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Recent advances in genetically modified animal models of glaucoma and their roles in drug repositioning

Chikako Harada, Atsuko Kimura, Xiaoli Guo, Kazuhiko Namekata, Takayuki Harada

Visual Research Project, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan

Correspondence to
Professor Takayuki Harada, Visual Research Project, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan; harada-tk@igakuken.or.jp

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ABSTRACT

Glaucoma is one of the leading causes of vision loss in the world. Currently, pharmacological intervention for glaucoma therapy is limited to eye drops that reduce intraocular pressure (IOP). Recent studies have shown that various factors as well as IOP are involved in the pathogenesis of glaucoma, especially in the subtype of normal tension glaucoma. To date, various animal models of glaucoma have been established, including glutamate/aspartate transporter knockout (KO) mice, excitatory amino acid carrier 1 KO mice, optineurin E50K knock-in mice, DBA/2J mice and experimentally induced models. These animal models are very useful for elucidating the pathogenesis of glaucoma and for identifying potential therapeutic targets. However, each model represents only some aspects of glaucoma, never the whole disease. This review will summarise the benefits and limitations of using disease models of glaucoma and recent basic research in retinal protection using existing drugs.

INTRODUCTION

Glaucoma is a neurodegenerative disease of the eye which involves degeneration of retinal ganglion cells (RGCs) and their axons, namely, the optic nerve. It is the second leading cause of blindness,[1 2] and with the current growth in the ageing population, one expects a robust increase in patients with glaucoma, resulting in an urgent need for a cure for this disease. It is estimated that by 2020, more than 80 million people will be affected worldwide.[3] The factors associated with the pathogenesis of glaucoma include high intraocular pressure (IOP), ageing,[4] decreased blood flow,[5] oxidative stress,[6–9] glutamate neurotoxicity,[10 11] decreased trophic factors,[12] low cerebrospinal fluid pressure,[13] myopia,[14] eye movements[15] and susceptibility genes, such as optineurin and myocilin (figure 1).[16] Currently, pharmacological intervention for glaucoma therapy is limited to eye drops that reduce IOP. Recently, a concept of neuroprotection against RGC death induced by multiple disease pathogenesis as well as IOP lowering is receiving a lot of attention.[11 17–19] However, the development of a new drug can take an enormous amount of time, money and effort. One alternative to this is an approach known as drug repositioning, which is the application of an existing drug to a different disease. The beneficial effects of drug repositioning are immense because the drug has already gone through vigorous toxicity and other tests required for bringing a drug to market; thus, it saves vast amounts of time and money. Basic research using animal models of glaucoma has shown some beneficial effects of such existing drugs in neuroprotection.[20–24] These results suggest the possibility that drug repositioning is a safe and cost-effective approach for the treatment of glaucoma.

ANIMAL MODELS OF GLAUCOMA

In preclinical research, animal models of glaucoma have contributed greatly to understanding glaucoma pathology and examining potential therapeutic candidates. There are several experimentally induced animal models,[25 26] such as a simple acute optic nerve crush,[27] intraocular injection of excitotoxic agents such as *N*-methyl-D-aspartate (NMDA),[28] anterior chamber injection of sterile saline at high pressure,[29] microbead injection,[30] episcleral vein injection of hypertonic saline[31] or external laser photocoagulation.[32] In addition, there are several inherited animal models of high IOP glaucoma (table 1).[26 33] For example, the DBA/2J mouse is an age-dependent, inherited model of a high IOP glaucoma with many similarities to the human disease.[34] The DBA/2J mice develop anterior segment anomalies, iris atrophy, peripheral anterior synechiae and pigment dispersion, leading to an elevated IOP, and they are used in many studies worldwide. However, the model presents significant challenges for drug studies in glaucoma, as there are many confounding factors: difficulty with accurate IOP measurement due to corneal calcification, in vivo imaging and electrophysiology recording due to poor pupil dilation and 22% of the animals developed major systemic complications leading to a high dropout rate.[35] In addition, there may be an underlying neurodegenerative process independent of IOP.[36] For example, approximately 8%∼10% of the older DBA/2J mice had one normal, non-diseased retina and optic nerve, and one eye had undergone complete degeneration.[25] Nevertheless, DBA/2J mice made significant contribution to advances in glaucoma research and served as a valuable tool for translational research in glaucoma.[24 37] Recently, a study reported ameliorated optic nerve degeneration in transgenic mice overexpressing Norrin crossed with DBA/2J mice (DBA/2J/Pax6-Norrin mice), providing a possible therapeutic target for glaucoma.[38] Norrin is a secreted signalling molecule activating the Wnt/β-catenin pathway and protects retinal neurons after NMDA injection,[39] suggesting that an activator of Norrin and its downstream

