

# Glaucoma: Validated and Facile *In Vivo* Experimental Models of a Chronic Neurodegenerative Disease for Drug Development

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**Abstract:** Glaucoma is a neurodegenerative disorder that affects the optic nerve and the inner layers of the retina. Increased intraocular pressure is a major risk factor in the disease. Chronic elevation of intraocular pressure specifically induces the death of retinal ganglion cells. By developing animal models of the disease, the scientific community has been able to make progress in understanding the mechanisms leading to the death of retinal ganglion cells, the molecular mechanisms of the pathology, and developing new pharmacological interventions. In this report, we review and compare animal models of glaucoma. We find that the episcleral cauterization model offers many advantages over other *in vivo* models. Its feasibility and lack of frequent complications make it the most extensively used animal model of glaucoma. Furthermore, we discuss features related to the pathogenesis of the disease and compare it with other models of retinal ganglion cell damage (*e.g.* optic nerve axotomy and excitotoxicity). In the last section, we focus on drug candidates for neuroprotective treatment of glaucoma, and discuss their likely mechanisms of action.

**Keywords:** Glaucoma, animal model, optic nerve, neurodegeneration, neuroprotection, episcleral, cauterization, photocoagulation, treatment.

## INTRODUCTION

Glaucoma is the third leading cause of blindness worldwide [1] and it accounts for ~9% of patient visits to the ophthalmologists' office in North America. Open angle glaucoma (the most frequent form of glaucoma) is currently defined as a multifactorial optic neuropathy characterized by (*i*) high intraocular pressure, (*ii*) open anterior chamber angles and (*iii*) characteristic patterns of visual field loss accompanied by progressive optic nerve fiber loss [2]. Clinically the disease is suspected when a patient presents glaucomatous optic disc changes and/or high intraocular pressure (IOP). High IOP is a major risk factor in glaucoma [3], and can be detected in five out of six patients with glaucoma at one point in the progression of their disease [2].

Mechanistically, IOP increases are generally due to a decrease in outflow of aqueous humor (AH) and not due to its overproduction. Normally, AH is produced in the ciliary body, circulates into the anterior chamber and then exits the eye (Fig. 1). Exit of AH occurs through two pathways. The conventional pathway, represented by the trabecular meshwork/Schlemm's canal system is responsible for most of the AH outflow in humans (Fig. 1, bottom left), and where more than 75% of the resistance to AH outflow is exerted [4]. A second pathway is the non-conventional or uveoscleral pathway. In this pathway, AH exits the eye through bundles of the ciliary muscle. In normal conditions the uveoscleral pathway is responsible for 5 to 25 % of the AH draining

in humans [5, 6], but outflow can increase up to four fold during inflammation [7].

