ARTICLE

In glaucoma, should enthusiasm about neuroprotection be tempered by the experience obtained in other neurodegenerative disorders?

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Abstract

Some *in vitro* and *in vivo* evidence, as well as rare observations in human eyes with glaucoma, suggests that retinal ganglion cells could be lost by apoptosis during the course of glaucomatous optic neuropathy. There exist also observations indicating that in the vitreous of patients with glaucoma it is possible to measure an increased concentration of glutamate (an excitotoxic amino acid known to induce neuronal apoptosis in animal models). These observations, among others, suggest the possibility of an excitotoxicity mechanism in the pathogenesis of glaucoma and as a consequence the potential for a neuroprotective approach to treating this disorder. Amazingly, not only in glaucoma but also in other neurodegenerative disorders (Parkinson's disease, amyotrophic lateral sclerosis, stroke, etc.) it has been postulated that neurons could be lost through an excitotoxic mechanism. In these non-glaucomatous disorders, quite a large number of clinical trials have already been conducted to determine the potential benefit of different neuroprotective therapies. Unfortunately, with a few rare exceptions, the results of these clinical studies have been very disappointing (in contrast to encouraging results obtained in preclinical trials). The experience acquired in other neurodegenerative disorders should probably be kept in mind when addressing the question of neuroprotection in glaucoma. In particular, the hope raised by preclinical studies showing that drugs could have a beneficial effect on the survival of retinal ganglion cells should certainly be tempered until such an effect is confirmed by clinical trials conducted in patients with glaucoma.

Key words Glaucoma pharmacology, Glaucomatous excavation, Intraocular pressure, Normal tension glaucoma, Optic nerve head, Visual field defects

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Retinal ganglion cell loss in glaucoma

Loss of retinal ganglion cells is a characteristic histological feature of glaucomatous eyes.[1–5] Although in glaucoma no retinal ganglion cell seem to be spared from damage, some reports have suggested that those with an axon of a diameter that is larger than the mean could be lost earlier.[1,2,5]

It is assumed that it is the loss of retinal ganglion cells which is responsible for the irreversible visual field defects that can be observed in patients with glaucoma,[6–9] defects which can eventually lead to blindness.[10] In patients with glaucoma, the natural course of the progression of the irreversible visual field defects is usually slow and irregular with marked inter-individual variability.

A direct correlation between the progression of retinal ganglion cell loss and the progression of visual field defects does not necessarily occur in patients with glaucoma. Indeed, there exist some clinicopathological observations suggesting that the loss of retinal ganglion cells can occur before the appearance of any visual field defects.[1,8] Nevertheless, because a direct assessment of retinal ganglion cell loss can not easily be done in the clinic, campimetry is commonly used as an indirect way of monitoring the progression of retinal ganglion cell loss.

It should also be mentioned that in patients with glaucoma, neurological defects are not necessarily limited to a loss of retinal ganglion cells. Indeed, the existence of an association between glaucoma and neurosensorial

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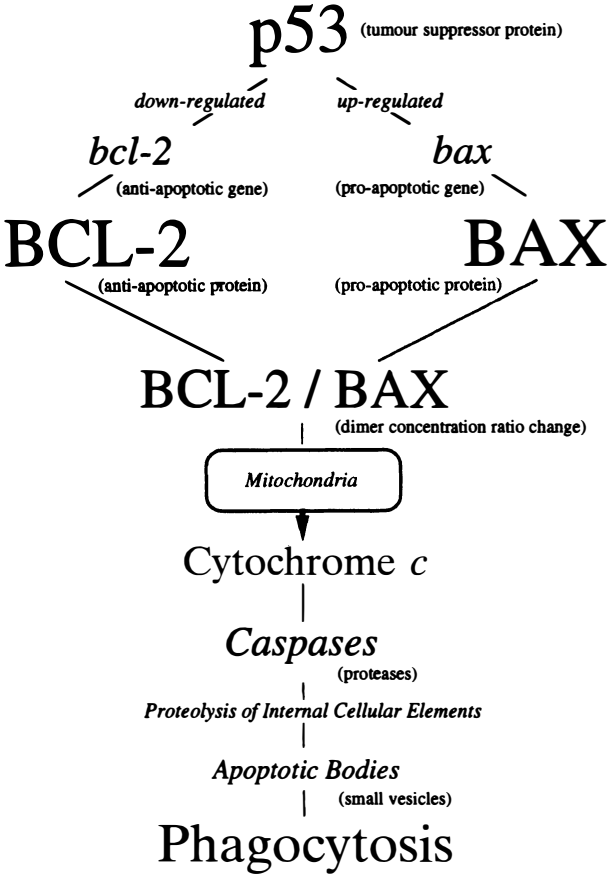

