Research article

Genetic modification of glaucoma associated phenotypes between AKXD-28/Ty and DBA/2J mice

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Abstract

Background: Glaucoma is a common disease but its molecular etiology is poorly understood. It involves retinal ganglion cell death and optic nerve damage that is often associated with elevated intraocular pressure. Identifying genes that modify glaucoma associated phenotypes is likely to provide insights to mechanisms of glaucoma. We previously reported glaucoma in DBA/2J mice caused by recessive alleles at two loci, *isa* and *ipd*, that cause iris stromal atrophy and iris pigment dispersion, respectively. A approach for identifying modifier genes is to study the effects of specific mutations in different mouse strains. When the phenotypic effect of a mutation is modified upon its introduction into a new strain, crosses between the parental strains can be used to identify modifier genes. The purpose of this study was to determine if the effects of the DBA/2J derived *isa* and *ipd* loci are modified in strain AKXD-28/Ty.

Results: AKXD-28/Ty mice develop glaucoma characterized by intraocular pressure elevation, retinal ganglion loss, and optic nerve excavation. In AKXD-28/Ty, *isa* causes an iris stromal atrophy phenotype as in DBA/2J. However, the iris pigment dispersion phenotype associated with *ipd* in DBA/2J does not occur in AKXD-28/Ty. Additionally, a greater severity and speed of retinal and optic nerve damage following intraocular pressure elevation in AKXD-28/Ty compared to DBA/2J mice suggests that AKXD-28/Ty is more susceptible to pressure-induced cell death.

Conclusions: The consequences of the *ipd* and *isa* mutations are modified in the AKXD-28/Ty background. These strains provide a resource for the identification of modifier genes that modulate pigment dispersion and susceptibility to pressure-induced cell death.

Background

Glaucoma is a prevalent group of retinal and optic nerve neuropathies that currently renders approximately 67 million people worldwide at risk for developing significant vision loss, including blindness [1]. Glaucoma involves the death of retinal ganglion cells (RGCs) and their axons, and is characterized by atrophic excavation of the optic nerve [2,3,4]. Glaucoma is usually associated with high intraocular pressure (IOP) resulting from an increased resistance to drainage of aqueous humor. Not all people with high IOP develop glaucoma [5], however, suggesting that other factors, such as genetic susceptibility to pressure-induced damage, interact with IOP to cause damage. The occurrence of glaucomatous damage in individuals without high IOP and the benefit of lowering IOP in some of these individuals further suggest mul-

tiple factors determine an individual's susceptibility to pressure-induced damage [6]. The nature of the factors participating in glaucomatous events, particularly those influencing disease progression and modifying disease severity in different individuals, remain largely unknown. Increased understanding of factors contributing to glaucoma will suggest new therapeutic strategies and will likely lead to improved clinical management.

Glaucomatous phenotypes have been observed in a number of mammalian species, including mice [7,8,9]. Mice can be clinically and histologically analyzed throughout the course of a disease and their genes can be altered to study the molecular framework underlying pathology [10]. As a consequence, mouse models are useful for identifying and characterizing the effects of causative genes necessary for glaucoma development, and for characterizing the genes and molecular pathways that participate in or modify disease progression [9]. A collection of glaucomatous mouse strains with phenotypic differences would facilitate research to understand the complexity of glaucoma. As part of our efforts to understand glaucoma, we are screening for and characterizing glaucoma in aged mice of various strains. We are particularly interested in documenting differences in glaucoma phenotypes between strains of mice that share the same causative genes. This information will provide an experimental basis for identifying genes and mechanisms that modify the progression or severity of glaucoma. Here we report differences in genetic susceptibility to glaucoma-associated phenotypes between two related inbred strains of mice, AKXD-28/Ty (AKXD28) and DBA/2J (D2).

D2 mice develop glaucoma involving a harmful increase of IOP followed by RGC loss and optic nerve damage [11]. The increase in IOP is associated with an iris disease involving iris pigment dispersion (IPD), iris stromal atrophy (ISA), and the formation of synechiae that block aqueous humor drainage. The iris pigment dispersion and iris stromal atrophy phenotypes are caused by distinct recessive alleles at the *ipd* and *isa* loci, respectively [12]. The IPD phenotype in D2 mice is similar to human pigment dispersion syndrome, a condition that often leads to pigmentary glaucoma [12, 13], and involves degeneration of the iris pigment epithelium [12, 14]. Causative genes for pigment dispersion remain to be identified [12, 15]. The gene responsible for the ISA phenotype is tightly linked to the tyrosinase related protein 1 gene (*Tyrp1*) [12], which also regulates coat color. D2 mice have a mutant allele of this gene ($Tyrp1^b$) and a brown coat color [16]. In previously studied genetic backgrounds, homozygosity for D2 alleles of both *isa* and *ipd* results in iris atrophy that severely affects both the iris stroma and iris pigment epithelium, leading to a severely atrophic and largely transparent iris in old mice [11, 12].

AKXD28 is a recombinant inbred strain derived by inbreeding offspring from an intercross between mice of the D2 and AKR/J strains [17]. AKXD28 mice, therefore, have a genetic background that is a mix of the D2 (glaucomatous) and AKR/J (normal eyes with no obvious disease) genomes. Due to extensive inbreeding, all AKXD28 mice are genetically identical except for the sex chromosome difference between males and females. This genetic uniformity is important for the analysis of complex diseases because it allows repeated and therefore accurate assessment of phenotypes associated with the AKXD28 mix of D2 and AKR/J genomes. AKXD28 mice inherited the glaucoma causing *ipd* and *isa* alleles from strain D2. This is evident by their brown coat color and the inheritance of D2 derived microsatellite markers flanking these loci [18,19,20,21,22,23]. To further assess the phenotypic consequences of *ipd* and *isa* and whether their effect is modified in the AKXD28 genetic background, we performed a detailed characterization of the ocular phenotype in AKXD28 mice.

As demonstrated here, AKXD28 mice develop an age related glaucoma involving increased IOP and optic nerve damage, and they are a useful tool for glaucoma research. The AKXD28 disease has a number of similarities to that observed in D2 mice. Importantly, however, two major differences exist between the disease in AKXD28 and D2 mice housed in the same environment. First, AKXD28 mice do not develop the IPD phenotype. The absence of the IPD phenotype is surprising considering AKXD28's inheritance of the D2 chromosomal region containing the *ipd* gene, and likely results from genetic differences between these strains. Understanding these differences will be important for understanding pigment dispersion, a common cause of human glaucoma. Second, although D2 and AKXD28 strains demonstrate similar magnitudes of IOP, AKXD28 mice develop more severe and more extensive retinal damage and are more prone to optic nerve head excavation than D2 mice. This suggests that compared to D2 mice, AKXD28 mice have an increased genetic susceptibility to pressure-induced damage. Identifying the genes that differ between AKXD28 and D2 mice and that modify the progression and severity of pressure-induced retinal cell death will be important for understanding mechanisms killing cells in glaucoma and may ultimately lead to improved patient care.