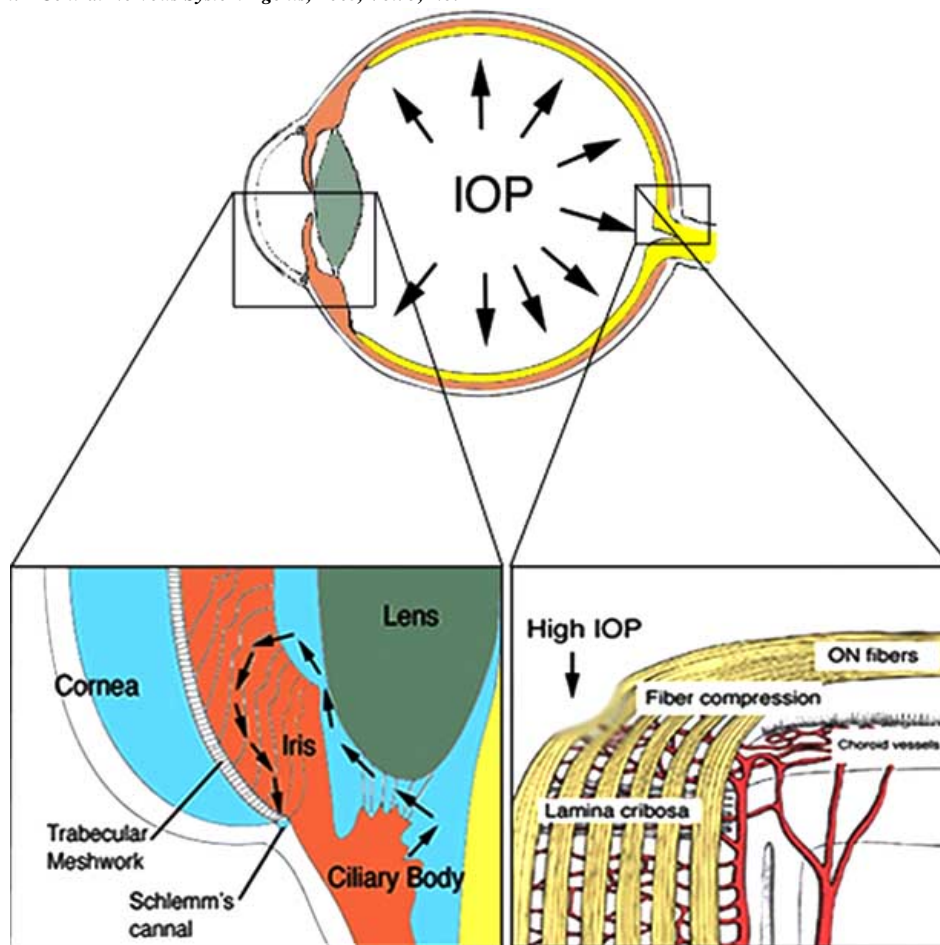
If left untreated, patients with chronic high IOP will develop changes at the inner layers of the retina and in the optic nerve head (ONH), specifically, a chronic and progressive death of retinal ganglion cells (RGC), that translates clinically as visual field loss. RGCs form the innermost nuclear layer of the retina. In the retina, RGC axons are grouped as nerve fibers in the nerve fiber layer (NFL) and exit the eye through the ONH, forming a well defined neural rim (Fig. 1, bottom right). Diagnosis and evolution of glaucoma can be easily performed by direct examination of the ONH [2].

Two theories have been postulated to explain RGC damage, and both focus on ONH compression during high IOP [8]. The mechanical theory suggests that optic nerve fibers are compressed by high IOP, when traversing a rigid structure called lamina cribosa, leading to an effective physiological axotomy and problems in axonal transport (Fig. 1, bottom right). The ischemic theory points to alterations in ONH blood supply as the main factor in the progressive death of RGCs. These hypotheses are non-exclusive, and both could be correct.

In addition to mechanical compression, current work also points to other molecular mechanisms that directly damage RGCs: release of neurotoxic substances such as glutamate and nitric oxide [9-11] and pro-inflammatory processes [12]. Genetic factors and suppression of protective genes may play a role in glaucoma [13] particularly in normal tension glaucoma.

Indeed, even though IOP lowering agents decrease or partially reverse IOP, RGC death and clinical evolution towards glaucoma can continue. This suggests that molecular

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**Fig. (1).** Bottom left: Aqueous humor (AH) is produced at the ciliary body in the posterior chamber of the eye. It circulates into the anterior chamber through the pupil. The AH play important roles in the structural and metabolic activities of both chambers. When exiting the eye, the AH traverses the trabecular meshwork (TM), into the Schlemm's canal, finally reaching systemic circulation after draining by limbal episcleral veins (in rats) and by collector channels (in humans).

Bottom right: RGC axons grouped as nerve fibers (NFL) exit the eye through the optic nerve head (ONH). This place is relevant for two reasons. First, the ONH is the place where RGC axons are hypothetically initially damaged by high IOP when traversing the lamina cribrosa. Second, diagnosis and evolution of the disease can be performed by looking at the ONH.

events triggered by high IOP may still be active even after normalization of IOP.

We have recently shown that even short-lived high IOP regulates gene expression in retina, and one particular interest is the pressure-specific regulation of genes encoding for neurotrophic growth factors and their receptors [14] because these factors and their receptors are key players in supporting neuronal phenotype, maintenance, and function.

Although it may seem a simple minded comparison, a parallel has been drawn between glaucoma and other chronic neurodegenerative disorders [15]. It is intriguing to note that both glaucoma and Alzheimer's Disease are slow and chronic neurodegenerative disorders, both activate caspases, both involve the dysfunction of neurotrophic growth factors and their receptors, and current therapeutic approaches target both diseases with pharmacological application of neurotrophins, N-methyl-D-aspartate (NMDA) receptor antagonists, or immune regulators. Overall, this suggests that although

the etiology is different, some neurodegenerative mechanisms may overlap.

