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Inducible Rodent Models of Glaucoma

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Abstract

Glaucoma is one of the leading causes of vision impairment worldwide. In order to further understand the molecular pathobiology of this disease and to develop better therapies, clinically relevant animal models are necessary. In recent years, both the rat and mouse have become popular models in glaucoma research. Key reasons are: many important biological similarities shared among rodent eyes and the human eye; development of improved methods to induce glaucoma and to evaluate glaucomatous damage; availability of genetic tools in the mouse; as well as the relatively low cost of rodent studies. Commonly studied rat and mouse glaucoma models include intraocular pressure (IOP)-dependent and pressure-independent models. The pressure-dependent models address the most important risk factor of elevated IOP, whereas the pressure-independent models assess “normal tension” glaucoma and other “non-IOP” related factors associated with glaucomatous damage. The current article provides descriptions of these models, their characterizations, specific techniques to induce glaucoma, mechanisms of injury, advantages, and limitations.

Keywords

Glaucoma; Animal Model; Rodent; In Vivo; Intraocular pressure; Pathogenesis

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1. Introduction

1.1. Glaucoma

The glaucomas are a heterogeneous group of optic neuropathies that have in common characteristic damage to the optic nerve head (ONH) (progressive cupping and excavation) and loss of vision (characteristic progressive changes to the visual field). This group of diseases are the leading cause of irreversible vision loss and blindness worldwide, affecting more than 80 million individuals by 2020 (Tham et al., 2014). There are a number of subtypes of glaucoma, but primary open-angle glaucoma (POAG) is the most prevalent. Among a long list of risk factors that have been associated with the development of human glaucoma in a number of studies include: elevated intraocular pressure (IOP), increasing age, ethnicity, family history of glaucoma, and responsiveness to glucocorticoids (GCs) (Weinreb et al., 2014; Quigley 2011; Kwon et al., 2009; Jonas et al., 2017). It should be noted that for space limitations, this list of risk factors is not exhaustive. Elevated IOP is the most important risk factor for both the development and progression of POAG (AGIS, 2000; Kass et al., 2002), and lowering IOP pharmaceutically or surgically is the major and currently only method to treat glaucoma. Family history of glaucoma as an important risk factor supports the role genetics in the development of glaucoma (Weinreb et al., 2014; Quigley 2011; Kwon et al., 2009; Jonas et al., 2017; Worley and Grimmer-Somers 2011). *Myocilin (MYOC)* was the first glaucoma gene identified, which is autosomal dominant and responsible for ~4% of POAG (Alward et al., 1998; Stone et al., 1997). A large number of genome wide association studies (GWAS) show that the majority of glaucoma is polygenic with a number of minor risk alleles identified (Liu and Allingham, 2017; Wiggs and Pasquale, 2017). Many individuals receiving prolonged anti-inflammatory GC therapy develop the side effect of elevated IOP that can cause iatrogenic open-angle glaucoma, which is clinically very similar to POAG. These “steroid responders” are at greater risk for developing POAG (Kitazawa and Horie, 1981; Lewis et al., 1988).

Glaucoma pathogenesis involves: (1) damage to the trabecular meshwork (TM) that is responsible for decreased aqueous outflow and IOP elevation; (2) damage to the ONH, which thereby damages the unmyelinated optic nerve (ON) axons; (3) progressive death of retinal ganglion cells (RGCs); and (4) progressive loss of neurons in the vision centers of the brain (Figure 1). Detailed studies on the molecular causes of glaucoma in human are complicated by accessibility to human donor eyes with clearly defined glaucoma histories, limitations on death to preservation time (very important for neuronal tissues), and that the majority of glaucoma donor eyes are at later stages of disease, etc. Despite these limitations, a number of pathogenic signaling pathways associated with human glaucoma have been discovered, including transforming growth factor (TGF)β2, gremlin (GREM1), connective tissue growth factor (CTGF), endoplasmic reticulum (ER)/protein stress, Wnt, GC, CD44, COCH, among others. However, to better understand the molecular pathobiology of glaucoma and to develop better disease modifying therapies, new animal models are required that realistically phenocopy clinical glaucoma in man.

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1.2. Need for relevant rodent models of glaucoma

Nonhuman primate (NHP) models of glaucoma have been developed to study IOP-related damage to the ONH (Yang et al., 2017) and to discover novel IOP lowering therapeutics (Hellberg et al., 2001). However, NHPs are very expensive and ocular hypertension (OHT) models laser and sclerose the TM, making this unsuitable to better understand glaucomatous damage to the TM. Both the rat and mouse have become popular in glaucoma research due to improved methods to induce glaucoma as well as to measure and quantify glaucomatous damage to the eye (see below). Mice and rats are much less expensive to purchase and maintain, and larger numbers can be used to obtain statistically meaningful data. The clear advantage in using mice is the power of mouse genetics, both in the wide variety of congenic mouse strains available and in the capabilities to modify (increase or decrease) gene expression in specific tissues. Relevant glaucoma rodent models should accurately phenocopy glaucomatous damage in the human eye, and fortunately a number of these rodent models develop glaucoma that mimic many features of the human disease. However, many of these rodent models artificially occlude the aqueous outflow pathway leading to acute IOP elevation, which does not mimic glaucomatous damage to the aqueous outflow pathway but does model pressure induced damage to the optic nerve and retina. To address this, efforts have been made to generate more relevant models that mimic POAG-like damage to the trabecular meshwork (see Section 3.7). Other “non-pressure” rodent glaucoma models attempt to address other glaucoma pathogenic pathways that may be more relevant to “normal tension” glaucoma.

1.3. Comparative ocular anatomy of the rodent eye

The NHP eye is the closest in anatomica! structure to the human eye, but NHPs are very expensive to use in the laboratory setting. Surprisingly, rat and mouse eyes have a number of similarities to the human eye. The TM of rats and mice has laminar trabecular beams surrounded by TM cells and a true Canal of Schlemm (Smith et al., 2002). The aqueous outflow physiology is also similar to man in that ~80% of outflow is through the TM pathway (Millar et al., 2011; Millar and Pang, 2015). The majority of IOP lowering glaucoma drugs in man also lower IOPs in rat (Morrison et al., 1998; Pang et al., 2005b) and mouse (Aihara et al., 2002; Akaishi et al., 2009; Yang et al., 2012) eyes. Although mouse and rat eyes do not have laminar sheets of connective tissue supporting the ONH as seen in man, these rodent eyes have a cellular lamina that serves a similar role (Morrison, 2005; Sun et al., 2009). There is a pressure sensitive block of axonal transport at the ONH in mouse and rat eyes (Howell et al., 2007; Vidal-Sanz et al., 2012) similar to that seen in human and NHP eyes with glaucoma (Quigley et al., 1981; Quigley et al., 1979; Sakugawa and Chihara, 1985). There is a “wedge” shaped organization of RGCs and RGC axons that bundle together at the ONH in mouse eyes that mimic the arcuate bundle arrangement in RGC axons in human eyes, and initial glaucoma damage occurs at the ONH leading to sectoral loss of RGC axons, similar to arcuate bundle loss in man. There are a number of subsets of RGCs in mouse eyes (Rheaume et al., 2018), again similar to man. The RGC dendritic arbors in the inner plexiform layer are stratified as seen in human eyes (El-Danaf and Huberman, 2015; Sumbul et al., 2014). Mouse and rat RGC axons bifurcate at the optic chiasm and target ipsilateral and contralateral neurons in the visual centers in the brain.

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Among the major differences in ocular anatomy of rat and mouse eyes is the very large lens that accounts for a majority of the ocular volume compared to human eyes.

1.4. Classes of rodent glaucoma models

Rat and mouse glaucoma models generally can be classified as either pressure (IOP) dependent or pressure independent (Table 1). Many of the pressure dependent models address the most important and causative risk factor of elevated IOP, although the manner in which IOP is raised in the rodent eyes varies. Pressure independent models attempt to address "normal tension" glaucoma in which IOPs remain in the normal range. These models also address other "non-IOP" related factors that have been associated with glaucomatous damage to the ONH and/or RGCs. Both pressure dependent and independent models will be addressed in this review. It should be noted that many of these models are specific to a particular lab and oftern require considerable skill to develop reproducibility. Unfortunately, there may be some "reporting bias" because labs that are unable to reproduce one of these models generally do not report negative data. If possible, it is best to receive detailed methods and/or visit the lab where the model is routinely run in order to learn the "tricks of the trade". Still, a major challenge with many of the inducible rodent models of glaucoma is this variability in success between laboratories (science needs to be independently reproducible) and the need to properly convey all the details required to accurately and independently validate a model in another laboratory.

1.5. Genetic rodent models of glaucoma

There are a number of interesting and informative genetic mouse models of glaucoma (Table 2). However, these models are beyond the scope of our current review and have been previously covered in other very good review articles (Fernandes et al., 2015; Howell et al., 2008; John et al., 1999; Lindsey and Weinreb, 2005; McKinnon et al., 2009).

2. Experimental techniques and end points

One of the major advances in the growing use of mice and rats in glaucoma research has been the development of relevant experimental techniques that allow measurement and quantification of glaucomatous damage to the rodent eye (Table 3). Both invasive (Avila et al., 2001; John et al., 1997) and non-invasive techniques have been used to measure IOP in rodent eyes. Cannulation of the rodent eyes with microneedles provide the most accurate measurement of IOP but has the disadvantage of requiring anesthesia (that affects IOP), and the invasive nature prevents multiple, frequent measurements of IOP in the same eye. Non-invasive tonometry using the Tonopen in rat eyes (Moore et al., 1993) and the rebound tonometer (e.g. TonoLab) in mouse and rat eyes (Danias et al., 2003a; Wang et al., 2005) provide accurate IOPs as long as these tonometers are properly calibrated for each rat or mouse strain being evaluated (Figure 2). Both rats and mice (and human operators!) can be behaviorally trained to record conscious IOPs, which eliminates the confounding effects of anesthesia on the IOP readings. With extensive experience in using both the Tonopen and Tonolab in the rat and mouse, our research team feels that the TonoLab is a much easier equipment to learn and use. Prolonged training sessions for new users are typically not required before satisfactory data are obtained. Most importantly, because of the very light

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force when the probe touches the cornea, the animals tolerate it very well and appear comfortable. Their IOP values are not affected after multiple (even up to 90) consecutive measurements (Wang et al., 2005).

Glaucomatous damage to the retinal nerve fiber layer (RNFL), retinal ganglion cell layer (RGCL), and interplexiform layer (IPL) can be measured non-invasively and progressively in the same eyes using optical coherence tomography (OCT) imaging (Dietrich et al., 2019; Kim et al., 2016; Liu et al., 2014) (Figure 3). Likewise, RGC function also can be measured non-invasively using electroretinography (ERG) technologies that specifically measure RGCs, including pattern ERG (PERG) (Figure 4) and the positive scotopic threshold response (pSTR) (Liu et al., 2014; Perez de Lara et al., 2014; Zode et al., 2012; Salinas-Navarro et al., 2009; Cuenca et al., 2010). (Figure 4). It should be noted that some rodent glaucoma models report changes to the ERG a- and b-wave amplitudes and/or latencies, which indicates damage to photoreceptors and bipolar cells, respectively. ERG a- and b-wave deficits generally are not associated with glaucomatous damage to the retina in humans. RGCs or subsets of RGCs have been labeled with fluorescent proteins that allow visualization of RGS in live mice (Leung et al., 2008; Tosi et al., 2010) or help distinguish RGC subset sensitivity to glaucomatous insults by immunofluorescent staining of retinal flatmounts (Daniel et al., 2018; El-Danaf and Huberman, 2015). RGCs counting in retinal flatmounts often use immunofluorescent staining of Brn3a or Brn3b, RBPMS, NeuN (Figure 5), or b-tubulin-III (Tuj1). It should be noted that anti-Brn antibodies do not label the entire RGC population, so alternatives such as RBPMS and NeuN are commonly used. It often is difficult to see individual RGC soma with Tuj1 because this antibody also labels the overlying RGC nerve fiber layer. Commercial availability of these antibody reagents is somewhat unpredictable and may change with time. RGCs in specific regions of the retina can be counted manually or automatically. There are several automated programs that perform total RGC counts (Danias et al., 2003b; Geeraerts et al., 2016; Salinas-Navarro et al., 2009) (Danias et al., 2003b; Geeraerts et al., 2016; Salinas-Navarro et al., 2009; Rovere et al., 2015). However, many of the systems used to count RGCs have not been validated in multiple models and independently confirmed by other labs. In addition, in some models the retina can expand in hypertensive eyes (i.e. in eyes of young animals with high IOPs), so RGC number vs RGC density needs to be considered. RGC numbers can vary among different mouse and rat strains, so this needs to be considered when comparing results between strains. Most studies do not address whether their models also damage other retinal neurons (e.g. displaced amacrine cells in the RGC layer or other cells in the inner and outer retina). In addition, it is highly likely that both retinal astrocytes and microglia play an important role in pathogenic damage to the retina and optic nerve. In addition, quantitative assessment of retina! mRNA levels of RGC-specific biomarkers, such as Thy-1 and neurofilament-L, can be used as indices of RGC injury (Nash & Osborne, 1999; Chidlow et al., 2005).

RGC numbers and axonal transport function can be determined using intravitreal injection of cholera toxin B (CTB) attached to a fluorescent dye for anterograde transport or injection of a membrane impermeable fluorescent dye (e.g. di I, fluorogold) into the superior colliculus to measure retrograde transport. Optic nerve function also can be measured *in vivo* by recording light-induced electric currents in the visual cortex using visual evoked potentials

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(VEPs) (Domenici et al., 2014; Heiduschka et al., 2010; Porciatti et al., 1999). Histological damage to the ON can be assessed by counting axons, using either manual or automated imaging techniques (Ebneter et al., 2012; Oglesby et al., 2012; Quigley et al., 2011; Zarei et al., 2016). Paraphenylenediamine (PPD) staining of ON cross-section allows easy identification of degenerating axons, and a number of "clinical" scoring procedures have been used to very quickly assess ON damage in large numbers of samples in both rat and mouse models of glaucoma (Chauhan et al., 2006; Fortune et al., 2004; Libby et al., 2005a) (Figure 6). Loss in vision can be measured behaviorally using optokinetics (Dietrich et al., 2019; Stahl, 2004).

Recently, there has been more emphasis on issues related to rigor and reproducibility as requirements for both publications and grant proposals, and these should be carefully considered when using inducible rodent models of glaucoma. Genetic backgrounds for each group should be carefully matched and "n"s per group should be designed based on preliminary data to provide statistically significant data at the end of the study. Also, proper controls need to be incorporated (i.e. sham injections/procedures; anesthetics; post-surgical care). Care also needs to be considered when using contralateral eyes as controls, because these eyes are not naïve and often are also affected (Liu et al. 2014). Age and gender also should be addressed. Although it is less expensive to use young rodents, in some cases ocular tissues and neuronal connections within the brain are still developing, and the majority of human glaucoma occurs in older adults.

3. Pressure-dependent models

Since OHT is a major risk factor for glaucoma, it makes sense to develop and use animal models with elevated IOP for glaucoma research. In rodents, most pressure models involve blockade of the aqueous humor outflow either by intracameral injection of occluding materials or by sclerotic damage of the outflow structures/vasculatures. As previously mentioned in Section 1.2, this acutely elevates IOP and does not mimic the natural course of glaucomatous damage to the aqueous outflow pathway, but other models attempt to more accurately model glaucoma pathogenesis in the TM (Section 3.7). Both mice and rats are most active at night, and A aqueous humor production as well as IOP are higher at nighttime (Haddadin et al., 2009; Valderrama et al., 2008), and therefore damage to the outflow pathway leads to even higher IOPs at nighttime (Zode et al. 2014; Patel et al. 2017; Jia et al., 2000). Therefore, many studies reporting daytime IOPs are under-estimating the peak IOP and the full magnitude of IOP exposure. In addition, IOPs need to be carefully circadian matched. For some inducible glaucoma models, careful aqueous humor dynamics studies have not been performed, so we do not know whether the method of IOP induction has any influence on these natural circadian variations.

3.1. Microbead occlusion models

A number of laboratories run various versions of the microbead occlusion model, which appear to work well in specific laboratories. In addition, these models appear to evolve over time, even within the same laboratory. Unfortunately, no "head-to-head" comparisons have been reported, so it is difficult to determine which is the "optimal" version of these models.

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3.1.1. Intracameral injection of microbeads—Intracameral injection of microbeads (microspheres) to elevate IOP was first described in larger mammals, such as the primate (Weber and Zelenak, 2001). This method was later successfully adapted and optimized for mice and rats. Polystyrene or latex microbeads injected into the anterior chamber accumulate in the TM and Schlemm's canal (Cone et al., 2010) and cause a physical blockade of the aqueous outflow pathway leading to OHT.

Because of the small size of the rodent eye, injecting fluid into the anterior chamber itself is sufficient to produce an IOP spike. If the injected volume is too large, the excessive increase in IOP may reduce retinal circulation transiently or induce other types of unintended ocular injuries, which will confound data analysis and interpretation. Thus, most laboratories limit their ocular injection volume to 2 μL for the mouse and 10 μL for the rat, although higher volumes have been reported.

Researchers have also evaluated effects of the size and number of the injected microbeads on the induced OHT. In the mouse, Chen et al (2011) demonstrated that using 1.4×10^4 beads of 10 μm diameter (in a volume of 2 μL) produced a higher IOP than 15 μm beads. They further showed that a second injection prolongs the IOP change for more than 8 weeks. In the rat, Sappington and coworkers (Sappington et al., 2010) carefully compared amounts of injected 15 μm microbeads and concluded that 5×10^3 beads (in a volume of 5 μL) produced consistent elevation of IOP and, similar to the mouse, a second intracameral injection lengthens the duration of OHT to more than 8 weeks. Urcola et al (2006) showed that weekly injections of latex microbeads ($2–4 \times 10^5$; in a volume of 20 μL) produces a very prolonged IOP elevation lasting for more than 30 weeks.

3.1.2. Intracameral injection of microbeads with viscoelastic material—While intracameral injection of microbeads suspended in an aqueous solution, such as PBS or balanced salt solution, is sufficient to induce OHT, others have modified the technique by injecting a viscoelastic formulation, either 1% sodium hyaluronate (Healon®, Proviso®) or 2% hydroxypropylmethylcellulose (Methocel®), following the bead suspension. Addition of viscoelastic solution enhances the mean IOP elevation and perhaps the duration of hypertension (Cone et al., 2012; Urcola et al., 2006). It is believed that the viscoelastic solution minimizes the leakage/egress of microbeads when the injection needle is removed. Furthermore, because of their physical properties, the viscoelastic material may also directly contribute to additional blockade of aqueous humor outflow. Cone et al (2012) and Frankfort et al (2013) further used a mixture of microbeads of two different sizes to attempt to improve the OHT effect.

3.1.3. Intracameral injection of magnetic microbeads—In the rat, this technique was also optimized by intracameral injection of ferromagnetic microbeads. As first described by Samsel et al (2011), at the completion of injection of the beads, a hand-held magnet is used to draw the magnetic beads away from the injection site, such that bead leakage can be minimized when the needle is withdrawn. The magnet can also help to spread the beads to all quadrants of the iridocorneal angle to maximize their distribution, and presumable trabecular outflow blockade. Bunker and colleagues (2015) further refined the procedure by placing a cylindrical magent over the anterior segment to encourage fast

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transfer of magnetic beads to the TM, which was reported to produce a very high IOP in the rat.