Table 1: Clinical and histological findings in AKXD-28/Ty eyes.

	Clin			Histo						
Age (months)	ISA	TRAN	DP	ISA	PAS	PS	RGCD	ONA	ONC	INL
6-8	0/12	12/12 12 per	0/12	0/8	0/8	0/8	0/8	0/8	0/1	0/8
9-14	52/78 36 mi 16 mod	78/78 70 mi 8 mo	0/78	2/8 2 mi	7/8 7 mi	6/8 4 mi 2 mo	2/8 2 mi b	0/4	0/3	0/8
15-22	60/60 a 24 mi 36 mo	60/60 a 20 mi 40 mo	0/60 a	11/12 2 mi 7 mo 2 sev	12/12 4 mi 4 mo 4 sev	9/12 3 mi 1 mo 5 sev	11/12 1 mi c 10 sev d	8/10 1 mi c 3 mo d 4 sev d	7/7 1 mo c 6 sev d	7/12
23-28	26/26 26 sev	26/26 26 sev	1/26	25/25 25 sev	23/23 1 mi 15 mo 7 sev	24/25 2 mi 9 mo 13 sev	25/25 25 sev	23/23 1 mo 22 sev	21/21 21 sev	24/25

Clin = clinical, Histo = histological. a All were 15 months old; b All were 14 months old; c All were 17 months old; d All were 18 to 22 months old.
Summary of Clinical and histological phenotypes in AKXD28 eyes. Clinical and histologic examinations were performed with different cohorts of mice. The age groups are arranged so that the overall prevalence and severity of specific disease features within each group are similar. In general, age groups are not skewed with mice of a particular age, except that the 15-22 month old group examined clinically consists of 15 month old mice. Mice less than 6 months old were characterized by normal clinical exams and histology but are not included in Table 1. The numerator indicates the number of eyes exhibiting the lesion, whereas the denominator indicates the number of eyes successfully analyzed for that lesion. The number of eyes with a finding of a specific severity is shown in italics. Phenotypes were ranked as peripheral (per), mild (mi), moderate (mo), or severe (sev) (see Materials and methods, and ref 11). Clinical abbreviations: iris stromal atrophy (ISA), iris transillumination (TRAN), and dispersed pigment (DP). Histologic abbreviations: iris stromal atrophy (ISA), peripheral anterior synechiae (PAS), posterior synechiae (PS), retinal ganglion cell depletion (RGCD), optic nerve atrophy (ONA), optic nerve cupping (ONC), and inner nuclear layer cell loss (INL). Other phenotypes not listed here include corneal neovascularization and corneal calcification that occurred in some mice, and cataracts whose incidence increased with age and that were present in all 23 month old or older mice. Despite gentle handling and great care, clinical examination induced subconjunctival hemorrhage in approximately 30-50% of AKXD28 mice. Though less frequent, spontaneous and handling induced vascular hemorrhage into the anterior chamber (hyphema) also was observed in a number of AKXD28 mice. These bleeding phenotypes occur only rarely in other strains we have studied.

Results

AKXD28 is a recombinant inbred mouse strain that inherited the glaucoma associated *isa* and *ipd* alleles from strain D2. To investigate the effects of these alleles in AKXD28 mice, and the potential presence of modifier loci, we examined the eyes of AKXD28 mice at ages from 2 to 28 months, as summarized in Table 1. Although the presence and severity of specific disease features were variable between mice of the same age, the disease severity clearly increased progressively with age. To ensure accurate phenotypic comparisons between the AKXD28 and D2 strains both strains were housed at the same time in the same environment and fed the same diet. The phenotype of D2 mice was previously reported and is not presented in detail here [11,12]

AKXD28 develop ISA but not IPD

Consistent with the inheritance of *isa* from strain D2, the iris stroma of AKXD28 mice atrophied with age (Figure 1). The first signs of ISA were small focal peripheral transillumination defects at 6 to 8 months of age. Iris stromal atrophy was histologically evident in most eyes by 15 months. ISA became progressively more severe, profoundly affecting all eyes by 23 months (Table 1, Figure 1).

AKXD28 mice contain D2 derived alleles for markers *Mtv23* and *D6Nds3* which are located approximately 9.5 cM proximal and 5 cM distal to *ipd*, respectively [12, 22, 23]. Additionally, AKXD-28/Ty mice contain D2 derived microsatellite markers for *D6Mit207*, *D6Mit33*, and *D6Mit93* which subdivide this region and are tightly linked to *ipd* (data not shown). Despite inheritance of the *ipd* chromosomal segment from strain D2, both histolog-