### GENERAL FEATURES OF ANIMAL MODELS

Good animal models are key for pharmacological studies. In addition to reproducing human disease as closely as possible, the experimental model should have the following characteristics:

- Reproducibility
- Easily induced disease
- Avoiding neighboring tissue damage and side effects, such as pro-inflammatory reactions
- Low cost, so sufficient animals can be used to obtain statistically significant results

When referring specifically to glaucoma, the model should also possess the following features:

- Increased IOP should be sustained chronically (months)
- Frequent IOP measurements should be done to follow kinetics
- Easy assessment of retinal neuronal damage

The resemblance between primate and human glaucoma stimulated the use of primates for research purposes during the 80s and early 90s [16]. Economical and ethical issues led to a decrease in their use and partial replacement by rodents. Rats have anatomical similarities with primates, regarding the anterior segment blood supply and aqueous humor drainage [17]. Increased IOP can be easily induced in rats by a number of different mechanisms (see below). In rats and in humans, chronic high IOP progressively damages RGCs [18-20]. Long-lived glaucomatous rats seem to present optic disc changes similar to those described in late stage human glaucoma [20-22].

## RAT GLAUCOMA MODELS

A few rat models of glaucoma are currently used for research purposes, each one with its own advantages and disadvantages. Here we describe and classify these models based on their mechanisms that cause increased IOP.

### PRE-TRABECULAR MECHANISMS

#### Blocking the Trabecular Meshwork

Increased IOP after a blunt trauma or cataract surgery is a common clinical scenario [23,24]. Mechanistically, the accumulation of viscous substances at the TM, precludes normal AH outflow and consequently raises the IOP. Ocular hypertension has been successfully induced in rabbits and rats after the intraocular injection of viscoelastic substances such as hyaluronate [25-27].

Feasibility and reproducibility are the main advantages of this experimental model, since high IOP is obtained in the majority of the animals. However, this model has several disadvantages. First, the IOP increase is transient because viscoelastic substances are cleared from the eye a few days after the procedure, thus to maintain high IOP weekly intraocular injections are necessary [25]. Second, the increases in IOP are hard to control and can cause very high IOP values (*e.g.* 4-fold over normal IOP) that lead to retinal ischemia. Note that in glaucoma high IOP is generally *less* than 2-fold increase over normal pressure, and typically ~1.8-fold. Thus, this model is less suitable for studying retinal changes specifically induced by high IOP.

### TRABECULAR MECHANISMS

#### (a) Corticosteroid Treatment

Ocular hypertension is a common clinical secondary effect of corticosteroid anti-inflammatory treatment. In dogs and cats, daily topical application of 1% dexamethasone causes a gradual increase in IOP, within 2 weeks of initiation of treatment [28,29]. Accumulation of cellular debris at the TM (with a consequent decreased AH outflow) was shown to be the mechanism [30]. The model has been extensively used in the past decades due to its simplicity. It has a few

drawbacks: not all rodent species increase IOP after topical corticosteroid treatment and undesired secondary effects such as cataracts may be induced.

#### (b) Laser Photocoagulation Treatment

Laser photocoagulation of the TM is another approach that has been successfully used to increase IOP in primates and rats [16,31-33]. Reduction of AH outflow by a transient network of cells and debris as well as disorganization of the TM architecture have been indicated as the cause of increased IOP [31,34].

Laser TM photocoagulation can be performed either directly at the TM [35,33,36] or by a translimbal approach [37]. Direct TM photocoagulation in rats increases IOP to values seen in glaucoma (26-56% higher than the normal value) [32,33,35,38]. A 40-50% reduction in RGC density was shown in rats that maintained high IOP for at least 8 weeks [32,33,38]. A more recent report using more sensitive RGCs measurements showed a significant reduction in RGC density as early as 3 days after ocular hypertension [35], confirming that RGC loss is slow, chronic and progressive.

Photocoagulation of only the TM by a translimbal approach is not very effective at increasing IOP. Significant increases in IOP values were obtained, only if additional photocoagulation of the episcleral vessels were performed [37]. Up to a 60% decrease in RGC density after 6 weeks of treatment was reported using this combined surgical treatment.