Fig. 1. *Schematic and simplified cascade of events that can lead to cell death by apoptosis. A cell that is stimulated to die by apoptosis will activate the tumour suppressor protein p53. The p53 protein will then up-regulate the pro-apoptotic gene* bax *and down-regulate the anti-apoptotic gene* bcl-2, *leading to an increase in the pro-apoptotic protein BAX and a decrease in the anti-apoptotic protein BCL-2. The change in the intracellular concentration ratio of these proteins will affect the mitochondrial membrane, leading to a release of cytochrome* c. *This will activate a series of proteases called* caspases *that induce proteolysis of the internal elements of the cell. The fragmented cellular elements are stored in small vesicles called apoptotic bodies that are ultimately phagocytosed by surrounding cells or macrophages.*

hypoacousia,[11] or lesions of the central nervous system (detected by magnetic resonance imaging),[12] has been reported.

The progression of the visual field defects observed in patients with glaucoma has been shown to be associated with the presence of different risk factors such as a high intraocular pressure or systemic functional blood flow dysregulation.[13] Because consistent evidence has been provided that a high intraocular pressure is a causative factor for the development of glaucomatous optic neuropathy, the accepted treatment of the progression of glaucomatous damage is nowadays essentially limited to a reduction of the intraocular pressure.

Recently, evidence obtained from *in vitro* and *in vivo* animal models has led to the hypothesis that glaucoma patients could potentially benefit from a neuroprotective therapeutic approach in order to slow down the progression of their retinal ganglion cell loss. The aim of the present article is to provide a brief and simplified overview of some of the current knowledge on the potential of neuroprotection in glaucoma[14–20] and to draw some parallels with other neurodegenerative diseases in which this approach has already been investigated.[21–23]

Retinal ganglion cell loss by apoptosis

Apoptosis is a type of cell death that can occur in all organs during development or in some pathological conditions. It is a form of cell death (often described as a kind of cell 'suicide') that has the characteristics of being controlled by the genes of the cell which is dying, and of involving the activation of a series of well-regulated biochemical reactions.[24,25]

In a nutshell and very schematically (Fig. 1), a cell that has been stimulated to die by apoptosis will often first activate the tumour suppressor protein p53. This protein can in turn modulate the expression of two different genes: the pro-apoptotic gene *bax* (that will be up-regulated) and the anti-apoptotic gene *bcl-2* (that will in contrast be down-regulated). This can lead, on the one hand, to an increase in the intracellular concentration of the protein BAX (which is pro-apoptotic), and, on the other hand, to a decrease in the intracellular concentration of the protein BCL-2 (which is anti-apoptotic). The change in the intracellular concentration ratio of the BCL-2 and BAX proteins can ultimately affect the mitochondrial membrane, leading to release of the electron transporter cytochrome *c*. In turn, cytochrome *c* can activate a series of proteases called caspases that can induce proteolysis of the internal elements of the cell.[24,25]

The degradation of DNA is an early feature of apoptosis.[26] Initially the chromatin structure is still fairly intact and the degradation of DNA occurs between the nucleosides. The DNA that is wrapped around the histone core of each nucleosome will thus be protected from enzymatic degradation. When purified and analysed by gel electrophoresis, the remaining undigested DNA has the appearance of a ladder of fragments in multiples of about 180 base-pair units.

With the progression of the apoptotic process, it can be observed by electron microscopy that the chromatin becomes electron dense, begins to accumulate at the inner surface of the nuclear envelope, and eventually fills the entire nucleus.[24] The cell then begins to break up into smaller membrane-bound fragments. These disintegrated cellular elements are stored in small vesicles called apoptotic bodies, which in turn are usually phagocytosed either by macrophages or, more often, by surrounding cells that are stimulated to act like macrophages although it is not necessarily their primary function (Fig. 2).

