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Mouse models of retinal ganglion cell death and glaucoma

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Abstract

Once considered too difficult to use for glaucoma studies, mice are now becoming a powerful tool in the research of the molecular and pathological events associated with this disease. Often adapting technologies first developed in rats, ganglion cell death in mice can be induced using acute models and chronic models of experimental glaucoma. Similarly, elevated IOP has been reported in transgenic animals carrying defects in targeted genes. Also, one group of mice, from the DBA/2 line of inbred animals, develops a spontaneous optic neuropathy with many features of human glaucoma that is associated with IOP elevation caused by an anterior chamber pigmentary disease. The advent of mice for glaucoma research is already having a significant impact on our understanding of this disease, principally because of the access to genetic manipulation technology and genetics already well established for these animals.

Keywords

experimental glaucoma; retinal ganglion cell; optic nerve disease; animal models; DBA/2J mouse glaucoma; transgenic mice

1. Introduction

The past decade and a half has seen dramatic advances in the field of glaucoma research. A great deal of this explosion of knowledge can be attributed to the introduction of rodent models of experimental glaucoma, and the description of chronic inherited disease in inbred mice. Smaller rodent models have allowed access to glaucoma research by numerous research programs that before had neither the expertise nor financial clout to study this disease in the classic and still important non-human primate model.

A description of rat models of experimental glaucoma is given by several other contributions to this special issue (Johnson et al., 2009). These models have yielded tremendous new information on the cellular and molecular pathology of retinal ganglion cell death and optic nerve disease. Studies on mice, however, have one distinct advantage over rat studies, in that these animals enable researchers to conduct complex genetic manipulations in order to study the function of critical genes and biochemical pathways. This short review provides a description of current technologies that have been applied to mice to experimentally stimulate ganglion cell death or induce ocular hypertension. We also provide a discussion of various transgenic mice, and the DBA/2J mouse and associated substrains that naturally

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develop a form of pigmentary glaucoma with similar phenotypes to the human condition. Finally, we briefly touch on specific studies that have utilized genetic manipulations in mice to examine the roles of different genes in the ganglion cell death pathway, and have identified ganglion cell death susceptibility alleles.

2. Acute models of retinal ganglion cell death

Several methods of inducing rapid and relatively uniform retinal ganglion cell death, originally developed in animals with larger eyes, have been adapted for use in mice. These include direct intraocular injections of toxins that facilitate ganglion cell death such as staurosporine (Maass et al., 2007), or *N*-methyl-D-aspartate (NMDA), the non-hydrolyzable analog of glutamate (Li et al., 1999). NMDA injections were considered particularly relevant to the study of glaucoma during the 1990s when glutamate toxicity was enthusiastically considered a mechanism for ganglion cell loss in this disease (Dreyer and Lipton, 1999). Mouse retinal ganglion cells demonstrate a dose-dependent loss in response to a single intravitreal injection of NMDA (Fig. 1), but early studies clearly showed that other cells, probably amacrine cells, were also affected (Li et al., 1999).

Ganglion cell loss in the mouse retina can also be induced by a direct lesion to the optic nerve. Severe lesions, including axotomy, can be carried out in mice. Complete axotomy is a difficult procedure due to the small size of the orbit and the close proximity of the central retinal artery and vein. Thus, unlike in rats, it is difficult to access the nerve posterior to these vessels using an intraorbital approach without damaging the retinal blood flow. Because of this, some studies have utilized intracranial approaches in which the optic nerve is accessed after a small portion of the brain is aspirated (Cenni et al., 1996). Alternatively, optic nerve crush is a viable option that can be conducted using an intraorbital approach to access the nerve, which is then crushed using self-closing watchmakers forceps (Li et al., 1999). The surgery is simple and rapid, resulting in synchronous loss of 70–90% of the ganglion cells over a 3-week period in most strains of mice (Fig. 1). Because the crush is relatively mild, ocular blood flow is not compromised. The procedure is also amenable to genetics studies involving large numbers of mice (see Section 5). Although the damage to the nerve is likely more severe than damage induced by elevated intraocular pressure (IOP) to the optic nerve head, optic nerve crush appears to elicit many of the same molecular events in ganglion cells as elevated IOP (Schlamp et al., 2001; McKinnon et al., 2002; Huang et al., 2005a,b, 2006; Libby et al., 2005a; Yang et al., 2007).