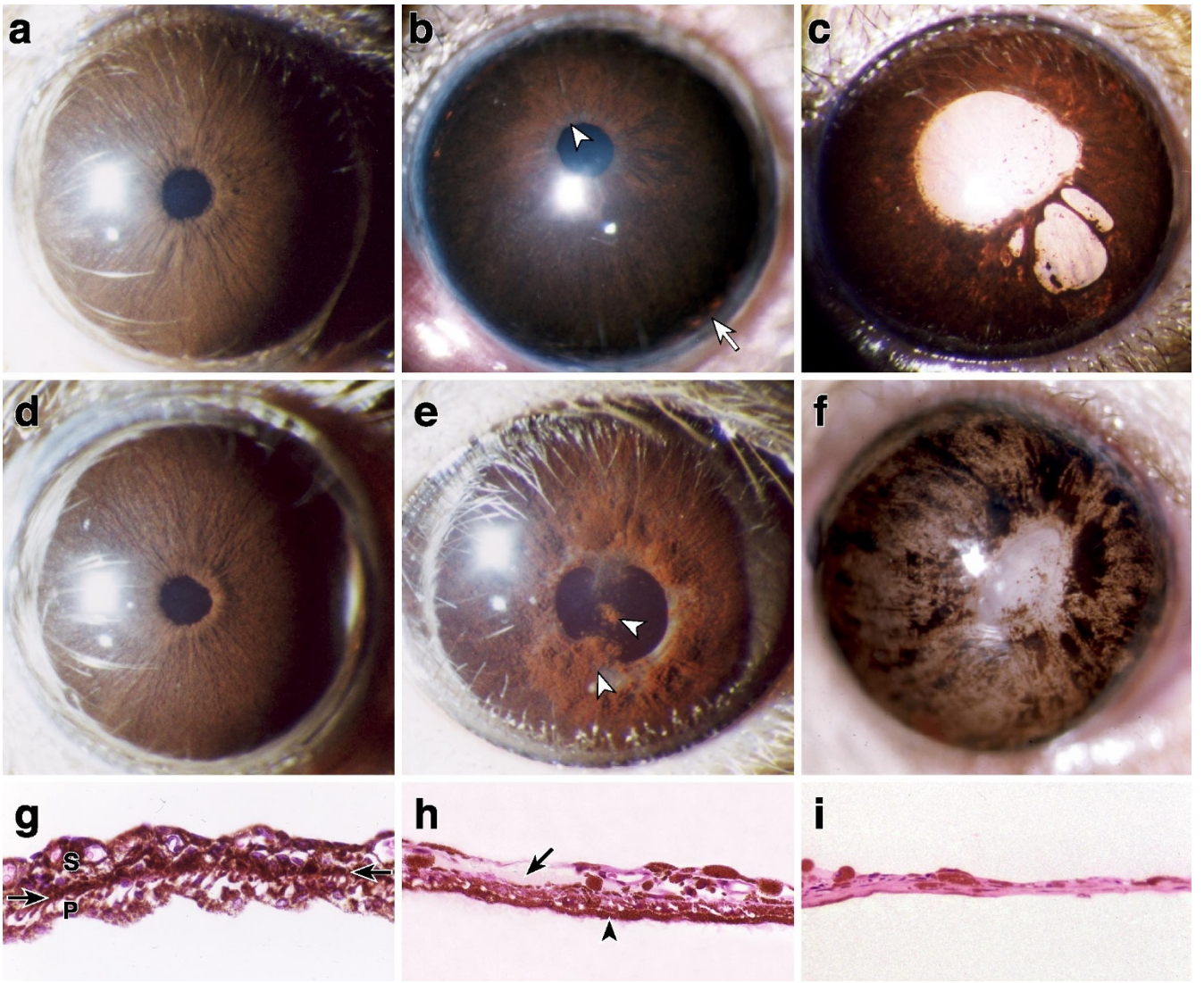

Figure 1
AKXD28 develop ISA but not IPD. Representative iris phenotypes in aging AKXD28 (**a-c** and **g**, **h**) and D2 (**d-f** and **i**) mice are shown. Age and disease severity increase from left to right. (**a**, **d**) Two month old AKXD28 and D2 mice have a normal, complex iris morphology with clearly evident iris details including crypts, small central pupil, prominent peripupillary sphincter muscle, and pupillary ruff. (**b**, **e**) At 12 months AKXD28 (**b**) have iris stromal atrophy characterized by loss of iris detail, thinning of the iris stroma with exposure of the sphincter muscle (arrowhead in **b**), and mild transillumination defects (arrow). Dispersed pigment is not prominent in the anterior chamber. The eyes of similarly aged D2 mice (**e**) are more severely affected with both stromal atrophy and iris pigment dispersion. The stromal atrophy is characterized by abnormally shaped pupils, exposure of the sphincter, and loss of iris detail while the pigment dispersion is evidenced by the prominent accumulation of pigment on the front of the iris and lens (arrowheads in **e**). (**c, f**) 26 month old AKXD28 and D2 mice have marked stromal atrophy with enlarged irregular pupils, iris holes, and increased transillumination. In D2, severe loss of both iris stromal and iris pigment epithelium pigmentation due to the ISA and IPD phenotypes results in large transparent iris regions. In contrast, the AKXD28 iris remains generally pigmented due to the lack of IPD and retention of much of the pigment epithelium. Histologic analysis confirms the presence of ISA and lack of IPD in AKXD28 mice. (**g**) In a young AKXD28 mouse the iris has a robust stroma (S) and pigment epithelium (P) that are separated by the dilator muscle (masked by pigment in this section but located at the level of the arrows). (**h**) Although severity varies locally, the stroma of old AKXD28 mice is severely atrophied and almost non-existent in many places (arrow). The iris pigment epithelium (arrowhead) of old mice has a flattened morphology but remains remarkably intact considering the overall condition of the iris. (**i**) In old D2 mice, both the iris stroma and iris pigment epithelium are severely atrophic.

ical and clinical analysis of AKXD28 irides and anterior chambers failed to detect the presence of IPD at all ages examined. IPD is characterized by deterioration of the iris pigment epithelium with accumulation of dispersed

pigment and pigment filled cells in the anterior chamber and on the front of the iris and lens [12] (Figure 1e). No pigment dispersion or accumulation was observed in AKXD28 eyes (Figure 1a,1b,1c), and the iris pigment epithelium remained remarkably intact considering the severe iris stromal atrophy (Figure 1h). The overall condition of the iris in old AKXD28 versus old D2 mice also indicated the lack of the IPD phenotype in AKXD28. D2 mice succumb to both ISA and IPD, and develop iris atrophy that progresses to the point of transparency over most of the iris (Figure 1f). The irides of AKXD28 eyes maintain substantially greater tissue integrity and pigmentation (compare Figure 1c to 1f and 1h to 1i).

Anterior synechiae and increased IOP in AKXD28

The ISA phenotype in D2 mice involves deterioration of the iris stroma and release of cellular debris into the anterior chamber. This leads to the formation of adhesions (anterior synechiae) that block aqueous humor access to its drainage pathways including the trabecular meshwork and Schlemm's canal in the iridocorneal angle [11, 12]. AKXD28 mice similarly develop iris stromal atrophy, anterior synechiae, and IOP elevation (Figure 2). In AKXD28, the synechiae were first evident at 9 months, and were extensive in most eyes by 15 to 22 months of age (Table 1).

To assess the functional relationship between these morphological abnormalities and aqueous drainage, we determined the IOPs of AKXD28 mice at various ages (Figure 2). IOP increased significantly with age ($P=0.0001$) and pressure elevation followed a time course consistent with obstruction of aqueous humor drainage by extensive anterior synechiae formation. Although there were no obvious histologic differences between males and females, gender had a significant effect on the timing of IOP elevation ($P=0.008$). Among females, the mean IOP increased from 12.9 ± 0.3 mmHg at 7 to 10 months (when synechiae were absent or mild) to a peak value of 19.8 ± 1.8 mmHg at 15-18 months (when synechiae were often moderate or severe). The IOP of males showed an increase from 14.9 ± 0.4 mmHg at 7 to 10 months to a peak of 18.9 ± 1.0 mmHg at 19-21 months. The magnitudes of IOP elevation were similar to those in D2 mice [11].