Apoptosis of retinal ganglion cells can be observed in different conditions: for example during the normal development of the retina (chick)[27] or after an optic nerve transsection (rat, rabbit, monkey, etc.).[28–30] In experimental animal models of glaucoma evidence has also been provided that retinal ganglion cells can die through a process that seems to be closely related to apoptosis (rat, monkey).[30–34]

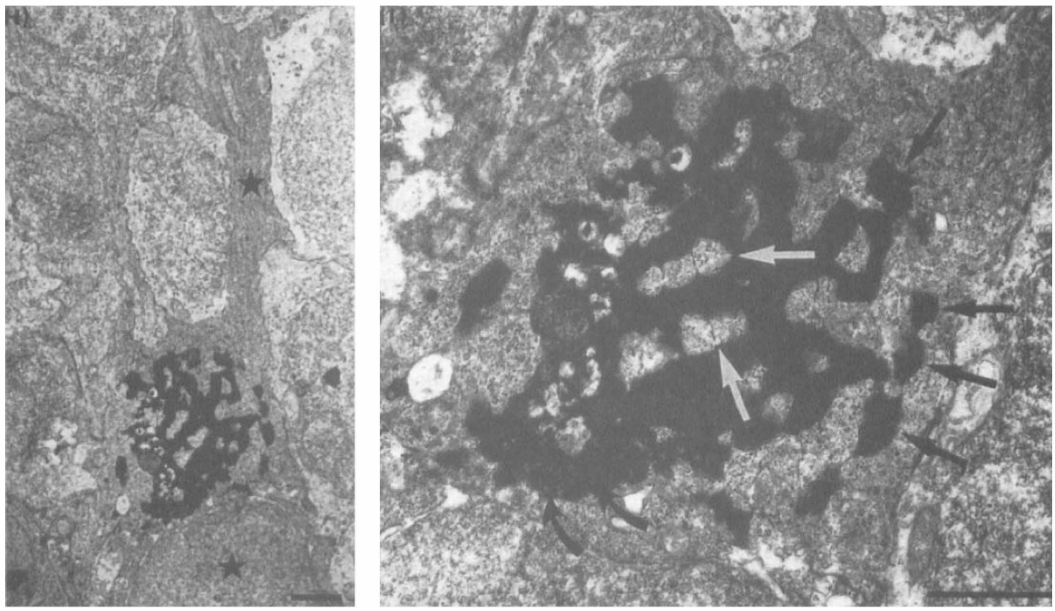

Fig. 2. *Left: Electron microscopy of an apoptotic retinal ganglion cell (fragmented cellular elements) that is in the process of being phagocytosed by a Müller cell (black star) 3 days after 90 min ischaemic episode of the retina followed by reperfusion. Right: Higher magnification of the same area. Müller cell processes appear to penetrate the condensed material (white arrows), subdividing it into small pieces. These smaller fragments then appear to be transformed into inclusion bodies (straight black arrows) by coalescence of the processes. Even at this stage, however, condensed mitochondria can still be identified (curved arrows). Scale bar represents 1 μm. From Büchi.*[34]

It has to be borne in mind that, until now, only rare observations have been able to provide hints that retinal ganglion cells do actually die by apoptosis in human eyes with glaucoma.[35] A suggested explanation given for the difficulty of providing such evidence in human eyes is the fact that in patients with glaucoma the rate of retinal ganglion cell loss is slow and often irregular and therefore the probability of detecting an apoptotic retinal ganglion cell might be very low (in contrast to experimental animal glaucoma models).[16,20] Furthermore, in human eyes apoptosis of retinal ganglion cells does not seem to be limited to glaucoma; for example it has also been possible to describe morphological changes compatible with apoptosis of retinal ganglion cells shortly after anterior ischaemia optic neuropathy.[36]

Stimuli that could trigger retinal ganglion cell apoptosis in glaucoma

A wide variety of stimuli can trigger apoptosis.[24] In the context of the loss of retinal ganglion cells observed in glaucoma patients, probably by apoptosis, three stimuli might be of relevance (Fig. 3); (1) a deficiency of neurotrophic factors after an interruption of the axoplasmic flow at the level of the lamina cribrosa; (2) the generation of reactive oxygen species after transient ischaemia episodes; and (3) NMDA-receptor-mediated excitotoxicity evoked by glutamate.