Increasing IOP by laser TM photocoagulation is a method that presents some disadvantages, such as the need for specialized equipment to perform the photocoagulation, and the reported multiple laser treatments needed to maintain high IOP values for several months [35]; most importantly, anterior chamber complications induced by laser treatment (hyphema and corneal opacities), especially with the translimbal approach [37].

### POST-TRABECULAR MECHANISMS

After traversing the TM, the AH leaves the anterior chamber through the Schlemm's canal and drains into a venous plexus [17]. This plexus encircles the entire limbal area and is drained by multiple episcleral veins. A decrease in AH outflow with a consequent increase in IOP can be obtained by modifying the post-trabecular anatomy.

#### (a) Episcleral Vein Injections

Here, the outflow system is retrogradely sclerosed by injections of hypertonic saline solutions in the episcleral veins. Forceful injections of a solution of 2 molar saline increase IOP in 60% of treated eyes [19,39]. Single injections of less hypertonic solutions compromise the efficiency of the technique to cause high IOP in only ~40% of injected eyes [19].

This technique is less invasive than laser TM photocoagulation, and hence, it induces less anterior chamber complications. However, it does present some disadvantages. First, sequential hypertonic saline injections are needed to maintain chronic high IOP for a sustained period of time.

**Table 1. Glaucoma Models Classified by Mechanisms Used to Increase IOP**

Mechanism	Procedure	Species	Advantages	Disadvantages
Pre-trabecular	AC injection of viscoelastic substances	Primates, rats	High IOP is easily induced	Short lasting effect IOP spikes
Trabecular	Steroid induced glaucoma	Cat, dogs	High IOP is easily induced	Induction of cataract Refractory in some rodents
	TM laser photocoagulation	Primates, Rats	Resistance to AH outflow occurs in the TM as in humans	Repeat laser treatments to maintain high IOP Hyphema Corneal opacities
Post-trabecular	TM sclerosis induced by saline injections*	Rats	No inflammation	Repeated injections to maintain high IOP High variability between rats
	Episcleral venous cauterization	Rats	Easy to perform Reproducible Long term reliable high IOP	Choroid vein stasis?

AH: Aqueous humor; TM: Trabecular meshwork, Hyphema: blood in the anterior chamber. \*Saline solutions are injected through the episcleral veins (post-trabecular), scarring all the outflow system including the TM.

Second, a high inter-animal variability in IOP values was reported and consequently a high variability in ON fiber degeneration in the glaucomatous eye [19,40,41].

#### (b) Cauterization of Episcleral Limbal Veins

Another experimental model of glaucoma increases IOP by cauterization of the episcleral limbal veins [14,18,20,42-44]. Cauterization of three of the four episcleral veins increases IOP 2.0–2.5 fold the normal value one day after surgery. Then IOP decreases slightly, and plateaus to 1.5 to 1.8 times the normal, which mimics the pressure seen in glaucoma. These desired high IOP values are sustained chronically for more than 3 months in 70% of cauterized eyes [14]. This moderate and chronic increase in IOP causes the progressive death of RGC, from 2 to 6% loss of the RGCs per week [14,18,20,42,43].

The episcleral cauterization model has advantages over other *in vivo* models. First, it is a feasible glaucoma model that does not require sophisticated equipment. Second, complications secondary to surgical treatment are rare. Third, inter-animal variability of IOP values are lower than that reported for the episcleral saline injections, resulting in homogeneous groups of animals for study. For example, in our hands more than 70 % of the rats with cauterized episcleral veins maintained high IOP values of 1.5 to 1.8 times the normal for more than 3 months, and we rarely observed anterior chamber complications [14].

#### COMPARISONS WITH ACUTE MODELS OF RGC DEATH

In addition to glaucoma few other rat models of RGC damage are used for research purposes. These models damage RGCs acutely, in contrast to glaucoma which is a chronic disease.

#### (a) Optic Nerve Axotomy

The optic nerve axotomy/crush model is probably the most extensively studied and used model of acute RGC damage. RGC axons are mechanically damaged in the orbit (at myelinated ON fibers) and due to retrograde degeneration, massive neuronal death (at the RGC soma) occurs within 2 weeks [45,46]. Lack of trophic support from the CNS has been indicated as the cause of the centripetal degeneration [45,47].

