

# Dependency of Intraocular Pressure Elevation and Glaucomatous Changes in DBA/2J and DBA/2J-Rj Mice

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**PURPOSE.** In this study parameters relevant for glaucoma in DBA/2J (D2J) mice were compared with those in age-matched DBA/2J-Rj (D2Rj) mice, to challenge the postulated role of D2J mice as a model for secondary high-tension glaucoma.

**METHODS.** Genotyping for three known short nucleotide polymorphisms (SNPs) in the *Tyrp1* gene and the *Gpnmb* gene by MALDI-TOF-MS and immunohistochemical staining for Gpnmb was performed in D2J and D2Rj mice. Twelve C57Bl/6 (B6), 8 D2Rj, and 11 D2J mice between 1 and 4 months of age were screened qualitatively and quantitatively for morphologic differences within the anterior eye segment. The IOP progression of 25 D2Rj and 18 D2J mice were investigated between 4 to 10.5 months after birth. At the end of this study, in 10 randomly selected individuals of each D2J and D2Rj cohort, correlation of IOP progression and optic nerve damage were determined in each eye.

**RESULTS.** D2J and D2Rj strains were homozygous for both *Tyrp1* amino acid substitutions, so far only described in D2J mice. The *Gpnmb*<sup>R150X</sup> point mutation present in D2J mice was not detected in D2Rj. Accordingly, immunoreactivity (IR) for Gpnmb was present only in D2Rj and B6 eyes, but not in D2J. Compared with B6, both DBA/2 mice (D2) showed a significantly narrowed chamber angle caused by an anteriorly displaced ciliary body. IOP measurements showed an average IOP of approximately 14 mm Hg between age 4 and 7 months in D2Rj, which decreased to approximately 11 mm Hg in the period from 8 to 10.5 months. In D2J the average IOP showed a steady increase in the observed period from 4 to 10.5 months (from 8.65 to 15.58 mm Hg). Individuals with IOP peaks up to 30 mm Hg were detected in D2Rj, but none of these mice showed signs of an optic neuropathy after 10.5 months. In contrast, 30% of the investigated D2J mice at the age of 10.5 months showed a severe optic neuropathy. Individual data analyses, however, showed no significant correlation between elevated IOP and glaucomatous changes within the D2J population.

**CONCLUSIONS.** Individual correlations of IOP course with axon loss in the single eyes confirmed that in D2J mice, hypertension is not the only causative factor in glaucomatous optic neuropathy. For further investigations on the pathogenesis of

glaucoma in D2J mice, the D2Rj strain without a *Gpnmb*<sup>R150X</sup> mutation and without glaucomatous changes, but with individual IOP elevation, can be used as an interstrain control for D2J. (*Invest Ophthalmol Vis Sci.* 2008;49:613–621) DOI:10.1167/iov.07-0745

The D2J mouse is commonly used as a model of secondary angle-closure glaucoma accompanied by an age-dependent increase of IOP.<sup>1–4</sup> D2J mice show severe changes in eye phenotype, including iris stroma atrophy, pigment dispersion, and glaucomatous changes with age.<sup>5,6</sup> Iris stroma atrophy in D2J is caused by two homozygous amino acid substitutions in the *Tyrp1*<sup>b</sup> gene (*Tyrp1*<sup>R326H</sup>, *Tyrp1*<sup>C110Y</sup>). Iris pigment dispersion is caused by a homozygous point mutation in the *Gpnmb* gene, introducing a stop codon at codon 150 (*Gpnmb*<sup>R150X</sup>), leading to a truncated protein.<sup>7,8</sup> It has been postulated that pigment dispersion leads to a partial occlusion of the aqueous humor drainage pathways, followed by an increase of IOP and subsequent glaucomatous changes in the retina and the optic nerve.<sup>9,10</sup> Introduction of the above-mentioned mutations, present in the D2J and presumed to be responsible for the development of glaucoma, into the genetic background of B6 mice led to similar iris pigment dispersions and stroma atrophies in these animals.<sup>2</sup> Despite this similarity, the IOP elevation in most of the B6 double-congenic mice, however, occurred significantly later than in D2J mice. Notably, no glaucomatous changes were found in the examined B6 mutants, indicating that the homozygous *Gpnmb* and *Tyrp1* mutations alone are sufficient to cause IOP elevation but not for the development of glaucomatous changes.<sup>2</sup> These conclusions were corroborated by findings describing slight inflammatory changes in the irides<sup>9</sup> and modulated expressions of immune relevant cytokines in D2J compared with control mice,<sup>11,12</sup> suggesting an involvement of the immune system in the onset of this disease.