Axoplasmic flow interruption and glaucoma

As is the case for other neurons of the central nervous system, a disruption of retrograde axonal transport, and thus a deprivation of some growth factors, can lead to a loss of retinal ganglion cells. For example, it has been shown that brain-derived neurotrophic factor (BDNF), when injected in a rat eye, can delay retinal ganglion cell death after an axotomy, or improve the survival of rat ganglion cells in culture.[37,38]

Obstruction of axoplasmic flow at the level of the lamina cribrosa was observed in monkeys after an acute increase in intraocular pressure.[39–45] It has also been reported in monkeys that after a chronic rise in intraocular pressure axoplasmic flow was preferentially decreased in the magnocellular layers of the dorsal lateral geniculate nucleus, to which retinal ganglion cells with larger axons project.[46]

It has further been reported that not only an increase in intraocular pressure but also occlusion of the short posterior ciliary artery, or of the central retinal artery, could induce an interruption of retinal ganglion cell axoplasmic flow.[47–50] Apparently, the blockage of axonal transport induced by an increase in intraocular pressure

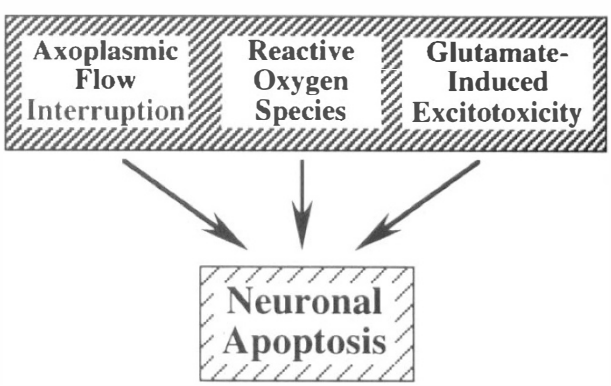

Fig. 3. *Stimuli that could potentially lead to apoptosis of retinal ganglion cells in glaucoma.*

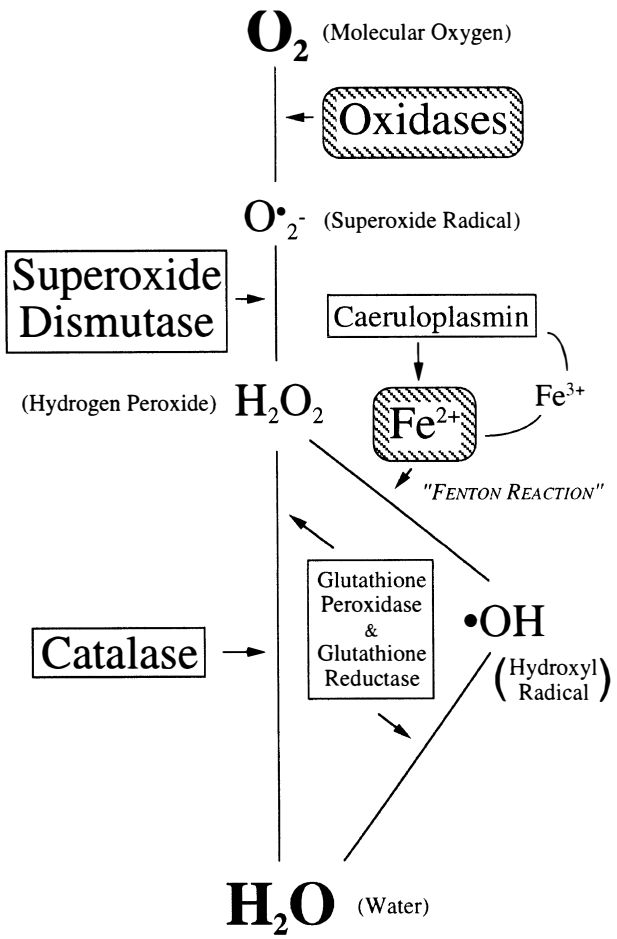

Fig. 4. *Formation of free radicals by the reduction of oxygen to water. Important reactive elements that lead to the generation of oxygen radicals are oxidases and Fe^{2+}. To defend against these free radicals, different enzymatic oxidants (i.e. superoxide dismutase, catalase, glutathione peroxidase and reductase) and non-enzymatic antioxidants (i.e. caeruloplasmin, vitamin E, metal chelator, etc.) exist.*

