by the NMDA blocker.
- NMDA receptors *are* present on unmyelinated ON axons.
- Blockade of NMDA receptors located on the extra-ocular, myelinated portion of the RGC axons[154] aids survival of these cells.

#### 6.1.4 Summary

The evidence in support of an involvement of excitotoxicity in glaucoma is, at present, somewhat inconsistent. The findings that glutamate levels are not elevated in the vitreous of glaucomatous eyes and that retinal glutamate transporters appear to function normally in eyes with experimental glaucoma argue against an involvement of glutamate in the pathogenesis, yet the neuroprotective properties of memantine in models of glaucoma are impressive. Neuroprotection-based human studies can provide invaluable insights into the pathogenesis of glaucoma. If memantine proves to be clinically useful, then it will not only have provided the first pressure-independent, neuroprotective treatment, but will have also proved that excitotoxicity plays a role in the pathogenesis of the disease.

### 6.2 Boosting Protective Autoimmunity

T-cell-mediated autoimmunity is traditionally considered to be an attack on host components by activated T cells leading to pathological changes and ultimately self-destructive autoimmune disease; however, an impressive body of work generated by the group of Schwartz has shown that boosting T cells weakly recognising self antigens following injury to CNS tissue can, under the right circumstances, protect against further degeneration and play a role in recovery.[155] They propose that boosting autoimmunity by vaccination is a realistic treatment option for glaucoma.[156,157]

The first convincing evidence that stimulating the immune system aids the survival of injured RGCs was the finding that systemic injection of encephalitogenic T cells directed to myelin basic protein in rats that had undergone partial ON crush resulted in an accumulation of these T cells at the site of injury, and histological and functional preservation of RGCs.[158,159] The protection afforded by this passive immunisation was, however, accompanied by transient symptoms of autoimmune disease. In a follow-up study, it was ascertained that active immunisation, achieved by injection of various myelin-associated proteins, also increased survival of RGCs after ON injury, and perhaps more importantly that cryptic (non-encephalitogenic) myelin-associated peptides were equally protective of RGCs, but induced no signs of autoimmune disease.[160] In further studies, Schwartz and colleagues found evidence consistent with the idea that the neuroprotective effects they had achieved by experimental manipulation of the number of autoimmune T cells were merely the augmentation of an endogenous response that occurs in stressful situations, i.e. that protective autoimmunity is a physiological phenomenon that can be boosted in a manner that is analogous to preconditioning. The experimental results that allowed this conclusion to be reached were as follows:

- in rats devoid of mature T cells, increased numbers of RGCs died after ON crush compared with normal rats;[161,162]

- giving a prior unrelated CNS injury to a group of ON crush rats resulted in the loss of fewer RGCs than in animals that underwent just the ON injury;[161,162]
- transfer of splenocytes from rats with the unrelated CNS injury to normal rats protected against an ON crush injury.[161]

Initiation of an endogenous, autoimmune T cell-mediated self-protective mechanism is not exclusive to axonal injuries, but also occurs after excitotoxicity[115] and chronic elevation of IOP.[163] Interestingly, immunisation targeted at myelin-associated proteins failed to prevent RGC death after these injuries,[115,116] from which the authors concluded that the myelin antigen is not specific to a RGC body injury. Immunisation with self-peptides derived from retinal neurons did result in beneficial effects.[164] In glaucoma, the site of injury includes the RGC body and axon, which immunologically can be considered to be distinct tissues. One way of circumventing the need for tissue specificity and protecting against both axonal and cell body injuries is to use a synthetic antigen such as glatiramer acetate, which cross-reacts with multiple self-antigens.

#### 6.2.1 Glatiramer Acetate

Glatiramer acetate (Copolymer-1; Cop-1; Copaxone) is a synthetic, random oligopeptide composed of tyrosine, glutamate, lysine and alanine residues. It is a low-affinity antigen that cross-reacts with a wide range of self-reactive T cells. This property allows glatiramer acetate to mimic self-antigens at various sites of injury by evoking neuroprotective autoimmune responses.

*Activity*: Glatiramer acetate emulsified with Freund's adjuvant increased survival of RGCs after glutamate-induced excitotoxicity in mice,[115] after an acute increase in IOP[165] and following ON crush in mice[115] and rats,[166] but not, surprisingly, after partial or complete transection of the ON in rats.[167] The reason for the lack of effect of glatiramer acetate in the latter models is unclear. In the lasered rat model of chronic hypertension, Schwartz and colleagues demonstrated that immunisation with glatiramer acetate plus adjuvant resulted in a highly significant preservation of RGCs, irrespective of whether the drug was administered at the time of laser cauterisation or 10 days later.[115,116] In a follow-up study,[117] the same group extended their findings. They showed, firstly, that glatiramer acetate was an effective neuroprotectant, even when administered without adjuvant, and secondly, that glatiramer acetate imparted functional as well as histological protection to RGCs.

*Mode of action*: The available evidence indicates that glatiramer acetate induces neuroprotection of RGCs via recruitment of systemic T cells to the site of injury:

- administration of glatiramer acetate caused a transient accumulation of T cells and microglia/macrophages in the retina;[117]
- transfer of glatiramer acetate-reactive T cells elicited the same protective response as glatiramer acetate itself;[115,166]
- glatiramer acetate was ineffective in animals devoid of mature T cells.[117]

The precise mechanisms by which T cells confer resistance to injury have not yet been elucidated, although the release of trophic factors and cytokines may well be involved.[166]

*Penetration*: Unlike other neuroprotective compounds, glatiramer acetate is an immune-based therapy and penetration of significant amounts of the drug to the site of injury is unnecessary.

*Clinical trials*: Glatiramer acetate is a US FDA-approved drug for multiple sclerosis, which elicited positive effects when administered daily in clinical trials.[168] The compound is expected to enter phase II trials for use in glaucoma in the near future.

#### 6.2.2 Summary

Boosting autoimmunity is an exciting and novel approach to treat glaucoma, and results obtained by Schwartz and colleagues using glatiramer acetate in a rat model of chronic hypertension are encouraging. It is important that other research groups investigate whether boosting autoimmunity is neuroprotective in rodent and primate models of glaucoma.

### 6.3 Neurotrophin Deprivation

The neurotrophin deprivation hypothesis, as expounded by Quigley,[30] is essentially an adaptation of the traditional mechanical theory: raised IOP and/or defects in the extracellular matrix cause(s) compression and displacement of the lamina cribrosa. The resultant damage to the ON axons leads to

blockage of retrograde delivery of neurotrophic survival factors to the cell bodies and the initiation of RGC apoptosis. This section considers the evidence for obstructed axoplasmic transport and compromised neurotrophic support of RGCs in glaucoma.

#### 6.3.1 Obstructed Axonal Transport

The first data in support of the theory came from Anderson and Hendrickson in a 1974 study in which they showed that acute elevation of IOP in monkeys obstructed rapid orthograde axoplasmic transport.[169] Subsequent experimental studies in *monkeys* and rats have confirmed and extended these findings:

- retrograde as well as orthograde axonal transport is blocked by acutely elevated IOP;[170-172] the extent of axonal transport obstruction appears to be proportional to the magnitude and duration of the increase in IOP;[170-175]
- axonal transport obstruction is evident in eyes with mild, chronic elevations in IOP;[60,176]
- blockade of axonal transport can be reversed in some axons if the IOP is lowered and the duration of hypertension is relatively short-lived;[170,176]
- there is an accumulation of dynein, a protein fundamental to retrograde axonal transport, in the ONH of rodents with chronic IOP elevation.[177]

Histological analysis of human glaucomatous ONHs has revealed an accumulation of mitochondria and vesicles within axons where they cross the lamina cribrosa.[49,178] These findings, although consistent with blockade of axonal transport at the ONH, are not conclusive. An alternative explanation has recently been postulated, which suggests that this increased mitochondrial density exists to serve the higher energy requirements necessary for electrical conduction in unmyelinated axons in the prelaminar and laminar ON.[179]