Vitreous glutamate levels increase when IOP is elevated in various species and this increase is associated with RGC death [24,25,26]. As an initial effort to assess glutamate levels in AKXD28 mice, vitreous was collected from mice of different ages and subjected to amino acid analysis. In this study, we analyzed two pools of samples from 14 month old mice and two pools of samples from 16 month old mice. Pooling was necessary due to the small volume of vitreous collected, and each pool consisted of 2 to 4 eyes (see Materials and methods). In this study, vitreous glutamate levels were 13.2 ± 1.7 μM in 14 month old mice (when IOP is generally normal) and 29.2 ± 4.7 μM in 16 month old mice (when IOP is often elevated).

AKXD28 develop glaucomatous neuropathy

AKXD28 mice develop several glaucomatous abnormalities. All retinas had a normal appearance until 14 months when mild loss of retinal ganglion cells was observed in some mice (Table 1). All mice over 18 months of age had severe ganglion cell loss both peripherally and close to the optic nerve (Figure 3, Table 1). Atrophic excavation of the optic nerve head was evident histologically and by clinical fundus examination in old but not in young mice (Figure 3, Table 1). Fundus examination was not possible in many old mice due to corneal opacities and cataracts.

To further examine the progression of optic nerve damage, optic nerve cross sections from male and female mice of differing ages were analyzed (Figure 4). Optic nerve damage was detected closely following the period of increased IOP. Moderate or severe optic nerve damage was first observed in some 17 month old mice (3 of 12) and occurred in almost all 19-20 month old mice (20 of 22). Interestingly, optic nerve damage occurred earlier in females than in males. Damage was detectable in female but not male nerves at 17 months. By 19-20 months all female nerves were severely affected, whereas, approximately half of the male nerves were mildly or moderately affected ($P=0.02$, Chi-square) (Figure 4d).

Retinal and optic nerve damage is more severe in AKXD28 than D2

Retinal damage in AKXD28 eyes was more severe than in D2 eyes (Figure 3, Table 1, and Ref. [11]). In AKXD28 mice, IOP elevation was first noted in a few mice at 11 months and mild RGC depletion (defined here as loss of cells in the RGC layer) was initially observed at 14 months. By 18 months RGC loss was severe in all AKXD28 eyes. By contrast, D2 mice exhibit a slower and milder retinal disease following increased IOP (compare Figure 3c to 3f). In D2 mice, IOP elevation was first observed in some mice at 6 months. Mice at 18 or 19 months had no more than a moderate loss of RGCs, and only 1 of 11 mice older than 20 months had severe RGC loss. Paralleling RGC loss, optic nerve head excavation or cupping was also more severe and more frequent ($P < 0.0001$, Chi-square) in AKXD28 than D2 mice. Optic nerve excavation was histologically evident in 27 of 27 AKXD28 mice that were 18 months or older and was typically severe (Table 1). The severe atrophy extended to a level outside of the inner nuclear layer (INL) and often outside the choroid (Figure 3e). Optic nerve excavation occurred in only 11 of 24 D2 mice that were 18 months or