Recent studies have demonstrated that within the retinas of D2J mice, RGCs are almost exclusively affected. In addition, data about dimension and distribution of RGC degeneration and optic nerve damage were obtained.<sup>1,13–16</sup> Subsequently, different investigations concentrated on the effects of a subset of antiglaucoma compounds on the survival of ganglion cells in this model.<sup>17,18</sup> In several studies, it was pointed out that there is a correlation between IOP elevation and glaucomatous damage in D2J mice as a whole.<sup>1,3,10,19</sup> Unfortunately, in most approaches of the past, an individual screening of longitudinal IOP progression and corresponding glaucomatous changes in D2J eyes was not performed.

Recent studies referred to a variety of D2 substrains with different pheno- and genotypes.<sup>10</sup> In the recent past, new mouse strains with *Gpnmb* and/or *Tyrp1* genes of normal function and with a D2J genetic background were introduced as strain-matched control animals without IOP elevation and glaucoma.<sup>20</sup>

In the early 1980s, D2J mice from Jackson Laboratories (Bar Harbor, ME) were transferred to the Central Breeding Center for Laboratory Animals (Hannover, Germany). Then in 1988, several animals at F143 were again transferred to the Janvier

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TABLE 1. Primers Used for Primary PCR Amplification

Gene	Primer Name	Primer Sequence
<i>Tyrp1</i>	Tyrp1-PCR-110-for	5'-CCA GAC CTG CTC CCT TGC-3'
<i>Tyrp1</i>	Tyrp1-PCR-110-rev	5'-CTG TGA GAA TTT TCT GGT TGC-3'
<i>Tyrp1</i>	Tyrp1-PCR-326-for	5'-GCA CTG AGG GTG GAC-3'
<i>Tyrp1</i>	Tyrp1-PCR-326-rev	5'-TCC ACT GTA TTT CGA AAA-3'
<i>Gpnmb</i>	Gpnmb-PCR-150-for	5'-ATT TGG GAC TGA CCT CTG-3'
<i>Gpnmb</i>	Gpnmb-PC-150-rev	5'-CCA AGT GTG TGA AAG ACG-3'

Breeding Center (Le Genest St. Isle, France) and bred there for laboratory use as D2Rj. Whereas D2J mice display the morphologic changes just described, D2Rj mice as an interstrain counterpart, show a superficially unaffected eye morphology.

In this study, we investigated the geno- and phenotype of D2J and D2Rj mice in comparison to B6 controls to contrast possible predispositions for IOP elevation in D2 strains. Furthermore, we correlated individual IOP progressions from 4 to 10.5 months of age with corresponding glaucomatous optic nerve degenerations in eyes of two cohorts of D2J and D2Rj mice.

## MATERIALS AND METHODS

### Mice

All experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were housed in cages containing white pine bedding, covered with attachable nonwoven polyester filters, and maintained in a 12-hour light-dark cycle with standard rodent chow available ad libitum. D2J and B6 mice were initially obtained from Jackson Laboratories; D2Rj mice were delivered from Janvier Breeding Center. All animals were bred in a specific pathogen-free facility at the Institute of Anatomy 2, University of Erlangen-Nuremberg.

### PCR Amplification of Fragments of *Tyrp1* and *Gpnmb* Genes

Sequences were obtained from the gene data bank at the National Center for Biotechnology Information (NCBI) for *Tyrp1* (NM\_031202.2) and *Gpnmb* (NM\_053110.2). Selected fragments of the both genes of interest were amplified in a standard 50- $\mu$ L PCR reaction. One hundred nanograms of total genomic DNA was incubated with 12 picomoles of primers (Table 1), 200  $\mu$ M dNTPs, and PCR buffer containing  $MgCl_2$  and 2.5 U *Taq* DNA polymerase for 30 cycles (3 minutes 95°C initial, 30 seconds 95°C, 30 seconds 57°C, 1 minute 72°C). The PCR products were separated on an agarose gel and visualized under UV light.