#### 6.3.2 Neurotrophin Deprivation

The neurotrophin family consists of four members: BDNF, nerve growth factor, neurotrophin-3 and neurotrophin-4/5. To date, only a few studies have examined whether experimental glaucoma affects delivery of neurotrophins to, or expression of neurotrophins in, the retina. In rats with acutely elevated IOP, Quigley and co-workers found retrograde axonal transport of radioactive BDNF to the retina to be substantially inhibited.[56,57] The results indicated that during glaucoma RGCs may become depleted of BDNF. In two related studies, the chronology of neurotrophin changes after elevation of IOP were studied in models of chronic hypertension. Using the hypertonic saline model, Johnson et al.[59] found results consistent with a decrease of neurotrophin delivery; they showed a gradual depletion of BDNF and neurotrophin-4/5 in the proximal ON and in the superior region of the inner retina. In contrast, Rudzinski et al.,[180] using the episcleral vein cauterisation model, found a slow increase in BDNF protein expression in the retina over 4 weeks, no change in the level of neurotrophin-3, and a transient upregulation of nerve growth factor at 1 week, which had returned to normal by 4 weeks; these results do not imply a diminished delivery of neurotrophins. In these latter studies, differentiation between neurotrophins retrogradely transported from the superior colliculus and neurotrophins synthesised locally was not possible. New studies are needed to definitively address this issue.

#### 6.3.3 Brain-Derived Neurotrophic Factor

BDNF enhanced survival of RGCs after ON transection,[64,65] ON crush[68] and excitotoxic injury;[181] however, the effects after ON injuries were only transient. BDNF also promoted survival of RGCs in the episcleral vein cauterised-[63,118] and lasered-[67] rat models of chronic hypertension. Multiple intravitreal injections were needed to show positive effects in the former model, while in the latter model sustained delivery of BDNF was achieved by gene therapy. The effectiveness of BDNF in the episcleral cauterisation model was increased by co-administration of a free radical scavenger.[63] This was also the case after ON transection,[182] and it appears that the neuroprotective potential of BDNF, particularly at higher doses, is limited by adverse effects as a consequence of excessive free radical formation caused by BDNF itself.[63,182]

#### 6.3.4 Other Neurotrophic Factors

In models of ON crush or transection, RGC death can be delayed by a variety of other neurotrophins and trophic factors, including nerve growth factor, neurotrophin-4/5, glial cell-derived neurotrophic factor, ciliary neurotrophic factor (CNTF) and fibroblast growth factor-2.[183,184] Of these compounds, only CNTF has thus far been tested in a

model of chronic hypertension. In the lasered rat model, a single intravitreal injection of CNTF enhanced survival of RGCs for up to 4 weeks and resulted in an increase in phosphorylated STAT3.[119] It is not known whether activation of the JAK-STAT signalling pathway accounted for the observed neuroprotection.

#### 6.3.5 Summary

Although there is no direct evidence from clinical studies that reduced delivery of neurotrophins is a contributory factor in the apoptotic death of RGCs in glaucoma, work performed in animal models does support the hypothesis. The majority of investigators have thus far concentrated their efforts on BDNF and the results are promising, but a number of questions remain unanswered. Are BDNF and its receptor TrkB downregulated in glaucomatous retinas? Does BDNF only afford transient neuroprotective effects in models of chronic glaucoma as is the case after ON transection? Will delivery of BDNF exacerbate any free radical-mediated injury in glaucoma? Does sustained delivery of BDNF lead to downregulation of TrkB and loss of trophic responsiveness? Will it be necessary to deliver BNDF and TrkB to achieve long-lasting neuroprotection? Is BDNF the most suitable candidate of the nerve growth factors/neurotrophins/neurotrophic factors to be used for neuroprotection? What is the most appropriate route of delivery for nerve growth factors?

### 6.4 Nitric Oxide Synthesis

It has been suggested that excessive NO, released by reactive astrocytes and microglia in the ONH, causes damage to ON axons at the level of the lamina cribrosa.[83] Furthermore, it has been hypothesised that pharmacological inhibition of NOS-2 represents a novel strategy for the treatment of glaucoma.[87] The available data is reviewed in this section.

The first evidence supporting the involvement of NO in the pathogenesis of POAG came from studies that localised the NOS isoforms in the ONH of POAG patients and healthy individuals. Neufeld et al.[84] showed that NOS-1 and NOS-3 were upregulated in POAG, and NOS-2, which was absent in the normal ONH, was expressed in the ONH of POAG patients. In follow-up studies, it was ascertained that reactive astrocytes and some microglia were the cells responsible for expressing NOS-2 in the ONH.[52,89] These findings were strengthened by the results of a genetic analysis of glaucoma patients and controls, which showed an association between NOS-2 and POAG.[185] A rationale for the expression of NOS-2 in the ONH of POAG patients was provided by the finding that when human ONH astrocytes were placed in culture and subjected to elevated hydrostatic pressure they synthesised large amounts of NOS-2.[88] Experiments performed using the episcleral vein cauterisation model of chronic hypertension supported the association between NOS-2 expression and ONH pathophysiology. Shareef et al.[85] showed that in rats with elevated IOP there was an expression of NOS-2 in ONH astrocytes, while Neufeld et al.[120] demonstrated that an inhibitor of NOS-2, aminoguanidine, increased survival of RGCs compared with controls (see section 6.4.1).

The involvement of NO in ONH injury and RGC death was, however, recently questioned in a detailed paper by Pang et al.[86] They found no evidence for the expression of NOS-2 by astrocytes in the ONH of POAG patients or in the ONH of rats with chronic ocular hypertension. Moreover, they documented no protection of RGCs by aminoguanidine.

#### 6.4.1 Aminoguanidine

*Activity*: Aminoguanidine, when administered orally to rats (2 g/L), afforded protection to RGCs in the episcleral vein cauterised model of chronic hypertension.[120] However, when administered in an identical fashion to rats injected with hypertonic saline to elevate IOP, it yielded no protection.[86]

*Mode of action*: Aminoguanidine is a relatively potent inhibitor of NOS-2 (the concentration that produced 50% inhibition [$IC_{50}$] was 31 μmol/L), but it is also an inhibitor of NOS-1, with an affinity less than one order of magnitude lower. The drug is not specific for NOS; it is also a prototype α,β-dicarbonyl scavenging agent that inhibits formation of advanced glycosylation end products ($IC_{50}$ ≈200 μmol/L), and has antioxidant activity – at 100 μmol/L it decreased lipid peroxidation and the formation

of reactive oxygen species in retinal Müller cells (for a review, see Thornalley[186]).

*Penetration*: The concentration of aminoguanidine in the retina following oral administration of 1 g/L to rats has been measured at approximately 50 μmol/L;[187] the concentration in the studies highlighted above is likely to be closer to 100 μmol/L, given that twice the dose was used. As such, it is doubtful that aminoguanidine functioned solely as an inhibitor of NOS-2 in the models of chronic hypertension.

#### 6.4.2 Summary

The concept that reactive astrocytes/microglia release NO, which is toxic to ONs is an attractive one for two reasons: firstly, it agrees with the widely accepted viewpoint that the primary insult in POAG occurs at the level of the ONH; secondly, it offers a relatively straightforward means of treatment. However, to date, the evidence is ambiguous. It is not yet clear whether NOS-2 is expressed by cells within the ONH of glaucoma patients, and there is no consensus between the results that have been obtained from two different rodent models of ocular hypertension. As a further complication, truly isoform-specific NOS inhibitors are not yet available, which is problematic when all three isoforms of NOS are found in various cell types in the retina and at least two of the isoforms are present in the ONH region. Since there is no agreement as to which of the rodent models for glaucoma most closely simulates POAG, the most pressing need is for the role of NOS-2 to be explored in other laboratory paradigms of glaucoma, such as the lasered model of chronic hypertension.