The microbead-induced OHT in the mouse and rat causes pathological changes similar to glaucomatous retinopathy and optic neuropathy: thinning of the retina (Chen et al., 2011) (especially the combined thickness of the RNFL, RGCL, and IPL), reduction in ERG parameters (such as PERG amplitude), RGC loss (Cone et al., 2010; Frankfort et al., 2013; Urcola et al., 2006; Wu et al., 2019; Yang et al., 2012) or apoptosis (Bunker et al., 2015), and ON axonal damages (Bunker et al., 2015; Cone et al., 2010; Frankfort et al., 2013; Sappington et al., 2010). However, decreased a- and b-wave ampltiudes on ERG have also been observed (Wu et al., 2019), suggesting that in addition to affecting RGCs, there also is damage to photoreceptors and bipolar cells.

3.2. Intracameral injection of viscous agents

In addition to microbead injection, physical occlusion of the aqueous humor outflow can also be achieved by intracameral injection of viscous substances. For example, a single injection of hyaluronic acid into the anterior chamber increased IOP lasting for more than a week in the rat (Benozzi et al., 2002). With weekly repeated injections, OHT reached a consistent, elevated plateau for at least 10 weeks (Benozzi et al., 2002). The prolonged IOP increase caused losses of axons in the ON and cells in the RGCL, as well as decreases in a- and b-wave amplitudes and oscillatory potentials of the scotopic ERG (Moreno et al., 2005).

3.3. Sclerosis of the outflow pathway

Other than intracameral injection of foreign substances, blockade of aqeuous outflow can also be accomplished by sclerosis of the outflow pathway. Two methods, episcleral injection of hypertonic saline and laser photocoagulation of the outflow structures, have been carefully characterized and used by multiple laboratories with reported successes in rapid and sustained increase in IOP.

3.3.1. Episcleral injection of hypertonic saline—First reported by Morrison et al (Morrison, 2005; Morrison et al., 1997), an ingenious way to induce OHT in the rat is by injection of hypertonic saline ([NaCl] ≈ 1.75 M) into one of the episcleral veins while occluding most of the other episcleral veins by a tight-fitting ring placed on the circumference of the globe immediately posterior to the limbus (Morrison et al., 2015; Morrison et al., 2018) (Figure 7). The injected saline is then retrogradely forced into the Schlemm's canal and related outflow structures, which induces sclerosis and consequently blocks aqueous humor outflow, leading to elevation of IOP, typically within a few days post-injection (Morrison, 2005; Morrison et al., 2015; Morrison et al., 2018; Morrison et al., 1997). An often encountered limitation of this technique is that not all treated eyes develop OHT and the level of IOP change can be variable among the injected eyes. However, once elevated, the IOP increase is usually sustained for a long period, reported to be up to 200 days (Morrison et al., 1997).

Hypertonic saline-induced chronic IOP elevation causes pathological changes in the rat retina, ONH, and ON similar to those described in glaucoma patients. In the hypertensive

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eyes, pressure-dependent apoptotic loss of RGC (Guo et al., 2005; Hanninen et al., 2002; Morrison et al., 1997; Schlamp et al., 2001), progressive cupping of the optic disc (Chauhan et al., 2002), and loss of nerve fibers (Huang et al., 2005) were evident. Disappearance of axoplasm, axonal swelling, and collapses of myelin sheath, indicating axonal degeneration were observed in ONs of injured eyes. When assessed by axon counts (Chauhan et al., 2002; Tezel et al., 2005), by quantifying the damaged area (Johnson et al., 2007; Morrison et al., 1997), or by ON injury scores (Ahmed et al., 2004; Fortune et al., 2004; Jia et al., 2000; Morrison et al., 2005; Pang et al., 2005a; Schlamp et al., 2001), the severity of ON injury correlated well with the magnitude and duration of IOP elevation. Importantly, these changes are obviously results of OHT, because treatment with glaucoma medications, betaxolol and apraclonidine, reduced the ON injury significantly (Morrison et al., 1998).

In addition to morphological changes, hypertonic saline-induced hypertension also produces functional changes in the rat retina in an IOP-dependent manner. In rats when a mild IOP elevation was achieved by this procedure, only their pSTR parameters were reduced, without affecting other ERG components, such as a-wave, b-wave, and oscillatory potential, indicating a selective RGC injury (Fortune et al., 2004). In contrast, rats with prolonged high IOP had diminished amplitudes of these other ERG components, suggesting damage to bipolar and photoreceptor cells, similar to late-phase glaucoma patients (Fortune et al., 2004).

This technique has been successfully adopted by many researchers. It is a very useful model for glaucoma research, generating IOP-dependent retina and ON pathophysiological changes. Nonetheless, it has several drawbacks: (1) The injection often produces various degrees of IOP elevation in different animals of the same cohort. Therefore, relatively large numbers of animals are usually necessary to allow sufficient sample sizes of similar IOP change. (2) The injection procedure is technically difficult and labor intensive, limiting the throughput of studies. (3) Because of the much smaller diameter of the mouse episcleral veins, this technique is too challenging to be practical in the mouse.

3.3.2. Laser photocoagulation of outflow pathway—Laser photocoagulation is another practical means to induce sclerotic damage to the outflow pathway. Several variations of this method have been reported. In the rat, Ueda et al first injected India ink into the anterior chamber, then lasered the TM to produce IOP elevation (Ueda et al., 1998). The procedure leads to apoptotic RGC death (Lam et al., 2003) and loss of ON axons (Ishii et al., 2003). A major inconvience of this technique is that, in order to sustain a prolonged OHT, frequent and repeated lasering is necessary.

WoldeMussie and coworkers directly lasered the rat limbal and episcleral veins, instead of the TM, to induce further downstream blockade of aqueous outflow and thus chronic OHT, which lasted for more than a year (Hare et al., 2001; WoldeMussie et al., 2001). Similar to TM laser photocoagulation, it caused RGC loss (WoldeMussie et al., 2001) and reduction in the PERG amplitude (Ben-Shlomo et al., 2005). The procedure does not seem to cause outer retina damage (Ben-Shlomo et al., 2005).

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Levkovitch-Verbin et al generated consistent IOP elevation in the rat translimbal laser treatments directed at the TM plus episcleral veins (Levkovitch-Verbin et al., 2002), which led to ON damage and RGC death (Levkovitch-Verbin et al., 2002; Martin et al., 2002; Martin et al., 2003). Importantly, the induced damage was rather expansive: all retinal layers became thinner (Grozdanic et al., 2004). Significant reductions in amplitudes of ERG a- and b-waves, together with a complete loss of oscillatory potentials, were observed (Grozdanic et al., 2004). These findings suggest that laser treatment of both the TM and episcleral veins produced unintended changes in addition to IOP increase.

Similar to the rat, laser photocoagulation of the mouse limbus also elevates IOP (Aihara et al., 2003a; Gross et al., 2003; Grozdanic et al., 2003b). The insult induces RGC loss and ON degeneration, comparable to changes seen in glaucoma patients (Gross et al., 2003; Grozdanic et al., 2003b; Ji et al., 2005; Mabuchi et al., 2003). However, in contrast to the rat, laser treatment of the mouse eye appears to produce more extensive retinal injuries; thinning of inner and outer nuclear layers, as well as reduction in ERG a- and b-wave amplitudes and oscillatory potentials were often reported (Gross et al., 2003; Grozdanic et al., 2003b; Ji et al., 2005; Mabuchi et al., 2003). It is likely that because of the smaller eye, every laser spot covers more mouse ocular structures than in the rat, and therefore additional retinal tissues are damaged.

Laser photocoagulation to induce OHT is generally regarded technically challenging to perform to the rodent eye. It requires precise delivery of approximately 80 laser burns around the limbus in order to produce a meaningful increase in IOP. Inprecision can lead to insufficient IOP elevation or unintended damage to other ocular tissues or both. Post-operation high IOP spikes were also observed. Despite these cautions, this technique is a useful research model; it produces many pathological changes similar to those seen in glaucoma patients.

3.4. Cautery of extraocular veins

Shareef et al reported that cauterization of three of the four “episcleral veins” of the rat increased IOP (Shareef et al., 1995). However, based on the descriptions by the authors, it is likely that vortex veins were mistaken as episcleral veins, since there are four vortex veins but more than 30 episcleral veins. Cauterization of vortex veins produces additional biological effects that are not solely due to OHT, such as ocular ischemia and congestion, leading to outer retina damge, in addition to apoptotic RGC death, optic disc excavation, and ON degeneration (Garcia-Valenzuela et al., 1995; Grozdanic et al., 2003c; Ko et al., 2000; Mittag et al., 2000; Neufeld et al., 1999; Sawada and Neufeld, 1999). Involvement of IOP-indedpent insult is further corroborated by Shi et al (Shi et al., 2007), who reported continuous RGC degeneration even after the cauterization-induced OHT was ameliorated by betaxolol. Hence, users of this method are advised to be cautious in the interpretation of results.

Cauterization of extraocular veins is relatively easy to perform in the rat, and it is effective in raising IOP. Nonethess, it has been observed that, in some instances, likely due to new growth of blood vessels, IOP may return to baseline in a few weeks after surgery (Grozdanic et al., 2003c; Kanamori et al., 2005; Mittag et al., 2000).

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3.5. Circumlimbal suture

Recently, He and coworkers decribed an interesting mechanical method to induce chronic ocular hypertension (He et al., 2018). In this method, a purse-string suture (7/0 nylon for the rat, 10/0 nylon for the mouse), without penetrating the sclera, is weaved around the globe on the bulbar conjunctiva posterior and parallel to the limbus. The purse-string is tightened by tying a slipknot. The tightness of the slipknot is adjusted to achieve the targeted IOP. The authors reported that a stable elevation in IOP could be maintained for at least 8 weeks in the rat and 12 weeks in the mouse, concomitant with reductions in pSTR, RNFL thickness, and RGC density (He et al, 2018).

3.6. Transient/intermittent IOP elevation

All of the above OHT models are intended to generate sustained and presumably relatively constant elevation in IOP. However, in the early stages of glaucoma, the retina and ONH are exposed to repeated fluctuations of IOP. Some of the biological changes induced by acute IOP changes may not be captured by the prolonged OHT models. To address this discrepancy, transient elevation of IOP models, without significant retinal ischemia, have been developed and characterized.

Sun et al induced an acute rat IOP elevation to 45 mmHg for up to 7 h by compression of the conjunctival limbus (Sun et al., 2011). They found that this insult caused thinning of the inner retinal layers, ON damage, and a time-dependent loss of RGC (Figure 8). It did not affect thickness of outer retina layers, nor a- or b-wave responses (Sun et al., 2011).

Crowston et al elevated rat IOP to 50 mmHg for 30 min by cannulation of the anterior chamber of anesthetized rats. Suprisingly, with such a relatively short period of OHT, loss of RGC was observed, together with reduction of photoptic negative response and pSTR, both being indicators of RGC function, but not a- or b-wave amplitudes (Crowston et al., 2015). Morrison and coworkers used a similar technique to assess IOP elevation to 60 mmHg for 8 h, and found significant focal axonal degeneration 10 days after insult. pSTR was also significantly depressed. In contrast, a- and b-waves fully recovered at 2 weeks after the acute OHT (Morrison et al., 2016).

He et al, by cannulation of the anterior chamber, increased IOP to 70 mmHg repeatedly (up to four episodes of 15 min each). Based on ERG recovery, they concluded that repeated IOP insults lead to cumulative dysfunction in the bipolar cell and RGC, but not photoreceptor cells (He et al., 2008).

At this time, effects of transient IOP elevation are difficult to compare among different studies. The main reason is the different parameters used: different levels of IOP elevation, different durations, and different techniques. Further studies are needed to clarify and perhaps unify some of the various results.

3.7. OHT induced by transduction of TM with glaucoma related genes

In order to better understand glaucomatous damage to the trabecular meshwork, viral vectors have been used to transduce the TM with genes that have been associated with the development of ocular hypertension in POAG (Table 4). While these models have been

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mainly used to validate the association of specific transgene expression with the development of OHT in rodents, additional studies need to address whether this OHT leads to glaucomatous damage to RGCs and the optic nerve.

Family history is an important risk factor for the development of POAG as well as other forms of glaucoma. Although there are a few forms of glaucoma with Mendelian heredity (e.g. *MYOC* glaucoma), the majority of glaucomas are multigenic and multifactorial (Kwon et al., 2009; Liu and Allingham, 2017; Quigley, 2011; Weinreb et al., 2016). GWAS studies have identified risk alleles in a wide variety of genes (Liu and Allingham, 2017; Wiggs and Pasquale, 2017), but each of these provide only very modest risk and often are difficult to experimentally model. Another approach is to compare transcriptome and proteome expression differences between age-matched control and glaucoma (largely POAG) trabecular meshwork tissues and cells. This research has identified a number of genes with altered expression and potential pathogenic signaling pathways to further test to determine whether these genes and pathways cause the glaucoma phenotype of elevated IOP (Table 1). We have used viral delivery of transgenes associated with glaucoma to the mouse TM *in vivo* in order to determine their effects on IOP, the aqueous outflow facility, and TM cellular and molecular biology. Not only does this validate the specific gene/pathway in the regulation of IOP, but this also allows detailed study of glaucoma pathogenesis as well as development of new glaucoma models in which to discover new disease modifying therapies.

To test the effects of glaucoma related genes discovered by “omics” comparisons of normal and glaucoma TM cells and tissues, it is imperative to use transducing reagents that effectively and selectively target rodent TM *in vivo*. Several groups have shown that adenovirus 5 (Ad5) uniformly transduces the TM of mouse eyes, although there also are small amounts of corneal endothelium and iris transduction (Hoffman et al., 1997; Junglas et al., 2012; Millar et al., 2008). We have found that administration of the Ad5.transgene by intravitreal injection provides greater TM transduction in the mouse eye compared to intracameral injection, perhaps due to a slower release of the viral construct from the vitreous, while the virus appears to be quickly “washed out” of the anterior chamber after intracameral injection. However, effective TM transduction can be accomplished by slowly injecting the transducing virus into the anterior chamber using a perfusion pump (Li et al., 2013).

An additional issue with Ad5 delivery has been the short duration of transgene action, lasting only 3–6 weeks in young mice (Millar et al., 2008; Shepard et al., 2010). An immune response to the virus and/or transgene appears to be involved because concurrent systemic treatment with anti-CD40L antibodies significantly prolongs transgene expression (Millar et al., 2008). Some groups report that this inflammation appears to be transgene dependent, with very little to no inflammation using just the Ad5.null vector to measurable anterior segment inflammation with other transgenes (Millar et al., 2008; Shepard et al., 2010; Shepard et al., 2007; McDowell et al., 2015). Some of the controversy in the literature may be due to the final purity of the viral vector preparation, as we have discovered that impurities in the viral preparations can cause significant anterior segment inflammation (unpublished results). In the past, we have used direct ophthalmoscopy to grade

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inflammation (Millar et al. 2008) but now routinely use slit lamp examination as a more sensitive assessment of inflammation. Although the CMV promoter drives strong transgene expression, this expression can be down-regulated within a month (Everett et al., 2004). For reasons that still are unclear, prolonged CMV mediated transgene expression in the TM can occur when older mice (>5–6 months old) are transduced with Ad5 (McDowell et al., 2012; Giovingo et al., 2013; Hernandez et al., 2017). There appears to be ocular toxicity induced by some AAV2 promoters (Xiong et al., 2019), and this should be carefully examined for each promoter in each viral vector.

3.7.1. MYOC—*Myocilin (MYOC)* was the first POAG gene identified and accounts for approximately 4% of POAG worldwide. There is a strong clinical genotype/phenotype correlation with some point mutations casing early glaucoma with high IOPs, while the Q368X mutation is associated with adult onset and modest but significant IOP elevation (Alward et al., 1998). Transduction of the mouse TM with Ad5.*MYOC* expression vectors were used to better understand the molecular mechanisms involved in *MYOC* glaucoma showing that the carboxy terminal 3 amino acid peroxisomal targeting signal-1 (PTS1) was essential for mutant MYOC-induced OHT (Shepard et al., 2007) (Figure 9). Interestingly, these *MYOC* transduced mice also demonstrate a genotype/phenotype correlation that matches that seen clinically. This work showed why over-expression of wild type *MYOC* (Gould et al., 2004) or introduction of glaucoma mutations into mouse *Myoc* do not effectively induce OHT (Gould et al., 2006). These data were the impetuous for developing the transgenic *MYOC.Y437H* mouse model of glaucoma (Zode et al., 2011). We also identified mouse strain differences in the ability to develop Ad5.*MYOC.Y437H* induced OHT and glaucomatous optic neuropathy (McDowell et al., 2012), suggesting that modifier genes also are present in certain mouse strains. Although the glaucoma phenotype has been independently reproduced by another laboratory (Bechel et al., 2014), rederivation of this *Tg.MYOC.Y437H* line has reported no glaucoma phenotype (Lynch et al., 2019). To date, it is not clear why there are phenotypic differences in this transgenic line between these laboratories. Significant advances in genome editing technology have allowed modifications of specific genes. We used CRISPR/Cas9 and RNA guide strand targeting *MYOC* packaged into Ad5 to knockout *MYOC* expression in the TM of Tg.*MYOC.Y437H* mice. Genome editing and elimination of mutant *MYOC* expression prevented IOP elevation in young mice and decreased IOP in older mice (>9 months old) (Jain et al., 2017).

3.7.2. TGFβ2—The profibrotic cytokine TGFβ2 is elevated in the aqueous humor and TM of POAG eyes (Inatani et al., 2001; Ozcan et al., 2004; Picht et al., 2001; Tovar-Vidales et al., 2011; Tripathi et al., 1994). Initial attempts using Ad5.TGFβ2 to induce OHT in mice and rats were unsuccessful until the generation of a mutant bioactivated form of TGFβ2 (TGFβ2 $^{C226/228S}$) was used (Shepard et al., 2010). Ad5.TGFβ2$^{C226/228S}$ increased TGFβ2 expression in the aqueous humor and TM as well as mediated statistically significant IOP elevation in both mice and rats (Shepard et al., 2010) (Figure 10). This TGFβ2-induced OHT was due to a significant reduction in the aqueous outflow facility in mouse eyes. Prolonged TGFβ2-induced OHT (4–6 weeks) caused glaucomatous optic neuropathy in A/J mice as assessed by PPD stained axons in ON cross sections (Figure 11). This specific vector also has been used to determine the *in vivo* signaling pathways responsible for TGFβ2-induced

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OHT. In cultured human TM cells, TGFβ2 signals via both Smad dependent (Smad2/3/4) and Smad independent (p38, ERK1/2, and Jnk) pathways (Sethi et al., 2011). In order to determine which pathway(s) were involved in TGFβ2 OHT, we used Ad5.TGFβ2$^{C266/228S}$ to transduce the TM of wild type C57/BL6J mice and *Smad3* knockout mice. Although the WT mice developed significant TGFβ2 OHT, *Smad3*$^{-/-}$ mice did not (McDowell et al., 2013). A recent study has suggested that TGFβ2 induced DAMPs such as the EDA isoform of fibronectin in the TM (Medina-Ortiz et al., 2013) serve as ligands for the TLR4 receptor to promote fibrotic damage to the TM and thereby may elevate IOP. This hypothesis was tested in WT (C3H/HeOuJ) and *Tlr4* mutant mice (C3H/HeJ). The lack of functional TLR4 in the *Tlr4* mutant mice prevented TGFβ2-induced OHT and the associated reduced outflow facility (Hernandez et al., 2017). It should be noted that other approaches have been used to generate TGFβ2 OHT, including multiple injections of TGFβ2 into the anterior chamber of rat eyes (Hill et al., 2018).