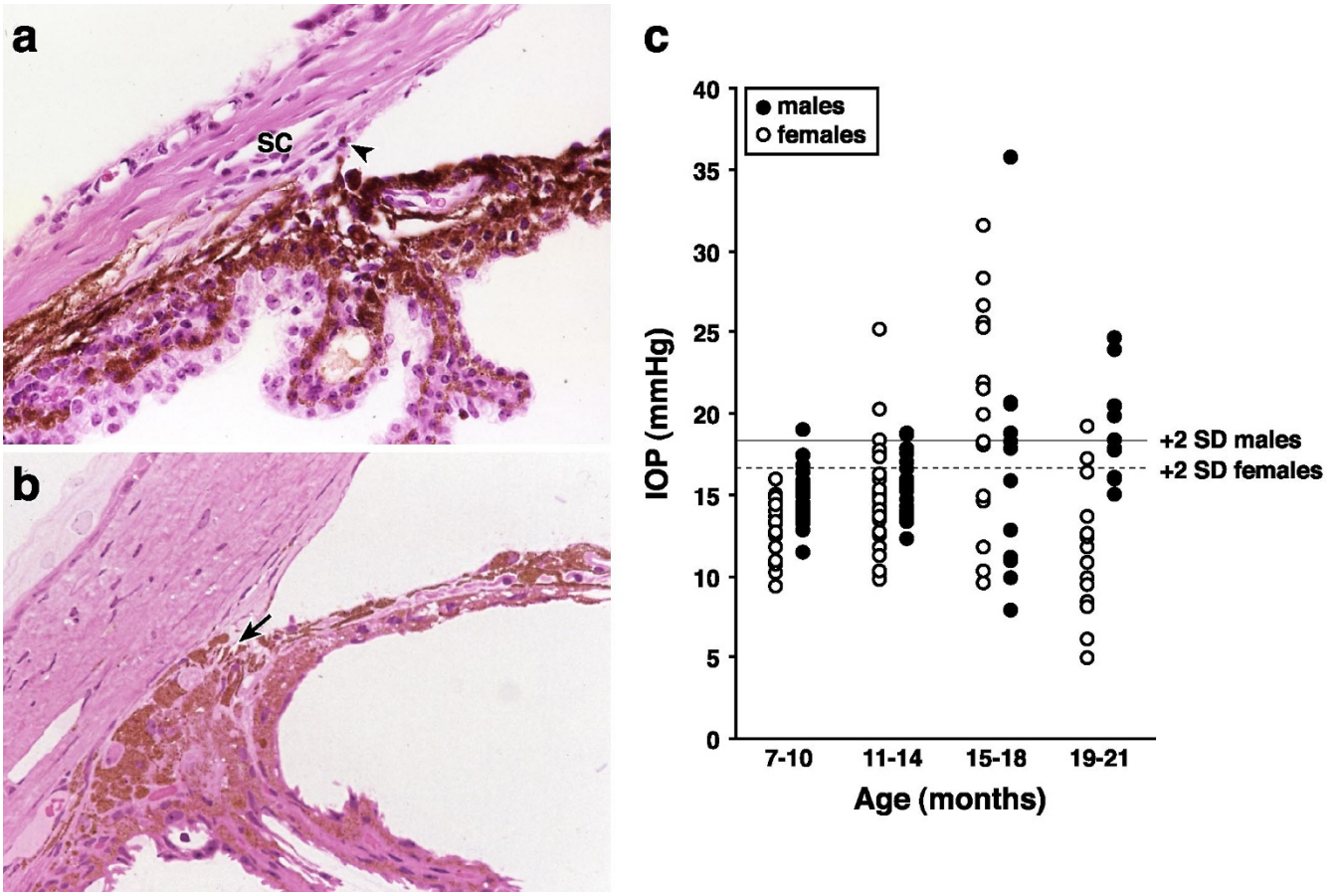

Figure 2
Anterior synechiae and elevated IOP in AKXD28. (**a**) Young mice have normal, unobstructed iridocorneal angles. The angle has a well developed trabecular meshwork (TM, arrowhead) and open Schlemm's Canal (SC). (**b**) A large anterior synechia (arrow) has occluded the TM and SC in a 14 month old mouse. As shown in **c** (and see Table 1), the formation of synechiae is followed by IOP elevation. Two standard deviations (2 SD) above the mean IOP for 7-10 month mice is indicated by the dashed line (females) and a full line (males). Ciliary body atrophy occurs in some mice and its incidence increases with age. Although histologic analysis and IOP measurement were not performed on the same eyes, it seems likely that ciliary body atrophy explains the drop in female IOP at 19 to 21 months. Original magnifications 400X.

older. The excavation in these mice never extended deeper than the INL and was never rated severe. Clinical fundus examinations agreed with histological findings showing obvious, severe optic nerve damage in successfully analyzed old AKXD28 eyes but not in D2 eyes.

In addition to the typical glaucomatous changes mentioned above, an obvious loss of cells in the INL occurred in almost all AKXD28 mice 23 months old and older (Table 1, Figure 3c). In some AKXD28 mice older than 26 months, the disease progressed to affect the entire retina with substantial loss of both INL cells and photoreceptors. This is in contrast to the phenotype in D2 mice, in which INL cell loss sometimes occurred but extensive damage throughout the retina was never observed (Fig. 3f).

Discussion

Identifying genes that modify glaucoma associated phenotypes is likely to provide insights to mechanisms of glaucoma. An approach for identifying modifier genes is to transfer mutations that produce characterized phenotypes into different mouse strains [27,28,29,30,31,32]. When the phenotypic effect of a mutation is modified upon its introduction into a new strain, crosses between the parental strains can be used to identify modifier genes. Also, the phenotypic characteristics of the new mutant strain may make it a valuable new model of the human disease. In this study, we determined the effects of the D2 derived *ipd* and *isa* alleles in the AKXD28 genetic background. We show that AKXD28 mice develop elevated IOP and glaucoma with similarities to the disease observed in D2 mice [11, 12]. However, there are also significant phenotypic differences between the disease in D2 and AKXD28 mice. This suggests that the ge-