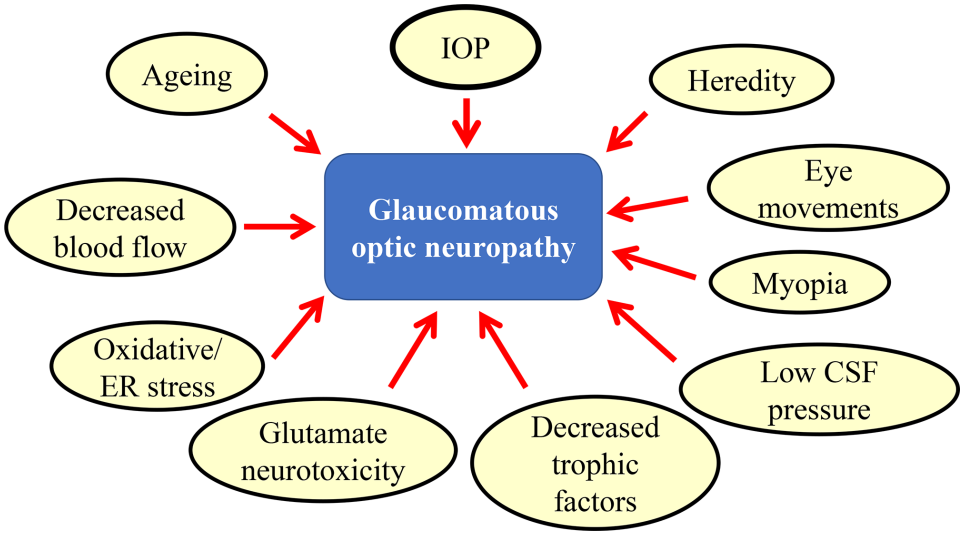

Figure 1 Multiple factors that are involved in the onset/progression of glaucoma. CSF, cerebrospinal fluid; ER, endoplasmic reticulum; IOP, intraocular pressure.

signalling might be useful to prevent glaucomatous damage in human patients.

Other high IOP glaucoma models include Vav2/3 and Vav2-deficient mice that show ocular hypertension due to ocular anterior chamber abnormalities and, consequently, cause glaucomatous optic neuropathy.[40] Although high IOP and enlarged eyeballs are not detected in all mice, Vav-deficient mice may serve as a valuable animal model of spontaneous ocular hypertension. We recently reported that the purinergic $P2Y_6$ receptor is critical for lowering IOP and that ablation of the $P2Y_6$ gene in mice ($P2Y_6$ KO) results in hypertensive glaucoma-like optic neuropathy.[41] Topically applied uridine diphosphate, an endogenous selective agonist for the $P2Y_6$ receptor, decreases IOP. The $P2Y_6$ receptor is expressed in the non-pigmented epithelial cells of the ciliary body, and its activation results in suppression of the aqueous humour production. $P2Y_6$ KO mice exhibited sustained IOP elevation, age-dependent RGC damage and impairment in visual functions, which are similar to the phenotypes of hypertensive glaucoma. We also found that the expression and function of $P2Y_6$ receptors in wild-type mice was significantly reduced by ageing, another important risk factor for glaucoma (figure 1). These data suggest that dysfunctional purinergic signalling causes IOP dysregulation, resulting in glaucomatous optic neuropathy, and the $P2Y_6$ receptor may be a target for the treatment of glaucoma.