3.7.3. GREM1—Several studies have shown that the profibrotic activity of TGFβ2 in the TM is blocked by bone morphogenetic protein (BMP) signaling. Both BMP7 (Fuchshofer et al., 2007) and BMP4 (Wordinger et al., 2007) inhibit TGFβ2 induced expression of a number of extracellular matrix (ECM) genes and proteins in cultured human TM cells suggesting that TGFβ2 and BMP crosstalk signaling regulates TM ECM homeostasis and normal IOP. Expression of the BMP antagonist *Gremlin (GREM1)* is elevated in TM cells derived from POAG compared to control eyes (Wordinger et al., 2007). There appears to be a “feed-forward” profibrotic response between TGFβ2 and GREM1 because TGFβ2 increases GREM1 expression and vice versa in cultured TM cells (Sethi et al., 2011). In order to determine whether GREM1 was able to affect IOP and aqueous outflow, Ad5.*GREM1* was used to transduce the TM of mouse eyes. Over-expressing *GREM1* significantly elevated IOP and reduced the outflow facility (McDowell et al., 2015). Interestingly, this GREM1-induced OHT was blocked in *Smad3*$^{-/-}$ mice, further supporting the role of Smad3 signaling in the profibrotic activities of both TGFβ2 and GREM1 (McDowell et al., 2015).

3.7.4. CTGF—An important mediator of the profibrotic effects of TGFβ2 is connective tissue growth factor (CTGF). CTGF induces ECM deposition in cultured TM cells (Junglas et al., 2009; Wallace et al., 2013) and increases TM cell viability (Kuespert et al., 2015). Expression of CTGF is elevated in the aqueous humor of patients with exfoliation glaucoma (Browne et al., 2011). Ad5.CTGF transduction of mouse eyes increased expression of CTGF, fibronectin, and α-smooth muscle actin in the TM as well as deposition of extracellular material in the cribriform region, which was associated with the development of OHT and ON damage (Junglas et al., 2012). Based on these results, a very useful transgenic *Ctgf* mouse model of glaucoma was developed (Junglas et al., 2012).

3.7.5. Secreted frizzled-related protein 1 (SFRP1)—Transcriptomics and proteomics comparisons between TM cells isolated from POAG donors and age-matched controls have shown increased expression of SFRP1 mRNA and protein in GTM cells (Wang et al., 2008a). SFRP1 is an antagonist of the Wnt signaling pathway. Wang and colleagues have shown that TM cells and TM tissues express Wnt agonists, Wnt receptors

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(FZLD), as well as Wnt antagonists (Wang et al., 2008a). This group also demonstrated that TM cells and tissues have a functional canonical Wnt β-catenin signaling pathway that regulates IOP (Mao et al., 2012; Wang et al., 2008a). Ad5. *SFRP1* transduction of mouse eyes statistically elevated IOP and reduced aqueous outflow facility (Mao et al., 2012; Wang et al., 2008a), which was reversed by topical ocular administration of a GSK3β inhibitor, further supporting the role of the Wnt β-catenin pathway in regulating IOP. However, SFRP1 directly binds Wnt ligands and inhibits all 3 major Wnt signaling pathways (including the β-catenin pathway). In order to more directly determine which Wnt signaling pathway is involved in lOP regulation, we evaluated the IOP effects of the Wnt antagonist DKK1, which is specific for the Wnt β-catenin pathway. Ad5. *DKK1* transduction of the TM in mice elevated IOP to the same extent as SFRP1 (Mao et al., 2012). This more conclusively demonstrates that the Wnt β-catenin signaling pathway regulates normal IOP, and perturbation of this signaling pathway causes OHT. Wnt stabilization of β-catenin leads to elevated cytosolic and nuclear levels of β-catenin (Mao et al., 2012). Nuclear β-catenin binds to TCF/LEF promoter regions, which mediate the expression of Wnt regulated genes. Cytoplasmic β-catenin binds to cadherin adhesion receptors and links them to the actin cytoskeleton. SFRP1 inhibition of Wnt signaling leads to the proteolytic degradation of β-catenin, thereby lowering both nuclear and cytoplasmic levels of β-catenin. This not only shuts down Wnt mediated gene expression, but also may interfere with cadherin junctions. The TM expresses a number of cadherins, including K-, OB-, and N-cadherins, and Wnt3a increased the expression of K-cadherin CDH, and this expression was decreased by SFRP1 (Webber et al., 2018). In order to determine whether SFRP1 suppression of K-cadherin expression was responsible for SFRP1-induced OHT, mouse eyes were transduced with Ad5. *SFRP1* with or without concomitant transduction with Ad5.K-cadhein. Co-expression of K-cadherin along with SFRP1 significantly decreased SFRP1-induced OHT suggesting that at least part of the IOP elevating activity of SFRP1 is mediated by decreased expression of K-cadherin in the TM (Webber et al., 2018) (Figure 12).

3.7.6. CD44—Comparison of protein expression in the aqueous humor of POAG patients and age-matched controls found significantly increased expression of the cell adhesion molecule CD44, which can be associated with the membrane (CD44S) or a soluble ectodomain form (sCD44) (Knepper et al., 2002). Increased levels of sCD44 were associated with progressive visual field damage in POAG patients (Nolan et al., 2007). In order to determine whether CD44 plays a potential pathogenic role in glaucomatous damage to the TM, mouse eyes were transduced with Ad5.CD44. Over-expression of CD44S significantly elevated IOP and reduced the aqueous outflow facility, suggesting a pathogenic role of this hyaluronan receptor in POAG (Giovingo et al., 2013).

3.7.7. Cre and inducible transgene models—In addition to using viral vectors to over-express specific glaucoma related transgenes, Ad5 also has been used to deliver Cre to the TM of conditional knockout (“floxed”) mice. This experimental approach does not require crossing a conditionally floxed mouse with a mouse strain having a tissue specific promoter driving Cre, which saves considerable time and expense. Also, no “TM specific promoter” has been identified to generate a specific TM Cre mouse line. Examples of this approach include Ad5.Cre delivery to the TM of *Bambi*$^{fl/fl}$ mice (Hernandez et al., 2018).

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These mice have increased ECM expression in the TM and develop OHT due to a decreased outflow facility (Figure 13), supporting the homeostatic role of TGFβ2/TLR4/BMP in the regulation of normal IOP. The role of the extracellular matrix cross-linking enzyme transglutaminase-2 (TGM2) in TGFβ2-induced OHT was confirmed using Ad5.Cre to knockdown TM TGM2 expression in *Tgm*$^{fl/fl}$ mice (Raychaudhuri et al., 2018). Knockdown of TGM2 expression in the TM significantly inhibited TGFβ2 OHT and reversed the decreased outflow facility.

Another interesting approach would be to use inducible transgene expression, in which the specific ocular transgene is turned on or turned off by systemic or topical ocular administration of a small molecule like tamoxifen. If the inducible transgene is expressed in all tissues, more selective ocular expression can be regulated by topical ocular administration of tamoxifen or in the case of transgene expression found only in specific subset of ocular cells that can be turned on/off by either systemic or local administration of tamoxifen.

3.7.8. Genome editing

Significant advances in genome editing technology have allowed modifications of specific genes. We used CRISPR/Cas9 and RNA guide strand targeting *MYOC* packaged into Ad5 to knockout *MYOC* expression in the TM of Tg.*MYOC*^{Y437H} mice. Genome editing and elimination of mutant *MYOC* expression prevented IOP elevation in young mice and decreased IOP in older mice (>9 months old) (Jain et al., 2017).

3.8. GC-induced OHT and glaucoma

Glucocorticoids (GCs) are unsurpassed in their anti-inflammatory and immunosuppressive activities and are very commonly prescribed medications for a wide variety of conditions, including ocular inflammation. Unfortunately, prolonged GC therapy can cause OHT and secondary iatrogenic open angle glaucoma, which clinically mimics POAG (Clark, 1995; Clark and Wordinger, 2009; Fini et al., 2017; Wordinger and Clark, 1999). The development of GC-induced OHT depends on the route of administration, GC potency, and duration of treatment. GC-induced OHT does not occur in all individuals receiving prolonged GC therapy. Approximately 30–40% of individuals receiving prolonged GC treatment develop GC-OHT (Clark, 1995; Clark and Wordinger, 2009; Fini et al., 2017; Wordinger and Clark, 1999). In contrast, almost all POAG patients are “steroid responders” (Armaly, 1963; Becker and Hahn, 1964) and relatives of POAG patients also are steroid responders (Bartlett et al., 1993; Becker and Chevrette, 1966). Elevated IOP is due to TM dysfunction, and GCs cause a number of changes to the TM (Clark and Wordinger, 2009; Wordinger and Clark, 1999), although it still is unclear which or all of these effects are responsible for the IOP elevation. The GC mediated changes to the TM are very similar to those seen in POAG (Clark and Wordinger, 2009; Wordinger and Clark, 1999). Therefore, a number of groups have developed rodent models of GC-OHT and glaucoma in order to better understand the molecular pathogenesis of these two diseases.

The first report of GC-OHT in mice used osmotic minipumps to delivery dexamethasone (DEX) systemically (Whitlock et al., 2010). The mice developed modest but statistically

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significant increases in IOP; however, there also were systemic side effects including progressive loss in body weight and leukopenia. Another group used the same approach to demonstrate that the DEX-induced OHT was associated with decreased aqueous outflow in enucleated eyes and corresponding ultrastructural changes to the TM, particularly the cribriform region, which mimic what is seen clinically in man (Overby et al., 2014). Unfortunately, approximately 40% of the mice did not reach the end of the study due to systemic toxicity.

In effort to reduce the systemic side effects, Zode and colleagues (2014) administered 0.1% DEX-21 phosphate eye drops three times/day and showed significantly elevated IOP beginning at week 2 through week 6. These GC-OHT mice developed glaucomatous damage to the ON and had functional deficits to the RGCs (assessed by PERG) (Figure 14).

Topical ocular administration of DEX 3 times/day for multiple weeks is labor intensive, so several groups have developed DEX slow release formulations for periocular injections. Patel and colleagues administered DEX 21-acetate suspensions by weekly fornix-based periocular bilateral injections and these mice developed reproducible OHT (Patel et al., 2017, 2018). Mice developed statistically significant IOP elevation starting 3 days post injection that lasted for more than 70 days. The IOP elevation was even more pronounced when measured at nighttime. This IOP elevation was correlated with a 50% reduction in the aqueous outflow facility, measured in live mouse eyes.

This model was used to explore potential therapies and to dissect the molecular signaling pathways involved in GC-OHT. The TM develops ER and protein stress after topical ocular DEX administration (Zode et al., 2014). To determine whether this TM protein stress was a cause of DEX-OHT, mice received the small chemical chaperone, 4-phenylbutyrate (PBA), in their drinking water (Zode et al., 2014) or by topical ocular administration (Zode et al., 2012). PBA reduced markers of ER/protein stress in the TM, and significantly lowered IOP as well as inhibited functional damage to retinal ganglion cells. There are 2 alternatively spliced isoforms of the glucocorticoid receptor (GR); GRα is the ligand activated biological receptor for GCs, while GRβ does not bind ligand and acts as a dominant negative regulator of GC activities. We showed that gene therapy with Ad5.GRβ totally reversed the DEX-OHT, even though these mice continued to receive weekly DEX-Ac injections (Patel et al., 2018) (Figure 15). GCs alter gene expression by two major signaling pathways involving ligand activated binding to GRα. In transactivation, the activated GR binds to GC response elements (GREs) to directly increase or decrease gene expression. In transrepression, activated GR monomers bind to other transcription factors such as AP1 and NFkB to prevent binding to their DNA response elements, thereby suppressing activation of their gene expression. It has been assumed that the major anti-inflammatory activities of GCs are mediated by transrepression, while the GC-OHT side effects are mediated by transactivation (Figure 16). This hypothesis was directly tested in GR^{dim} mice that have a mutation in the GR DNA binding and dimerization domains and are defective in GR transactivation but still are able to transrepress. Periocular DEX-Ac administration induced OHT in WT but not in GR^{dim} mice, demonstrating that GC-OHT is mediated by transactivation (Patel et al., 2019). These findings suggest that agents such as SEGRAs that specifically activate transrepression

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without causing GR transactivation may be suitable ocular anti-inflammatory agents without causing GC-OHT.

Another group developed a DEX nanoparticle formulation that was administered bilaterally to mouse eyes by subconjunctival/periocular injections every 1 or 2 weeks. These mice developed significant OHT beginning at 3 days that lasted to greater than 60 days (Wang et al., 2018b; Li et al., 2019). This IOP elevation was associated with an apparent decrease in the aqueous humor outflow facility, although this was not statistically significant. This model was used to determine the effect of DEX treatment on TM tissue stiffness, and both atomic force microscopy and SD-OCT evaluation of pressure-induced changes Schlemm's canal area indicated that the TM tissue was stiffer in the DEX treated animals (Wang et al., 2018b; Li et al., 2019). These findings correlate nicely with increased TM tissue stiffness in POAG donor eyes (Last et al., 2011) and increased ECM stiffness in TM cells cultured with DEX (Raghunathan et al., 2015).

Rats also develop DEX-induced OHT after topical ocular administration of 0.1% DEX four times/day for 4 weeks. lOPs increased by greater than 20% (~5–6 mmHg) starting at 2 weeks (Sawaguchi et al., 2005; Shinzato et al., 2007), and at 4 weeks proteomics analyses were conducted on TM tissue in attempt to understand pathways involved in the development of OHT. Additional studies used topical ocular DEX administered 2 times per day for 40 days as an experimental OHT model to study the IOP lowering activities of a variety of test compounds, including *trans-resveratrol* and ROCK inhibitors (Razali et al., 2018; Marcus et al., 2019).

It should be noted that there has been mixed success between labs in generating GC-induced OHT in rodents. Nakazawa and colleagues treated Sprague Dawley rats with topical ocular 0.1% DEX three times as day for 4 weeks, and the rats progressively lost body weight as well as showed decreased IOP, perhaps due to the considerable systemic side effects in these animals (Sato et al., 2016). In addition, Faralli and colleagues reported that topical ocular administration of 0.1% DEX-phosphate to C57/BL6 mice induced a modest elevation in IOP by 3 weeks that returned to baseline at 5 weeks (Faralli et al., 2018). The DEX treated mice lost approximately 20% body weight, and DEX treatment did not appear to significantly increase FN protein expression in whole eye lysates. It is likely that a number of additional labs have attempted to develop a GC-OHT model in their own labs and have not reported negative results.

4. Pressure-independent models of glaucoma

Although IOP is a risk factor for the progression of both ocular hypertensive and normotensive glaucoma, other factors also appear to play important roles (Table 5). A number of other rodent models have been developed to address and study these non-pressure related factors. They are intended to address specific pressure-independent, pathophysiological mechanisms of the disease. It should be noted that many of these models do not necessary mimic exact features of glaucomatous damage, but they have provided important insights into pathways and mechanisms of damage to RGCs and the optic nerve. The most prevalent ones are described below.

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4.1. ON transection or ON crush

By selectively protecting the RGC soma versus the ON axons of a spontaneous glaucoma mouse model, the DBA/2J mouse, Howell and colleagues demonstrated that local insult to axons in the ON is the initial site of glaucomatous damages (Howell et al., 2007). This observation corroborates with important findings in human and non-human primates with glaucoma, which also indicate that degenerative changes of axons in the lamina cribrosa area is a primary early site of injury (Anderson and Hendrickson, 1974; Anderson and Hendrickson, 1977; Quigley and Anderson, 1976; Quigley and Addicks, 1980; Quigley et al., 1981; Quigley and Anderson, 1977; Quigley et al., 1980; Quigley et al., 1983). Because of these results, ON injury is expected to represent certain characteristics of optic neuropathy seen in glaucoma and is therefore an often-used animal model for glaucoma research.

ON transection or ON crush of the rat or mouse produces ON injury. In the rat, transection causes complete axotomy of the ON (Kielczewski et al., 2005), producing a complete and specific apoptotic RGC loss in approximately two weeks (Isenmann et al., 1999; Ju et al., 2000; Kermer et al., 2000; Kittlerova and Valouskova, 2000). Correspondingly, ON transection also completely obliterates the PERG (Domenici et al., 1991; Kittlerova and Valouskova, 2000), significantly diminishes the scotopic threshold response (Bui and Fortune, 2004), as well as abolishes visual function (Kittlerova and Valouskova, 2000), without affecting a- and b-waves (Bui and Fortune, 2004). Inhibition of the apoptosis pathway is efficacious in protecting RGC against ON transection-induced cell death (Chaudhary et al., 1999; Heiduschka and Thanos, 2000; Lingor et al., 2005). Similar damages were also reported in the mouse; most RGC are lost 2 weeks post injury (Kilic et al., 2002). Total disappearance of RGC, ON axons, and PERG response occurs within 2 months after transection (Chierzi et al., 1998).

Some argue that total damage of the ON axons may be too severe to represent glaucomatous changes. Levkovitch-Verbin et al (2003) developed a partial transection model, in which only one third of the rat ON is cut. In doing so, the model allows the study of direct axotomy-induced changes, as well as indirect, secondary insults to the uncut axons of the ON and RGCs (Levkovitch-Verbin et al., 2003). It is believed that the secondary insult plays an important role in glaucomatous retinopathy and optic neuropathy.

Crushing the ON with forceps or clamps is another technique to induce ON injury. In both the rat and mouse, ON crush injures ON axons (Minzenberg et al., 1995; Yoles and Schwartz, 1998; Zalish et al., 1993) and causes RGC death (Choudhury et al., 2015; Liu et al., 2014) (Figure 17), concomitant with reduction in PERG (Choudhury et al., 2015; Liu et al., 2014) (Figure 4). It is important to note that, because of differences in techniques and magnitudes of the applied crush force, levels of RGC damage can vary, ranging from minimal to practically total loss (Gellrich et al., 2002; Klocker et al., 2001; Liu et al., 2014). The time course of apoptotic, and sometimes necrotic, RGC loss can also be different from a few days to several weeks (Allcutt et al., 1984; Barron et al., 1986; Buys et al., 1995; Freeman and Grosskreutz, 2000; Levkovitch-Verbin et al., 2000; Libby et al., 2005b; Maeda et al., 2004; Misantone et al., 1984; Naskar et al., 2002; Schlamp et al., 2001; Schmitt and Sabel, 1996; Schuettauf et al., 2000; Swanson et al., 2005; Tezel et al., 2004a). Similarly,

various degrees of decreases in VEP and pattern VEP amplitudes have also been reported (Klocker et al., 2001; Liu et al., 2014). However, based on our experience, which appears to be true in other laboratories, the RGC and ON damage are typically consistent within each research group.