### SNP Genotyping Using MALDI-TOF-MS

SNP genotyping was performed as described elsewhere.<sup>21</sup> Briefly, aliquots of the PCR products obtained in the primary PCR reaction were purified by using magnetic beads (genopure ds) as specified by the supplier (Bruker Daltonik, Bremen, Germany). Purified PCR DNA was used for the primer-extension reactions after addition of 10  $\mu$ L extension mix, containing 12 picomoles extension primer, 1 U thermostable DNA polymerase, reaction buffer, and 2 nanomoles of the appropriate dNTPs and ddNTPs (for primer sequences and NTP composition, see Table 2). Extension reactions were performed for 45 cycles (2 minutes 95°C initial, 30 seconds 95°C, 30 seconds 57°C, and 3 minutes 72°C). To confirm the MALDI-TOF-MS-based genotyping, samples without DNA were used as the negative control and samples with known genotypes as the positive control. Primer extension products were purified with magnetic beads (genopure oligo; Bruker Daltonik) and the aliquots spotted onto matrix crystals of 3-hydroxyisobutyric acid on a chip device (Anchor Target; Bruker Daltonik) and air dried. Mass determinations were performed on a MALDI-TOF mass spectrometer (Autoflex; Bruker Daltonik) equipped with a nitrogen laser ( $\lambda = 377$  nm) and delayed extraction. Laser desorbed positive ions were evaluated after acceleration by 20 kV in the linear mode. External calibration was performed with a standard oligonucleotide mixture. Generally 30 individual spectra were averaged to produce a mass spectrum. Genotypes were determined by analyzing the signals observed in the spectra (see Table 2 for expected masses according to SNP).

### Immunohistochemistry

To confirm the biochemical results, we performed immunohistochemical staining of D2J, D2Rj, and B6 eyes at 3 months of age. We used an affinity-purified mouse osteoactivin/Gpnmb/DC-HIL antibody, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Immunoreactivity was visualized on paraffin-embedded sections. Ten- to 20- $\mu$ m sagittal sections through the eye bulbs were placed on poly-L-lysine-coated slides and initially incubated with dry milk solution (Blotto; Santa Cruz Biotechnology, Heidelberg, Germany) at room temperature for 30 minutes, to reduce nonspecific background staining. Incubation with the primary antibody was performed in a moist chamber for 12 to 36 hours at room temperature. The sections were rinsed in Tris-buffered saline (TBS) three times for 10 minutes each and

TABLE 2. Primers Used for Extension Reactions and Expected Masses According to Allele

SNP	Primer-Sequence	NTPs	Expected Masses
<i>Tyrp1</i> <sup>C110Y</sup> = G 328 A	Tyrp-Ext-110-for 5'-CTC AGG ACA CAA CT-3'	dG, ddT, ddA	Primer: 4216Da A-allele: 4513Da G-allele: 4833Da
<i>Tyrp1</i> <sup>R326H</sup> = G 976 A	Tyrp-Ext-326-for 5'-GAC CAG CAG TGC AGC-3'	dG, ddT, ddA	Primer: 4583Da A-allele: 4880Da G-allele: 5225Da
<i>Gpnmb</i> <sup>R150X</sup> = C 447 T	Gpnmb-Ext-150-for 5'-GAA GAT GGC ACC AGC-3'	dT, ddG, ddC	Primer: 4607Da C-allele: 4880Da T-allele: 5224Da

SNP, short nucleotide polymorphism.

then incubated for 1 hour with the appropriate secondary antibody (Alexa 488; 1:400; Invitrogen-Molecular Probes, Eugene, OR). After they were rinsed in phosphate-buffered saline (PBS), the sections were mounted in Kaiser's glycerin jelly (KGaA; Merck, Darmstadt, Germany). The sections were viewed either with a fluorescence microscope (DMR; Leica Microsystems GmbH, Wetzlar, Germany) or with a confocal laser scanning microscope (LSM 5 Pascal; Carl Zeiss Meditec, GmbH, Jena, Germany).