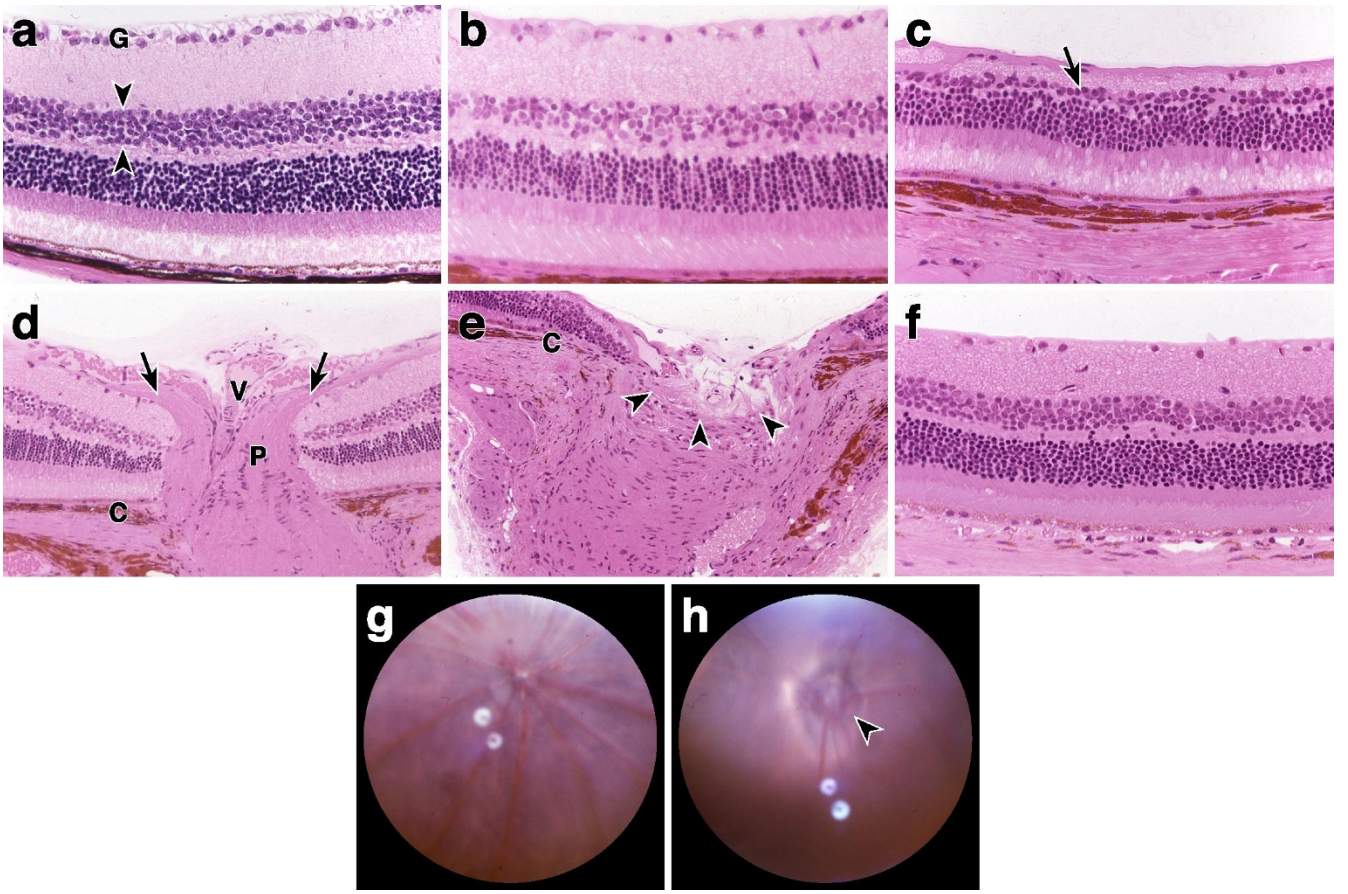

Figure 3
Severe retinal and optic nerve damage in AKXD28 mice. The panels are arranged to display the progressive increase in severity from left to right. All images are from strain AKXD28 mice except for **f**, (D2) and **g,h** (AKXD28B6F1 X AKXD28, backcross N2). (**a**) Young AKXD28 retinas have normal morphology. The retinal ganglion cell layer (G) is continuous and 1-2 cells thick. The inner nuclear layer is approximately 5 to 6 cells thick (flanked by arrowheads). (**b**) Moderately affected AKXD28 retinas contain fewer retinal ganglion cells while the inner nuclear layer has some cell loss but remains relatively normal. (**c**) Severely affected AKXD28 retinas have very few retinal ganglion cells, the inner nuclear layer (arrow) is only 1-2 cells thick, and the total thickness of the retina is greatly reduced. Focal loss of photoreceptors is also present. The image represents the severe phenotype attained by all old AKXD28 eyes; some old eyes have even more cell loss with severe photoreceptor depletion and the remnants of retina are very thin. This severe atrophy does not occur in D2 mice (see **f** for a typical severe D2 retina). (**d**) Normal optic nerve head of a young mouse characterized by a thick nerve fiber layer entering the optic nerve (arrows), a central vessel (V), and well organized pial septae (P). (**e**) Advanced optic nerve excavation (arrowheads) with atrophy extending to a level external to the choroid (C). There is severe peripapillary atrophy with thinning of most retinal layers near the nerve. Although not prominent in this image, gliosis was frequently observed in severely damaged nerves. (**f**) Representative retina from a D2 mouse exhibiting advanced end stage retinal disease typical for that strain. Note that the inner nuclear layer is relatively unaffected and overall retinal thickness is maintained. (**g**) Normal fundus. (**h**) Glaucomatous fundus with an asymmetric and severely excavated optic nerve head (arrowhead). Peripapillary chorioretinal atrophy is also distinctly recognizable in this eye. These fundi are from backcross mice since all old AKXD28 mice had severe cataracts that made photography very difficult. The appearance of these backcross fundi closely resembles those of age matched AKXD28. Original magnifications 400X (**a**,**b**,**c**,**f**,) and 200X (**d**,**e**).

netic constitution of these strains result in differing genetic susceptibilities to glaucoma associated phenotypes and may allow the identification of modifier genes.

An AKR/J modifier(s) likely suppresses IPD in AKXD28

A significant difference between D2 and AKXD28 mice is the lack of an overt IPD phenotype in AKXD28, despite inheritance of the *ipd* allele from D2. In AKXD28, there is no obvious pigment dispersion, the iris pigment epithelium remains remarkably intact, and the irides of old mice remain pigmented. Evidence that the *ipd* containing chromosomal region in AKXD28 is derived from D2 includes inheritance of D2 alleles of microsatellite markers spanning an approximately 14.5 cM chromosomal region surrounding the *ipd* locus (delimited by the markers *Mtv23* and *D6Nds3*) [22, 23]. Though unlikely, the pos-

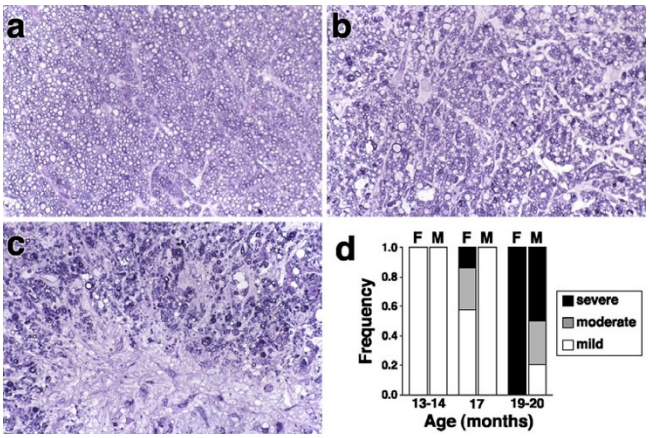

Figure 4
Optic nerve damage in AKXD28 mice. Representative nerve sections scored as (**a**) mild, (**b**) moderate, and (**c**) severe are shown. (**d**) Nerve damage is first evident in females. At 19 to 20 months all females are severely affected whereas approximately half of the males are. Original magnifications 630X.

sibility of an undetected recombinational rearrangement within this region that placed an AKR/J allele of the gene that causes IPD on the AKXD28 chromosome cannot be ruled out. The presence in AKXD28 of the D2 allele of *D6Mit33*, which maps to the same position as *ipd* (25.5 cM), argues against such a rearrangement [12, 21]. Other explanations for the absence of an IPD phenotype in AKXD28 include: inheritance of an AKR/J modifier gene(s) that suppresses the IPD phenotype, a spontaneous reversion of the *ipd* mutation in AKXD28 mice, or the spontaneous occurrence and fixation of the *ipd* mutation in strain D2 after the derivation of the AKXD28 strain. Inheritance of an AKR/J modifier seems the most likely explanation but elucidation of the molecular nature of the *ipd* mutation or the mapping of a modifier gene(s) is necessary to distinguish among the possibilities.

IOP elevation occurs earlier in females

Female AKXD28 mice exhibit increased IOP earlier than males. Nerve damage was also first observed in females. Our clinical and histological studies identified no obvious gender differences in anterior segment pathology that account for this. It must be remembered, however, that our histological analysis accounts for a small portion of the eye and so differences in the prevalence or severity of anterior synechiae may have been missed. Further experiments including more detailed analysis of the aqueous drainage system are necessary to understand why IOP elevation occurs earlier in females than males. Interestingly, D2 females also exhibit increased IOP prior to males [11]. Sex-specific differences in human glaucoma susceptibility have been reported in some, but not all human studies [33,34,35,36,37]. In general, sex-specific differences in human glaucoma may be difficult to identify or interpret because of genetic differences and uncontrolled environmental factors between patients that may alter the phenotype. Since they are inbred and environment can be highly controlled, AKXD28 mice provide an experimental resource for examining the biological significance of gender for susceptibility to events that elevate IOP.