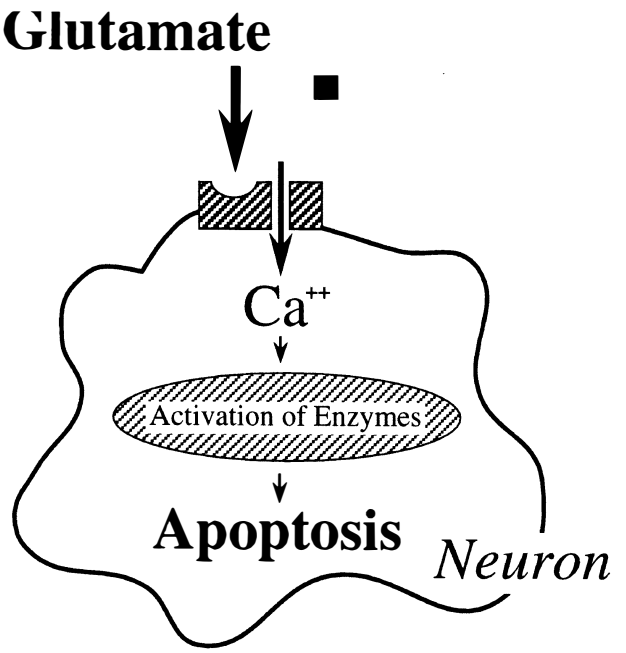

Fig. 5. *Simplified scheme of neuronal apoptosis that can be induced by activation of the* N-*methyl*-D-*aspartate (NMDA) membrane receptor channel. Stimulation of the receptor by glutamate induces an extracellular influx of calcium (Ca^{2+}) which can in turn activate various Ca^{2+}-dependent enzymes and can lead to cell death by apoptosis.*

was greater in the presence of the vasoconstrictive drug angiotensin which is used to induce systemic hypertension.[51]

Thus it appears that, at least in monkeys, either an increase in intraocular pressure or ischaemia can lead to an impairment of axoplasmic flow, two conditions which, to a certain extent, are also known to be risk factors associated with the type of neuropathy observed in patients with glaucoma.

Oxidative stress and reactive oxygen species

A free radical is defined as any atom or molecule with one or more unpaired electrons in its outer shell. Numerous radicals exist but some of the most potent are formed in the reduction of molecular oxygen to water (Fig. 4). Reduction of molecular oxygen by one electron yields the superoxide radical ($O^{\bullet}_2{}^-$), which has limited reactivity with some proteins but is not reactive with lipids or DNA. Under the influence of superoxide dismutase, hydrogen peroxide (H_2O_2) is formed by addition of an electron and two H^+ ions. Although H_2O_2 does not have an unpaired electron and is not a free radical, it is an effective oxidant for many biological molecules. Reduction of H_2O_2 yields the hydroxyl radical (•OH), which is the most reactive oxygen radical and is capable of oxidising lipids, carbohydrates, protein and DNA. These oxygen free radicals together with H_2O_2, singlet oxygen and hypochloric acid, are spoken of as reactive oxygen species (ROS).

Iron is an important reactive element that catalyses oxidative reactions and the generation of oxygen radicals. Iron contains a loosely bound electron and has the ability to exist in more than one valence state. The stable redox state of iron is Fe^{3+}, but its bivalent form Fe^{2+} is capable of transferring one electron and facilitating free radical generation. The reaction of Fe^{2+} with H_2O_2 produces ·OH and is termed the Fenton reaction.

To defend against free radicals, living organisms have developed antioxidants and repair enzymes to remove and/or repair molecules that are oxidised. A few enzymatic antioxidants are synthesised by cells. These include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH) and glutathione reductase (GSSG). Other non-enzymatic antioxidants and metal chelators include vitamin E, vitamin C, beta-carotene and caeruloplasmin.

Oxidative stress can be defined as the increased production of free radicals capable of damaging DNA, protein and lipids. It occurs when free radicals and their products are in excess of the antioxidant defence mechanisms, because of either an increased production of free radicals or a decrease in antioxidant defences.