### 6.5 Voltage-Gated Sodium Channels

The rationale for using $Na^+$ channel antagonists as therapeutic agents in glaucoma stems from the knowledge that preventing $Na^+$ influx through voltage-gated $Na^+$ channels is an effective means of inhibiting axonal degeneration and preserving function in the CNS during periods of physiological stress, irrespective of whether the stress is caused by limited oxygen and nutrient supply, mechanical/compression injury or NO toxicity.[153,188] Although there is little direct evidence that excessive influx of $Na^+$ into ON axons contributes to the death of RGCs in glaucoma, the hypothesis is logical for the following reasons:

- each of the physiological stresses referred to above is implicated in the pathogenesis of POAG;
- the ONH is particularly vulnerable to insults involving $Na^+$ influx;
- experimental studies show that $Na^+$ channel blockers can protect the ON from various insults.

The evidence for the involvement of ischaemia, mechanical compression and NO in the pathogenesis of POAG is discussed earlier (sections 3.1, 3.2 and 6.4), but the latter two statements are worth expanding upon.

The ONH is particularly vulnerable to injuries involving $Na^+$ influx because of the very high density of $Na^+$ channels, and in particular the $Na_V$ 1.6 subtype, which is present in the unmyelinated prelaminar and laminar ON.[179] $Na_V$ 1.6, which produces a persistent, use-dependent, non-inactivating sodium current, is probably important for propagation and maintenance of action potentials;[189] however, in pathological situations, these same properties are thought to trigger an uncontrolled influx of $Na^+$ into the axoplasm, causing reversal of the $Na^+$-$Ca^{2+}$ exchanger, reversal of glutamate transport and, ultimately, irreversible $Ca^{2+}$-mediated axonal injury.[153]

In the isolated ON, $Na^+$ channel antagonists have been demonstrated to preserve axonal function against injury caused by NO,[188] anoxia,[190,191] and the more damaging combination of oxygen and glucose deprivation.[192] Blockade of voltage-gated $Na^+$ channels has also been shown, *in vivo*, to protect against degeneration of the ON in models of inflammation,[193] ON compression and in a chronic hypertensive glaucoma model (see section 6.5.1).

#### 6.5.1 Phenytoin

Phenytoin (5,5-diphenylhydantoin) is an effective and commonly prescribed anticonvulsant used for the treatment of epilepsy.

*Activity*: Systemic administration of phenytoin reduced RGC death following partial ON crush[149] and in the episcleral vein cauterised model of chronic hypertension.[121] Phenytoin, at concentrations lower than those used clinically for the treatment of

epilepsy, has also been shown to protect the isolated rat ON from anoxia-induced injury.[194]

*Mode of action*: Phenytoin binds to voltage-gated $Na^+$ channels in the neuronal cell membrane and inhibits $Na^+$ influx. It is especially effective in reducing neuronal excitability in neurons undergoing repetitive firing. In addition to its well documented effects on $Na^+$ ion conductance, phenytoin has also been shown to interact with $Ca^{2+}$ channels, ionotropic glutamate receptors, sigma binding sites and glatiramer acetate$BA_A$ receptors.[195]

*Penetration*: Systemic administration of phenytoin to rats has been shown to affect the a- and b-waves of the electroretinogram,[196] indicating that the drug crosses the blood-retinal barrier, but the exact concentrations reached after short- or long-term administration are not known.

*Clinical trials*: In a small-scale, short-term study of glaucoma patients, oral phenytoin produced a beneficial effect on visual fields.[197]

#### 6.5.2 Summary

The involvement of voltage-gated $Na^+$ channels in the pathogenesis of acute ON injury, such as that caused by ischaemia, mechanical trauma and inflammation, is unequivocal; their involvement in the chronic optic neuropathy of POAG is at present uncertain. One of the difficulties of using $Na^+$ channel antagonists as neuroprotectants in POAG is finding a dose of drug that will reduce persistent, damaging $Na^+$ currents but have no effect on normal ion conductance. Phenytoin is considered to be one such drug, yet systemic administration of clinical doses to rats does lead to electrophysiological changes in the visual system,[196] which may be related to its effects on other transmitter systems. In contrast, flunarizine and the β-adrenoceptor antagonist betaxolol (see section 7.2 for more detail), which both inhibit $Na^+$ influx, have no adverse effects on the visual system, although whether either drug reaches the ONH at a sufficient concentration to reduce $Na^+$ influx is unknown.

### 6.6 Voltage-Gated Calcium Channels

Calcium overload plays a central role in ischaemia- and excitotoxicity-induced death of white and grey matter, but the mechanisms by which $Ca^{2+}$ is elevated in white and grey matter differ markedly. This is exemplified by the retina/ON. In the retina, drugs that inhibit $Ca^{2+}$ entry into cells through voltage-gated $Ca^{2+}$ channels (termed $Ca^{2+}$ channel antagonists) attenuate RGC death caused by ischaemia and excitotoxicity.[73] Conversely, in the ON, $Ca^{2+}$ channel antagonists are relatively ineffective against anoxic or ischaemic injuries; reversal of the $Na^+$/$Ca^{2+}$ exchanger rather than activation of voltage-gated $Ca^{2+}$ channels is thought to be the route for $Ca^{2+}$ entry into the axoplasm.[153] Therefore, it has been hypothesised that $Ca^{2+}$ channel antagonists would be effective as neuroprotectants in glaucoma if ionic fluxes in RGC bodies/dendrites are adversely affected, for example by hypoxic/ischaemic episodes or by elevated glutamate, but not if RGCs die only as a consequence of axonal injury.[198]

*Activity*: To date, the role of retinal $Ca^{2+}$ influx and the potential effectiveness of $Ca^{2+}$ channel antagonists in chronic hypertensive models of RGC death have not been explored, although the results of these studies would not be straightforward to interpret since $Ca^{2+}$ channel antagonists reduce IOP[199-202] and increase ONH blood flow[203,204] in laboratory animals.

*Clinical trials*: A number of studies have examined the effects of $Ca^{2+}$ channel antagonists on ONH perfusion and visual parameters. The combined results suggest that some members of this class of drugs may increase ONH blood flow and improve vision in normotensive glaucoma patients.[13,205] Because of their effects on blood flow, the mechanism of action of $Ca^{2+}$ channel antagonists in normotensive glaucoma is thought to be primarily due to vasoprotection rather than neuroprotection. However, all of the studies performed thus far are short-term, and there is a real need for long-term clinical outcome data from randomised, placebo-controlled studies.

### 6.7 Oxidative Stress

It is hypothesised that oxidative stress plays a role in RGC death in POAG by causing damage at three discrete sites in the eye: the trabecular meshwork, the ONH and the retina. The involvement of oxidative stress in trabecular meshwork pathophysiology and obstruction of aqueous outflow is beyond the scope of this review and readers are directed to recent articles by Veach[206] and Izotti et al.[207] The

current review is concerned with the evidence supporting a role for oxidative stress in ON and RGC pathophysiology, and the ability of antioxidants to act as neuroprotectants to these tissues. As stated earlier (section 3.2), free radicals produced by activated astrocytes and by mitochondria within the ON, perhaps following ischaemia-reperfusion, are thought to contribute to extracellular matrix remodelling and atrophy in the lamina cribrosa, while in the retina free radicals are implicated in the death of RGCs caused by toxic mediators such as glutamate.

Because of the difficulties in analysing the ON or retina from POAG patients, direct evidence for an involvement of oxidative stress in ON atrophy and RGC death is sparse; however, there is an increasing body of indirect evidence that does support the hypothesis. The first strand of evidence comes from studies that have shown POAG patients to have reduced antioxidant defences. Birich et al.[208] demonstrated that patients with POAG exhibited a higher concentration of serum lipid peroxidation products, Ferreira et al.[209] conducted a prospective case control study and reported a lower total antioxidant capacity in aqueous humour from POAG patients than from controls, while Gherghel et al.[210] measured total and reduced levels of the water-soluble antioxidant glutathione in the blood of untreated POAG patients and controls, and revealed that glaucoma patients exhibited lower levels of circulating glutathione. These studies suggest a general compromise of antioxidant defences in POAG patients, but which is not specific to the ON or retina.