Recent studies have reported that there is an unexpectedly high prevalence of normal tension glaucoma (NTG), a subset of glaucoma that is not associated with increased IOP, especially in Japan and other Asian countries.[42 43] Interestingly, IOP-unrelated genetic mutations have been found in NTG, and the optineurin E50K mutation was the first one identified in familial NTG.[44] Optineurin is a multifunctional protein, and its mutations are associated with neurodegenerative diseases, such as primary open-angle glaucoma (POAG) and amyotrophic lateral sclerosis (ALS).[45] It is reported that an E50K mutation-carrying transgenic mouse shows RGC loss and the reduction of retinal thickness from 12 to 16 months of age under normal IOP.[46] Transgenic mice with global overexpression of high levels of the E50K mutant optineurin showed a diffuse loss of photoreceptors and non-RGCs, features not observed in POAG.[46 47] This phenotype of E50K mutant was improved in another E50K transgenic mouse, in which the overexpression was lower and was driven by an optineurin promoter,[48] and in E50K knock-in mice.[49] At the molecular level, the E50K mutant strongly interacted with TANK-binding kinase 1 (TBK1), which prohibited the proper oligomerisation and solubility of optineurin, both of which are important for the intracellular transition of optineurin.[50] These findings suggest that alternation of the optineurin sequence can initiate significant retinal degeneration in mice. This model mimics the gene mutation that is detected in patients with glaucoma, but one problem is that mutations in glaucoma-associated genes, including optineurin, myocilin and CYP1B1, account for about 10% of cases worldwide.[51–53] Thus, more applicable models that are not necessarily associated with

Table 1 Genetic models of glaucoma

	IOP	Incidence	Onset and duration of RGC degeneration	Characteristics	References
DBA/2J	High	Dependent on colonies	8~15 months	Region specific RGC loss and systemic complications	24 25 34–38
Vav2/3 KO	High	Almost 75%	3~8 months	Buththalmos	40
$P2Y_6$ KO	High (POAG)	100%	6~13 months	Enhanced production of aqueous humour	41
Optineurin E50K knock-in	Normal	100%	3~12 months	Same as human genetic mutation	49
EAAC1 KO	Normal	100%	1~3 months	Early onset and short disease course	21 23 54 62 69 86
GLAST KO	Normal	100%	KO: 3~5 weeks Heterozygous: 1~4 months	Decrease in glutathione concentration in the retina and increased oxidative stress	22 54 68 70 80

EAAC1, excitatory amino acid carrier 1; GLAST, glutamate/aspartate transporter; IOP, intraocular pressure; KO, knockout; POAG, primary open-angle glaucoma; RGC, retinal ganglion cell.

glaucoma-associated genes, but can cover a wider range of the NTG population, are required (figure 1).