4.2. Reti nal ischemia/reperfusion injury

Many studies showed a decrease in ocular blood flow in glaucoma patients and an association between glaucomatous changes versus blood flow decrease (Hwang et al., 2012; Jia et al., 2012; Kim et al., 2012). The reduced blood flow may be a result of elevated IOP, vascular dysregulation, systemic hypotension, or other vascular disorders. Regardless the etiology or exact mechanism, abnormality of retinal blood supply is one of the probable mechanisms for glaucoma pathophysiology (Harris et al., 1994; Kaiser et al., 1993; Rader et al., 1994; Waldmann et al., 1996).

4.2.1. Ocular cannulation—Retinal ischemia models have been used to study disease changes and evaluate neuroprotective approaches. A very common method to induce retinal ischemia is to cannulate the eye with a cannula connected to a raised reservoir, which generates hydrostatic pressure above the systolic blood pressure and temporarily stops blood flow in the eye (Buchi et al., 1991; Hughes, 1991; Li et al., 2002; Smith and Baird, 1952). The success of the technique is typically evidenced by blanching of the iris and retina, which indicates complete and global ocular ischemia. Transient ischemia for approximately 60 minutes is commonly used, followed by reperfusion.

Retinal ischemia/reperfusion produces apoptotic loss of RGC in the rat (Inoue-Matsuhisa et al., 2003; Joo et al., 1999; Junk et al., 2002; Kaneda et al., 1999; Lafuente et al., 2002; Lai et al., 2002; Lam et al., 1994; Selles-Navarro et al., 1996; Wood et al., 2003) and thinning of the RNFL (Chidlow and Osborne, 2003; Chidlow et al., 2002; Dijk et al., 2004a; Nash and Osborne, 1999). Retinal ischemia/reperfusion also induces ON injury, including axonal degeneration, disruptions of mitochondria, and disordered myelin sheaths (Adachi et al., 1996). The damages produced by retinal ischemia/reperfusion are not specific to the RGC or ON. Instead, it produces panretina damage. Photoreceptors and inner nuclear layer cells are affected, too (Buchi et al., 1991; Chun et al., 1999; Dijk and Kamphuis, 2004; Dijk et al., 2004b; Grozdanic et al., 2003d; Hughes, 1991; Katai and Yoshimura, 1999; Singh et al., 2001). Amplitudes of scotopic a- and b-waves of the ERG were significantly reduced (Ettaiche et al., 1999; Grozdanic et al., 2003a; Inoue-Matsuhisa et al., 2003; Junk et al., 2002; Katano et al., 2001; Lai et al., 2002; Wood et al., 2003). In addition, ischemic injury changes amplitude, latency, and maximum velocity of the pupillary light reflex (Barnett and Grozdanic, 2004; Grozdanic et al., 2003a), showing ocular functional deficits.

Although cannulation of the mouse eye is technically more challenging to perform due to its smaller size, successful operations with similar pathological changes were reported. Apoptosis of cells in the inner nuclear layer and GCL, thinning of retinal IPL and INL (Figure 18), as well as diminished ERG a- and b-wave as well as pSTR amplitudes have been observed after retinal ischemia/reperfusion in the mouse (Kim et al., 2016; Nashine et al., 2014; Wang et al., 2002) (Figure 19). Unilateral retinal I/R injury also causes loss of

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RGC synaptic connections and atrophy of target neurons in the contralateral superior colliculus (Kim et al., 2016) (Figure 20).

4.2.2. Photothrombosis—Another technique to produce retina! ischemia is by photothrombosis. This is achieved by a green light irradiation of the central retinal artery after an intravenous rose bengal injection. Upon green light irradiation, rose bengal releases singlet molecular oxygen, which initiates platelet activation and consequently local thrombosis (Mosinger and Olney, 1989). Reperfusion can be accomplished by intravenous injection of tissue-type plasminogen activator to dissolve the thrombus. Apoptosis of RGC and cells in the inner nuclear layer by photothrombosis-induced transient ischemia was reported (Daugeliene et al., 2000; Romano et al., 1993). Some noted drawbacks of this technique are that, in addition to ischemia, phototoxicity may contribute to the resulting injuries, and it produces highly variable morphological damages (Buchi et al., 1994). These limitations make data analysis and comparison among studies challenging.

4.2.3. Ligation—A more invasive procedure, ON ligation, has also been described to produce retinal ischemia. This procedure places a suture around a surgically exposed ON and ligates it to stop blood flow in the central retinal artery and posterior ciliary arteries, which produces ischemia of the retina and other ocular structures (Stefansson et al., 1988). Removal of the suture causes reperfusion. This method, similar to other retinal ischemia/ reperfusion methods, causes apoptotic death of the RGC (Chintala et al., 2002; Vidal-Sanz et al., 2001; Zhang et al., 2003; Zhang and Chintala, 2004) and other retinal cells (Nonaka et al., 2000; Osborne et al., 1995; Rosenbaum et al., 2001). Functionally, it reduces a- and b-wave ERG amplitudes (Chiou and Li, 1993; Nonaka et al., 2000; Osborne et al., 1995; Rosenbaum et al., 2001). A major limitation of this technique is that ligation may produce mechanical injury to the ON in addition to ischemia. Cautious interpretation of results is advised. To overcome this limitation, Lafuente et al. described a technique of selective ligation of rat ophthalmic vessels. They used nylon sutures to constrict the dural sheath and blood vessels surrounding the ON, without mechanical damage to the axons (Lafuente et al., 2001, 2002b). This method induced RGC loss; the extend of loss was dependent on the duration of ischemia, ranging from approximately 50% RGC loss with a 30-min ischemia to 100% loss with 120-min ischemia.

4.2.4. Suture-Pulley System—A less common, but interesting, approach to induce transient retinal ischemia in the rat is by placing a suture around the equator of the eye immediately behind the limbus; the suture is then tightened by fixed weights through a pulley system (Li et al., 2002). The tightening of the suture elevates pressure inside the eye and, if sufficiently high, can produce ocular ischemia. Loss of a- and b-wave amplitudes and cell density in the RGCL have been reported (Li et al., 2002).

4.3. Intra vitrea! injection of excitotoxic amino acid

In the retina of vertebrates, glutamate is the essential excitatory neurotransmitter, having major roles in transmission of vision signals. However, overstimulation of a subtype of glutamate receptors, the *N*-methyl-D-asparate (NMDA) receptor, leads to excessive calcium influx into neurons (Lipton and Rosenberg, 1994; MacDermott et al., 1986), and

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consequently neuronal cell death. In the retina, NMDA receptor-mediated RGC apoptosis has been demonstrated both *in vitro* or *in vivo* (Furuya et al., 2012; Lucas and Newhouse, 1957; Luo et al., 2001; Sucher et al., 1991). Based on the observation that glutamate level in the vitreous was elevated in glaucomatous monkey and human eyes (Dreyer et al., 1996), excitotoxicity was proposed to play a role in glaucomatous retinopathy (Dreyer and Grosskreutz, 1997). However, this critical piece of evidence could not be substantiated in subsequent studies (Wamsley et al., 2005). Despite the controversy, changes in levels of glutamate metabolite, transporters, and receptors suggest an involvement of glutamate in glaucomatous retinopathy (Carter-Dawson et al., 2002; Dreyer et al., 1996; Naskar et al., 2000; Park et al., 2009). It is important to note that excitotoxicity is not limited to the RGC, nor unique in glaucoma or glaucoma study models (Ju and Kim, 2011; Levinger et al., 2012).

As a model for retinal diseases, researchers have injected NMDA or glutamate intravitreally to induce excitotoxicity in the retina. In both rats and mice, intravitreal administration of NMDA or glutamate (typically 20 to 200 nmol) induces RGC apoptosis in a dose-dependent manner. In the rat, several days after NMDA injection, apoptosis (El-Remessy et al., 2003; Kwong et al., 2003; Ma et al., 2010; Sun et al., 2001) and depletion of more than 80% of RGC (Schuettauf et al., 2004; Vorwerk et al., 1996), as well as reduction of retinal Thy-1, an RGC marker, immunoreactivity and mRNA (Chidlow and Osborne, 2003; Nash and Osborne, 1999; Osborne et al., 1999) were reported. Nevertheless, the NMDA-induced toxicity affects not only the RGC, but also other retinal neurons, such as amacrine cells, as evidenced by a dose-dependent reduction of choline acetyltransferase (Casson et al., 2004; Siliprandi et al., 1992). Intravitreal injection of NMDA causes apoptosis of neurons in the inner nuclear layer, too (Lam et al., 1999).

Functionally, NMDA injection in the rat diminishes the negative scotopic threshold response (Bui and Fortune, 2004) and the pattern VEP (Kermer et al., 2001). The electrophysiological changes correspond to a complete but transient loss of the rat's visual function as evaluated by behavioral studies. Intriguingly, the visual discriminatory behavior recovers gradually to close to baseline level even though more than half of RGCs are lost by the injection (Sabel et al., 1995; Sisk and Kuwabara, 1985; Sisk et al., 1984; Vorwerk et al., 1996). Consistent with morphological results, intravitreal injection of NMDA also significantly reduces the b-wave response (Bui and Fortune, 2004; Casson et al., 2004; Maruyama et al., 2002).

In the mouse, NMDA and glutamate produce similar injuries as in the rat retina. RGC apoptosis occurs as soon as 1 h and peaks 2 days after NMDA injection (Li et al., 2002; Li et al., 1999). Similar to the rat, NMDA also causes apoptosis of mouse inner nuclear layer neurons (Kumada et al., 2005; Li et al., 1999).

Intravitreal administration of excitotoxic amino acid is relatively easy to perform and its effects can be assessed in a few days. However, this technique as a study model for glaucoma is somewhat controversial because of the uncertainty of the role of excitotoxicity in disease pathophysiology.

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4.4. infra vitrea! injection of tumor necrosis factor alpha

Tumor Necrosis Factor Alpha (TNFα) has been implicated to play a role in glaucomatous neurodegeneration (Tezel, 2008). The level of TNFα in aqueous humor is typically very low and often below the sensitivity threshold of many immunoassays. Despite that, Sawada and coworkers were able to detect TNFα in a significantly higher percentage of glaucoma patients compared to cataract patients (Sawada et al., 2010). Using a highly sensitive bead-based immunoassay, Balaiya et al found that the average TNF-α level in aqueous samples of POAG patients was approximately 70% higher than the control samples (Balaiya et al., 2011). Recently, the mean plasma TNFα was also reported to be higher in POAG patients than the controls. In this study, logistic regression analysis suggests that the risk of POAG correlated most significantly with plasma TNFα level but not with gender or age (Kondkar et al., 2018). In donor eyes of glaucoma patients, expression of TNFα and its receptor TNF-R1 are upregulated in both the RGCs (Tezel et al., 2001) and in astrocytes at the ONH (Yuan and Neufeld, 2000).

Intravitreal injection of TNFα causes retinal changes akin to glaucoma damages, notably, the decline of survival of RGCs and oligodendrocytes (Nakazawa et al., 2006). The insult also causes significant degeneration and loss of ON axons (Kitaoka et al., 2006). It has been used as a research model to assess potential neuroprotective strategies for glaucoma therapy.

4.5. infra vitrea! injection of endothelin-1

Endothelin-1 (ET-1) has been implicated in glaucoma pathogenesis (Prasanna et al., 2011; Yorio et al., 2002). ET-1 levels in plasma and aqueous humor samples of POAG patients (both normal tension and OHT) are significantly higher than those of control subjects (Emre et al., 2005; Noske et al., 1997; Sugiyama et al., 1995; Tezel et al., 1997). Initially, because of its vasoconstrictive action, it was used to induce chronic ON ischemia (Cioffi et al., 1995). The peptide was subsequently discovered to cause injuries directly to the RGC and ON, unrelated to its effect on ocular circulation (Chauhan et al., 2004; Krishnamoorthy et al., 2008; Lau et al., 2006; Stokely et al., 2002; Wang et al., 2008b). For example, intravitreal injection or local administratioin of ET-1 to the rat ON reversibly reduces axonal transport (Stokely et al., 2002; Wang et al., 2008b) and stimulates astrocyte proliferation (Prasanna et al., 2002). Furthermore, delivery of ET-1 continuously by an osmotic pump over a prolonged period to the intraorbital ON effects a time-dependent (21 to 84 days of treatment) losses of RGC and ON axons (Chauhan et al., 2004), without obvious injuries to other retinal structures. ET-1-induced optic neuropathy may be an interesting model for glaucoma research (Prasanna et al., 2011; Blanko et al., 2017).

5. Future studies and challenges

5.1. Future studies

It is clear that there are a number of different mouse and rat models of glaucoma, each of which has its own strengths and weaknesses (Table 1). Some of these models have been successfully used by numerous laboratories, with consistant results (e.g. the Morrison hypertonic saline-induced ocular hypertension models and the mouse model of optic nerve crush). However, additional models need to be better characterized and independently

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reproduced in other laboratories. The also will be opportunities to develop additional rodent models of glaucoma given advances in better understanding the molecular pathogenesis of glaucoma in man and new molecular techniques to selectively alter the expression of specific genes in specific tissues.

Recent advances in genome editing has quickly changed the ability to specifically modify individual nucleotides in genes and/or alter gene expression (Patsali et al., 2019). CRISPR/Cas9 and related systems can examine the functions of specific genes, study the epigenetic regulation of transcription, introduce disease associated mutations to generate new disease models, and be used therapeutically. This relatively new technology has allowed the generation of new relevant disease models, although potential off target editing needs to be seriously addressed. In addition, using viral delivery of Cre to specific cells based on the tropism of the transducing virus has taken advantage numerous "floxed" (fl/fl) mouse strains to selectively knockout/knockdown gene expression (Hernandez et al., 2018; Raychaudhuri et al., 2018). Several organizations are involved in increasing the number of genes that are being floxed to expand the availability of floxed mouse strains (http://www.mousephenotvpe.org/; https://www.komp.org/).

The generation of new rodent glaucoma models is not only being used to study molecular pathogenesis, but also provides more relevant models in which to test new therapies and therapeutic approaches. For example, several new approaches to treat GC-induced OHT including gene therapy with GRβ (Patel et al., 2018) and the topical ocular administration of the small chemical chaperone PBA (Zode et al., 2012) were tested in new mouse models of GC-OHT. Topical ocular administration of a small molecule GSK-3β inhibitor was used to lower OHT induced by inhibition of Wnt signaling by overexpressing SFRP1 in the trabecular meshwork (Wang et al., 2008a). Although there already are a number of inducible rodent models of glaucoma, we fully expect to see the development of more relevant models based on new discoveries on the molecular pathogenesis of human glaucoma, including subtypes of glaucoma (e.g. exfoliation glaucoma). This in turn, will lead to the discovery and testing of new therapeutic approaches.

The power of mouse genetics is being used to identify genes associated with glaucoma endophenotypes. Recombinant inbred mouse strains such as the BXD set (C57BL/6 crossed with DBA/2 mouse strains) have allowed fine mapping of a number of glaucoma endophenotypes (Geisert et al., 2009). For example, elevated IOP is one of the most important risk factors for the development and progression of glaucoma (Kass et al., 2002) and therefore is a major endophenotype. IOP was measured in over 500 eyes of 38 BXD recombinant inbred strains of mice, which showed a wide range of IOPs (King et al., 2015). QTL mapping identified nonsynonymous SNPs in two cadherin genes, *Cdh8* and *Cdh11,* as candidates for modulating IOP. Interestingly, inhibition of the canonical Wnt signaling pathway decreased the expression of several cadherins in the as well as elevated IOP (Webber et al., 2018). This OHT was reversed by over-expressing Cdh6 further implicating cadherins in the regulation of IOP (Webber et al., 2018). In addition, there is a 3-fold difference in the ability of different BXD strains to regenerate ON axons after ON crush, showing that genetic background regulates regeneration (Wang et al., 2018a).

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5.2. Challenges

The similarity of an animal model to its corresponding human conditions determines its relevance.

Despite rodents' ocular structures that are anatomically very similar to those of human, their biochemical, physiological, or pharmacological responses may still differ. Therefore, a major challenge is the relevance and "translatability" of these rodent models of glaucoma to glaucoma in man. A number of pathogenic pathways initially discovered in glaucomatous human TM tissues and cells (Table 4) also elevate IOP in mice (see section 3.6.), so it appears that there is some overlap in these pathogenic pathways between mouse and man. However, the new glaucoma therapies that have worked in mouse models have not been evaluated in man, leaving the issue of translatability to be determined.

Furthermore, because some of the IOP-independent pathophysiological mechanisms of glaucoma are still controversial, non-pressure models may not represent the disease changes accurately. For example, involvement of excitotoxicity in glaucomatous retinopathy is still regarded as uncertain, results obtained using models based on this theory may have minimal clinical relevance if excitotoxicity is proven non-essential in the disease.

Therefore, the translatability of discoveries in rodent glaucoma models to human glaucoma is still in question. Future studies on taking compounds or biologics that work well in mouse models into the clinic will determine the overall importance of these inducible mouse models of glaucoma.

Another challenge is the size of the eye, blood flow rate, drug metabolism, and other pharmacokinetic factors are very different between rodents and human. The pharmacokinetics of administered compounds or biologics to rodent eyes will be much different than in human eyes. Topical ocular administration in rodents generally also leads to more systemic exposure often due to the relatively large volume delivered vs the ocular surface area as well as the grooming behavior of rodents, who rub the remaining dose from their eyes and then lick their forelimbs. The vitreous volume is very low in rodent eyes due to the very large lenses, so intravitreally administered therapies will have very different dilutions and distribution kinetics. There also are species differences in metabolism of these administered compounds between rodent eyes and human eyes.

For drug discovery and development, preclinical animal models are important for their generation of meaningful data ready for translational studies. In that regard, rat and mouse glaucoma models are useful and relevant. However, caution is necessary in interpreting the rodent findings because of biological divergent among the species, uncertain pathophysiological mechanims, and pharmacokinetic parameters.