The second strand of evidence concerns the detection of NOS-2 and the 27 kDa HSP in the ONH of glaucomatous eyes, and the elevated levels of iron-regulating genes in the retina of POAG patients. NOS-2, which is expressed by ONH astrocytes in glaucomatous eyes but is not present in healthy eyes,[89] is a source of the free radical NO. Similarly, the redox chaperone HSP27, which serves as a cytoplasmic 'antioxidant',[211] is present at higher levels in the ONH of glaucoma patients than healthy individuals.[91] Transferrin, ceruloplasmin and ferritin regulate the availability of active iron as part of the antioxidant system and increased levels of these proteins are indicative of oxidative stress; all three proteins have been detected at elevated levels in glaucomatous retinas relative to controls.[212]

The third line of evidence comes from laboratory models of glaucoma. In both the episcleral vein cauterisation and the hyaluronic models of chronic hypertension, significant increases in the levels of reactive oxygen species and lipid peroxides have been detected in hypertensive eyes together with changes in the activities of antioxidant enzymes.[213,214]

#### 6.7.1 α-Tocopherol

α-Tocopherol (vitamin E) is quantitatively the major lipid-soluble antioxidant present in cell and mitochondrial membranes of the CNS and is present in high amounts within the rod photoreceptor outer segments and the retinal pigment epithelium. It acts as a powerful chain-breaking agent by scavenging peroxyl radicals.

*Activity*: α-Tocopherol is an effective neuroprotectant in models of retinal ischaemia/reperfusion,[215,216] but has not, to date, been tested in the chronic hypertensive models.

*Clinical trials*: In a short-term, nonblind, uncontrolled study, glaucoma patients receiving α-tocopherol had a reduced concentration of serum lipid peroxidation products and an expanded visual field,[217] while in a separate study, glaucoma patients taking a complex containing α-tocopherol for 3 months displayed improved visual fields.[218] Conversely, the results of a prospective, large-scale, long-term, epidemiological study that analysed the relationship between dietary antioxidant intake and POAG found little evidence that intake of α-tocopherol reduced the risk of developing POAG.[219]

#### 6.7.2 *Ginkgo biloba*

EGb 761 is a standardised extract of the leaves of the Chinese tree *Ginkgo biloba*, which improves blood flow in the whole body, including the eye,[220] and which has been suggested to be beneficial in the treatment of mild to moderate dementia. EGb 761 contains two major groups of substances, the flavone glycosides and the terpene lactones, with >60 bioactive compounds in total.

*Activity*: EGB 761 has been demonstrated to protect the retina against ischaemia/reperfusion injury induced by transient ligature of the central retinal artery[221,222] and acute elevation of IOP.[223] In addi-

tion, pre-and post-treatment of rats with EGb 761 increased survival of RGCs in the episcleral vein cauterised model of chronic hypertension.[122] In combination with other herbs, EGB 761 enhanced survival of RGCs in a model of ON injury.[224] In retinal cultures, EGB 761 afforded protection against glutamate neurotoxicity.[225]

*Mode of action*: In addition to its well documented vasotropic effect, the constituents of EGB 761 have been shown to have multiple cellular effects, which may account for the neuroprotective properties of the extract. EGB 761 is a potent antioxidant that has been shown to scavenge free radicals and to inhibit lipid peroxidation in the rat retina.[226-228] It is also a potent inhibitor of platelet-activating factor, and has been shown to interfere with the glutamatergic NMDA receptor, to inhibit NOS activity and to stabilise the redox state of cells.[229] Other as yet incompletely characterised mechanisms may also be involved.

*Penetration*: EGB 761 is taken orally, but information regarding the subsequent concentrations of its constituents in the retina is lacking. Several pharmacokinetic studies have been performed on EGb 761 and results indicate that extensive metabolism takes place following oral intake with bioavailability reaching <35%;[230] thus, improved formulations are probably needed.

*Clinical trials*: In a small-scale, prospective, randomised, double-blind, crossover trial, EGb 761 administration effected an improvement in pre-existing visual field damage in some individuals with normal tension glaucoma.[231]

#### 6.7.3 Other Free Radical Scavengers

Aside from EGB 761, only one other study has assessed the effect of a free radical scavenger in a rat model of chronic hypertension. Ko et al.[63] showed that systemic administration of the nonspecific free radical scavenger *N-tert*-butyl-(2-sulfophenyl)-nitrone in the episcleral vein cauterisation model did not significantly improve survival of RGCs, although it enhanced the protection afforded by BDNF.

#### 6.7.4 Summary

Free radical scavengers have proved effective retinal neuroprotectants in laboratory models of ischaemia/reperfusion and excitotoxicity; however, the evidence to date in favour of their effectiveness in laboratory models of glaucoma, or more importantly in POAG patients, is at best tentative. Although recent studies have documented increased oxidative stress in the ONH and retina, only two free radical scavengers have been tested in models of chronic hypertension, and the beneficial effects of EGB 761 on RGC survival may not be due to its antioxidant properties. More basic and clinical research is needed into the role of oxidative stress in glaucoma.

### 6.8 Heat Shock Proteins

HSPs are ubiquitous, highly conserved molecular chaperones. They have multiple functions under normal conditions, such as facilitating proper assembly and intracellular folding of nascent polypeptides, and regulating protein degradation. HSPs are classified by their molecular weights (in kDa), comprising five main families: HSP110, HSP90, HSP70, HSP60 and the small HSPs. Induction of HSP expression occurs in response to a variety of stress situations, where it is believed to play an important role in the re-establishment of normal tissue function and in providing tolerance against further trauma. HSP70, often referred to as HSP72 owing to its apparent molecular weight, and HSP27, a small HSP, are of particular interest in the CNS because of their highly inducible nature in glia and neurons, and the established connection between their induction and subsequent neuroprotection from various injuries.[232,233]

Recent studies have shown increased expression of HSPs in the eyes of glaucoma patients and animals with chronic hypertension. In glaucomatous human eyes, Tezel et al.[91] found increased immunostaining for HSP60 and HSP27 in RGCs and the nerve fibre layer, together with increased labelling for HSP27 in astrocytes of the lamina cribrosa. A rationale for the latter finding was provided by the results of a study that showed cultured human ONH astrocytes exposed to elevated hydrostatic pressure increased their expression of HSP27.[234] In monkeys with laser-induced glaucoma, Sakai et al.[93] showed markedly enhanced labelling for HSP90, HSP60 and HSP27 in the RGC and nerve fibre layers, and moderately increased staining intensity for HSP70. In rats with chronic hypertension,

a robust and sustained increase in HSP27 was noted in RGCs,[92] but only a moderate and transient increase in HSP70.[95]

Increased expression of HSPs in the glaucomatous retina and ONH might indicate that these proteins form part of an endogenous defence mechanism in response to trauma. Support for this idea has been provided by the studies of Caprioli and colleagues[95,123] who demonstrated that prior augmentation of HSP70 expression by RGCs, achieved by heat stress or pharmacological intervention, protected against RGC loss in rats caused by long-term elevation of IOP (see section 6.8.1). Since HSP27 is strongly upregulated in glaucomatous retinas, it would be interesting to determine if RGC loss in animals with chronic hypertension is accelerated in animals lacking the ability to synthesise this HSP.

#### 6.8.1 Geranylgeranylacetone

Geranylgeranylacetone (GGA) is an anti-ulcer drug developed in Japan, which is used clinically for the treatment of gastric disorders. GGA has been shown to induce HSP70 synthesis in multiple tissues and to confer protection from various types of toxicity.