3. Inducible glaucoma in mice

3.1. Measurements of IOP in the mouse eye

IOP measurement is a necessary and important feature in any experiment employing murine glaucoma models. Both invasive and non-invasive methods have been employed, each with their advantages and disadvantages. Invasive methods include the use of manometric techniques in which a water-filled microneedle is attached to a pressure transducer, and servo-null systems in which a micropipette filled with electrolyte solution is inserted into the anterior chamber and changes in electrical resistance due to aqueous humor displacement are converted to IOPs. John et al. used a manometric method to measure IOPs in ketamine/xylazine anesthetized males of strains C3H/HeJ, C57BL/6J, A/J, and BALB/cJ. Significant inter-strain differences were noted, with mean IOPs ranging from 7.7 mmHg in BALB/cJ mice to 13.7 mmHg in C3H/HeJ mice (John et al., 1997). Aihara et al. used a manometric method to show that topical latanoprost reduced mouse IOP in a dose-dependent manner in NIH Swiss mice (Aihara et al., 2002). Avila et al. used the servo-null method to show that topical pilocarpine 1%, latanoprost 0.005% and timolol 0.25% lowered IOP in black Swiss outbred mice (Avila et al., 2001). Invasive methods have the advantage of direct access to

the pressurized environment of the anterior chamber, and the disadvantage of producing anatomic changes in the cornea, limiting the number of measurements that can be taken in an individual mouse. The servo-null system uses a pulled micropipette tip with a smaller bore than that of the microneedle used in the manometric method, lessening but not eliminating scarring due to corneal puncture.

Non-invasive methods to measure murine IOP employ adaptations of technologies currently used in clinical practice, such as pneumotonometry or applanation. Avila et al. used a pneumotonometer (Paradigm Medical Industries, Inc.) with a customized probe tip to measure IOP in anesthetized C57BL/6 mice, and demonstrated good correlation with servo-null IOP measurements (Avila et al., 2005). Cohan and Bohr used a miniaturized biprism tip to perform Goldmann applanation on restrained awake mice, and showed good correlation of IOP with manometric measurements (Cohan and Bohr, 2001). Repeated applanations caused plateauing of the IOP measurement, similar in magnitude to that seen in humans (Whitacre and Stein, 1993). The Tono-Pen XL (Medtronic Solan) has a long history of use in measuring IOP in rats (Moore et al., 1993; Mermoud et al., 1994) and has subsequently been successfully used in mice. Reitsamer et al. used anesthetized C57BL/6 mice to show good correlation of Tono-Pen IOPs with those obtained by servo-null (Reitsamer et al., 2004). Tono-Pen IOPs were overestimated at low pressures and underestimated at high pressures, a phenomenon previously noted in rats (Moore et al., 1993). These results were confirmed by Pang et al. in anesthetized C57BL/6 mice (Wang et al., 2005). Danias et al. have recently developed an induction-impact (“rebound”) tonometer in which a magnetized probe was contacted with the mouse cornea and the deceleration during rebound of the probe was measured with a sensing coil. Deceleration times were correlated with manometric IOPs in male C57BL/6 mice yielding a logarithmic IOP calibration curve (Danias et al., 2003a). Wang et al. validated the TonoLab rebound tonometer (Colonial Medical Supply) in conscious and anesthetized mice of four different strains, and showed excellent correlation with manometry (Wang et al., 2005). A significant IOP-lowering effect was noted after induction of anesthesia (Wang et al., 2005; Johnson et al., 2008) as has been previously reported in rats (Jia et al., 2000). In our hands, TonoLab tonometry is technically easier than Tono-Pen tonometry, and has become the IOP measurement technique of choice. These non-invasive IOP measuring methods are advantageous in that they do not cause damage to the corneal surface, are rapid and reproducible, and allow serial measurements over extended time periods in chronic models. Non-invasive methods typically display greater variability than invasive methods (Morris et al., 2006), so proper training is critical to ensure accuracy.

3.2. Experimental glaucoma in mice

Measurements of IOP, episcleral venous pressure (EVP), aqueous humor production (F_a) and outflow facility (C) in NIH Swiss mice compare quite favorably with human IOP and aqueous production and outflow values, and further support the use of mice as a model for human glaucoma experimentation (Aihara et al., 2003a). Given these facts, inducible models of glaucoma in mice have been developed that cause chronic ocular hypertension by obstruction of aqueous humor outflow. These methods mimic those used successfully in rats, namely laser photocoagulation of episcleral veins (WoldeMussie et al., 2001), episcleral vein occlusion (Garcia-Valenzuela et al., 1995), and sclerosis of episcleral veins by injection of hypertonic saline (Morrison et al., 1997). In these models, one eye is treated, leaving the other to serve as an internal control that sets the baseline of effect for untreated eyes.