An analogy has been proposed for RGC death in ON axotomy/crush and glaucoma, based on two observations. First, a comparable retrograde pattern of axonal damage were described for both axotomy and glaucoma [8]. Second, a blockade of growth factor transport was described during high IOP [33]; this was deemed comparable to a “physiological axotomy”. According to this concept, reduced retrograde transport of growth factors can be caused by pressure in glaucoma, as well as by physical injury to the optic nerve.

However, recent data may challenge the hypothesis of a retrograde centripetal damage in glaucoma. Evidence of RGC death early than 24 hrs after a so-called “moderate” increase in IOP has been obtained. This fast RGC degeneration would seem incompatible with the time that a retrograde axonal degenerative mechanism would require to reach the soma [35,48]. There are three possible and overlapping explanations for these seemingly incompatible results.

First, a subset of RGCs may be extremely sensitive to axonal transport blockade and die very fast with increased IOP; these are the RGCs that die after 24 hrs. Second, high IOP may activate a mechanism different than retrograde axonal degeneration. Third, high IOP may cause direct damage to the RGC soma rather than just at the optic nerve.

**(b) Excitotoxicity**

The excitotoxicity model uses N-methyl-D-aspartate (NMDA) receptor agonists as initiators of damage. NMDA receptor agonists are well-known for mediating secondary death after neuronal ischemia and trauma [49,50]. In the retina, glutamate induces the selective death of RGCs and severe inflammation of the inner retinal layers 48 hrs following intravitreal injection [49,51]. A few days later, retinal thickness is severely decreased due to a significant loss of cells at the inner retinal layers.

Excitotoxic damage has been suggested to be one mechanism of RGC damage during glaucoma, and “overactivity” of NMDA receptors has been described in glaucoma [52]. Moreover, both NMDA and high IOP induce RGC apoptosis selectively without significant damage to other cell types. On the other hand, it is difficult to reconcile a role for glutamate in both acute and in chronic neuronal apoptosis without suggesting an indirect or secondary mechanism. Nevertheless, a partial NMDA receptor antagonist is being tested as a potential therapeutic agent in glaucoma.

We believe that both the ON axotomy and the excitotoxic animal models of acute RGC damage are suitable for pharmacological studies because they share some of the epiphenomena described in glaucoma (increased levels of glutamate and lack of trophic support). However, they are less helpful for understanding the initial mechanisms triggered by high IOP, hence they are less suitable for finding a specific glaucoma neuroprotective treatment.

**NEUROPROTECTIVE DRUGS FOR GLAUCOMA TREATMENT**

Throughout the last decade, a new concept in glaucoma therapy has emerged involving RGC protection in addition to lowering IOP. Drug candidates have RGC death reduction as a common effect when tested in animal models of RGC

damage. Most of them target indirect mechanisms of neuronal damage, such as glutamate release, NO<sub>2</sub> production, immune system-mediated damage, etc.

We present a list of candidate agents that have been shown to be RGC protective in at least two different animal models of RGC damage (Table 2).

NMDA antagonists are known for effectively reducing neuronal death in experimental models of brain trauma and cerebral ischemia [53-55]. Recently, memantine and dexanabinol (both drugs with NMDA antagonistic properties) were shown to protect RGC after ON axotomy and high IOP damage [22,52,56,57]. Both drugs are currently under clinical trials for glaucoma. Side effects, especially those related to CNS will probably restrict their use to specific cases.

A second group of drugs for potential glaucoma neuroprotective treatment are the inhibitors of the inducible nitric oxide synthase (NOS-2). Nitric oxide is a well-known mediator of neuronal damage and chronic oral administration of NOS-2 inhibitors has recently demonstrated to be RGC protective after retinal ischemia and high IOP [58,59].

Neurotrophins (NTFs) are soluble growth factors that affect survival and differentiation of neurons by binding specifically to cell surface tyrosine kinase receptors called “Trk” [60,61]. The NTFs comprise Nerve Growth Factor (NGF, which binds TrkA), Brain Derived Neurotrophic Factor (BDNF, which binds TrkB) and Neurotrophin-3 (NT-3, which binds TrkC). All NTFs also bind to a shared receptor termed p75. The p75 receptor can function as pro-apoptotic or pro-survival depending on the cellular environment and ligands that bind to it.