### Tonometer Measurements of IOP

For noninvasive IOP measurements, we used a rebound tonometer, kindly provided by Thomas W. Mittag (Department of Ophthalmology, Mount Sinai School of Medicine, New York, NY). The calibration procedure of the tonometer is described elsewhere.<sup>22</sup> The impact-rebound tonometer (Tonopen; Medtronic, Jacksonville, FL) was developed and tested specially for IOP measurement in murine eyes.<sup>22-24</sup> Therefore, accurate measurements are possible, because of the small sensory probes of approximately 1 mm in size. Animals were restrained in a custom-made device that allows IOP measurements without causing an increase in intrathoracic pressure. This method made it possible to gain IOP data without anesthetizing the animals, and thus any effects of anesthetics on IOP could be excluded in advance. To assess valid data, we took five measurements from each eye and averaged them.

It has been demonstrated that D2J mice have high IOP from 9 months on.<sup>10</sup> To exclude age-dependent degeneration and detect glaucoma-relevant IOPs, we chose a study duration of 4 to 10.5 months. IOP measurements were performed on the two eyes of 18 D2J and 25 D2Rj awake, nonsedated female mice. The females were chosen because it has been shown that female D2J mice tend to develop elevated IOP earlier than males and to maintain IOP elevation longer.<sup>10</sup>

During the first months of this study, we included IOP measurements of 15 B6 mice at the age of 4 months to validate the tonometer measurement and to gain a control distribution of IOPs at this age. To obtain IOPs, the eyes were measured weekly on the same day at a definite time, beginning at 10 AM. Therefore, all data were collected during daylight. After first detectable IOP elevations in D2J eyes at 7 months, further IOP measurements in both D2 cohorts were performed once in a fortnight up to 10.5 months. IOP measurements were temporarily interrupted at the age of 8 to 9 months, because of technical difficulties with the experimental setup.

### Ocular Morphology and Optic Nerve Analysis

Mice were killed with a sublethal exposure to CO<sub>2</sub>, followed by cervical dislocation. To investigate the anterior eye morphology, we placed enucleated eye bulbs of 12 B6, 8 D2Rj, and 11 D2J mice from 1 to 4 months of age in Ito's fixative (2.5% glutaraldehyde, 2.5% paraformaldehyde, and 0.01% picric acid in 0.1 mM cacodylate buffer) and embedded in Epon. Semithin sagittal sections of the eye globe were obtained from each block and stained with toluidine blue.

To assess optic nerve damage, we investigated morphologically 18 D2J and 25 D2Rj female mice at 10.5 months of age. Optic nerves of each eye were fixed in Ito's solution and embedded in Epon. Semithin cross sections were stained with toluidine blue. To determine glaucomatous changes in the optic nerves of both D2 strains, we quantified the number of axons in optic nerve cross sections of 2 B6, 14 D2J, and 8 D2Rj eyes at the ages of 2 to 7 months and 2 B6, 5 D2J, and 4 D2Rj eyes at the ages of 8 to 12 months. We counted each axon in the myelinated portion of the nerve identified within a 1000  $\mu\text{m}^2$  area. To determine the whole number of optic nerve axons, we defined five squares in the superior, inferior, temporal, nasal, and central optic nerve region and estimated subsequently the numbers of axons for the whole optic nerve. This time-consuming quantitative optic nerve analyses revealed that at the age of 10.5 months, mice either showed obvious glaucomatous optic neuropathy or the optic nerve was almost unaffected. Therefore, we decided subsequently to classify the optic nerve states (ONS) into two groups: ONS 1 nerve sections showed no or only a few damaged axons, whereas ONS 2 nerve sections were

defined by a loss of axons of more than 50% and by a clearly increased glial cell activation.

To study corneal differences, corneal thickness measurements of the investigated D2J and D2Rj eyes at 10.5 months of age were performed. One central and two peripheral and vertical measurements (from the outer corneal epithelium to the inner endothelial cell layer) were obtained from sagittal cross sections. To obtain comparable data, only those eyes were evaluated that displayed an open iris and the optic nerve head.

All sections were viewed in a light microscope (Aristoplan; Ernst Leitz GmbH, Wetzlar, Germany), and quantitative and qualitative measurements were performed with scientific analysis software (QWin; Leica Microsystems GmbH). All photography was performed with a digital camera (DC500 camera; Leica).