We previously discovered that deletion of the glutamate transporters, glutamate/aspartate transporter (GLAST) or excitatory amino acid carrier 1 (EAAC1) exhibits spontaneous RGC death and optic nerve degeneration without elevation of the IOP, pathology that is similar to NTG.[54] GLAST is expressed in Müller glia in the retina and clears excess glutamate from the synapse, thus preventing excitotoxic damage on surrounding retinal neurons.[29] We now understand that the glutamate concentration is not upregulated in the vitreous of patients with glaucoma, but glutamate neurotoxicity may still be involved in the pathology of glaucoma as GLAST expression levels may be decreased with ageing, especially in patients with glaucoma.[55–58] Glutamate neurotoxicity may also be involved in the RGC loss in DBA/2J mice.[59 60] In addition, glutamate, which is transported into Müller glia by the glutamate uptake process, together with cysteine and glycine, is converted to glutathione (GSH), a major antioxidant in the retina.[61] Increased oxidative stress is one of the risk factors in glaucoma (figure 1) and, consistently, the plasma GSH level is decreased in patients with glaucoma.[6 7] In GLAST knockout (KO) mice, decreased glutamate uptake into the Müller glia leads to reduced GSH production,[54] indicating that GLAST KO mice exhibit the key pathological features of NTG as a result of chronic glutamate neurotoxicity and increased oxidative stress, which are independent of glaucoma-associated genes. On the other hand, EAAC1 is mainly localised to RGCs, and cultured RGCs from EAAC1 KO mice are more susceptible to H_2O_2 insults compared with wild-type RGCs. In addition, oxidative stress markers in EAAC1 KO mice are significantly higher than those in wild-type mice.[54 62 63] Recent large-scale association analysis in a Japanese population showed the genetic link between POAG and myocardial infarction and ischaemic stroke.[64] Since both oxidative stress and glutamate neurotoxicity are involved in the pathogenesis of various neurodegenerative diseases and neurological dysfunction, including ischaemic conditions,[65] GLAST/EAAC1 KO mice seem to reproduce some aspects of sporadic, age-dependent NTG pathology. However, there are some limitations to using these mouse models. For example, the RGC loss in GLAST/EAAC1 KO mice is distributed across the entire retina, rather than in specific regions, as seen in human glaucoma and in some mouse models of ocular hypertension-induced glaucoma,[66 67] and retinal degeneration in these mice starts at 3∼5 weeks of age, an earlier and faster time-course than one may expect in human glaucoma. Despite some limitations, these mice all develop NTG-like phenotypes in a consistent time course without affecting non-RGCs[54 63] and have been providing useful information regarding NTG therapy easily and quickly (table 1). In addition, RGC degeneration in GLAST heterozygous mice occurs slowly (postnatal 1∼4 months), and this strain was available for 1 year to test the effects of a new drug.[54 68] We recently reported that some widely prescribed drugs suppressed RGC death in GLAST/EAAC1 KO mice without altering IOP. We will summarise these findings in the following section.

EFFECTS OF EXISTING DRUGS ON GLAUCOMA MODEL MICE

At present, glaucoma cannot be cured, but it can be treated. For example, there are many eye drops that suppress IOP elevation, and some of them may have additional neuroprotective effects,[63 69] but some patients do not respond to this type of treatment. It is a better option for patients if we could prevent or delay glaucomatous neurodegeneration using existing drugs. For example, we recently reported that widely prescribed drugs, such as candesartan,[21] valproic acid[22] and geranylgeranylacetone (GGA),[70] may have neuroprotective effects and preserve visual function in genetically modified animal models of glaucoma (table 2). Candesartan is an angiotensin II receptor antagonist, which is clinically used for the treatment of hypertension, and it has demonstrated neuroprotective effects by suppressing the upregulation of Toll-like receptor 4 (TLR4) in EAAC1 KO mice.[21] Since TLR4 polymorphisms are associated with NTG,[71 72] targeting TLR4 may be a promising strategy for the treatment of glaucoma. Valproic acid is a short-chain fatty acid, which acts as an epigenetic modifier by inhibiting histone deacetylases, and it is already established for use in treatment of epilepsy and others. Treatment with valproic acid improved visual fields in patients with retinitis pigmentosa, and data from a pilot study are encouraging.[73 74] Valproic acid may also have therapeutic

Table 2 Existing drugs with possibility of drug repositioning for glaucoma

	Existing indications	Characteristics	Side effects	Usage models	References
Candesartan	Hypertension Heart failure	Angiotensin II receptor antagonist	Birth defects Low blood pressure High blood potassium	EAAC1 KO mouse	21
Valproic acid	Epilepsy Migraines Bipolar disorder	Histone deacetylase inhibitor	Birth defects Liver dysfunction Thrombocytopaenia	GLAST KO mouse NMDA injection	22 28
Geranylgeranyl acetone	Gastric ulcers	Antioxidant Hsp70 inducer	Liver dysfunction Jaundice	GLAST KO mouse	70
Edaravone	Acute stroke ALS	Free radical scavenger	Acute renal failure Liver dysfunction	EAAC1 KO mouse Optic nerve injury	23 77
Nicotinamide	Pellagra High cholesterol Skin diseases	Vitamin B3 antioxidant and antipruritic	Skin flushing Low blood pressure Hyperglycaemia Liver dysfunction	DBA/2J mouse	24
Amlexanox	Aphthous ulcers Bronchial asthma Allergic rhinitis	Antiallergic immunomodulator and TBK1 inhibitor	Skin rash Headache Nausea Diarrhoea Liver dysfunction	Optineurin E50K knock-in mouse	49