Argon laser has been applied to the limbus in Swiss mice (Aihara et al., 2003b). In one study, more persistent and reproducible IOP elevation was found after mydriasis and removal of aqueous humor from the anterior chamber by micropipette. This appeared to

bring the trabecular meshwork into closer proximity to limbal areas targeted with the laser, causing complete angle closure. Laser parameters were: wavelength 532 nm, spot size 200 μm , power 100 mW and duration 0.05 s. The number of laser applications was 64 ± 6 (mean $\pm$ SD), given in a single treatment. IOP was measured weekly by pressure transducer measurement after microcannulation of the anterior chamber. One week after laser treatment, 15 of 22 eyes (68%) had significant IOP elevations of over 30% (compared to the untreated contralateral eye). However, the IOP elevation was not sustained in the treated eyes, slowly declining to baseline by 8 weeks post-treatment. Axons were counted from electron microscope (EM) images taken of ultrathin optic nerve cross-sections stained with 1% uranyl acetate (Mabuchi et al., 2003). Axon survival in 13 mice was determined by taking the ratio of axon counts of the treated and control eyes. Axon survival in 13 mice was $68.1\% \pm 38.1\%$ (mean $\pm$ SD) and correlated significantly with IOP exposure measured in mm-weeks ($r^2 = 0.36$) and with decreased optic nerve cross-sectional area ($r^2 = 0.79$). Astrocyte numbers were also significantly increased in treated optic nerves. Complications of laser photocoagulation in this study included corneal edema, corneal opacity, and cataract.

Other studies used argon laser photocoagulation to the episcleral and limbal veins in C57BL/6 mice (Gross et al., 2003; Ji et al., 2005). Laser parameters were: wavelength 532 nm, spot size 200 μm , power 100 mW, and duration 0.05 s. IOP was measured using a modified indentation tonometer. At 4 weeks post-treatment, IOP in the treated eyes was 20.0 ± 2.8 mmHg (mean $\pm$ SD) compared to 13.0 ± 1.8 mmHg in the untreated contralateral eyes. IOPs in the treated eyes also declined to baseline levels approximately 8 weeks post-treatment. Survival of RGCs was performed by immunohistochemical staining of retinal cross-sections with an antibody to Thy1.2 and subsequent confocal imaging and RGC counting. At 4 weeks post-treatment, RGC loss was rated at $22.4\% \pm 7.5\%$ (mean $\pm$ SD). TUNEL-positive RGCs were also seen in significantly greater number in the retinas from treated eyes, further implicating apoptosis as the primary RGC death mechanism due to elevated IOP.

Using a variation of the laser photocoagulation technique, 22 eyes of C57BL/6 mice were first treated with 4% pilocarpine to induce miosis. Indocyanine green (ICG) was then injected into the anterior chamber followed by diode laser treatment of the limbus delivered by fiber optic probe (Grozdanic et al., 2003). Laser parameters were: wavelength 810 nm, spot size 50 μm , power 350 mW and duration 1.5 s. The number of laser applications was between 30 and 50, given in a single treatment. IOP was measured approximately every 3 weeks using a modified Goldmann tonometer. IOP in treated eyes 10 days post-laser was 33.6 ± 1.5 mmHg (mean $\pm$ SD) compared to 17.0 ± 0.7 mmHg in the untreated contralateral eyes. As was seen in the previously discussed laser studies, IOPs in the treated eyes returned to baseline approximately 8 weeks post-treatment. RGCs and optic nerve axons were not counted in this study, so no correlation between IOP exposure and RGC or axon survival could be determined. However, electroretinography (ERG) revealed significant correlation between IOP exposure and reduction in a-wave, b-wave and oscillatory potential. Histological analysis of optic nerve cross-sections revealed loss and degeneration of larger diameter axons, swelling of myelin sheaths, and activation of glial cells proximate to degenerating axons.

Overall, since the elevation of IOP is transient in these laser models, and the level of cell loss is modest, there may be some difficulty in using this model for molecular studies. Nevertheless, one group was able to use a laser glaucoma model to evaluate the role of the R2 receptor for tumor necrosis factor alpha. In this study, *Tnfr2*^{-/-} mice with experimental ocular hypertension showed minimal evidence of developing glaucoma compared to similarly treated wild-type mice (Nakazawa et al., 2006).

Different from the laser ablation techniques, IOP elevation was achieved in 23 CD1 mice by occlusion of 3 episcleral veins using hand-held cautery (Ruiz-Ederra and Verkman, 2006). IOP was measured bi-weekly using a rebound tonometer. 20 of the 23 mice (87%) displayed IOP elevation. IOP in treated eyes 1 week post-surgery was 28 ± 1.5 mmHg (mean $\pm$ SEM), an increase of 104% compared to untreated contralateral eyes. IOP in treated eyes returned to baseline approximately 6 weeks after surgery. RGC counts were performed on retinal cross-sections retrogradely labeled with FluoroGold applied to the superior colliculus. RGC survival was significantly lower (80.2%) in the treated eyes 2 weeks post-surgery. Complications of episcleral vein cautery in mice included thermal damage to sclera, intraocular inflammation and ocular surface damage.