## RESULTS

### Genotyping and Immunohistochemistry of D2Rj and D2J

To detect genetic differences between both D2 strains, we genotyped the mice with MALDI-TOF-MS. We checked for three known SNPs in the *Typr1* and the *Gpnmb* gene.<sup>5,8</sup> Both strains were homozygous for the *Typr1*<sup>R326H</sup> and for the *Typr1*<sup>C110Y</sup> mutation. D2Rj mice did not have the homozygous *Gpnmb*<sup>R150X</sup> point mutation present in D2J mice (Figs. 1A-C). These results were confirmed by  $\alpha$ -Gpnmb immunostaining on sagittal sections of 3-month-old D2Rj, B6, and D2J mouse eyes (Figs. 1D-F). The D2Rj eyes (Fig. 1D) showed a normal Gpnmb-IR within the pigmented epithelia layers and the melanocytes of the iris, identical with the B6 eyes (Fig. 1E). In the D2J eyes, no signal was detected (Fig. 1F). Thus, the D2Rj mice showed a normal iris phenotype without iris pigment dispersion. Both D2 strains, however, showed a deterioration of the iris stroma, when compared with the B6 control (Figs. 1D-F; arrows).

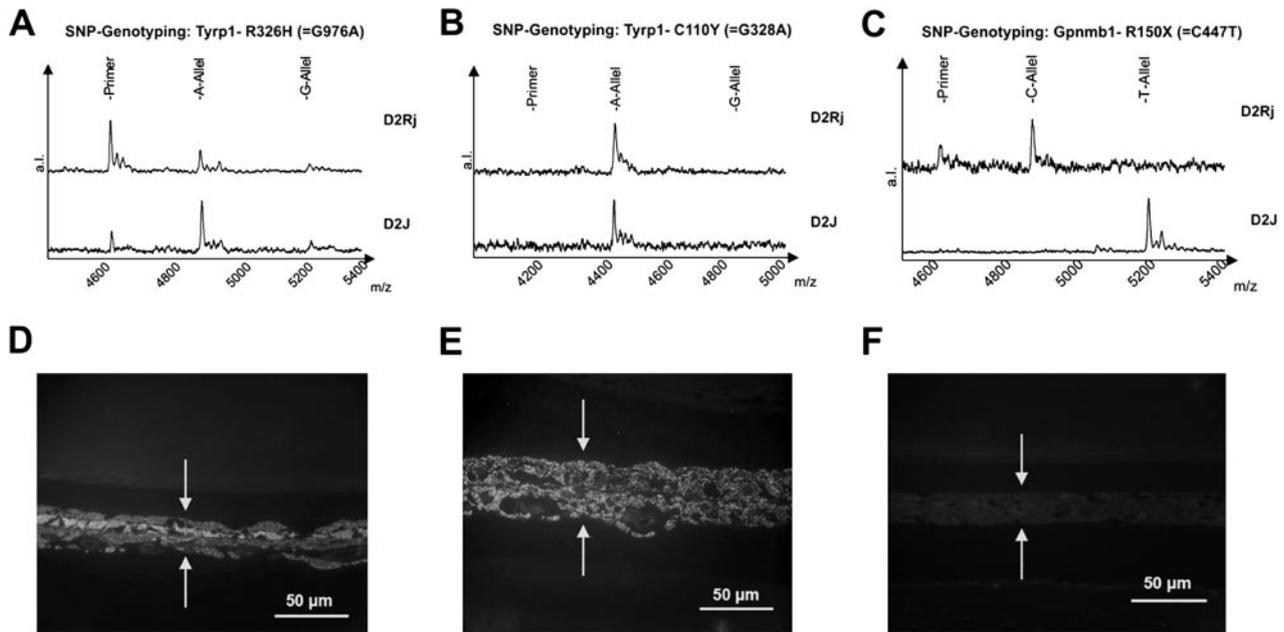
### Longitudinal IOP Measurements

Corneal calcifications, which can be observed in D2J mice with increasing age,<sup>6</sup> were not a limiting factor for valid IOP measurements in this study. Because of the chosen study duration of up to 10.5 months, we found only mild changes in two corneas of 36 in the D2J mice and no corneal changes in the D2Rj mice. Longitudinal IOP progression studies were performed in the two eyes of 18 D2J and 25 D2Rj females between the ages of 4 and 10.5 months. At 4 months, we additionally measured IOP in 15 B6 mice as a control distribution. IOPs of all eyes at the stage of 4, 6, 7, and 10.5 months are given in Figure 2. At 7 months, we found a significant IOP elevation in 25% of D2J mice. The average IOP increased from 7.73 mm Hg at age 4 to 12.74 mm Hg. At this age, some individual IOPs for the first time exceeded 21 mm Hg, which is commonly regarded as suspected glaucoma.<sup>2,25</sup> At 10.5 months the average IOP in the D2J eyes was further increased up to 15.58 mm Hg (Fig. 2).