*Activity*: Repeated, systemic administration of GGA (200 mg/kg) reduced RGC loss and ON damage in the laser-induced model of chronic hypertension.[123]

*Mode of action*: The authors demonstrated increased expression of HSP70 in RGCs after administration of GGA. The effects of GGA on HSP70 expression and RGC survival were prevented by inclusion of a recognised inhibitor of HSP expression, quercetin.

*Clinical trials*: GGA has not been tested in glaucoma patients. The compound is widely used as an oral antiulcer drug and has extremely low toxicity; thus, a clinical trial warrants consideration.

#### 6.8.2 Summary

Overexpression of HSPs has been shown to afford protection to RGCs from injury caused by ischaemia-reperfusion, excitotoxicity, ON transection and experimental glaucoma, and pharmacological manipulation of HSP expression is an attractive therapeutic approach for use in glaucoma. Nevertheless, there are certain issues that need to be addressed if such a strategy is to be successful. Perhaps the issue of most importance is the possibility that HSPs may contribute to, rather than retard, the progression of glaucoma. HSPs possess a number of immunological activities and their enhanced expression in RGCs of glaucomatous eyes may be a signal to the immune system of the location of stressed cells that need to be removed. The idea that HSPs may be immune targets was proposed by Wax and colleagues[38] and arose from their findings of increased titres of HSP autoantibodies in the blood of glaucoma patients.[235] Other issues to be addressed include delivery of HSPs to RGCs, and whether single or multiple HSPs should be upregulated in the tissue. There are many ways to increase HSP levels in a tissue, including physiological, pharmacological and genetic induction of HSP expression, and direct administration of the proteins. Treatment for a chronic, neurodegenerative disease such as glaucoma based on HSP induction will require a suitable delivery mechanism that takes into consideration the site of damage, the time course of the injury and the specificity of the response desired. Although studies to date have tended to concentrate on HSP70, evidence from the CNS suggests that HSP27 may have an equally potent and more wide-ranging protective effect.[232] With regard to the retina, Kretz et al.[236] recently showed that overexpression of HSP27 enhanced RGC survival following ON transection.

### 6.9 Retinal Ganglion Cell Death and Apoptosis

Apoptosis has been described as the opposite of necrosis (accidental, unregulated cell death) and is a genetically regulated cellular death process reliant upon a distinct temporal sequence of events such as mobilisation and post-translational activation of enzymes (see figure 1 and the excellent review by Hengartner[237] for a summary of the basic pathways). During apoptosis, cellular constituents are internally degraded meaning that there is condensation and shrinkage of the nucleus and cytoplasm, but no associated tissue inflammation.

#### 6.9.1 Evidence for Apoptosis in Glaucoma

Studies conducted over the last decade have suggested that apoptosis plays a major role in the process of RGC death. Cells dying by apoptosis were initially identified in the axotomised rat retina[58,62]

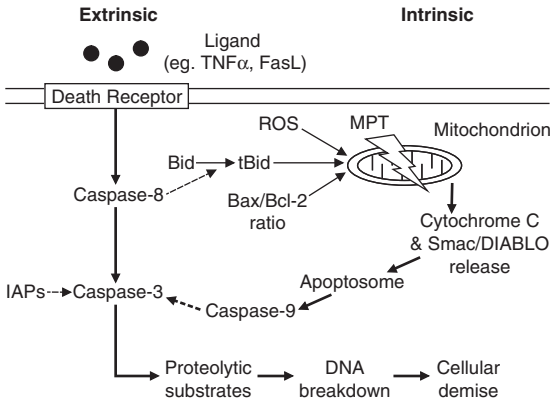


**Fig. 1.** Simplified scheme of events underlying the process of apoptotic cell death. Two major pathways have been described: the intrinsic pathway and the extrinsic pathway. The extrinsic pathway is stimulated by either the presence or absence of ligand-binding at cell surface receptors (e.g. FasR, tumour necrosis factor-αR [TNFαR]). This leads to the aggregation and activation of caspase-8 proforms and subsequent proteolytic cleavage and activation of caspase-3, which is instrumental in the dismantling of cellular structure and machinery. The intrinsic route to cell death, on the other hand, is stimulated by unknown events and results in a homeostatic imbalance in mitochondrial function, leading to mitochondrial permeability transition, and a release into the cytoplasm of apoptotic inducer proteins such as cytochrome C and Smac/DIABLO. This process also culminates in the activation of caspase-3. The roles of other caspase family members remain unclear. It is also likely that the cellular balance between pro-apoptotic Bax and anti-apoptotic Bcl-2, the cleavage by caspase-8 of pro-apoptotic Bid to truncated Bid (tBid) and the local levels of reactive oxygen species (ROS) contribute to stimulation of the mitochondrial-mediated intrinsic pathway. Major pathways to cell death are shown with thick arrows, speculative pathways in thin arrows and activation events by dotted arrows. **IAP** = inhibitor of apoptosis protein; **MTP** = mitochondrial permeability transition.

and in a monkey model of chronic IOP.[238] Subsequent studies have confirmed the occurrence of apoptotic RGC death after axotomy,[57,239-242] and similarly shown it to be a feature of central retinal artery occlusion,[243] excitotoxicity[244,245] and retinal ischaemia.[246,247]

Apoptotic RGC death has been observed in rats with chronic ocular hypertension,[58-60,248-253] as well as in quail with a glaucoma-like mutation.[134] Furthermore, the quantities of TUNEL-positive cells in the RGC layer of human eyes with both POAG and secondary glaucoma have been shown to be significantly elevated with respect to control retinas.[32,254] Apoptotic cells have also been detected by visualisation of apoptotic chromatin condensation in eyes from patients diagnosed with normal pressure glaucoma[255] and anterior ischaemic optic neuropathy.[256]

#### *6.9.2 Apoptotic Pathways in Glaucoma*

Over recent years, intensive research has delineated many of the crucial signalling pathways involved in the process of apoptosis; figure 1 provides a summary of the major events that have been described in relation to RGC death in glaucoma models. It has become apparent that it is important to distinguish between the extrinsic, or receptor-activated pathways, and the intrinsic or mitochondrial-mediated events. Although triggers for the extrinsic pathway (e.g. TNFα, FasL, TNF-related apoptosis-inducing ligand [TRAIL]) have been well described and understood, the relationship between intracellular signals and mitochondria is not so clear.[237] For example, it is apparent that the cellular balance between levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 (and related family members) as well as the local availability of reactive oxygen species (ROS) play central roles in determining how and when mitochondria become stimulated to release their contents, initiating formation of the intrinsic pathway apoptosome (figure 1). However, the basis for mitochondrial opening, via the mitochondrial permeability transition (MPT), is unknown. Furthermore, there is an apparent redundancy in caspase-mediated substrate proteolysis, with many caspase enzymes appearing to perform very similar tasks. Regardless of these caveats, however, it is clear that there are certain key signalling mediators involved in RGC apoptosis (Bax/Bad, Bcl-2/Bcl-$X_L$, p53, caspases-1, 3, 8, 9, Bid and PKB/Akt) and these elements have been subject to increasing levels of scrutiny in order to determine their exact roles in the process.