Some NTFs are known to delay RGC death after ON axotomy when injected intraocularly [62]. Because RGCs depend on NTFs for their survival, and lack of efficient trophic support has been suggested as a cause of RGC death [33], NTFs binding to Trks emerged as natural candidates for

**Table 2. Candidate Drugs for Neuroprotective Treatment of Glaucoma**

Drug	Tested models	Hypothesized mechanism
Dexanabinol	ON crush	Antagonist of NMDA receptors Anti-TNF properties
Memantine	Optic nerve crush, glaucoma	NMDA receptor channel blocker
Inhibitors of Inducible NO <sub>2</sub>	ON axotomy, glaucoma, retinal ischemia	Inhibition of retinal NO <sub>2</sub> production
BDNF	ON axotomy, glaucoma	Activation of MAPK/Akt pathway in RGC
Brimonidine	ON crush, glaucoma, retinal ischemia	Upregulation of b-FGF, BDNF and bcl2/bclx Activation of ERK and Akt survival pathways
Betaxolol	Retinal ischemia, glaucoma	Upregulation of BDNF Suppression of RGC ionic channels Downregulation of NO <sub>2</sub> synthase
Copaxone	Excitotoxicity, glaucoma	Protective T- cell phenotype induction
Gabapentin-lactam	Retinal ischemia	Open ATP sensitive potassium channels
geranylacetone	Glaucoma	Induction of heat shock proteins by RGCs

neuroprotective treatment of glaucoma, although NTF binding to p75 may be considered problematic.

If NTFs are going to be employed therapeutically for glaucoma, which drugs should be used? TrkB agonists are the most likely candidates, although BDNF alone did not significantly reduce RGC death during glaucoma, unless it was added concomitantly with a scavenger of free radicals [63].

TrkA agonists are also candidates because the TrkA receptor is upregulated after RGC damage [64,14]. Whether to use agonists or antagonist of p75 and TrkC receptors as drug candidates, is a matter of debate. Both receptors are upregulated by Muller cells [14] and hyperactivity of Muller cells has been suggested to be detrimental to RGCs [65]. Thus, antagonism of p75 and TrkC receptors may be reasonable.

Brimonidine (alpha-2 adrenergic agonist) and betaxolol (beta-blocker) are known for their IOP lowering capacity and which is why they have been used for many years in glaucoma treatment. Recently, RGC protection was demonstrated for both drugs using different models of RGC damage [66-69]. The protective effect of Brimonidine is hypothetically mediated by upregulation of BDNF, Fibroblast Growth Factor (b-FGF) and the anti-apoptotic molecules bcl2/bclx [70,71]. In contrast, the protective effect of Betaxolol includes increased expression of BDNF, downregulation of NO<sub>2</sub> synthetase, and potent calcium and sodium channel blocking properties [72-75].

Copaxone (Cop-1), a small peptide that cross reacts with myelin antigen has effectively been used to suppress myelin-associated autoimmune disease [76]. Its neuroprotective effect is not limited to neuroinflammatory conditions; immunization with Cop-1 significantly reduces neuronal death after optic nerve axotomy, after intravitreal injections of glutamate and in ocular hypertension [77,12]. T- cells appear to be the mediators of this beneficial effect induced by Cop-1 vaccination, because passive adsorptive transfer of Cop-1 reactive T-cells confers protection. The hypothesis is that secondary damage induced by microglial cells and macrophages is reduced due to the regulatory activity of this T-cell phenotype [12]. Cop-1 is currently in the clinical trials for glaucoma and its results will be known in the following years.

Gabapentim-lactam [78] and geranylacetone [79] are drugs that target recently recognized mechanism activated upon RGC damage. Their potential use as pharmacological agents for glaucoma will become clearer in the next few years.

## CONCLUSIONS

The episcleral cauterization model of glaucoma is currently the most widely used animal model by glaucoma researchers. It presents many advantages over other models of this disease, and complications induced by the technique are rarely seen. A multifactorial origin in RGC death during glaucoma (trophic factor deficiency, excitotoxicity, immune mediated damage) suggests the possible need for combinatorial treatment of IOP lowering drugs and neuroprotective agents (neurotrophic agonists/ NMDA antagonists/ vaccination?). Some of these mechanisms are shared with other

chronic neurodegenerative disorders such as AD [80]. Availability of reliable animal models will shed more light on the pathogenesis and future treatments for glaucoma, and they may also be valid for drug screening in other neurodegenerative diseases.

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