Abbreviations

Ad5	Adenovirus serotype 5
BMP	Bone morphogenic protein

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CTB	Cholera toxin B
CTGF	Connective tissue growth factor
DEX	Dexamethasone
ER	Endoplasmic reticulum
ERG	Electroretinography
ET	Endothelin
GC	Glucocorticoid
GR	Glucocorticoid receptor
GWAS	Genome wide association studies
IOP	Intraocular pressure
IPL	Interplexiform layer
MYOC	Myocilin
NHP	Nonhuman primate
NMDA	*N*-Methyl-D-asparate
OCT	Optical coherence tomography
OHT	Ocular hypertension
ON	Optic nerve
ONH	Optic nerve head
PBA	4-Phenylbutyrate
PERG	Pattern electroretinography
POAG	Primary open-angle glaucoma
PPD	Paraphenylenediamine
pSTR	Positive scotopic threshold response
PTS1	Peroxisomal targeting signal-1
RGC	Retinal ganglion cell
RGCL	Retinal ganglion cell layer
RNFL	Retinal nerve fiber layer
SEGRA	Selective glucocorticoid receptor agonist
SFRP1	Secreted frizzled-related protein 1

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TGF	Transforming growth factor
TM	Trabecular meshwork
TNFα	T umor necrosis factor alpha
Tuj1	β-Tubulin-III
VEP	Visual evoked potential

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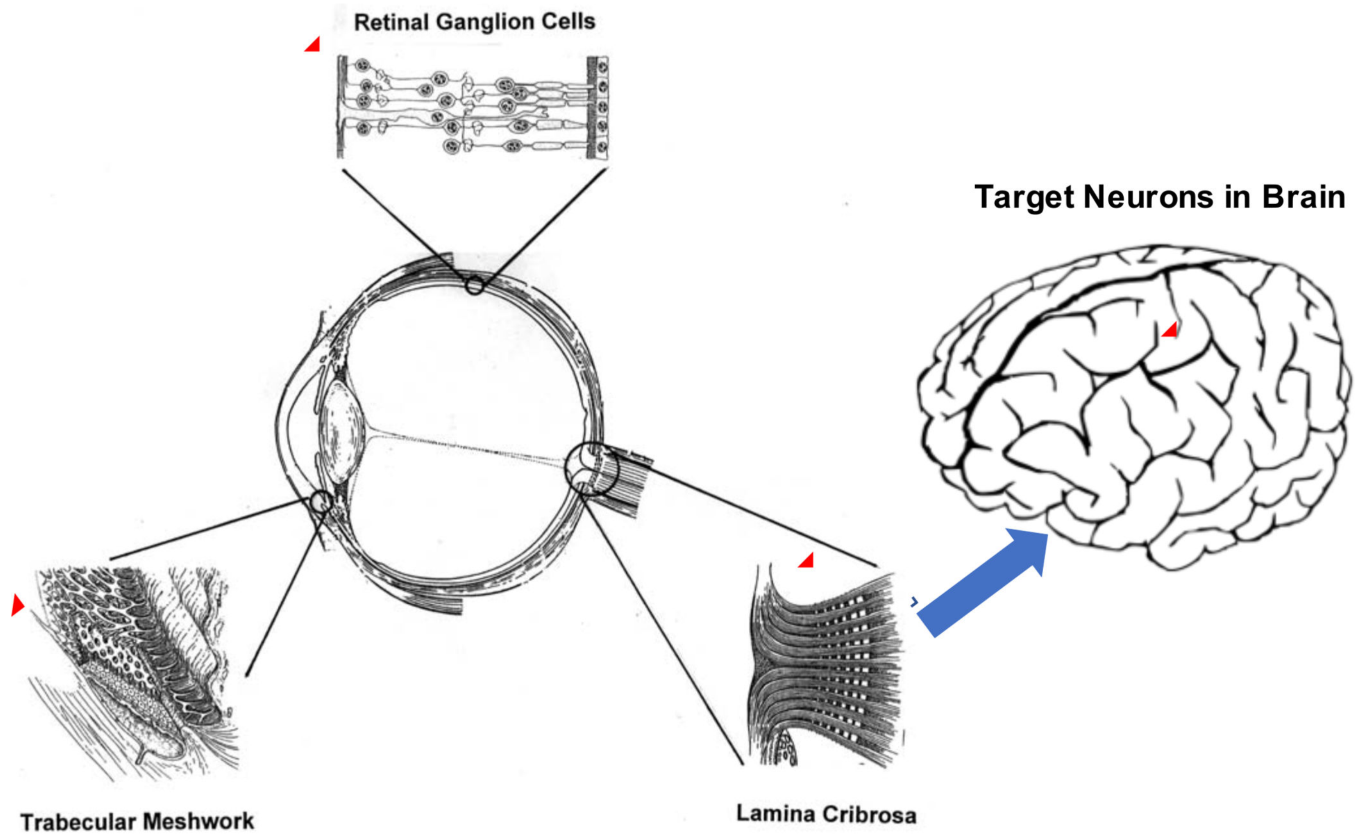

Figure 1.
Regions in the eye and brain damaged in primary open-angle glaucoma (POAG). Elevated IOP is due to damage to the aqueous humor outflow pathway (particularly the TM). Elevated pressure insults the ONH and damages the unmyelinated axons of the optic nerve, which eventually leads to RGC death and loss of target neurons in the brain.

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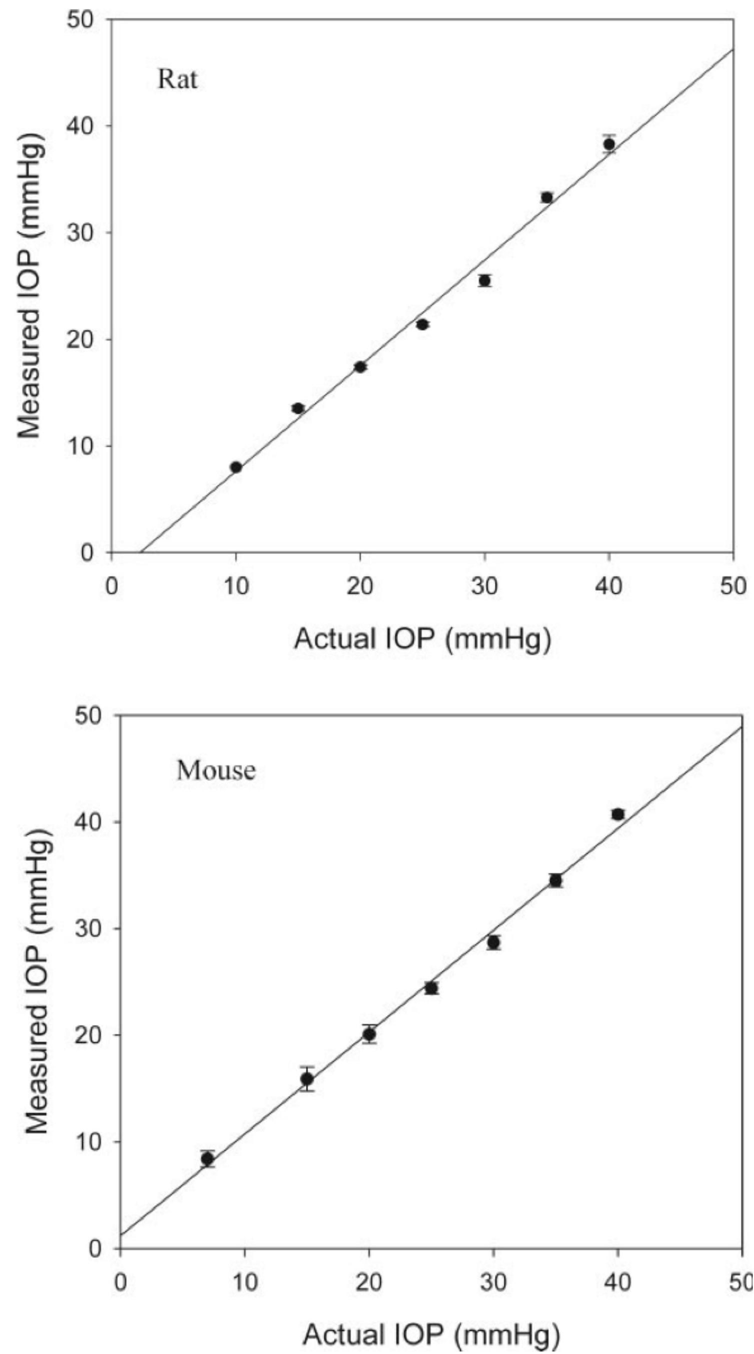

Figure 2.
TonoLab rebound tonometer measurements of IOP in rats and mice. TonoLab measured IOPs were highly correlated with actual IOPs of manometerically controlled IOPs in the anterior chambers (Figure from Wang et al., 2005).

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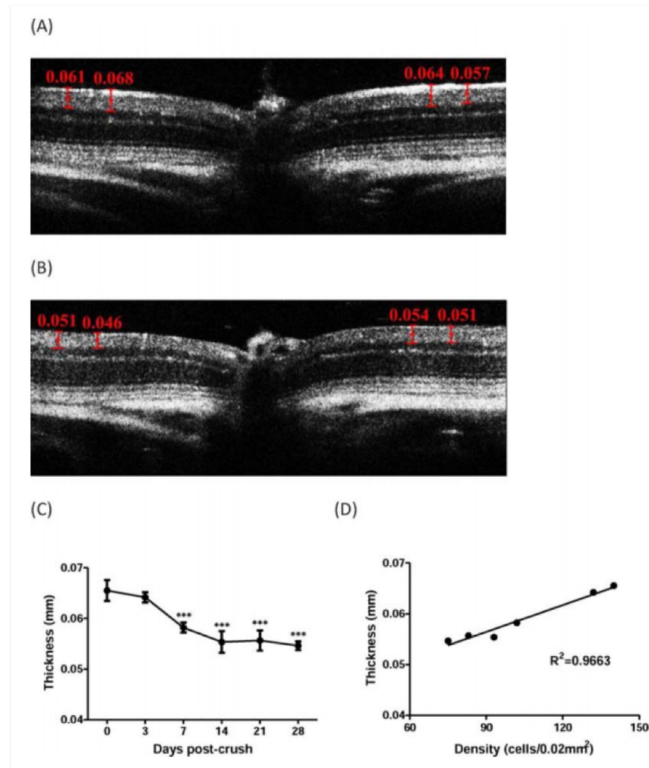

Figure 3.

Live SD-OCT imaging of retinal layers of contralateral control mouse eyes (A) and ONC injured eyes (B) 28 days post-injury. (C) The combinded RNF, RGC, and IP layer thickness progressively decreased over time ($p < 0.001$ for days 7–28). (D) There was a strong and statistically significant correlation between the OCT measured loss of RNFL/GCL/IPL thickness and GCGL cell density in this ONC model (R^2=0.9663, $p<0.001$) (Figures from Liu et al. 2014).

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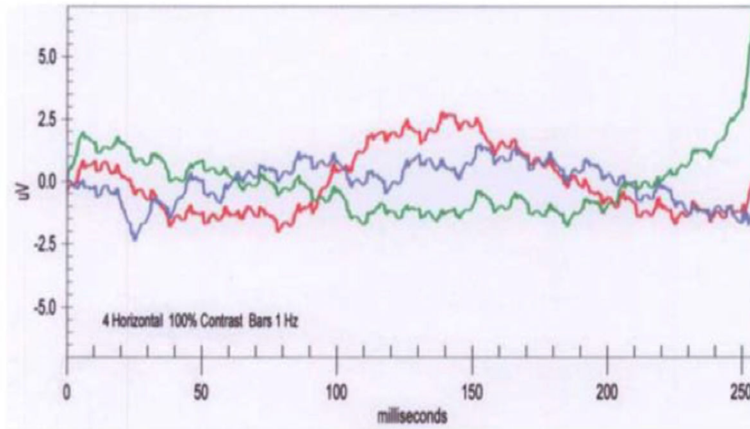
(A)



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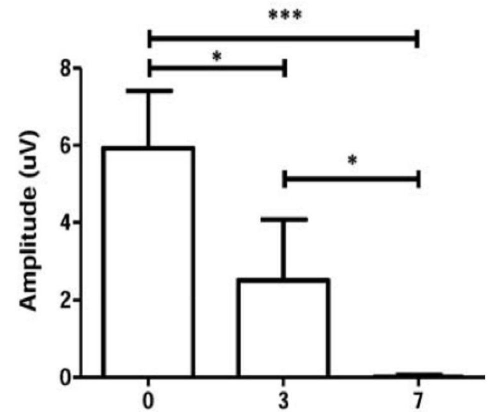

Figure 4.

Progressive loss of pattern electoretinography (PERG) in mice with ONC injury. (A) Representative PERG waveforms at 0 (red), 3 (blue), and 7 (green) days post ONC injury. (B) PERG amplitudes were significantly decreased at 3 days ($p<0.05$) and totally gone at 7 days ($p<0.001$) post ONC injury. (Figures from Liu et al. 2014)

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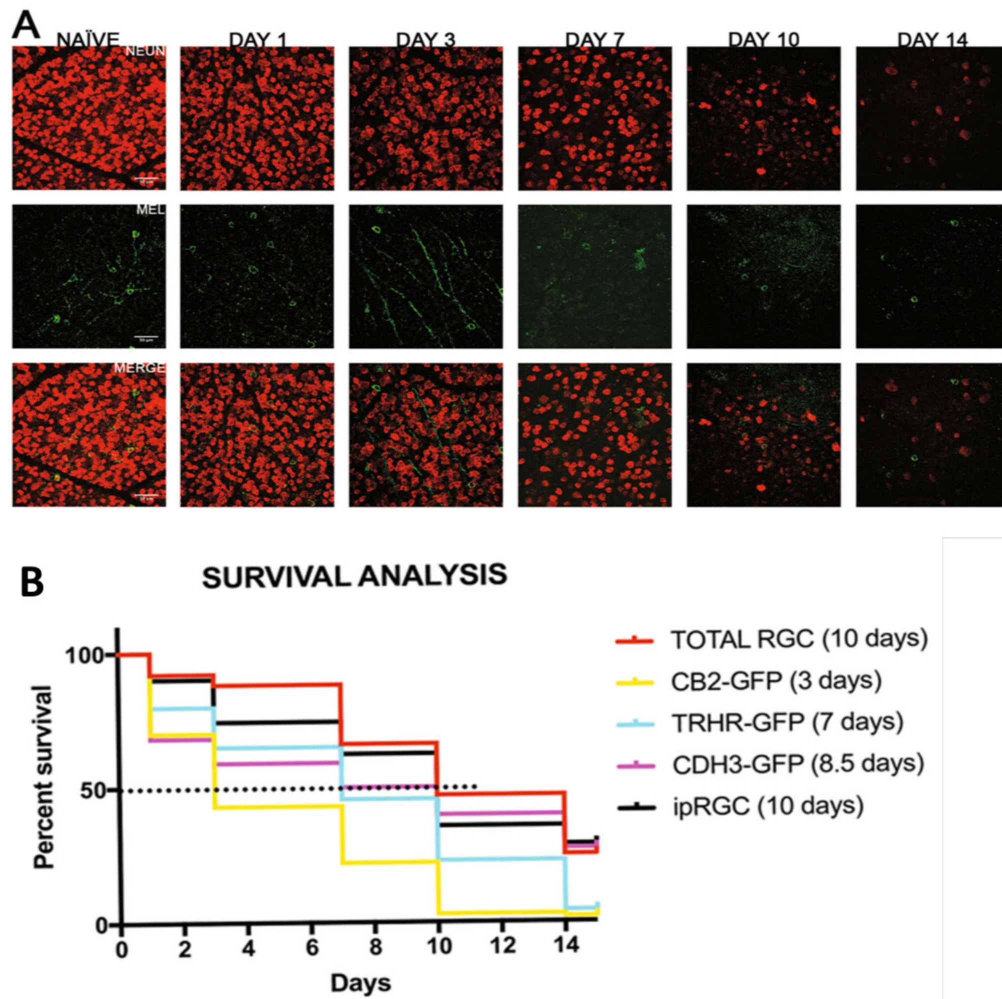

Figure 5.

Progressive loss of total RGCs (NeuN staining) and intrinsically photosensitive (ip) melanopsin (MEL) RGCs after ONC injury. (A) Upper panel = NeuN stained for total RGCs; middle panel = immunostained for melanoptsin (MEL) ipRGCs; lower panel = merged image. (B) Survival curve of total RGCs (red) and RGC subtypes (see figure legend) from 0–14 days post ONC injury showing differing RGC soma susceptibilities to ONC damage. (Figures from Daniel et al. 2018)

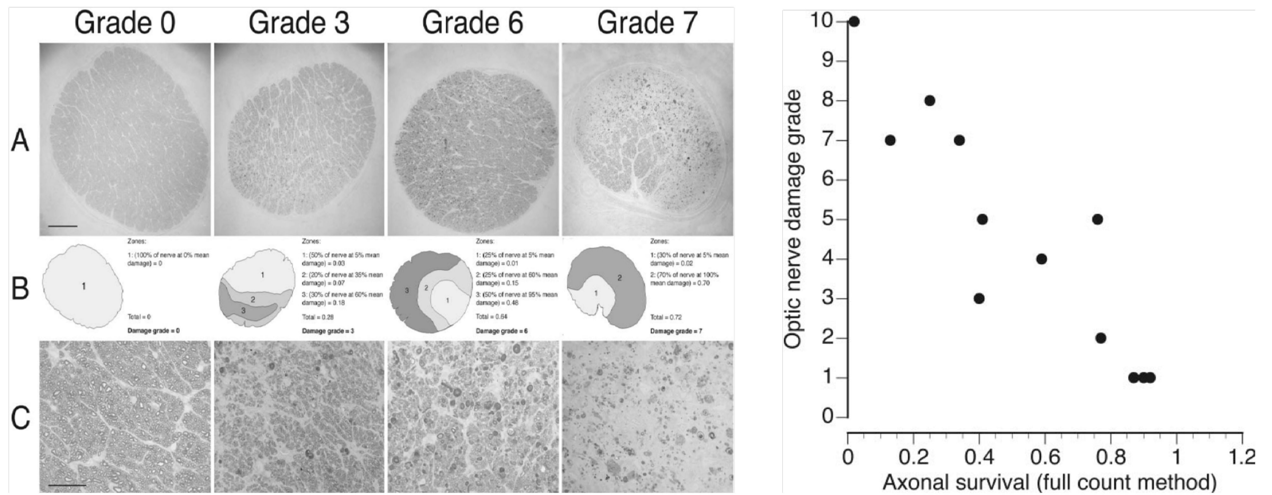

Figure 6.
Assessment of optic nerve damage in PPD-stained optic nerve cross-sections using a 10 point optic nerve damage score (ONDS). Examination of the entire PPD stained ON (A), outlining zones of injury (B), and higher magnification examination (C) allows the identification and semi-quantitation of darker stained damaged axons and areas of gliosis. 0 = no damage, 9 = total loss of ON axons. Examples of ONDS include grades of 0, 3, 6, and 7. Right side: correlation between ON damage grade and axonal survival determined by manual counting. (Figures from Chauhan et al. 2006)

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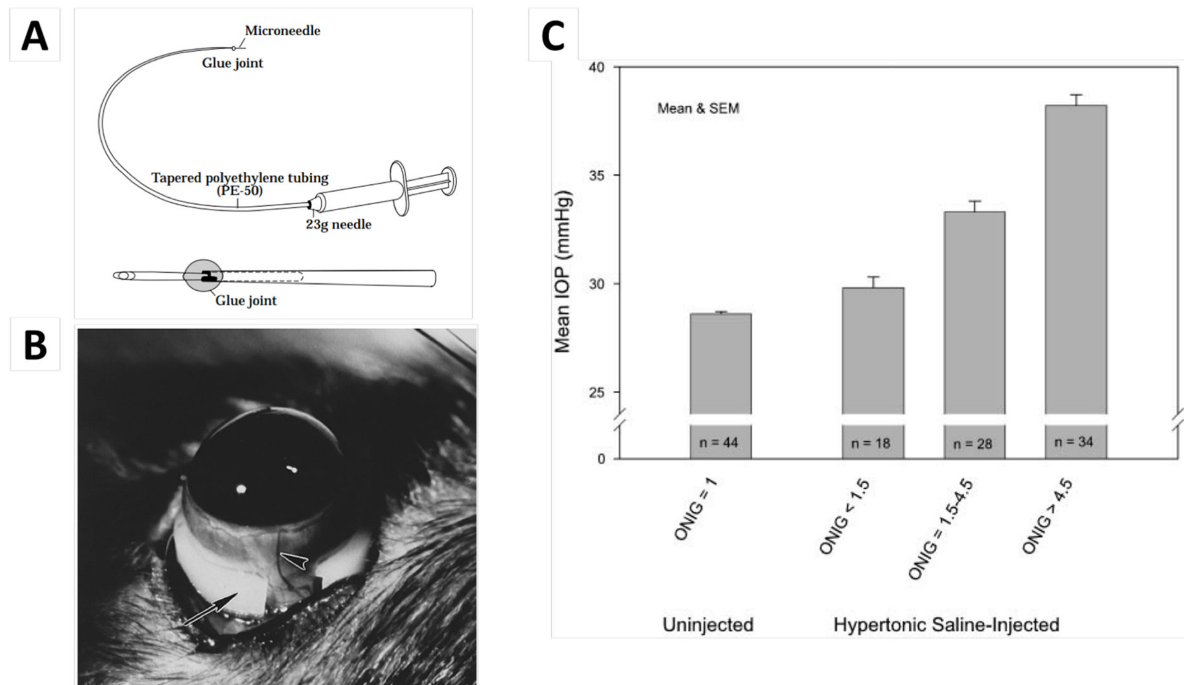

Figure 7.