A large volume of research information has been generated over the last decade concerning the involvement of intracellular mediators in experimental RGC death induced in models such as ON axotomy or acute elevation of IOP. However, information regarding the pathways involved in RGC death in experimental models of chronic IOP elevation is more limited. Activation of caspases contributes to cellular disassembly as a result of chronic ocular hypertension, with procaspases-3, 8 and 9 cleaved to

yield their active configurations.[250,257-260] Furthermore, caspase-3-mediated proteolytic degradation of amyloid precursor protein (APP)[258] and α-fodrin[125] increases in rodent models of chronic ocular hypertension. There are also transient elevations in RGC expression of both Bax and Bcl-2, which almost completely revert to pre-hypertension levels after 2 weeks of IOP elevation.[259] Bax is also involved in RGC death in the DBA/2J mouse model,[261] as are increased dephosphorylation of pro-apoptotic Bad and cytoplasmic elevation of cytochrome C, both known to be associated with the induction of apoptosis.[124] Bax is further elevated, along with Bak, p53, Fas, ICE and caspase-3 in RGCs[262] in the mouse episcleral vein photocoagulation model[263] and this is concurrent with a decrease in Bcl-2 expression.[262] Moreover, expression of *p53* gene family members *Gadd45a* and *Ei24* is also stimulated in RGC apoptosis after laser-induced chronic ocular hypertension in rats,[264] although interestingly, this study also showed that anti-apoptotic gene expression of the caspase inhibitor *IAP-1* is simultaneously upregulated, perhaps as part of an endogenous neuroprotective mechanism.[264] Finally, the PKB/Akt signalling pathway has been shown to have an involvement in RGC death in the episcleral vein model. PKB/Akt phosphorylation leads to its activation and this enzyme can subsequently promote cell survival by deactivating enzymes such as Bad, glycogen synthase kinase, caspase-3 and caspase-9, and influencing MAP kinase signalling pathways. Significantly, however, PKB/Akt undergoes dephosphorylation after episcleral vein cauterisation in the rat and this may prove to be a crucial early event in the initiation of RGC death in this model.[250,265]

Two things are apparent from the studies mentioned in the previous paragraph. First, pro-apoptotic cellular mediators (e.g. Bax, caspase-3) are *always* shown to be activated/upregulated in chronic ocular hypertension models. Secondly, the role of anti-apoptotic components in relevant signalling pathways is not clear. In some cases, for example, Bcl-2 is shown to be decreased,[262] which would be consistent with a downregulation event preceding initiation of death signalling. In other cases, Bcl-2 appears to be upregulated,[259] and this may indicate a role for promotion of survival of some cells and not others. Indeed, the role of anti-apoptotic signalling requires further investigation because knowledge of the function that these factors play in cell death may allow their manipulation in order to promote RGC survival.

#### 6.9.3 Pharmacological Intervention in Apoptosis

Specific biomolecular triggers for apoptotic RGC death in glaucoma remain elusive; suggestions have included reduced essential trophic factor[30,36] or cytokine[266] supply to neurons, alterations in intracellular levels of calcium or reactive oxygen intermediates,[267] or increased extracellular levels of certain neurotransmitters and neuromodulators.[40] It follows that interventions such as trophic factor delivery, excitatory receptor antagonists and antioxidants should prevent either the initiation or the enactment of apoptotic pathways, thereby offering some degree of protection in glaucoma. These treatment modalities do indeed offer some protection to RGCs in animal models of glaucoma, but relatively little mechanistic information has been uncovered regarding whether they interact directly with specific elements of apoptotic pathways.

Direct manipulation of intracellular apoptotic pathways in RGCs, for example by gene therapy or by pharmacological intervention using pro-apoptotic enzyme modulators, offers a potential way in which glaucoma may be treated in the future. To date, the majority of ocular studies that have described pharmacological anti-apoptotic strategies have fallen into two categories. The first approach has been to manipulate the cellular levels of proteins involved in apoptotic pathways. Administration of the $\alpha_2$-adrenoceptor antagonist brimonidine (see section 7.1), for example, which protects RGCs in ocular hypertensive models, has been shown to activate the anti-apoptotic ERK and Akt pathways, thereby increasing the synthesis of Bcl-2 and Bcl-$X_L$.[268] In cultured ganglion cells, lithium pretreatment also resulted in an upregulation of Bcl-2, and this was characterised to promote both cell survival and axon regeneration.[269] Furthermore, treatment of ocular hypertensive rats with a calcineurin inhibitor prevented Bad (a proapoptotic molecule of the Bcl-2 family) dephosphorylation and cytochrome C release and this resulted in a marked increase in RGC survival.[124]

The second approach has been to utilise caspase inhibitors to prevent RGC degradation; this has been the more widely utilised strategy. Intraocular injection of caspase-3 inhibitors has been demonstrated to increase the survival of RGCs after ON transection,[240,270-273] high IOP-induced ischaemia,[247] and ligature of the ophthalmic vessels.[272] Blocking caspase-3 also prevented glutamate-induced RGC death in retinal explants.[274] In rats with chronic ocular hypertension, inhibition of caspase-3 has been demonstrated to protect RGCs and to prevent cleavage of the neuronal cytoskeletal protein α-fodrin, a known target for caspase-3.[125,126]

Caspase-3 is a pivotal and relatively late-stage component of multiple apoptotic cascades; hence its involvement in RGC death is to be expected. To date, the effectiveness of other classes of caspase inhibitors at preventing RGC death in ocular models of hypertension has not been investigated. Some success would be anticipated for two reasons: firstly, that several cell death pathways appear to be activated, and proapoptotic genes upregulated, in retinas with experimental glaucoma;[250,260,264] and secondly, that inhibitors of other caspases have proved efficacious in alternative models of RGC injury. For example, inhibition of caspase-1 protected RGCs from NMDA-[244,275] and ischaemia-induced[247] apoptotic death, while caspase-8 inhibition aided survival of RGCs, *in vitro*, from simulated ischaemia, NMDA or HSP27 antibody application,[94] as well as preventing RGC death, *in vivo*, as a result of axotomy.[276]

#### 6.9.4 Summary

The strong protective effects of such different caspase inhibitors indicate that different intracellular pathways are likely to contribute to RGC death. However, the actual pathways involved in the death of RGCs in glaucoma remain uncertain, meaning that, at present, it is impossible to comment on the validity of such experimental data for treatment of patients in the clinic. Moreover, the majority of investigations targeting the effect of caspase inhibition on paradigms of RGC death have employed intravitreal injections to apply the compounds. Obviously, in the case of human glaucoma this is unlikely to offer a valid therapeutic approach. Therefore, the potential of using caspase inhibitors for human glaucoma treatment is, at present, also likely to be limited by potential pharmacokinetic constraints. Orally tolerated caspase inhibitor peptides have now been produced; for example, the Aventis compound pralnacasan (VX-740), which is a caspase-1 inhibitor that has recently been entered into a phase II clinical trial for patients with rheumatoid arthritis and other inflammatory diseases.[277,278] More recently still, a series of oxamyl dipeptide compounds have been generated that have been optimised for pan-caspase inhibition, anti-apoptotic cellular activity and *in vivo* efficacy.[279-281] On the basis of *in vitro* and *in vivo* experiments in murine models of liver injury, one inhibitor PF-03491390 [IDN-6556 (1)] has also been selected for two phase II clinical trials, evaluating its safety and efficacy for use in liver disease.[282,283] Therefore, the potential exists to apply such compounds to the retina in the treatment of glaucoma.

In his excellent recent review, Nickells[284] described the need for caution in interpretation and long-term significance of apoptotic findings in relation to glaucomatous RGC death. He astutely outlined that a number of points should be borne in mind when assessing the effects of anti-apoptotic strategies: first, that some events (e.g. mitochondrial dysfunction) are downstream in the overall apoptotic scheme and thus preventing these will not necessarily protect cells; second, that multiple pathways to cell death exist and shutting one or more of these down may, therefore, only lead to a circumvention of these specific processes; and third, that prevention of RGC apoptosis may leave behind a live but non-functioning cell. If these points are viewed in conjunction with the fact that not all RGC death is likely to be via classically defined apoptosis, then a note of caution must be applied to the possible anti-apoptosis strategy of treating RGC death in glaucoma.