Neurones depend on oxidative metabolism for their survival. Mitochondrial oxidative phosphorylation results in the production of adenosine triphosphate. The

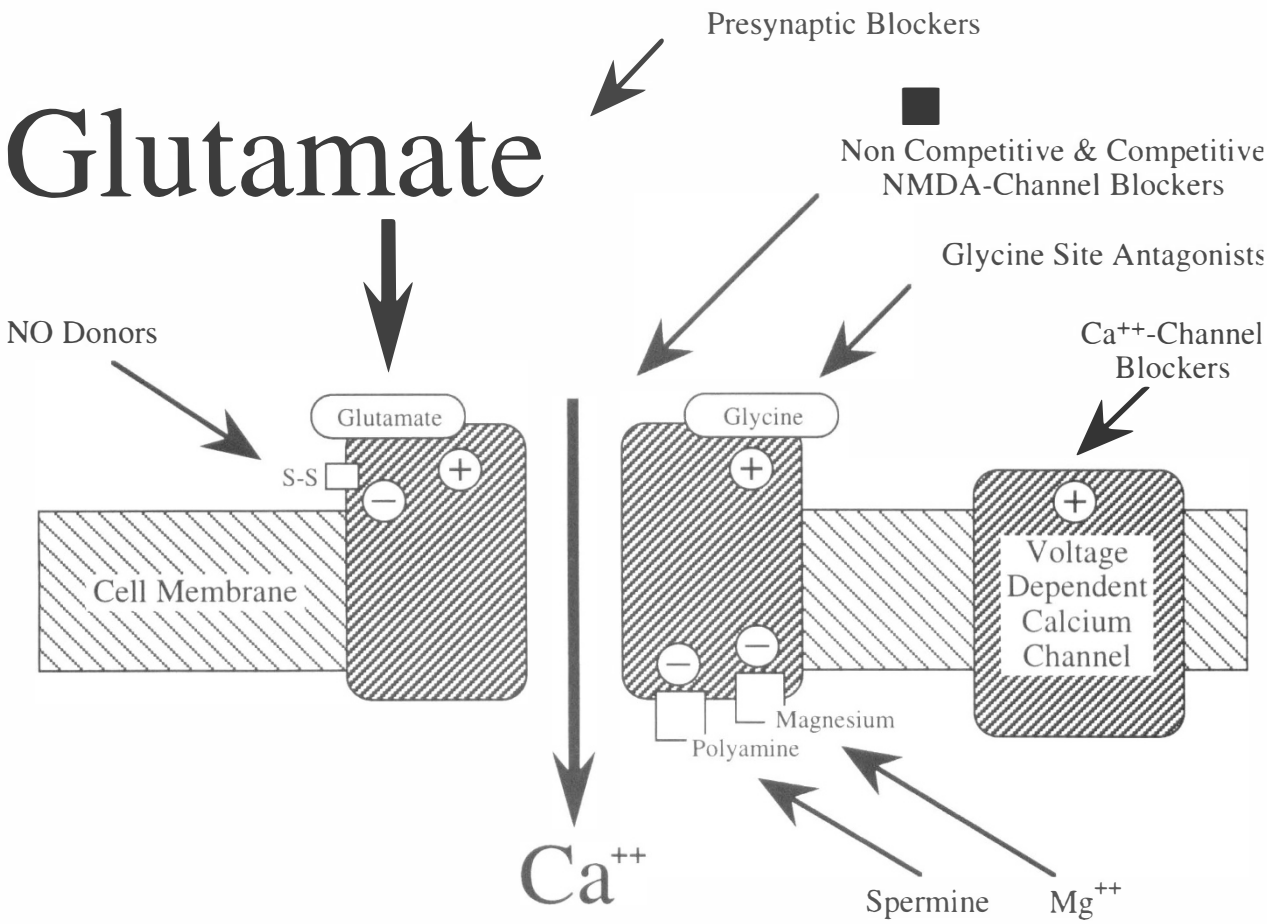

Fig. 6. *Simplified diagrammatic representation an* N*-methyl-*D*-aspartate (NMDA) receptor channel complex and some of the potential sites where it could be pharmacologically regulated. Activation of the complex by glutamate can eventually lead to a massive intracellular influx of calcium. Activation of the NMDA receptor channel complex by glutamate can be modulated by different drugs or classes of drugs which, by acting at different sites, can either potentiate (+) or attenuate (–) the effect of glutamate. Ca^{2+}, calcium; Mg^{2+}, magnesium; NO, nitric oxide; S–S, disulphide bond. Modified from Dreyer.*[19]