Hypertonic saline-induced OHT in rats (“Morrison model”). A glass needle is attached to flexible tubing (A) to inject hypertonic saline into an isolated episcleral vein (B) to sclerose Schlemm’s canal and trabecular meshwork, which significantly decreases aqueous outflow leading to elevated IOP. (C) Optic nerve damage (ONIG scaled from 1–5) is correlated with mean IOP in this model. (Figures from Morrison et al., 1997)

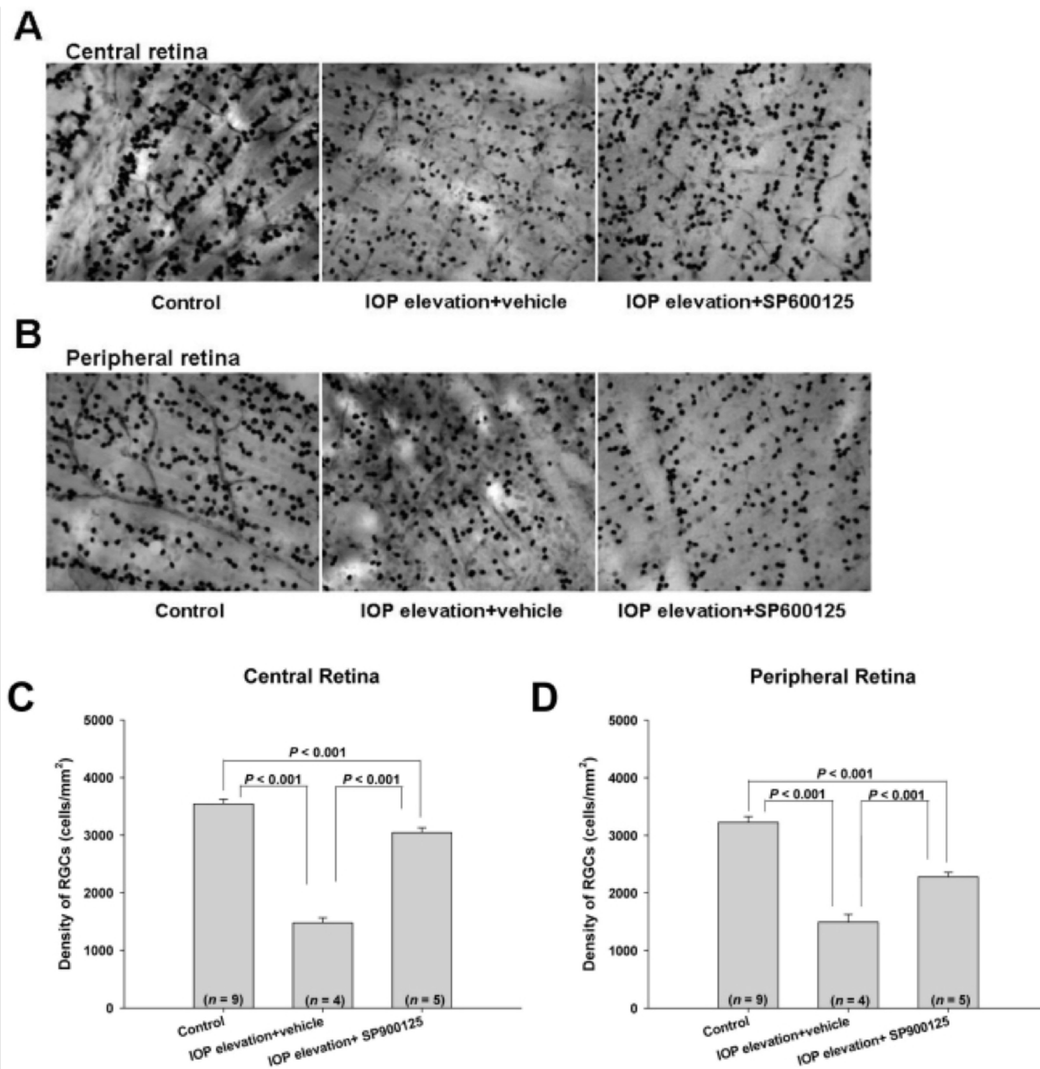

Figure 8.

Loss of RGCs in a transient IOP elevation model in rats. (A) Controllable compression of the rat eye globe using pulleys and weights (45 mmHg for 7 hours) caused loss of BRN3a labelled RGCs in both the central (A) and peripheral (B) retina, which were statistically significant ($p<0.001$ in C and D, respectively). Daily systemic treatment with the JNK inhibitor SP600125 significantly protected RGCs from transient IOP elevation ($p<0.001$, A-D). (Figures from Sun et al., 2011)

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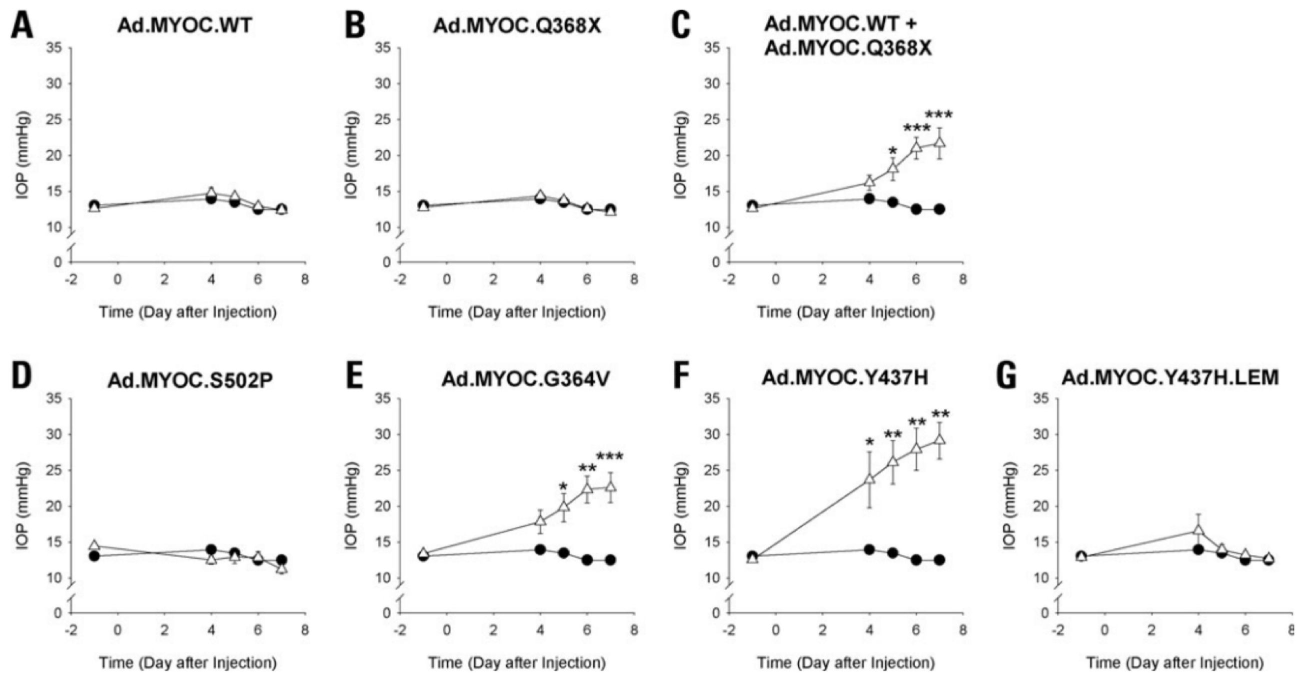

Figure 9.

Mouse eyes were transduced with Ad5 expressing wild type (WT) human *MYOC* as well as mutated forms of human *MYOC* associated with POAG to determine effects of IOP in mice. (A) Overexpression of normal (WT) *MYOC* in the mouse TM did not elevate IOP. Although when stop mutant *MYOC.Q368X* did not elevate IOP (B), co-expression with WT *MYOC* did (C). Point mutations G346V and Y437H also significantly elevated IOP ($p<0.01$), and the degree of IOP elevation for these mutations nicely correlated with the genotype severity of *MYOC* glaucoma (i.e. genotype/phenotype correlation Y437H > G364V > Q368X). The requirement for MYOC dimerization (C) as well as the carboxy terimal PTSR1 signal (SKM) for IOP elevation is nicely demonstrated using this transient transduction (B, D, G) OHT model. (Figure from Shepard et al., 2007)

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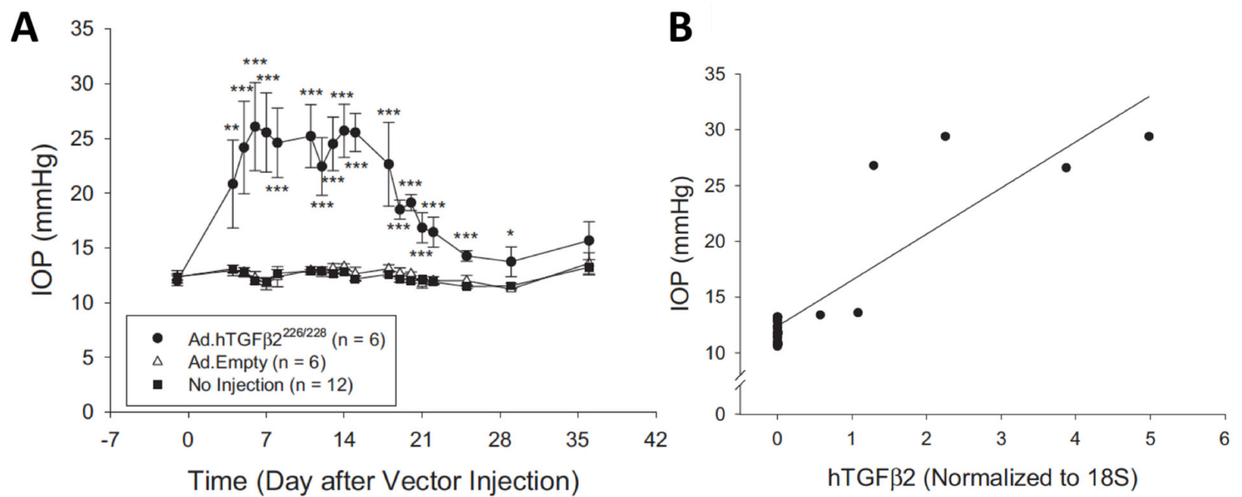

Figure 10.

TGFβ2-induced OHT in mouse eyes using Ad5 delivery of bioactivated TGFβ2$^{C226/228S}$. (A) Ad5.TGFβ2$^{C226/228S}$ transduction of the mouse TM significantly elevated IOP 5–28 days post-injection (* $p<0.05$; *** $p<0.001$) compared to Ad5.null or noninjected eyes, which did not elevate IOP. (B) TGFβ2-induced OHT was closely correlated with the amount of TGFβ2 mRNA in the mouse TM ($r = 0.88$; $p<0.0001$). (Figures from Shepard et al., 2010)

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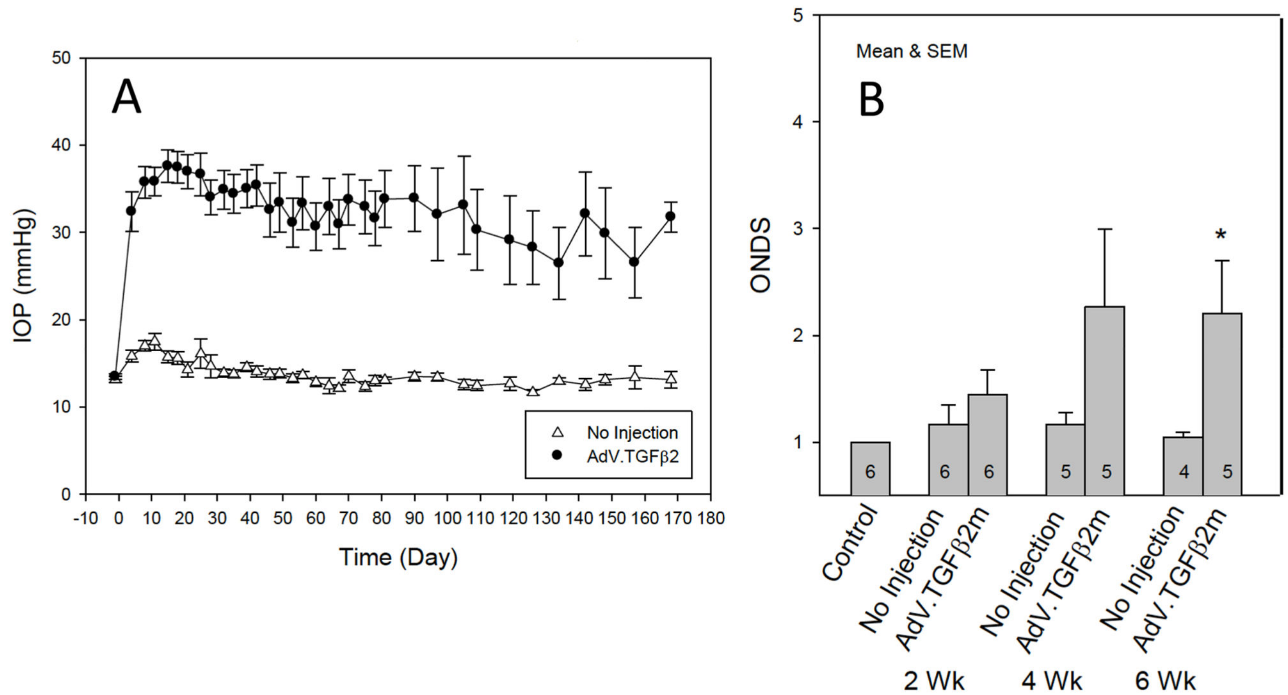

Figure 11.
TGFβ2-induced ocular hypertension and glaucomatous optic neuropathy. One eye of each BALB/cJ mouse was injected with Ad5.TGFβ2$^{C226/228S}$ and IOPs of both eyes were measured with a TonoLab tonometer over 168 days (n= 22; $p<0.001$ for days 4–168). Optic Nerve Damage Score (ONDS) was determined using the 5-point system (1 = no damage, 5 = severe damage) from PPD stained optic nerve cross-sections at 2, 4, and 6 weeks of ocular hypertension (n=4–6; $p<0.05$). (unpublished data from Luan T, Pang IH, and Clark AF)

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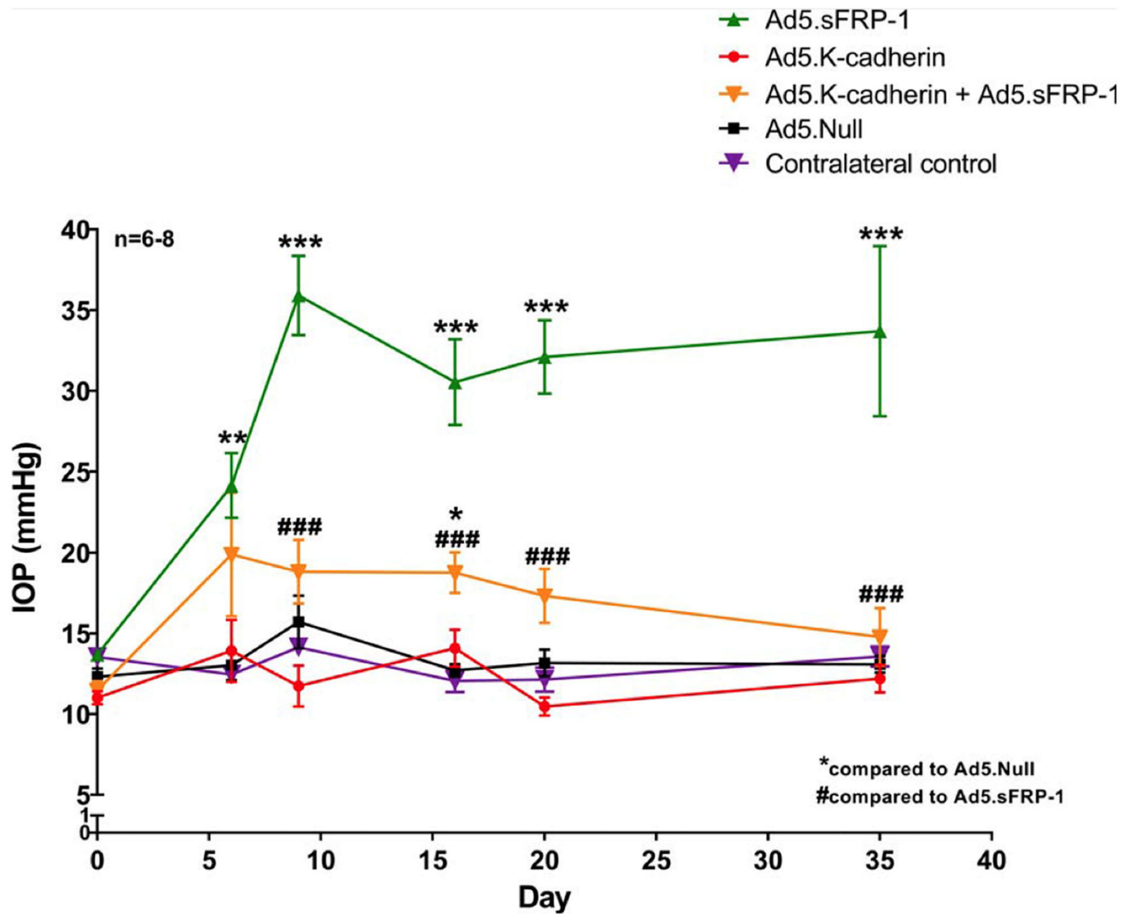

Figure 12.