ALS, amyotrophic lateral sclerosis; EAAC1, excitatory amino acid carrier 1; GLAST, glutamate/aspartate transporter; Hsp70, heat shock protein 70; KO, knockout; NMDA, *N*-methyl-D-aspartate; TBK1, TANK-binding kinase 1.

effects in glaucoma as it prevented RGC degeneration in GLAST KO mice by inhibiting the oxidative stress level in RGCs and stimulating the neurotrophic factor signalling.[22 28] Since valproic acid may promote survival and differentiation of human iPS cells,[75] this drug may be useful for transplantation therapy using iPS cells and/or iPS cell-derived RGCs to replace the degenerated RGCs[76] and benefit patients with glaucoma, as well as retinitis pigmentosa. In addition, GGA, which is used for treatment of gastric ulcers, can act as an antioxidant by directly inducing the cytoprotective heat shock protein 70 (Hsp70) expression. Oral administration of GGA also induced Hsp70 expression in the retina and suppressed RGC death in GLAST KO mice.[70] Therefore, targeting to reduce oxidative stress in the retina may be a novel therapeutic strategy for glaucoma.[9] We recently examined the effects of edaravone, a free radical scavenger, which is used for treatment of acute brain infarction and ALS, and found that this drug reduced oxidative stress and prevented RGC death in EAAC1 KO mice.[23] In addition, the intraocular injection of edaravone just after optic nerve injury (ONI) exerted neuroprotective effects by suppressing the production of reactive oxygen species and the stress-induced signalling of apoptosis signal-regulating kinase 1 (ASK1).[77] The ASK1-p38 mitogen-activated protein kinase (MAPK) pathway is activated in response to multiple types of stress, and it is implicated in diseases such as cancer, Alzheimer's disease and multiple sclerosis.[78 79] This signalling pathway also plays a role in RGC death and optic nerve degeneration in GLAST KO mice,[80] and post-ONI treatment with a p38 MAPK inhibitor injected into the eyeball was effective for RGC protection.[27] These results suggest that edaravone stimulates neuroprotection and may be useful for the treatment of glaucoma and post-traumatic complications.

In DBA/2J mice, oral administration of nicotinamide, the precursor of nicotinamide adenine dinucleotide and a key molecule in energy and redox metabolism, modulated mitochondrial vulnerability and prevented glaucoma in aged mice.[24 37] Nicotinamides have been used for many years in the attempted treatment of a variety of disorders, including pellagra and skin diseases.[81 82] Moreover, amlexanox, a clinically approved TBK1 inhibitor used to treat bronchial asthma and allergic rhinitis, protected RGCs in optineurin E50K knock-in mice.[49] These are relatively safe drugs and may be useful for the treatment of glaucoma (table 2).

In addition to the existing drugs, we are interested in the possibility that dairy food intake is positively related to neuroprotection. Spermidine is a natural component of our diet, and it is abundant in soybeans, mushrooms and so on. Spermidine is defined as a cationic polyamine, which exists in living cells and has been found to reduce the ageing process, at least partly, via autophagy activation.[83] We previously reported that spermidine in drinking water prevented RGC death following ONI and in EAAC1 KO mice by reducing oxidative stress levels in the retina.[62 84] Interestingly, spermidine inhibited activation of the ASK1-p38 MAPK pathway in RGCs and suppressed the expression of inducible nitric oxide synthase in microglia following ONI.[62 84] Spermidine may also stimulate optic nerve axon regeneration after ONI.[84 85] These findings indicate that the oral intake of spermidine and its antioxidative effects are beneficial in the treatment of glaucoma and that the beneficial effects of spermidine can be easily attained by making conscious food choices. We also reported that every-other-day fasting (EODF), a form of caloric restriction, increased histone acetylation and elevated expression levels of neurotrophic factors and catalase, whereas it decreased oxidative stress levels in the retina.[86] Consistently, EODF attenuated NTG-like retinal degeneration in EAAC1 KO mice in both the histological and functional aspects. Our findings suggest that caloric restriction, a safe, non-invasive and low-cost treatment, may be available as an auxiliary measure for a long-term therapy of glaucoma as well as other age-related eye diseases.[87 88]