production of adenosine triphosphate is coupled to the transfer of electrons to oxygen via the electron transport chain of the inner mitochondrial membrane. Impairment of this process causes an increase in free radical generation and thus oxidative stress,[52–55] and potentially a loss of retinal ganglion cells by apoptosis.[14]

Excitotoxicity and glaucoma

Retinal ganglion cells express *N*-methyl--D-aspartate (NMDA) ionotropic glutamate receptors[56] that can be stimulated by NMDA or glutamate.[57,58] It has been reported that in the rat eye, after an intravitreal injection of NMDA, a complete loss of the cells in the ganglion cells layer and a thinning of the inner nuclear layer can occur.[59–61]

In the vitreous body of patients undergoing surgery it has been observed that glutamate concentrations were apparently more elevated in eyes with glaucoma than in eyes without glaucoma (~25 μM vs ~10 μM).[18,62,63] A similar observation has also been reported in the vitreous humour of dogs and rabbits with primary open-angle glaucoma as well as in monkeys with secondary experimental glaucoma.[64,65] However, it has to be noted that increased concentrations of glutamate in the vitreous body do not seem to be specific to glaucoma, as a similar observation has also been reported in patients with proliferative diabetic retinopathy.[63]

It has also been observed that a moderate 2- to 3-fold increase (30 μM) in intravitreal glutamate over a period of 3 months (achieved by repeated intravitreal glutamate injections) resulted in a significant loss of retinal ganglion cells in rat eyes. This effect did not occur in eyes receiving vehicle injections, and could be prevented when the NMDA antagonist memantine was co-injected with glutamate.[66]

As mentioned earlier, it has also been suggested that larger retinal ganglion cells seem to be damaged earlier in the optic neuropathy observed in patients with glaucoma.[67–70] In this regard, evidence has also been reported *in vitro* and *in vivo* that larger retinal ganglion cells are more susceptible to glutamate-induced toxicity in rats.[71,72]

In mammalian central neurones it has been shown that stimulation of NMDA receptors can lead to excessive levels of calcium,[73,74] which in turn can activate different intracellular pathways and lead to cell death.[75,76] The association between excessive calcium

levels and cell death suggests a possible causal relationship between these two events that could be mediated through calcium-dependent enzymes.

Several sites on the NMDA receptor can modulate the activity of the receptor-channel complex.[77,78] For example there is a redox modulatory site which consists of multiple thiol (or disulphuryl) groups forming one or more disulphide bonds on the receptor.[79] Reducing agents, such as dithiothreitol, break disulphide bonds and enhance the activity of the NMDA receptor, whereas oxidising agents, such as 5,5-dithio-bis-2-nitrobenzoic acid, form disulphide bonds from vicinal or paired free thiol groups and decrease NMDA receptor function and neurotoxicity.[80]

Nitric oxide donors can also down-regulate NMDA receptor function, in part by oxidising the redox modulatory site via the generation of nitric oxide. Thus nitric oxide can thereby limit the activation of NMDA receptor and NMDA-receptor-mediated neurotoxicity.[81–83]

In a retrospective study comparing a population of glaucomatous patients taking nitrates for cardiac reasons with a control population of glaucomatous patients (who did not need to be treated with nitrates), it was observed that among patients taking nitrates the risk (Cox hazards model) of optic nerve deterioration was 20 times lower, and for visual field deterioration was 4 times lower, that in the control population.[84] Although there may be some confounding factors because the two populations are not really comparable (as the patients receiving nitrates had some cardiopathy), this observation has suggested a potential beneficial effect of nitric oxide donors for the eyes of glaucomatous patients, perhaps in part through a neuroprotective mechanism.