SFRP-induced ocular hypertension involves K-cadherin. Wnt signaling in the TM is involved in regulating IOP, and expression of the Wnt antagonist SFRP1 is elevated in glaucomatous TM cells and tissues, leading to IOP elevation (Wang et al., 2008). SFRP1 decreases K-cadherin expression in the TM (Webber et al., 2018). Ad5.SFRP1 transduction of the mouse TM significantly elevates IOP ($p<0.001$), but concomitant expression of K-cadherin significantly suppresses this SFRP1-OHT ($p<0.001$). (Figure from Webber et al., 2018)

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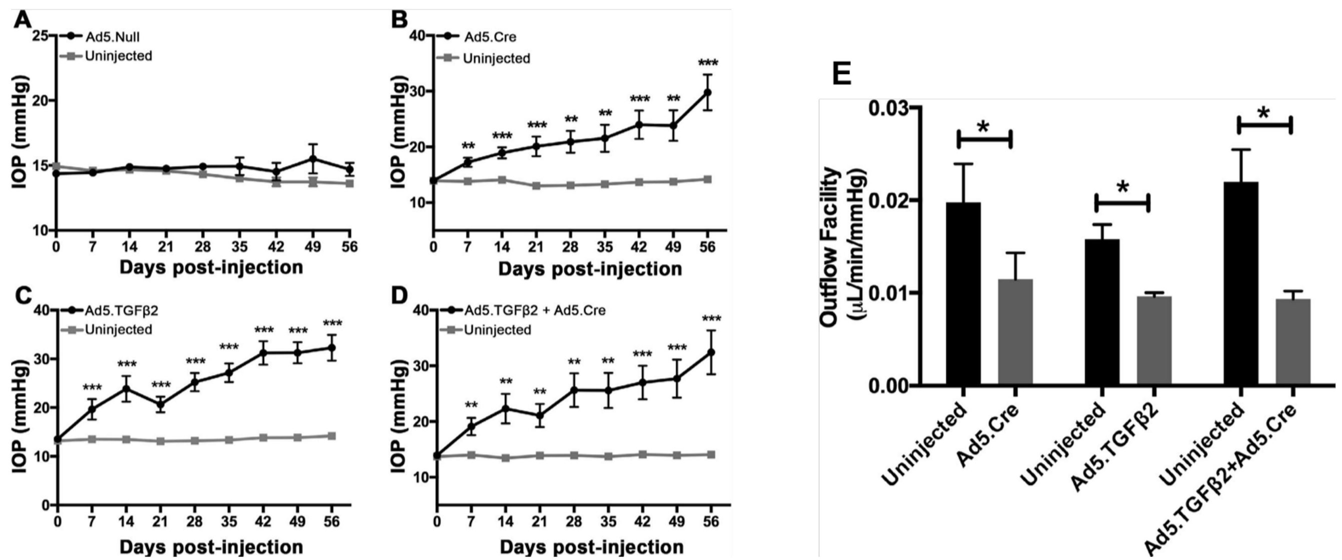

Figure 13.

Transduction of *Bambi*$^{fl/fl}$ mice with Ad5.Cre elevates IOP. Increased expression of bioactivated TGFβ2 in the mouse TM significantly elevates IOP (C) ($p<0.001$) compared to Ad5.null transduced eyes (A) (see also Fig 11). TGFβ2 inhibitor Bambi suppresses TGFβ2 signaling in the TM (Hernandez et al., 2018) as part of the homeostatic regulation of the profibrotic effects of TGFβ2. Ad5.Cre knockdown of *Bambi* in the TM of *Bambi*$^{fl/fl}$ mice inhibits this homeostatic balance, leading to significantly elevated lOP (B) ($p<0.001$), which was not further enhanced by co-treatment with TGFβ2 (D). (E) Elevated IOP due to the knockdown of *Bambi* or increased expression of TGFβ2 in the TM is due to significant decreases in the aqueous outflow facility ($p<0.05$). (Figures from Hernandez et al., 2018)

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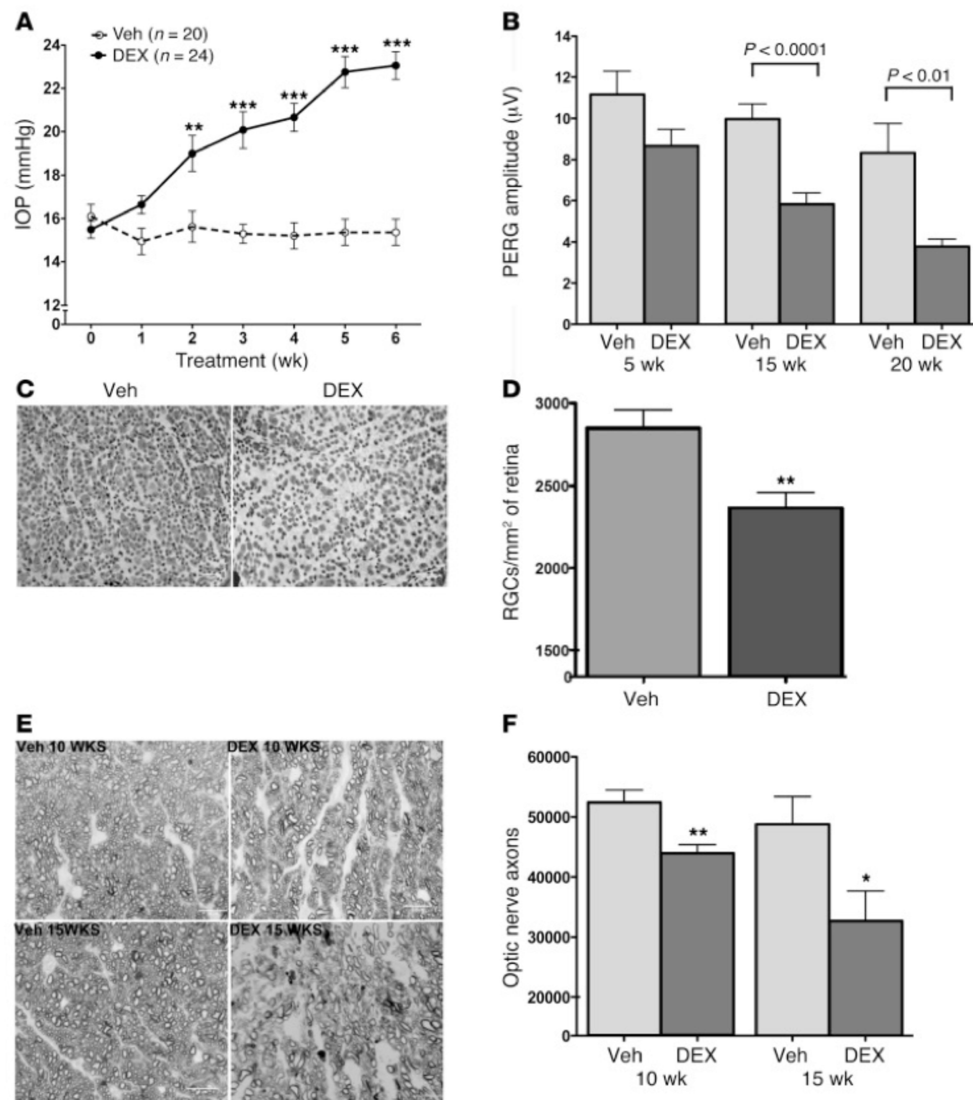

Figure 14.

Glucocorticoid (GC)-induced ocular hypertension and glaucomatous optic neuropathy/ retinopathy in mice. (A) Mice administered dexamethasone (DEX) 21-phosphate three times daily developed significantly elevated IOP compared to mice receiving vehicle control ($p<0.01$). The DEX treated mice also developed retinopathy due to significant loss of RGCs (C-D) ($p<0.01$) and optic neuropathy due to significant loss of optic nerve axons (E-F) ($p<0.05$). (B) In addition to structural loss, DEX treatment also caused signicant RGC functional loss assessed by PERG amplitudes ($p<0.01$). (Figures from Zode et al., 2014)

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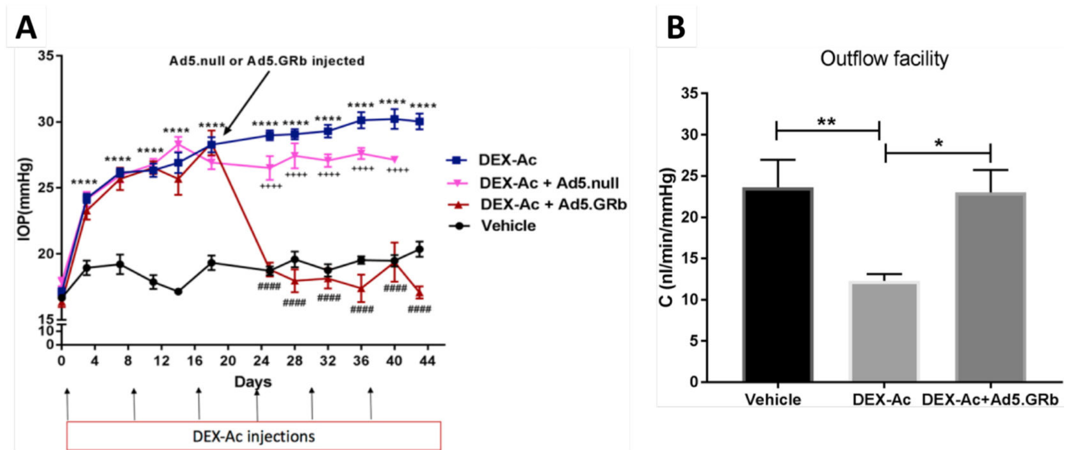

Figure 15.

DEX 21-acetate (DEX-Ac) induced ocular hypertension in mice is reversed by Ad5.GRβ transduction of the TM. The biological and pharmacological effects of glucocorticoids are mediated by the glucocorticoid receptor, GRα. An alternatively spliced isoform of the GR, GRβ acts as a dominant negative regulator of GC activities. (A) Weekly bilateral fornix based injections of DEX-Ac significantly elevate IOP ($p<0.0001$), which can be totally reversed by Ad5.GRβ transduction of the TM at day 19, despite continuous DEX-Ac treatment ($p<0.0001$). (B) DEX-Ac significantly decreased aqueous outflow in mouse eyes ($p<0.01$), which returned to normal after Ad5.GRβ treatment ($p<0.05$). (Figures from Patel et al., 2018)

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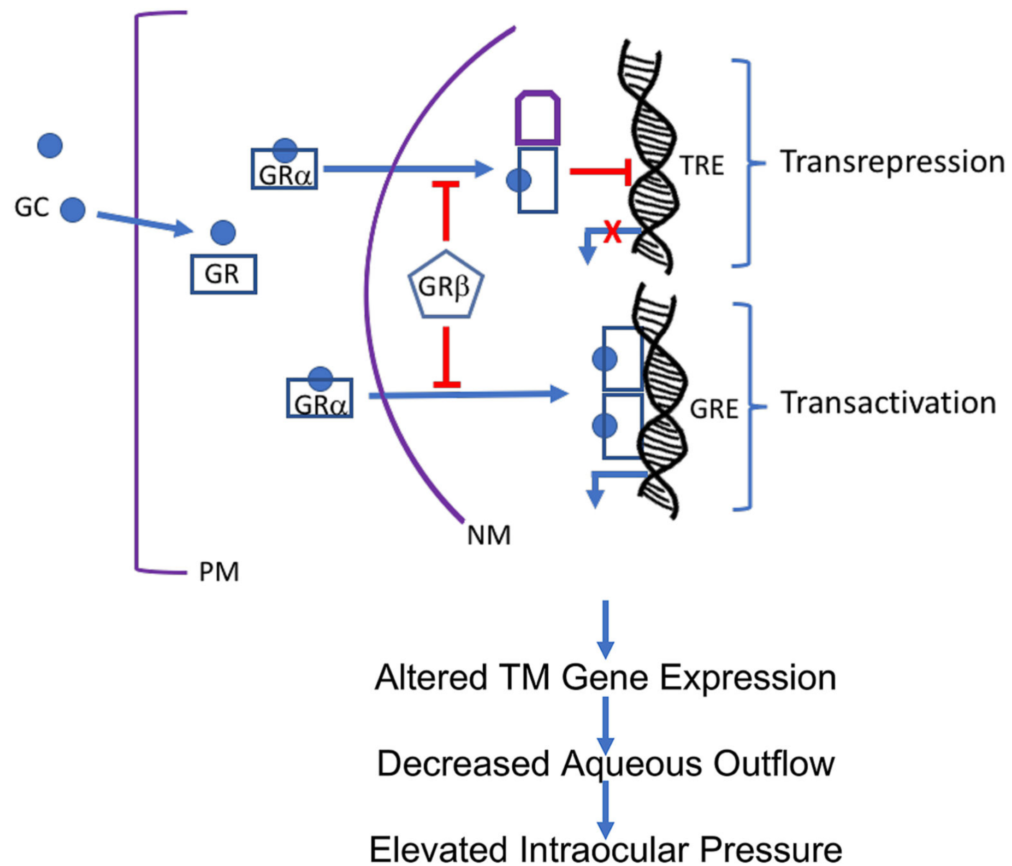

Figure 16.

Mechanism of glucocorticoid-induced ocular hypertension. Glucocorticoids (GCs) enter the cell and bind to glucocorticoid receptors (GRα). Ligand activated GRα undergoes a conformational change leading to dimerization and translocation to the nucleus. Once in the nucleus, the homodimerized GRα can bind to glucocorticoid response elements (GREs) to directly regulate gene expression in a process known as transactivation. Activated GRα monomers alsocan bind to other transcription factors (TF) such as AP-1 and NFkB to prevent these TFs from binding to their response elements (TREs) in a process known as transrepression. The alternatively spliced variant GRβ does not bind GCs and acts as a dominant negative regulator of GC activity for both transactivation and transrepression. Regulation of GC transcription can damage the trabecular meshwork (TM) leading to decreased aqueous outflow and elevated IOP.

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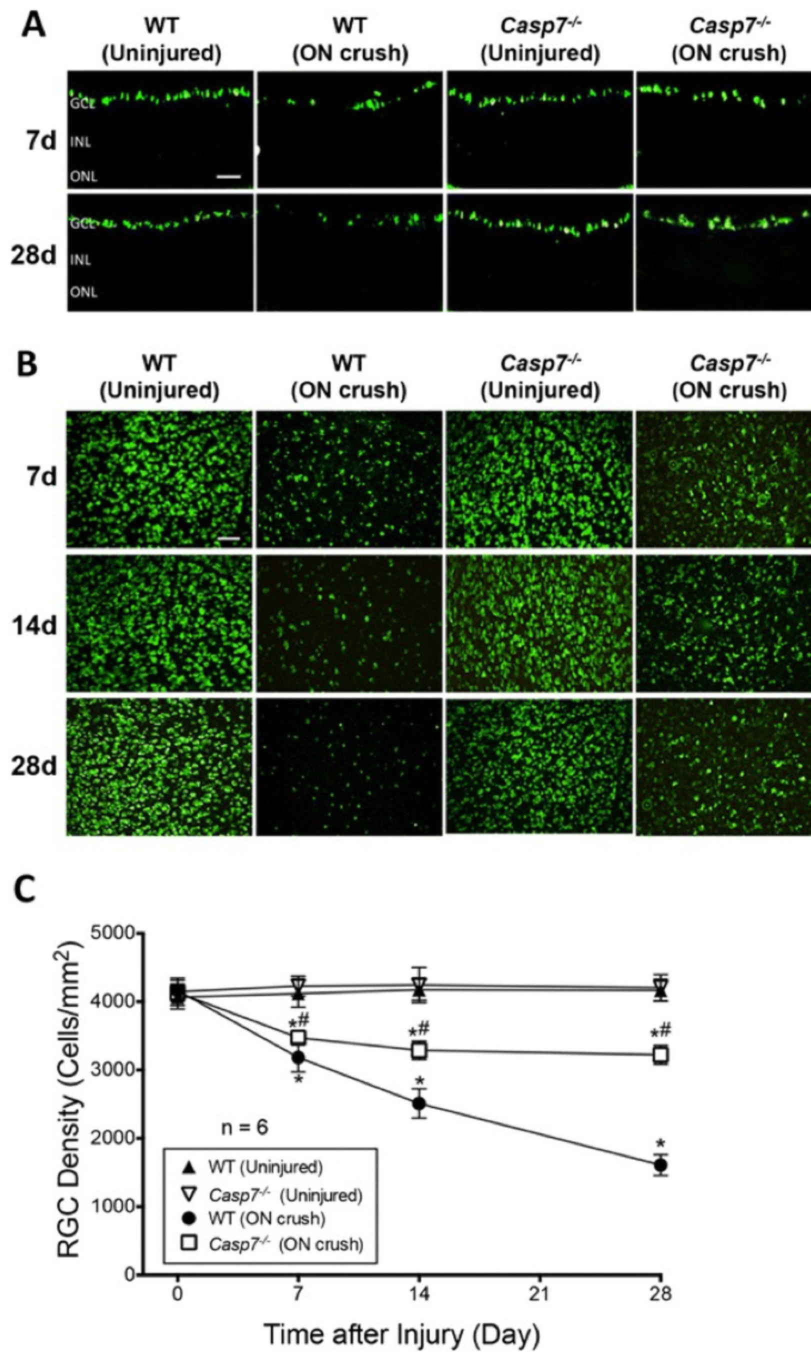

Figure 17.