AKXD28 and D2 have different susceptibilities to pressure-induced damage

Overall, the progression of optic nerve and retinal disease in AKXD28 mice supports a pressure-induced etiology. Optic nerve and retinal damage were only observed at ages after high IOP was detected. Following the period of increased IOP, the retinas and optic nerves of aged AKXD28 mice undergo substantially greater damage than those of D2 mice. The damage also occurs more rapidly after IOP elevation is first detected in AKXD28 compared to D2. Strikingly, all old AKXD28 mice have severe retinal ganglion cell loss typically associated with severe optic nerve excavation, whereas, few old D2 mice have severe RGC loss and optic nerve excavation is rarely, if ever severe (defined here as extending to a level out side of the inner nuclear layer). Additionally, following initial RGC loss, most AKXD28 eyes exhibit thinning of the inner nuclear layer, and some mice older than 26 months show severe depletion of all retinal layers. Such severe and extensive retinal damage never occurs in strain D2. The RGC layer is primarily affected in most old D2 mice with the occurrence of mild inner nuclear layer cell loss and possibly, very mild photoreceptor loss in only some mice. In our studies, the occurrence of outer retinal damage was only observed in old AKXD28 eyes with late stage disease, typically several months after signs of glaucoma were evident, and was a late consequence of disease progression. Although RGCs are generally accepted to be the major cell type affected in human glaucoma, and to our knowledge INL and photoreceptor cell loss similar to that in AKXD28 has not been reported, there is evidence for INL and photoreceptor cell involvement in some human and monkey cases [38,39,40,41,42,43].

Both AKXD28 and D2 have similar magnitudes and durations of increased IOP, with the duration appearing to last a little longer in the more mildly affected D2 strain. The IOP distribution of D2 females is significantly elevated from 8 to 12 months of age and AKXD28 females from 15 to 18 months. Although the duration of IOP elevation in AKXD28 males is not yet characterized, female AKXD28 mice are affected more severely than D2 mice of either sex. Thus, the duration and magnitude of IOP elevation does not seem to underlie the more severe

damage in AKXD28. The greater severity and speed of damage following IOP elevation in AKXD28 compared to D2 mice housed in the same environment, therefore, suggests that AKXD28 is more susceptible to pressure-induced cell death. Future experiments involving D2 and AKXD28 may allow identification of genes(s) that modulate susceptibility to pressure-induced damage and that may be relevant for human glaucoma. Identification of genes that modify pressure-induced cell death and characterization of the mechanisms by which they act will broaden the knowledge base from which informed decisions regarding patient counseling, screening, and treatment are made.

Systems that may alter susceptibility to pressure-induced damage

Known characteristics of the AKR/J and D2 strains offer few clues to AKXD28's increased susceptibility to pressure-induced damage and all explanatory attempts are speculative. The propensity for handling-induced hyphema and subconjunctival hemorrhage that we observe (see Table 1) in AKXD28 may indicate vascular abnormalities with relatively weak blood vessels and capillary beds. Weakened capillaries may not function optimally in the face of elevated IOP resulting in some degree of retinal ischemia that exacerbates retinal cell death. Retinal ischemia is known to affect cell viability in the INL and photoreceptor layers [44]. Ischemia would likely invoke glutamate induced neurotoxicity [45, 46].

Equally plausible is the possibility of differing neurotoxic responses to glutamate or differences in the ability of AKXD28 and D2 mice to buffer glutamate levels in the face of high IOP. Acting through N-methyl-D-aspartate receptors, glutamate is believed to be a potent initiator of RGC neurotoxicity [26] and, as described here, the vitreous of at least some AKXD28 mice contains elevated glutamate concentrations when IOP is elevated. Studies with human eyes have previously reported an increase in vitreous glutamate levels from approximately 10 μM in non-glaucomatous eyes to 23 μM in glaucomatous eyes [24]. Also, chronic elevation of rat vitreous glutamate levels from approximately 5-12 μM to 26-34 μM has previously been shown to be toxic to RGCs [25, 26]. The higher vitreous glutamate concentrations we observed in 16 month old (29.2 ± 4.7 μM) compared to 14 month old (13.2 ± 1.7 μM) AKXD28 mice would, therefore, be expected to damage the retina. Although these findings are promising and implicate glutamate in retinal neurotoxicity in AKXD28 mice, further experiments are needed to completely characterize vitreous glutamate levels and their relationship to IOP and glaucoma in this strain.

Molecules involved in glutamate metabolism, signaling, or buffering are interesting candidates to alter susceptibility to retinal cell death. An intriguing possibility is that AKXD28 and D2 mice have differing responses to platelet-activating factor (PAF). PAF is a biologically active phospholipid mediator. PAF acts by binding to a unique G protein coupled transmembrane receptor, PTAFR, that is broadly expressed in the eye [47]. Relevant activities of PAF include promoting ocular hypertension and enhancing neural glutamate release [48,49,50]. Interestingly, AKR/J airways have a strain specific hyper-responsive reaction to intravenously injected PAF [51]. A hyper-responsiveness of AKXD28 ocular tissues to PAF or increased activity of the PTAFR receptor mediated signaling pathway could, therefore, contribute to the pronounced retinal damage through potentiating glutamate neurotoxicity.

Conclusions

We have documented the development of a disease involving iris stromal atrophy and glaucoma in aged AKXD28 mice. These results add to our understanding of the natural history of glaucoma in mice and provide a new animal model for studying glaucoma involving increased IOP. Furthermore, careful phenotypic comparisons between the AKXD28 and D2 strains suggests that the AKXD28 genome contains genetic modifiers that suppress pigment dispersion and increase susceptibility to pressure-induced retinal damage. These results provide a basis for future research to identify glaucoma modifier genes and to dissect the molecular mechanisms by which they act.

Materials and methods

Animal husbandry

All experiments were performed in compliance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research. Mice were housed in cages containing white pine bedding and covered with polyester filters. The environment was kept at 21°C with a 14-h light:10-h dark cycle. Mice were fed NIH31 (6% fat) chow *ad libitum*, and their water was acidified to pH 2.8-3.2. All mouse strains used for this study were routinely screened for select pathogens by The Jackson Laboratory's routine surveillance program (see http://www.jax.org for specific pathogens). The mouse strains used in this study were DBA/2J (D2), AKXD-28/Ty (AKXD28), and C57BL/6J (B6). Cohorts of D2 and AKXD28 mice were housed in the same room and examined using identical protocols. The majority of AKXD28 mice were aged and analyzed at the same time as groups of D2 mice reported in a previous study [11]. Some AKXD28 mice were subsequently analyzed and so additional D2 mice were aged and analyzed at the same time. The disease characteristics of the additional D2 mice was the same as previously described [11].