We are also interested in the development of new drugs that have neuroprotective effects. As mentioned above, deletion of the ASK1 gene prevents RGC death in various mouse models of glaucoma, including retinal ischaemia, ONI and GLAST KO mice (GLAST/ASK1 double KO mice).[27 80 89] Together with recent studies demonstrating that an ASK1 inhibitor may be useful for patients with non-alcoholic steatohepatitis and kidney diseases,[90] our results suggest ASK1 inhibitors may be a good candidate for glaucoma.[78 79] On the other hand, Dock3 is an atypical guanine exchange factor for Rac1, which is specifically expressed in the central nervous system,[91] and its expression level is decreased in the brain of patients with Alzheimer's disease.[92] We found that overexpression of Dock3 protected RGCs in GLAST KO mice.[93 94] Interestingly, Dock3 binds to GluN2B, one of the subunits for NMDA receptors, and reduces NMDA receptor expression leading to RGC protection.[93 94] In addition, overexpression of Dock3 stimulates optic nerve regeneration by stimulating multiple pathways.[95–97] Thus, we are now trying to find a Dock3 activator that may be useful for neuroprotection and axon regeneration in Alzheimer's disease and glaucoma.

We believe that the various animal models of glaucoma previously mentioned should be available for future preclinical research to test these drugs, as well as any other new drugs.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

For glaucoma treatment, a strategy other than IOP reduction is required. We believe that neuroprotection is a promising alternative method and that animal models are useful for testing new therapies and pharmacological interventions, including drug repositioning. Pathogenesis of glaucoma is complex, and it involves multiple factors. Therefore, it is impossible for one animal model to satisfy all aspects of the disease. Moreover, when one of the factors is singled out, its contribution to pathogenesis may not necessarily be critical; for example, one should be aware that an animal model that possesses the same gene mutation as patients with glaucoma only represents a small percentage of total patients with glaucoma. In reality, the majority of glaucoma cases are sporadic and independent of genetic factors. This indicates that animal models whose pathogenesis is based on non-genetic factors, such as ageing and oxidative stress, are also significant and useful. In addition, there are fundamental anatomical and structural differences between mice and humans, for example, mice do not possess a lamina cribrosa or a macula. Therefore, it is unreasonable to expect that mouse models reproduce the same neurodegenerative patterns, namely, regional specificity of RGC loss or of visual field loss as observed in human glaucoma.

It is important to note that these animal models must be easy to use in practice, and this is a critical point to recognise for reviewers of scientific journals and research grants, as well as for scientists and medical doctors conducting experiments. In this respect, animal models that do not show 100% disease onset, have a lengthy disease course, or require extensive training for generation of disease may not be suitable from the reproducibility point of view and for clinicians and young researchers who, in recent years, have shown an increased tendency to leave research. However, research using disease models are essential for preclinical studies and for elucidating disease pathology, and

one would expect its value will remain unchanged. For example, recent advances in technologies using iPS cells could generate RGCs, but interaction between RGCs and the surrounding cells will not be reconstructed and, if a whole eye is generated, studying the effects of the blood flow and the entire body on the generated eye would be difficult.

Findings from studies using high IOP models are naturally useful for NTG and so employing findings from NTG models together with findings from high IOP treatments could lead to development of novel treatments targeting non-IOP factors. Furthermore, in addition to developing better and 'more useful' mouse models, establishment of non-human primate models is a challenge for the future. All in all, understanding the pros and cons of individual disease models and using them appropriately for advances in basic research will lead to producing clinically useful outcomes. There is no doubt that this is a common goal for everyone.

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