We (SJM) have successfully induced chronic ocular hypertension in mice by injection of hypertonic saline into a limbal vein, causing sclerosis of aqueous outflow channels and a subsequent elevation of IOP (manuscript in preparation). A pulled glass micropipette was inserted into a vein near the cornea and about 0.05 cc of sterile and filtered 1.3 M saline was injected into the limbal venous system. The injection was then repeated two weeks later. IOP was measured under topical anesthesia on a weekly basis post-operatively using a TonoLab rebound tonometer. In 12 treated eyes, eleven displayed elevations of IOP, for an overall conversion rate of 92%. Axons were counted from light microscope images taken of ultrathin optic nerve cross-sections stained with 1% toluidine blue. Axon survival was calculated by taking the ratio of axon counts of the treated and control eyes. IOP exposure was positively correlated with the degree of axon loss ($r^2 = 0.54$, $P = 0.01$). The IOP integral values revealed a linear dose-response effect of pressure exposure on optic nerve axon loss, although individual variability was seen.

4. Spontaneous glaucoma in the DBA/2J inbred strain of mice

In addition to inducible models of experimental glaucoma, some strains of mice have spontaneously developed mutations that result in chronic age-related glaucoma phenotypes (John, 2005). The most well characterized of these are the DBA/2J inbred line (John et al., 1997, 1998; Chang et al., 1999) and the related DBA/2NNia substrain (Sheldon et al., 1995; Danias et al., 2003b; Filippopoulos et al., 2006). DBA/2J mice are homozygous for mutations in two separate genes. The first is the b allele of tyrosine related protein (*Tyrp1^b*). This gene encodes a melanosomal protein that is suspected of both enzymatic and structural functions in these organelles. The second mutant gene in the DBA/2J background encodes a transmembrane glycoprotein found in multiple different cellular structures (*Gpnmb*). In these mice, the *Gpnmb* gene carries a stop codon mutation (*Gpnmb^{R150X}*). These genes clearly interact to initiate the disease in the DBA/2J strain. Mice homozygous for both mutant genes exhibit anterior chamber pathology characterized by pigment dispersion and iris stromal atrophy, while substitution of wild-type alleles on the DBA genetic background prevents this (Howell et al., 2007b). A distinguishing feature of the disease is the development of iris illumination defects associated with the loss of pigment granules (Fig. 2). As the pigment is dispersed into the anterior chamber, it accumulates in the mouse trabecular mesh-work leading to the formation of synechiae, blockage of the drainage structures, and the elevation of intraocular pressure.

Since both *Tyrp1^b* and *Gpnmb^{R150X}* gene products are associated with melanosomes, it has been proposed that the initial pathology of anterior chamber disease in these mice is caused by an abnormality in iris pigment synthesis and melanosome structure (John, 2005). In this model, cytotoxic byproducts from abnormal pigment synthesis may eventually leak out of structurally compromised melanosomes. This hypothesis is supported by evidence that DBA/2J mice engineered with tyrosinase mutations, making them albino, exhibit no disease phenotype (Chang et al., 1999). The pathology is more complex than just this,

however, and involves an immune component that can be modulated by genetically altering the bone marrow of standard DBA/2J animals (Mo et al., 2003). In recent experiments, for example, bone marrow transplants between DBA/2J mice, and congenic DBA/2J animals with a functional gene for *Gpnmb* (*Gpnmb*⁺) showed that transplanted bone marrow with the wild-type gene was able to suppress the iris phenotype (and the elevation of IOP) in *Gpnmb*^{R150X} animals (Anderson et al., 2008). Curiously, the reverse transplant of bone marrow with mutant *Gpnmb*^{R150X} into mice with the wild-type gene did not cause disease. These results are interpreted that two separate components affected by *Gpnmb*, one in the iris and the other being an immune component in the bone marrow, contribute to the overall pathology of anterior chamber disease. Further evidence supporting this hypothesis was revealed in studies showing that high dose irradiation of DBA/2J mice, followed by the transplant of native DBA/2J bone marrow, was completely able to abrogate glaucoma in the recipient mice (Anderson et al., 2005). Clearly, the roles that both *Tyrrp1^b* and *Gpnmb*^{R150X} play in the glaucoma phenotype are only partially resolved. Recent examination of a DBA/2 substrain from a European colony (DBA/2J-Rj), for example, show that mice with a variant of the *Gpnmb* gene develop typical anterior chamber pathology, elevated IOP, but no clear neurodegenerative phenotype (Scholz et al., 2008). This study is suggestive that *Gpnmb* may also play a role in the ganglion cell susceptibility to elevated IOP, but it should be noted that this particular substrain of the DBA/2 mouse has not been fully characterized for other genetic differences that may have arisen in this isolated colony.