## 7. Neuroprotective Properties of Drugs Already Used as Ocular Hypotensives

In recent years, a significant body of work has been conducted on the abilities of a range of drugs already used clinically as ocular hypotensives to act as retinal neuroprotectants. It is of course well documented that the best method of preventing RGC death in glaucoma or laboratory models of glaucoma

is to reduce the IOP; however, the concept of neuroprotection embraces the idea that a drug can protect RGCs independent of a reduction in IOP. If such a quality were to be demonstrated for a drug, this would enable it to be administered alongside a hypotensive agent to provide additional protection. The difficulty in determining the neuroprotective potential of drugs currently prescribed for the treatment of glaucoma in animal models of glaucoma is that the drugs are likely to lower IOP in the chronic hypertensive models, which makes it almost impossible to delineate whether any beneficial effect on RGC survival occurs via mechanisms that are independent of IOP. Bearing this in mind, in this review of the neuroprotective properties of hypotensive agents, particular attention has been paid to other models of RGC death, such as ischaemia/reperfusion, excitotoxicity and ON injury. As discussed previously (section 4), all of these paradigms of injury show some similarities to glaucoma, and information gained from them is, therefore, likely to have some relevance to the disease.

## 7.1 $\alpha_2$-Adrenoceptor Antagonists

### 7.1.1 Brimonidine

Brimonidine (Alphagan®)[1] is a highly selective $\alpha_2$-adrenoceptor agonist, which lowers IOP primarily by decreasing aqueous humour inflow. The evidence in favour of a neuroprotective action of brimonidine in the laboratory is convincing and has been covered in detail in a recent review article.[14] Nevertheless, the data are worth summarising as the thoroughness of the work performed using this compound sets a benchmark for other drugs.

*Activity*: Systemic and topical administration of brimonidine resulted in protective effects in various acute and chronic rodent paradigms of RGC death, including ischaemia/reperfusion induced by acute elevation of IOP,[285-287] ischaemia/reperfusion induced by transient ligature of the ophthalmic vessels,[288-291] mechanical injury to the ON,[292] and in the lasered[104] and episcleral vein cauterised[127] rat models of chronic ocular hypertension. Brimonidine also increased survival of retinal neurons in culture following exposure to the excitotoxin glutamate.[293]

*Target*: $\alpha_2$-Adrenoceptors have been identified in the ganglion cell layer of the retina,[107,294] and the protective effects of brimonidine in all of the models of injury are likely to be mediated via activation of these receptors since they can be blocked by co-administration of $\alpha_2$-adrenoceptor antagonists. Furthermore, indirect evidence for a receptor-mediated neuroprotective mode of action is provided by the knowledge that other $\alpha_2$-adrenoceptor agonists, such as clonidine and apraclonidine, are also neuroprotective in models of RGC death.[295,296]

*Mode of action*: Activation of $\alpha_2$-adrenoceptors provides neuroprotection to RGCs via complex but seemingly independent mechanisms. Evidence is accumulating that $\alpha_2$-adrenoceptor activation leads to inhibition of pro-apoptotic pathways (see review by Tatton et al.[268]), release of trophic factors, including BDNF[297] and basic fibroblast growth factor,[287] and inhibition of ischaemia-induced glutamate release.[286]

*Penetration*: Following multiple, topical application of brimonidine 0.2% in patients for up to 2 weeks, the mean vitreous concentration of drug was determined to be 185 nmol/L, as measured by gas chromatography.[298] Following multiple, topical administration of [$^{14}$C]brimonidine 0.2% in rabbits and monkeys, non-metabolised radioactivity concentrations of 82 and 170 nmol/L, respectively, were detected in the vitreous humour.[299] In the same study, the choroid/retina and ONH were found to contain even higher amounts of intact radioactivity. All of these levels of brimonidine are substantially higher than necessary to cause full activation of $\alpha_2$-adrenoceptors.

*Clinical trials*: Brimonidine has recently been shown to improve contrast sensitivity in glaucoma patients,[300] and the weight of evidence in favour of brimonidine as a potential neuroprotective agent has led to a large, multicentre, prospective randomised study comparing the effect of brimonidine to timolol on visual field changes in patients with normal tension glaucoma.[301]

**1** The use of trade names is for product identification purposes only and does not imply endorsement.

## 7.2 β-Adrenoceptor Antagonists (β-Blockers)

### 7.2.1 Betaxolol

Betaxolol (Betoptic®, Betoptic S®) is a relatively selective $\beta_1$-adrenoceptor antagonist (β-blocker), which is nevertheless considered to lower IOP via inhibiting $\beta_2$-adrenoceptors located in the non-pigmented ciliary epithelium, thereby decreasing aqueous humour inflow.

*Activity*: Racemic and levo-betaxolol have been demonstrated to reduce RGC death following ischaemia/reperfusion[79,80,302-306] and excitotoxicity.[303] Neuroprotection was evident irrespective of whether the drug was administered topically, intravitreally or systemically. Betaxolol also dramatically prevented RGC death and ON damage in the hypertonic saline rat model of chronic hypertension;[251] however, since betaxolol reduced IOP significantly in treated eyes, it was not possible to distinguish between any direct neuroprotective effects and the effect due solely to lowering of IOP. *In vitro* studies support the case for a neuroprotective role for betaxolol in the retina, where it has been shown that betaxolol increases survival of retinal neurons in culture following excitotoxic and anoxic insults.[293,305]

*Target*: Unlike brimonidine, the neuroprotective actions of betaxolol are not thought to be classically receptor-mediated. There is sparse evidence for the presence of $\beta_1$-adrenoceptors in retinal/ON tissue, and although the $\beta_2$ subtype has been localised to ON astrocytes,[307] it has not been identified in the retinal layers. Furthermore, studies have shown that betaxolol elicits a vastly superior neuroprotection to timolol or metoprolol,[293,305,308] yet all three compounds have similar affinities for $\beta_1$-adrenoceptors, and timolol a much higher affinity for the $\beta_2$ subtype. Instead, betaxolol is thought to increase RGC survival via direct inhibition of voltage-gated $Ca^{2+}$ and $Na^+$ channels and glutamate currents in the retina/ON (see following paragraph).

*Mode of action*: There is considerable laboratory evidence to indicate that betaxolol can prevent excessive influx of $Ca^{2+}$ and $Na^+$ ions into neurons, which occurs in pathological situations such as hypoxia, ischaemia and excitotoxicity.[15] Electrophysiological experiments have revealed that betaxolol inhibits voltage-gated $Ca^{2+}$ and $Na^+$ currents and glutamate-gated currents in RGCs,[309,310] while biochemical studies have shown the drug to inhibit NMDA- and/or glutamate-stimulated $Ca^{2+}$ influx in whole retina and isolated RGCs,[293,310-312] as well as $Na^+$ influx in rat brain.[313] The inhibitory actions of betaxolol are most likely explained by the fact that the drug binds allosterically to L-type voltage-gated $Ca^{2+}$ channels[314] and voltage-gated $Na^+$ channels;[313] however, it is also possible that betaxolol lowers the affinity of glutamate receptors for glutamate.[312] Betaxolol has also been shown to upregulate expression of trophic factors in the retina[304,315] and this too may play a role in the neuroprotection afforded by the drug.

*Penetration*: Studies in rabbits have shown that a significant portion of betaxolol reaches the retina when administered topically.[303,316] These data indicate that local ocular and systemic routes both play a significant role in delivering betaxolol to the retina. It is difficult to estimate the concentration of betaxolol at the site of action in the retina/ON following topical application because betaxolol is highly lipophilic and will accumulate in membranes. Since the ion channels affected by betaxolol are located in cell membranes, the actual concentration of the drug at the site of action is likely to be noticeably higher than the concentration measured in the vitreous or retina as a whole.

*Clinical trials*: Although no large-scale clinical trials have been conducted, a number of independent, small-scale clinical trials have compared the effect of betaxolol with timolol on visual fields in patients with open-angle glaucoma.[317-325]

The conclusion to be drawn from all of these studies is that betaxolol provides slightly better preservation of the visual field than timolol, despite its less efficacious effect on reducing IOP.

### 7.2.2 Timolol

Timolol (Timoptic®) is a non-selective β-blocker, which, like all β-blockers, lowers IOP by interacting with $\beta_2$-adrenoceptors in the ciliary epithelium causing a reduced secretion of aqueous humour.