Clinical examinations

The anterior chambers of male and female AKXD28 mice 2-27 months of age were examined with a slit-lamp biomicroscope (Haag-Streit, Mason, OH). Observed clinical phenotypes were recorded at the time of examination and rated as normal, mild, moderate, or severe based on the following criteria. Iris stromal atrophy (ISA) involves a loss of complex iris structure due to loss of iris stroma. It was ranked as mild (minor loss of iris complexity), moderate (notable lack of iris complexity and focal thinning of iris), or severe (iris lacking complex morphology and is thin, frequently with focal full thickness holes). Transillumination (TRAN) refers to light passed through the pupil and reflected off the back of the eye and through depigmented regions of the iris. TRAN was ranked as peripheral (transillumination limited to peripheral iris), mild (individual specks of transillumination across iris), moderate (individual specks and focal areas of mass transillumination), or severe (majority of iris transilluminates easily, frequently through holes). Dispersed pigment (DP) refers to clinically visible accumulations of pigment or pigment laden cells on anterior segment surfaces, most frequently observed on the lens or iris. Dispersed pigment is part of the iris pigment dispersion phenotype (IPD) that is caused by the D2 *ipd* allele. Fundus examination was performed with pupils dilated by a drop of 1% atropine sulfate and an indirect ophthalmoscope (American Optical, Rochester, NY) with a 60- or 90-diopter lens. Our method of fundus photography has been previously reported [52].

Ocular histologic processing

Enucleated eyes from AKXD28 mice 2-28 months old were fixed for plastic sectioning (0.8% paraformaldehyde and 1.2% glutaraldehyde in either 0.1 M phosphate buffer, pH 7.2, or 0.1 M cacodylate buffer, pH 7.2) or for paraffin sectioning (3.2% formaldehyde, 0.7 M acetic acid, 61% ethanol) as previously described [11, 12]. Serial sagittal sections passing through the optic nerve were collected, stained with hematoxylin and eosin, and analyzed for pathologic changes. To control for variation with position, various phenotypes were only assessed at specific ocular locations in high quality sections [11]. Thus, the number of eyes successfully analyzed differs with the phenotype assessed. Optic nerve cupping was only judged from sections containing central optic nerve. Optic nerve atrophy, retinal ganglion cell death, and inner nuclear layer cell loss were detectable in central sections and sections containing optic nerve that were slightly off center. Ocular lesions were rated as normal, mild, moderate, or severe using previously published criteria [11]. Since cell counts were not performed, inner nuclear layer cell loss was only rated as affected when obvious cell loss was evident (typically at least 2 to 3 cell layers missing). One D2 eye examined histologically was excluded from the study because of the complicating presence of a large corneal ulcer accompanied by severe intraocular inflammation; this eye had a very severe retinal phenotype and severe optic nerve damage.

Optic nerve histologic processing

Samples were processed as previously described [11]. After initial fixation for 24-48 hours, optic nerves were carefully dissected, postfixed with 1% osmium tetroxide, and subsequently processed by standard procedures. A few nerves were recovered from eyes previously embedded in Historesin (Leica, Heidelberg, Germany). Cross sections were cut at a thickness of 0.5-1.0 μm, stained with Toluidine Blue O, and analyzed for pathologic changes. Using this technique, the axoplasm of degenerating axons appears dark [53]. The number of females (F) and males (M) analyzed at each age (in months) were: F13-14, n=3; M13-14, n=13; F17, n=7; M17, n=5; F19-20, n=12; M19-20, n=10. The extent of nerve damage was independently rated by 3 investigators (MGA, RSS, SWMJ) who were unaware of the animals age and of each other's classification of the tissue. A single rating for each sample was then determined by consensus, although commonly the determination was unanimous. Based on nerve morphology, damage was rated using a broad scale consisting of mild, moderate, and severe categories. Nerves estimated to contain 0-25 dark staining axons per section and limited or no gliosis were rated as mild. Normal nerves with no damage were included in this category. Nerves estimated to contain greater than 25 dark staining axons per section but still maintaining a significant portion of live axons were rated as moderate. Nerves rated as severe contained numerous damaged axons throughout the nerve, had grossly disrupted nerve morphology and sometimes contained few, if any, healthy axons. Gliosis was present in some moderately and severely damaged nerves.

Intraocular pressure measurement

IOPs were measured using a microneedle system as previously described [11, 54]. The IOPs of B6 mice are consistent over time and so these animals were interspersed with experimental mice to ensure that calibration had not drifted and that the system was functioning optimally. The number of AKXD28 females (F) and males (M) successfully analyzed at each age were: F7-10, n=19; M7-10, n=8; F11-14, n=31; M11-14, n=21; F15-18, n=15; M15-18, n=12; F19-21, n=12; M19-21, n=10. All measurements were taken during the light-on phase of the recording room. IOP values are given as mean ± standard error of the mean. Reported P values are from multifactorial analysis of variance.

Measurement of glutamate levels

Aged mice were euthanized and eyes removed. Vitreous was collected by inserting a pulled glass microcapillary needle inserted approximately midway between the limbus and optic nerve, and gently pressed posteriorly into the vitreous at an angle avoiding the lens and retina. This technique cleanly collected the more liquified vitreous. Pilot experiments demonstrated that it is extremely difficult to harvest the more gelatinous vitreous that is intricately associated with the retina without retinal contamination. Vitreous was collected under a dissecting microscope and any samples containing blood or other visible debris were discarded. Collected vitreous was immediately transferred to dry ice and stored at -80°C until processed. Depending on the amount of vitreous collected per eye, samples from 2-4 eyes were pooled to obtain a total volume of 10-15 µl of vitreous. Sample pools for female (F) and male (M) mice were collected from mice 14 and 16 months old; the number of eyes contributing to each sample pool was: F14, n=3; M14, n=3; F16, n=2; M16, n=4. Each sample pool was diluted to a final volume of 70 µl with cold Beckman Li-S buffer (96.8% H_2O, 1% lithium citrate, 1% thiodiglycol, 0.7% hydrogen chloride, 0.5% benzoic acid, pH 2.2), transferred to an Amicon 30 microconcentrator (Amicon Inc., Beverly, MA), and centrifuged at 16060 × *g* for 60 minutes at 4°C. The concentrator was rinsed with 25 µl of Li-S buffer and centrifuged for 30 minutes. The combined filtrates were brought to a total volume of 90 µl with the same buffer.

The filtrates were divided into two and analyzed in duplicate on a Beckman 6300 Amino Acid Analyzer fitted with a 10 cm lithium column and the Beckman System Gold data collection and analysis system (Beckman Instruments Inc., Palo Alto, CA). Values for each filtrate were determined from the mean of the duplicate analyses and adjusted for the original volume of vitreous in each pool. The separation of glutamate and glutamine was achieved using Beckman's Li-A buffer (98% H_2O, 1% lithium citrate, 0.5% lithium chloride, 0.5% hydrogen chloride, pH 3.0). Standard curves validating the method and ensuring accuracy over the experimental range of concentrations measured were generated using 5 samples containing known quantities of glutamate (50-1000 pmoles) and glutamine (100-3200 pmoles). Values for glutamate levels at each age are reported as mean of the female and male sample pools ± the standard error of the mean.

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