The natural history of disease in DBA/2J mice can vary, depending on individual colonies and other environmental factors (such as diet). Generally, however, iris disease begins to manifest by 6–8 months of age. Shortly thereafter, mice exhibit increased IOPs that exceed 3 standard deviations over the mean IOP for younger animals (Libby et al., 2005b). In studies involving large numbers of animals at different ages, the majority of mice between ages of 8 and 13 months have elevated IOPs. The first evidence of glaucoma in these animals occurs as damage to the optic nerve. Independent studies show that aging mice exhibit axonal loss and optic nerve gliosis shortly after the onset of elevated IOP, around 8 months of age (Libby et al., 2005b; Schlamp et al., 2006). Overall, the mechanism of axonal loss in the optic nerve appears more similar to the “die-back” pattern, where the axons degenerate in a retrograde direction (Schlamp et al., 2006), as apposed to more classic Wallerian degeneration, where damaged axons degenerate at multiple points simultaneously along their length (Beirowski et al., 2005). This period of axonal degeneration coincides with compromised retrograde axonal transport (Jakobs et al., 2005; Buckingham et al., 2008), which precedes the degeneration of the ganglion cell soma in the retina.

Ganglion cell soma loss is first evident in glaucomatous DBA/2J mice at or near the time when animals are clearly exhibiting optic nerve disease, but the majority of animals exhibit retinal degeneration at approximately 10–11 months of age (Schlamp et al., 2006). Several studies have examined the process of ganglion cell death in these mice using both electron microscopic and biochemical techniques, indicating that apoptosis is the most likely mechanism of soma loss in these mice (Schuettauf et al., 2004; Libby et al., 2005a). Peak DNA fragmentation, monitored by TUNEL-staining and intercalating dyes, occurs during the period of 10–11 months, consistent with other methodologies that simply monitor the presence or the absence of ganglion cells (Libby et al., 2005a). Genetic crosses that have introduced a mutant allele for the pro-apoptotic gene *Bax* onto the DBA/2J background also dramatically demonstrate that the process of ganglion cell soma death in this model of glaucoma is completely *Bax*-dependent, clearly implicating the process of intrinsic apoptosis in these cells. More details of these latter experiments will be discussed below.

Interestingly, soma loss in DBA/2J mice often occurs in distinct fan-shaped patterns originating at the optic nerve head and spreading to the retinal periphery (Jakobs et al., 2005;

Schlamp et al., 2006) (Fig. 3). This unique pattern suggested that the initial site of damage to the ganglion cells in the mouse was at the level of the optic nerve lamina, the only region of the optic nerve where ganglion cell axons are organized into discrete bundles (May and Lütjen-Drecoll, 2002; Schlamp et al., 2006; Howell et al., 2007a). Careful histologic examination of both diseased retinas and their optic nerves subsequently revealed that fan-shaped patterns of ganglion cell loss were associated with corresponding regions of axon bundle loss (Schlamp et al., 2006; Howell et al., 2007a). Recently, detailed studies examining the pathology of the DBA/2J mouse optic nerve showed that the earliest focal damage to the ganglion cell axons occurred in the laminar region of the mouse eye, an area where axons are intimately associated with a high density of optic nerve astrocytes (Howell et al., 2007a). This finding supports the popular model that the first deleterious effects of elevated IOP are transmitted by glial changes in the optic nerve head (Hernandez, 2000; Neufeld and Liu, 2003; Morrison et al., 2005; Johnson et al., 2007; Nickells, 2007).

The initial site of damage at the optic nerve head may seem inconsistent with the pattern of axonal die-back observed by Schlamp et al. (2006), where the axons appear to be dying in a retrograde direction from the brain to the lamina. In reality, however, such a pattern of degeneration can be elicited by a distant lesion, if that lesion is relatively mild. Experimental damage to long axons from sciatic nerves showed that a moderate crush lesion induced retrograde die-back of axons, while a more severe lesion such as axotomy induced Wallerian degeneration (Beirowski et al., 2005). An acute crush lesion of the mouse optic nerve, for example, is substantially harsher than damage inflicted in the glaucomatous eye, and can stimulate a more Wallerian pattern of axonal breakdown (data not shown).