Caspase 7 deficiency *(Casp7$^{-/-}$)* protects mouse RGCs from ONC injury. (A) Retinal cross sections immunostained for RGC marker RBPMS 7 and 28 days post ONC show loss of RGCs in wild type (WT) mice, while there is little loss of RGCs in *Casp7$^{-/-}$* mice. (B) Retinal flat mounts immunostained for RGC marker RBPMS 7, 14, and 28 days post ONC show loss of RGCs in wild type (WT) mice, while there is little loss of RGCs in *Casp7$^{-/-}$* mice. (C) Quantification of RGC density in retinal flat mounts immunostained for RGC marker RBPMS post ONC show significant loss of RGCs in wild type (WT) mice at days 7,

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14, and 28 ($p<0.05$), while there was significantly less loss of RGCs in *Casp7*$^{-/-}$ mice ($p<0.05$). (Figure from Choudhury et al., 2015)

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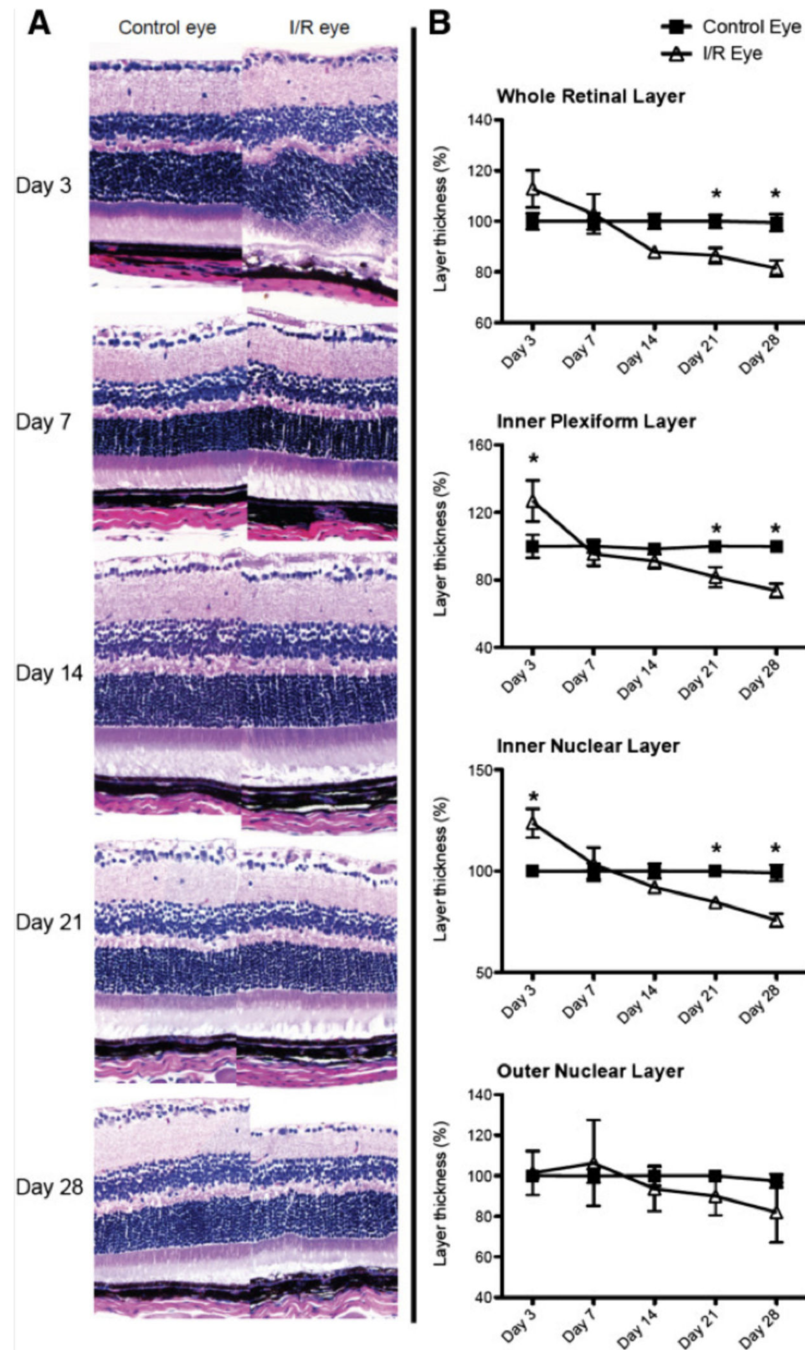

Figure 18.

Progressive thinning of inner retina in a mouse model of retinal ischemia/reperfusion (I/R) injury. One eye of each mouse was cannulated and pressure was elevated to 120 mmHg for 60 minutes, then normal pressure was restored when the needle was removed from the anterior chamber. The contralateral eye served as control. Mouse eyes were fixed 3, 7, 14, 21, and 28 days post I/R injury. Retinal cross sections were stained with H&E (A), and thickness of entire retina as well as individual retinal layers were measured (B). Total retina as well as inner plexifirm and inner nuclear layer thickness increased at 3 days due to

edema, and progressively thinned due to degeneration, with significant loss at 21 and 28 days ($p<0.05$). (Figure from Kim et al., 2016)

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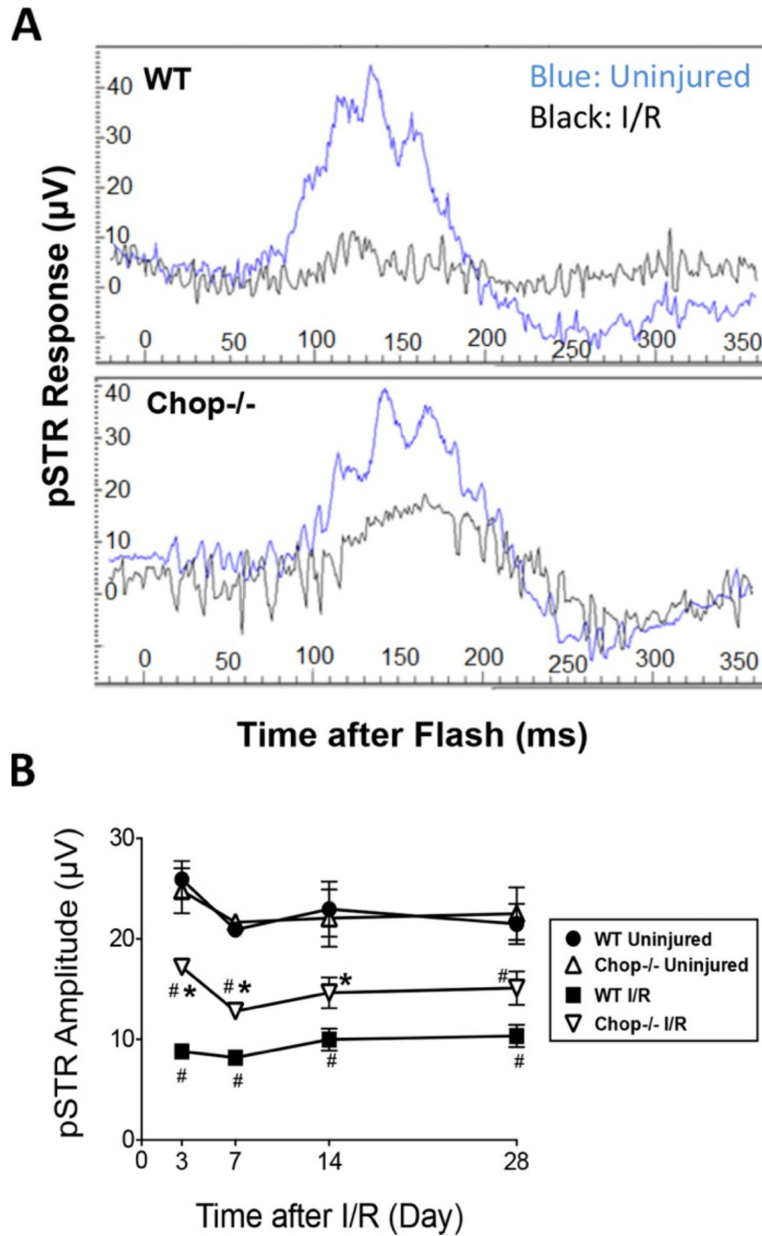

Figure 19.

Progressive loss of RGC function assessed by pSTR ERG in mouse model of retinal ischemia/reperfusion (I/R) injury was partially protected by *Chop* deficiency (*Chop*$^{-/-}$). One eye from each mouse in WT and *Chop*$^{-/-}$ strains was cannulated and had pressure was elevated to 120 mmHg for 60 minutes, then pressure was lowered when the needle removed from the anterior chamber. The contralateral eye served as control. Flash ERG at varying intensities were measured at 3, 7, 14, and 28 days post I/R injury, and pSTR amplitudes (A) were calculated and graphed (B). WT mice had significantly reduced pSTR amplitutes starting at 3 days which lasted throught 28 days ($p<0.05$). pSTR amplitudes were partially but significantly protected in *Chop*$^{-/-}$ mice ($p<0.05$). (Figure from Nashine et al., 2014)

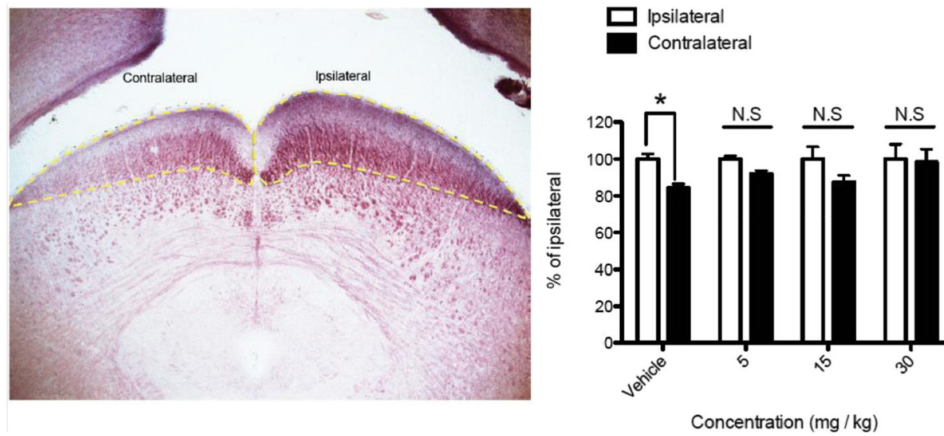

Figure 20.

Retinal I/R injury causes degeneration of target neurons in the contralateral superior colliculus (SC) that was protected by treatment with the JNK inhibitor SP600125. Mice were treated daily with vehicle control or SP600225 (5, 15, 30 mg/kg) for 28 days and brains were cryopreserved and serial sections through the superior colliculus were stained with black gold. The volumes of the black gold stained serial sections were determined for both ipsilateral and contralateral superficial layers of the SC. I/R injury caused an 16% volume loss in the contralateral SC ($p<0.05$) but the JNK inhibitor protected the SC with no significant volumes losses at all 3 doses. (Figure from Kim et al., 2016).

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Table 1.

Inducible rodent models of glaucoma

Models	Outcomes	Advantages	Challenges	References
Pressure-Dependent Models				
• Intracameral injection microbead				
○ Microbead	15 mmHg IOP increase; 23% RGC death	Relatively easy procedure	Duration of IOP elevation; multiple injections	Urcola et al, 2006
○ Microbeads with viscoelastic material	20 mmHg IOP increase; 27% RGC death	Relatively easy procedure; may improve IOP increase	Slow induction of OHT; multiple injections	Urcola et al., 2006
○ Magnetic microbeads	6 mmHg IOP increase; 36% RGCL cell loss	Direct microspheres to iridocorneal angle	Initial IOP spike; duration of IOP elevation; multiple injections	Samsel et al, 2011
• Intracameral administration of viscous agents	8–10 mmHg IOP increase	Relatively easy procedure	Duration of IOP elevation; multiple injections	Benozzi et al., 2002
• Sclerosis of the outflow pathway				
○ Episcleral injection of hypertonic saline	10–28 mmHg IOP increase; 10–100% ON axon loss	Spectrum of IOP responses	Surgical skills; Spectrum of IOP responses	Morrison et al., 1997
○ Laser photocoagulation of outflow pathway	6–24 mmHg IOP increase; 50–70% ON axon loss	Mimics a well-studied a primate IOP model; relatively high success rate.	IOP spike; transient IOP elevation; laser equipment needed	Ueda et al., 1998 WoldeMussie et al., 2001 Levkovitch-Verbin et al., 2002 Aihara et al., 2003a
• Cautery of extraocular veins	12 mmHg IOP increase; 4% RGC loss per week	Relatively easy procedure	Cautery of vortex veins; vasocongestion; IOP spike	Shareef et al., 1995; Laquis et al., 1998
• Transient/intermittent IOP elevation	50 mmHg for 30 min (mouse): decreased RGC function; 60 mmHg for 8 h (rat): ON axon degeneration & pSTR decrease	Mouse: reproducible and recoverable; responses age/diet dependent Rat: reproducible; model of early ON axon injury	Repeated/prolonged anesthesia; does not directly model glaucoma	Sun et al., 2011 Crowston et al., 2015 Morrison et al., 2016
• Transduction of the TM with glaucoma related genes				
○ *MYOC*	4–10 mmHg IOP increase; ON axon degeneration	Reproducible; genotype/phenotype correlation; data used to develop Tg mouse model		Shepard et al., 2007; McDowell et al. 2012
○ TGFβ2	10–15 mmHg increase; role of Smad signaling	Reproducible; prolonged IOP elevation with single Ad5 injection	Anterior segment inflammation; potential RGC and ON axon loss not reported	Shepard et al., 2010; McDowell et al. 2013
○ *GREM1*	10 mmHg IOP increase; role of Smad signaling	Reproducible	Potential RGC and ON axon loss not reported	Wordinger et al., 2007; McDowell et al. 2015
○ CTGF	5 mmHg IOP increase; 13% ON axon loss	Data used to develop Tg.CTGF mouse model		Junglas et al., 2012

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Models	Outcomes	Advantages	Challenges	References
○ *SFRP1*	10–15 mmHg elevation	Reproducible; proof of concept	Potential RGC and ON axon loss not reported	Wang et al., 2008a
○ CD44	15 mmHg IOP increase	Proof of concept	Potential RGC and ON axon loss not reported	Giovingo et al., 2013
○ Cre	15 mmHg IOP increase in *Bambi*$^{fl/fl}$ mice	Selectively targets TM; useful for wide variety of floxed genes	Potential RGC and ON axon loss not reported	Hernandez et al., 2018
○ Genome editing - *MYOC*	4–5 mmHg IOP lowering in *Tg.MYOC*^{Y437H} mice; improves aqueous outflow & PERG amplitudes	Knockout of *MYOC* confirmed; in vivo proof of concept for genome editing of TM		Jain et al., 2017
• Glucocorticoid-induced ocular hypertension	3–12 mmHg IOP elevation;	Reproducible within specific labs	Potential for systemic side effects	Whitlock et al., 2010 Zode et al., 2014 Patel et al., 2017, 2018, 2019 Li et al., 2019
Pressure-Independent Models				
• Optic nerve transection or crush	Up to 100% RGC death	Reproducible	Surgical skills	Allcutt et al., 1984 Barron et al., 1986 Domenici et al., 1991 Isenmann et al., 1999 Levkovitch-Verbin et al., 2003 Villegas-Perez et al., 1993
• Retinal ischemia/ reperfusion injury	30% RGC loss; significant damage to inner retina (in addition to RGCs)	Reproducible; longitudinal follow by SD-OCT and ERG deficits	Surgical skill required; prolonged anesthesia; damages more than RGCs (ERG b-wave deficits)	Mosinger and Olney, 1989 Buchi et al., 1991 Hughes, 1991 Li et al., 2002 Lafuente et al., 2002a,b; Kim et al. 2013
• Intravitreal injection of excitotoxic amino acid	Rapid apoptosis in RGCL; thinning of IPL	Reproducible; ivt injection	Damages cells in addition to RGCs; elevated vitreal glutamate hypothesis in glaucoma contradicted	Lucas and Newhouse, 1957
• Intravitreal injection of TNFα	40% loss of RGCs (8 wks); 50% loss of ON oligodendrocytes	Models OHT induction of retinal and ON TNFα		Nakazawa et al., 2006 Kitaoka et al., 2006
• Intravitreal injection of ET-1	30% RGC loss & thinning of RNFL & inner retina; defective ON axonal transport	Relatively easy procedure; model potential ET-1-mediated pathology	Need to assess potential effects on retinal blood flow; variable results between labs	Stokely et al., 2002 Nagata et al., 2014 Blanco et al., 2017

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Table 2.

Rodent genetic models of glaucoma

Models	References
DBA/2J mice	John et al., 1998 Anderson et al., 2002 Libby et al., 2005
Tg.*Col1a1*$^{r/r}$ (tm1Jae) mice	Aihara et al., 2003b Mabuchi et al., 2004 Dai et al., 2009
Nee mice	Mao et al., 2011
Tg.*MYOC*^{Y437H} mice	Zode et al., 2011
Tg.*Ctgf* mice	Junglas et al., 2012
sGCα1 KO	Buys et al., 2013
A1A2 KO	Thomson et al., 2014
Glast KO mice	Harada et al., 2007
EAAC1 KO mice	Harada et al., 2007
Tg.*TBK1* (CNV) mice	Fingert et al., 2017

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Table 3.

Experimental approaches to assess glaucoma in inducible rodent models

Techniques	References
Intraocular pressure	
• Invasive cannulation	John et al., 1997
• Tonopen tonometer	Moore et al., 1993
• Rebound tonometer (TonoLab®)	Danias et al., 2003a Wang et al., 2005
Aqueous humor dynamics	
• *In vivo*	Millar et al., 2011
• *Ex vivo*	Lei et al., 2011
Non-invasive imaging of the retina	
• OCT	Li et al., 2001 Ruggeri et al., 2007 Liu et al., 2014
• Fluorescent protein labeled RGCs	Leung et al., 2008 Tosi et al., 2010
RGC counting	
• Fluorescent protein labeled RGCs (and RGC subsets)	Daniel et al., 2018 El-Danaf and Huberman, 2015
• Retina flatmounts with immunohistochemistry (Brn3, Tuj1, NeuN, RBPMS)	Xiang et al., 1993 Danias et al., 2003b Kwong et al., 2010
• Retrograde fluorescent tracers (fluorogold, diI, etc.)	Vidal-Sanz et al., 1988 Hull and Bahr, 1994
ERG (PERG, pSTR)	Berardi et al., 1990 Bui and Fortune, 2004
ON damage	
• ON axon counts	Quigley et al., 2011 Ebneter et al., 2012 Oglesby et al., 2012
• ON damage score	Fortune et al., 2004 Libby et al., 2005a Chauhan et al., 2006
• Anterograde, retrograde transport	Aviles-Trigueros et al., 2003 Lambert et al., 2011
Visual function	
• VEP	Porciatti et al., 1999
• Optokinetics	Stahl, 2004 Dietrich et al., 2019

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Table 4.

Human glaucoma pathogenic pathways confirmed in mice

Gene	Pathway	Human	Mouse
MYOC mutations	ER stress	Stone et al., 1997	Shepard et al., 2007
TGFβ2	TGFβ2 fibrotic pathway	Tripathi et al., 1994	Shepard et al., 2010
CTGF	TGFβ2 fibrotic pathway	Junglas et al., 2009 Wallace et al., 2013	Junglas et al., 2012
GREM1	TGFβ2 and BMP pathways	Fuchshofer et al., 2007 Wordinger et al., 2007	Wordinger et al., 2007 McDowell et al., 2015
SFRP1	Wnt β-catenin pathway	Wang et al., 2008a	Wang et al., 2008a
CD44	To be determined	Knepper et al., 2002 Nolan et al., 2007	Giovingo et al., 2013

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Table 5.

Pressure-independent Factors Associated with POAG

Factor	Evidence	Rodent Model	Reference(s)
NT depletion	Obstruction of BDNF and TrkB transport in ON	ON crush/transection; acute chronic exp glaucoma	Pease et al., 2000
Ischemia/ reperfusion	Increased retinal and ON HIF-1; decreased retinal & ONH blood flow; disc hemorrhages	Retinal I/R injury	Tezal and Wax, 2004b; Krakau 1994; Flammer 1994
ON axonopathy	ONH site of initial OH injury; blockade of ON transport at ONH	ON crush/transection	Syc-Mazurek & Libby, 2019; Nickells et al., 2012
Toxic factors:			
Excitotoxic AAs	EAAs kill RGCs in vivo; defective glu uptake in glaucomatous retina	Ivt injection of EAA	Li et al., 1999; Martin et al., 2002
TNFα	Increased TNFα in glaucomatous ONH	Ivt injection of TNFα	Yan et al., 2000 Yuan et al., 2000
ET-1	Increased ET-1 expression; ET-1 kills RGCs; ET-1 blocks ON transport and activates ONH astrocytes	Ivt injection of ET-1	Prasanna et al., 2011; Blanco et a., 2017; Stokely et al., 2002