Neuroprotection in other neurodegenerative disorders

Glaucoma is not the only neurodegenerative disorder in which it has been proposed that neuronal loss could benefit from a neuroprotective approach. For example, in acute neurodegenerative diseases, such as in stroke[85,86] or after a traumatic brain injury,[87] good evidence has been provided for a rise in extracellular glutamic acid concentrations and subsequent NMDA-receptor-mediated neuronal death. In stroke it is even believed that the excitotoxicity process determines the extent of the long-term neurological sequelae. In these disorders, several trials have been conducted with anti-excitotoxic drugs. To date, apparently none of these has demonstrated sufficient benefit to warrant continued drug development.[88]

In chronic neurodegenerative diseases, such as human immunodeficiency virus encephalopathy, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (motor neurone disease), the evidence seems to be more circumstantial that an excitotoxic process takes place. In these disorders, for which therapies are limited, the potential of a neuroprotective therapeutic approach has raised major interest and hopes. Although preclinical animal studies were very promising and quite a large number of clinical trials have been conducted, with a few rare exceptions such as the effect of the anti-glutamate drug riluzole on amyotrophic lateral sclerosis,[89,90] the pursuit of effective neuroprotective therapies has been frustrating in neurological disorders.[21,88] In one of the studies conducted with riluzole (randomised, double-masked, placebo-controlled, multicentre study) 959 patients with a recent diagnosis of amyotrophic lateral sclerosis were given different concentrations of the drug. The primary outcome of the study was the survival rate without tracheotomy after 18 months of treatment. At the end of the study, the survival rate was 50.4% in the placebo-treated group of patients and 57.8% in the patients treated with 200 mg/day riluzole (the highest dosage tested). Although the results of the study were apparently very significant ($p < 0.0004$) the clinical relevance of the neuroprotective effect of the drug is more debatable. Nevertheless, on the basis of this study,[90] and another one,[89] riluzole has been approved in several countries (including by the Food and Drug Administration in the United States)[19] as a neuroprotective drug for the treatment of lateral amyotrophic sclerosis.

The lack of neuroprotective effect observed in other clinical trials has been attributed to the fundamental differences that can exist between some animal models and human neurodegenerative disorders. Other reasons evoked were the facts that clinical trials should be designed in order to reflect more accurately the experimental conditions under which efficacy was observed in preclinical studies (i.e. time frame, adequate brain levels of drugs, etc).[22]

It has also been suggested that due to fluctuations that can occur during the natural course of neurological disorders, the results should be stratified according to the clinical presentation of the disease when patients enter the study. Furthermore, in view of the relatively modest effect that can be expected from neuroprotective therapies, the sample size should be adjusted substantially upward so that a 3–5% absolute difference in outcome can be detected.[22]

Many phase III studies have also failed because safety and tolerability were not addressed carefully enough in phase II studies. Because of the frustrating issues regarding these clinical studies it has also been suggested that the rational combination of neuroprotective strategies might increase clinical efficacy.[22]

Neuroprotection potential in glaucoma

As mentioned earlier, the evidence tends to suggest that an excitotoxicity mechanism could lead to retinal ganglion cell death in glaucomatous neuropathy. However, this evidence is essentially based on observations made in *in vitro* systems and in *in vivo* animal models that do not necessarily reflect clinical conditions occuring among patients with glaucoma. Additional evidence comes from isolated studies reporting an increase in glutamate concentration in the

vitreous body of glaucoma patients and the presence of apoptotic retinal ganglion cells in the retinas of glaucomatous eyes.

Excitotoxicity certainly provides an elegant hypothesis to explain the loss of retinal ganglion cells observed in glaucoma, and as a consequence has raised the hope that glaucoma patients could benefit from neuroprotective therapies. However, this hope has not yet been supported by any clinical trials – clinical trials that might be difficult to conduct. Indeed, in view of the natural course of the disease, which is extremely slow, often irregular, and can show large variability in its progression from one patient to the other, this type of trial might require the study of large groups of patients over long periods of time.

In order not to mislead clinicians taking care of patients with glaucoma, the lack of clinical evidence for neuroprotection should perhaps be stressed when the neuroprotective properties of a drug have only been observed *in vitro* or in experimental animals. This also applies to some ophthalmic drugs (sometimes routinely used in clinic) for which some neuroprotective effects could only be demonstrated in preclinical studies.

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