There are advantages and disadvantages to the DBA/2J mouse model for the study of glaucoma. The principal advantage is that this is a spontaneously arising mouse disease with many similarities to the human disease. Thus, using well-established technology, DBA/2J mice can be genetically manipulated to investigate the roles of different genes and molecular pathways in the progression of the disease. Some of these studies are described in a latter section of this review. The disadvantages are not trivial, however. Despite significant advances in clinical evaluation of the mouse eye (Smith et al., 2002), its size poses difficulties in using a variety of conventional technologies, particularly the ability to conduct longitudinal measurements of IOP. Also, even though these are inbred mice, meaning that they have a fixed genetic background, they still exhibit a high degree of variability and asymmetry in developing the disease (Schlamp et al., 2006). At an age when most of the mice exhibit disease, for example, there will be a wide range of disease phenotypes within this age-matched group. At its extreme, we have documented that approximately 8–10% of the older DBA/2J mice in our colony have one normal, non-diseased retina and optic nerve, and one eye has undergone complete degeneration. This can be both an advantage and a disadvantage, but it generally means that relatively large cohorts of mice must be used in a single experiment. Lastly, the relevance of glaucoma in the DBA/2J mouse to the human condition is still controversial. Part of this is underscored by the unusual interplay of the *Tryp1* and *Gpnmb* genes in initiating the anterior chamber disease, and by the undefined role of the mouse immune system in the development of the ensuing optic neuropathy. Two things are certain, however. Discoveries precipitated by using the DBA/2J mouse have created exciting new avenues of research in glaucoma, and have helped to address some of the speculative nature of our understanding of glaucoma that existed before the introduction of this model.

5. Spontaneous IOP elevation in other transgenic mice

Transgenic mice with targeted mutations in the $\alpha 1$ subunit of collagen type I (*Col1a1^{+/r}*) develop spontaneous and gradual elevation of IOP (Aihara et al., 2003c). The mutations

block matrix metalloproteinase (MMP) cleavage by coding for five amino acid substitutions adjacent to the site recognized by MMP-1, causing accumulation of collagen type I within aqueous outflow pathways and subsequent IOP elevation. At 24 weeks of age, IOPs in the *Colla1^{tr}* transgenic mice were 23.5 ± 2.4 mmHg (mean $\pm$ SD) compared to 15.8 ± 0.8 mm in the wild-type (*Colla1^{+/+}*) mice. This represents a maximum IOP elevation (44%) in *Colla1^{tr}* mice compared to wild-type mice during the 36 weeks of this study. In a later study, optic nerve damage was assessed in these mice (Mabuchi et al., 2004). Axons were counted from EM images of ultrathin optic nerve cross-sections stained with 1% uranyl acetate. Axon loss was not significantly different at 24 weeks of age between *Colla1^{tr}* and *Colla1^{+/+}* mice, but was significantly lower in *Colla1^{tr}* mice at 54 weeks of age, representing 28.7% axon loss at this timepoint.

One of the first gene mutations found to be associated with glaucoma is myocilin, which is expressed and secreted in the trabecular meshwork. Myocilin mutations are identified in 3–4% of primary open angle glaucoma patients, and the Tyr437His mutation is associated with a much younger age of onset (Alward et al., 1998). At 18 months of age, transgenic mice expressing the Tyr437His mutation in myocilin showed a modest, but significant elevation in IOP (mean $\pm$ SD), when compared to wild-type litter-mates (18.3 ± 2.2 mmHg vs 14.9 ± 0.9 mmHg) (Zhou et al., 2008). RGCs were counted by retrograde labeling with FluoroGold. An approximate 20% loss of RGCs in the peripheral retina was noted in the Tyr437His transgenic mice compared to wild-type mice. Histological analysis of cross-sections of Tyr437His transgenic mouse optic nerves revealed loss of axons, degradation of myelin sheaths, and loss of fascicular organization.

With the advent of chronic spontaneous, inducible, and transgenic models of mouse glaucoma, it is important to discuss the relative merits of each. Comparatively, the natural history of glaucoma development in inducible models and models of transgenic mice all show that they are relatively mild forms of the disease with modest elevations in IOP and limited axon and ganglion cell loss. Alternatively, the spontaneous disease in DBA/2J animals often proceeds to end-stage. The spontaneous and transgenic models both require a prolonged period of time for glaucomatous damage to develop (over a year in some cases), making them equally expensive for long-range studies, while the inducible models can be conducted in a more time-compressed fashion. Similarly, although inducible models exhibit relatively mild disease, the spontaneous disease in DBA/2J is highly variable, which can complicate data collection and interpretation. Currently, however, the inducible and transgenic models of mouse glaucoma are still poorly characterized compared to the DBA/2J model, making the latter potentially more relevant for in depth drug and pathogenesis studies.