*Activity*: Scrutiny of the published literature relating to timolol reveals inconsistent results, wherein some studies show timolol to be moderately neuroprotective, yet others, using similar methodologies and models of injury, find little beneficial

effect. For example, Goto et al.[326] showed that timolol attenuated glutamate toxicity in both mixed retinal and purified RGC cultures; conversely, Baptiste et al.[293] found that timolol had no effect on neuronal survival in mixed retinal cultures treated with glutamate, and Kashiwagi et al.[327] similarly found no effect of timolol on glutamate-mediated toxicity in purified RGC cultures. With regard to models of chronic ocular hypertension, it appears likely that the neuroprotective effectiveness of timolol is related to its ability to lower IOP rather than via any direct action on neuronal/glial components. Systemically administered timolol reduced IOP only slightly and failed to provide any neuroprotection,[104] but topically applied timolol has been shown to reduce IOP efficaciously and counteract RGC loss.[328,329] Timolol was protective in the DBA/2J mouse model of glaucoma,[111] presumably via its ability to lower IOP in affected eyes; similarly in the episcleral vein cauterised model, timolol lowered IOP and caused a partial rescue of RGCs.[330] In an acute model of ischaemia/reperfusion, Wood et al.[305] found topically applied timolol to be only marginally neuroprotective against ischaemia/reperfusion injury, but Goto et al.,[326] who used twice the dose of timolol, found the drug to be more effective. Timolol afforded no protection in an acute model of ON injury.[331] The logical conclusion to be drawn from all of the results is that the compound is a weak neuroprotectant and needs to be used at high doses to exert direct neuroprotective effects. This conclusion is supported by studies that have analysed the mechanisms by which timolol might aid survival of RGCs.

*Mode of action*: Timolol does bind to voltage-gated $Ca^{2+}$ and $Na^{+}$ channels[313,314] and reduce NMDA-stimulated $Ca^{2+}$ influx,[311,312] but with a very much lower affinity than betaxolol. This is manifest from electrophysiological studies where timolol did not display any detectable action on $Ca^{2+}$ and $Na^{+}$ currents.[309,310] At present, other mechanisms of action of timolol are unknown. It does not act as a free radical scavenger[332] and has not been documented to increase expression of trophic/survival factors.

*Penetration*: Timolol reaches the retina in significant amounts after topical application. Tan et al.[333] suggest that absorption of timolol into the systemic circulation plays a significant role in delivering timolol to the retina, while Goto et al.[326] argue that periocular transscleral and/or vitreous routes are more important.

#### 7.2.3 Metipranolol

Metipranolol (OptiPranolol®) is a non-selective β-blocker with an IOP-lowering efficacy similar to that of other non-selective β-blockers. It is less widely prescribed than other β-blockers, primarily because of early reports that it was associated with granulomatous uveitis. Topically applied metipranolol has been shown to partially attenuate the effects of ischaemia/reperfusion induced by acute elevation of IOP in the rat and anoxia-induced neuronal loss in rat retinal cultures,[305] but the drug has not been tested in other paradigms of RGC death.

*Mode of action*: Metipranolol inhibits $Na^{+}$ and $Ca^{2+}$ influx into neurons in the same manner as betaxolol, albeit less potently.[305] Interestingly, metipranolol, uniquely of the ophthalmic β-blockers, has also been shown to act as a potent antioxidant, reducing lipid peroxidation in brain homogenates[332] and protecting the retina against damage caused by oxidants.[334,335] Since free radical formation is implicated in the pathogenesis of glaucoma, it will be of interest to test the effect of metipranolol in other models of RGC death.

*Penetration*: Metipranolol is assumed to reach the posterior segment of the eye in rats since it elicits some protection when applied topically, but no penetration studies have been conducted in rats or humans.

No studies have been conducted to determine the neuroprotective properties of the other commonly prescribed β-blockers carteolol (Ocupress®/Teoptic®) or levobunolol (Betagan®).

### 7.3 Prostaglandin Derivatives

Latanoprost (Xalatan®) and travoprost (Travatan®), bimatoprost (Lumigan®) and unoprostone (Rescula®) are derivatives of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that reduce IOP by enhancing outflow of aqueous humor. Although the drugs are classified as $PGF_{2\alpha}$ analogues, a prostamide and a docosanoid, respectively, they are fundamentally alike in terms of structure and largely with respect to their mechanisms of action. In recent years, this new class of

drugs has replaced the topical β-blockers as the most commonly prescribed first-line treatment for glaucoma. Prostaglandins, including $PGF_{2\alpha}$, are well documented vasoconstrictors; they are also implicated in the pathogenesis of ischaemic and inflammatory injuries and can potentially influence many cell signalling pathways. However, to date, there is no evidence to indicate that the prostaglandin derivatives used to lower IOP are neurotoxic to the retina/ON. In fact, there are very limited data to suggest the reverse is true.

*Activity*: Intravitreal administration of latanoprost to rats has been documented to increase survival of RGCs after NMDA injection and ON transection, by an unknown mechanism.[336] In a different study, systemically administered latanoprost partially prevented the accumulation of lactate induced by retinal ischaemia;[337] unfortunately, no electrophysiological or histological data were recorded. The drug also attenuated glutamate and hypoxia-induced cell death in retinal cultures. The authors hypothesised that latanoprost may exert neuroprotective effects via a negative feedback on cyclo-oxygenase-2 activity.

Unoprostone has also been shown to protect the retina in a model of ischaemia.[338] In this case, the mode of action of the drug is suggested to involve opening of maxi $K^+$ channels.

At present, no information is available with regard to travoprost or bimatoprost.

### 7.4 Carbonic Anhydrase Inhibitors

Dorzolamide (Trusopt®) and brinzolamide (Azopt®) are selective inhibitors of carbonic anhydrase isoenzyme II. Inhibition of this enzyme in cells of the ciliary epithelium causes a reduced formation of aqueous humour and a decrease in IOP.

*Activity*: Some studies have shown that carbonic anhydrase inhibitors increase choroidal and ONH blood flow via mechanisms that are independent of IOP;[113,339] however, little evidence has been presented to date to indicate that these drugs can protect the retina in models of RGC death.

Dorzolamide failed to rescue RGCs in the DBA/2J mouse model of inherited glaucoma[111] and, although the drug did afford protection in rat models of chronic hypertension,[328,329] it also reduced IOP in these eyes; the degree of damage/protection correlated with the level of IOP, indicating that the effect was entirely pressure-related and not caused by direct neuroprotection. Dorzolamide has not been tested in ischaemic, excitotoxic or ON injury models of RGC death.

### 7.5 Summary

Of the drugs that are currently prescribed to treat glaucoma, evidence to date indicates that brimonidine and betaxolol have significant neuroprotective properties. Small-scale clinical trials with betaxolol have shown that the drug performs slightly better than would be expected purely from its ability to lower IOP. The ongoing trial to identify whether brimonidine preserves visual fields more than timolol is of real importance for the future.

## 8. Conclusions

Despite the efforts of researchers to date, the specific site of the primary injury in glaucoma remains uncertain. The vast majority of clinical and experimental evidence indicates that the ONH is the primary pathological site, but there is limited evidence to suggest that RGC bodies may be primarily involved. The site of the primary injury has particular relevance to neuroprotection because different regions of the RGC may respond to different neuroprotective strategies. Indeed, it has been argued that a compartmentalised approach to neuroprotection in glaucoma may be the optimal strategy.[46]

In recent years, much progress has been made in identifying potential pharmacological targets for glaucoma. This process has been aided considerably by the development of rodent models of chronic ocular hypertension, which appear to display many similarities to glaucoma. At present, pharmacological neuroprotection for glaucoma remains a concept rather than a reality. In the not too distant future, however, it is highly likely that administration of neuroprotectants will form an adjunct to IOP-lowering drugs in the treatment of this debilitating disease.

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