6. The power of genetics: using mice to evaluate the roles of genes in glaucoma

6.1. Ganglion cell death and optic nerve degeneration

The ability to genetically alter mice to investigate gene function has helped to develop the concept that retinal ganglion cell death in glaucoma occurs as a series of compartmentalized self-destruct processes (Whitmore et al., 2005; Nickells, 2007). In this hypothesis, initial damage to the optic nerve axons leads first to the autonomous degeneration of the ganglion cell axon, and then, by virtue of impaired axonal transport, the apoptotic degeneration of the ganglion cell soma in the retina. The experiments that precipitated this concept are best exemplified by studies examining the function of the pro-apoptotic gene *Bax*. *Bax* is one member of a related gene family that shares homology to *Bcl2*, a gene that was initially discovered as having a critical role in preventing apoptosis of a wide variety of cells

(Bakhshi et al., 1985; Hockenbery et al., 1990; Knudson and Korsmeyer, 1997; Adams and Cory, 1998). Mice lacking *Bax* exhibit several populations of supernumerary neurons (White et al., 1998; Sun and Oppenheim, 2003), including retinal ganglion cells (Mosinger Ogilvie et al., 1998), suggesting that *Bax* is required for the execution of developmental programmed cell death. Using *Bax*^{-/-} mice in different models of acute ganglion cell death, we learned that ganglion cell soma death was absolutely dependent on *Bax* after optic nerve crush (Li et al., 2000). This proved to be a long-term effect, in that mice still exhibited a complete complement of ganglion cell somas as long as 18 months after the procedure. Interestingly, cell death was not abrogated in the model of NMDA toxicity, indicating that ganglion cells have both *Bax*-dependent and *Bax*-independent apoptotic pathways. The requirement of *Bax* was also investigated in the DBA/2J mouse model of glaucoma. For these experiments, the *Bax* mutant allele was crossed onto the DBA/2J genetic background through a series of backcrosses. Mice congenic for the mutant allele, were able to develop anterior chamber disease and elevated IOP, but showed complete resistance of the ganglion cell somas (Libby et al., 2005a), just as they had in the crush model. An important finding from this study, however, was that even though these mice exhibited no soma death, they still exhibited axonal degeneration, although it was delayed. These studies have revealed some critical information on the mechanism of ganglion cell pathology in glaucoma. First, ganglion cell soma death and axon degeneration can be genetically separated from each other. Clearly, axon death was not reliant on soma loss, although soma dysfunction may still be a critical component of this process, and these experiments were not able to rule out this possibility. Second, the role of excitotoxicity, mediated by glutamate release, appears to play a minor or secondary role in the pathology of glaucoma. This was implied by the fact that *Bax*^{-/-} cells are resistant in glaucoma, but are not rescued in acute experiments using the glutamate analog NMDA. It is possible that secondary effects may be precipitated by the initial loss of cells, but if this event is blocked, secondary events are also blocked. Third, it was learned that *Bax* may be a susceptibility allele for ganglion cell soma death because reducing *Bax* gene dosage to one wild-type allele from two, was also able to rescue these cells in both the crush and glaucoma models. In subsequent experiments to examine this phenomenon, it was learned that the level of *Bax* transcription was critically tied to a threshold for the cell death process. Essentially, the mechanism of cell death acts on an all-or-none switch depending on *Bax* expression (SJ Semaan, Y Li, and RW Nickells, unpublished observation). Ultimately, this feature of BAX protein function may make it a primary target for small molecule therapy aimed at reducing *Bax* expression levels or BAX protein function, in an effort to prevent ganglion cell apoptosis.

6.2. Reverse genetics to study glaucoma genes and identify susceptibility alleles

For the most part, glaucoma in humans is considered a complex genetic disease, in which susceptibility is linked to the inheritance pattern of multiple interacting loci (Wiggs, 2007). Although tremendous advances have been made in the field of glaucoma genetics, most of the genes or loci identified have been through the investigation of the genomes of families where there have been clear inheritance patterns. Finding susceptibility alleles, which are likely to contribute to disease in the majority of cases of primary open angle glaucoma, has been a much more difficult process. An alternative to direct human studies, however, is the use of reverse genetics involving mice. In this context, the term 'reverse genetics' refers to using animal models to examine and characterize the functions of genes initially identified in humans under controlled laboratory conditions (Kroeber et al., 2006; Senatorov et al., 2006; Paper et al., 2008; Skarie and Link, 2008).

Similarly, reverse genetics can also describe studies using mice to identify genes that affect an aspect of glaucoma in these animals, and then use that as a candidate gene to determine if the human homolog is also associated with disease in human population studies. This latter

approach may be useful to identify potential human susceptibility alleles. A recent example of this process was initiated in our laboratory. We speculated that the response of ganglion cell somas to a lesion of the optic nerve, which leads to the activation of the apoptotic program, may be one such aspect of glaucoma that could confer resistance or susceptibility to the damaging influence of elevated intraocular pressure. To test this hypothesis, we examined ganglion cell susceptibility to a standardized lesion of the optic nerve (crush) in 15 different inbred lines of mice. These experiments showed that some mice were more resistant to crush than others, and that this quantitative trait could be inherited in an autosomal dominant fashion (Li et al., 2007). Subsequent studies investigated the chromosomal region for this inheritance using conventional genome wide linkage analysis of a large population of mice generated by matings between the resistant and susceptible strains. This analysis revealed a single quantitative trait locus (QTL) on chromosome 5, which segregates with ganglion cell resistance to the crush lesion (Dietz et al., 2008). Because this QTL affects only a small percentage of the cell death phenotype (approximately 12%), it has been named Retinal ganglion cell susceptible 1 (*Rgcs1*) in the anticipation that other loci will be discovered that also influence this process. The next steps in this process are to narrow the region of the QTL from a region of chromosome 5 to a single gene and to evaluate its role in ganglion cell death in glaucoma.

The success of reverse genetics relies heavily on the basic technology to manipulate genomes now widely used in the field of mouse genetics. To examine the role of *Rgcs1* in glaucoma, for example, the susceptible allele found in BALB/cByJ mice is being bred onto the DBA/2J genetic background, where it is predicted that these mice will develop a more severe glaucoma phenotype. Ultimately, the causative gene will provide a candidate for high resolution mapping of DNA taken from well-defined datasets of patients with and without glaucoma. If successful, it will mark a powerful tool to identify susceptibility alleles in the human population.

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Fig. 1.

Acute induction of retinal ganglion cell loss in mice. (A) Histogram showing the reduction of cells in the ganglion cell layer after a crush lesion of the optic nerve. Quantification is measured as a percentage of the cells counted in the untreated fellow eyes of each mouse examined. The data shown here represents the decrease in cells observed in BALB/cByJ mice after crush. (B) Histogram showing the reduction of cells in the ganglion cell layer 4 days after intravitreal injection of the glutamate analog *N*-methyl-D-aspartate (NMDA). Cells are lost in a dose-dependent fashion in response to NMDA injection (total nmoles injected are shown). The data shown here represents cell loss in CB6F1 hybrid mice (F1 mice from a cross of a BALB/c female with a C57BL/6 male). (C, D) Nissl-stained flat-mounted retinas of a mouse 2 weeks after optic nerve crush in an SJL/J mouse. The untreated fellow eye (C) shows a high density of cells in the ganglion cell layer, which is mounted facing up on the slide. The crushed eye (D) shows a dramatic loss of cells. Scale bar = 80 μ m (E, F) Nissl-stained flat-mounted retinas of a C57BL/6J mouse 2 days after injection with NMDA. The uninjected eye (E) shows a typical complement of cells in the ganglion cell layer. In the fellow injected eye (F), however, fewer cells remain and there is evidence of apoptotic fragments from dead and dying cells. Scale bar = 10 μ m.

Fig. 2. Iris transillumination defects in aging DBA/2J mice. As these mice age, the deterioration of the iris stroma and the dispersion of sloughed pigment is readily detected as transillumination of light reflected off the lens. These defects first become evident by 6–8 months of age. Scale bar = 0.2 mm.

Fig. 3.

Optic nerve disease and retinal ganglion cell loss in DBA/2J mice. (A) DiI-labeled optic nerves of a young (3 months) DBA/2J mouse. In this method, nerves are labeled postmortem by introducing crystals of DiI into the optic nerve head of each eye. The dye is able to diffuse along the axons of the optic nerve, but since the dye does not transfer from one cell to another, only contiguous axons become labeled. In young mice, it is possible to label nerves all the way to the optic chiasm (arrow) by this technique. (OD – right optic nerve; OS – left optic nerve). (B) DiI-labeled optic nerves of an old (10 months) DBA/2J mouse, that shows signs of glaucomatous damage. In this mouse, the left nerve (OS) has yet to show significant signs of damage, while the right (OD) nerve is nearly completely degenerated. Scale bar = 250 μm (arrow – optic chiasm). (C–F) Nissl-stained flat-mounted retinas from

DBA/2J mice at different ages. A similar region of the retina from each eye is shown. Typically, as these mice age, cell density in the ganglion cell layer is diminished. The majority of eyes in mice between 10 and 13 months clearly show signs of cell loss. Scale bar = 10 μm . (G) Flat-mounted retina from a 10.5 month old DBA/2J mouse carrying the *Fem1c*^{Rosa3} marker transgene. This promoter trap marker fusion protein is inserted into the first intron of the mouse *Fem1c* gene and is predominantly expressed by ganglion cells in the retina (Schlamp et al., 2004). Healthy ganglion cells express the fusion protein, which contains β -galactosidase, and thus can be stained blue using X-gal as a substrate. During glaucomatous retinal damage, DBA/2J mice exhibit a loss of blue cell staining, typically in wedge-shaped patterns. (H) Computer enhanced image of the retina shown in (G), in which X-gal stain deposits are recognized on the basis of color parameters. Large to small cells are depicted as different colored spots on the computer two-dimensional plot. This retina clearly exhibits two distinct wedges of cell loss in different stages of completion. Scale bar = 100 μm .