In the D2Rj eyes we found the highest IOP average with 16.08 mm Hg as early as 4 months, caused by some very high individual IOPs of more than 30 mm Hg in this cohort. The average IOPs decreased in the D2Rj eyes with age to 10.87 mm Hg at 10.5 months (Fig. 2).

### Individual IOP Progression Analysis

For both D2 cohorts, IOP data sheets of each eye were plotted as a function of age in the range from 4 to 10.5 months. The slope of the linear regressions through these data yielded individual IOP progression (Fig. 3). The IOP progressions were summarized for D2J (Table 3) and for D2Rj (Table 4). We



**FIGURE 1.** SNP-genotyping for *Tyrp1*<sup>R326H</sup> (A), *Tyrp1*<sup>C110Y</sup> (B), and *Gpnmb*<sup>R150X</sup> (C) mutation in D2Rj and D2J mice. D2 strains were homozygous for both *Tyrp1*<sup>1b</sup> mutations. The *Gpnmb*<sup>R150X</sup> mutation was detected only in D2J mice. Anti-Gpnmb immunostaining at irides of the D2Rj (D), B6 (E), and D2J (F) mice at the age of 3 months. The D2Rj and B6 samples were positive for the wild-type protein, and the D2J specimens were not IR. Arrows: deterioration and thinning of the iris stroma in both D2 strains due to *Tyrp1*<sup>C110Y</sup> mutation in comparison to the B6 control.

defined an IOP as steady if the slope of the according regression line was between 0.4 and -0.4. Slopes higher than 0.4 were defined as progressively increasing IOP and slopes of -0.4 and lower as progressively decreasing IOP, for each eye.

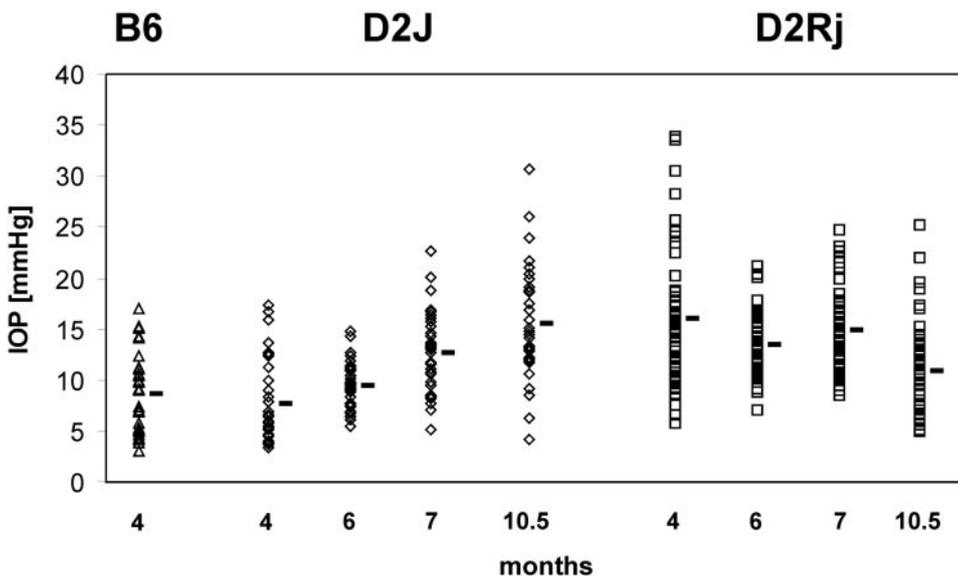
In the D2J cohort, only 42% of the investigated eyes showed an increasing IOP progression, whereas 58% revealed a steady IOP from 4 to 10.5 months of age (Table 3). In D2Rj mice, 60% of the eyes expressed a steady IOP course and 40% a decreasing IOP progression. In none of the eyes was progressively increasing IOP observed (Table 4).

**Correlation of IOP Progression and Glaucoma in D2J and D2Rj**

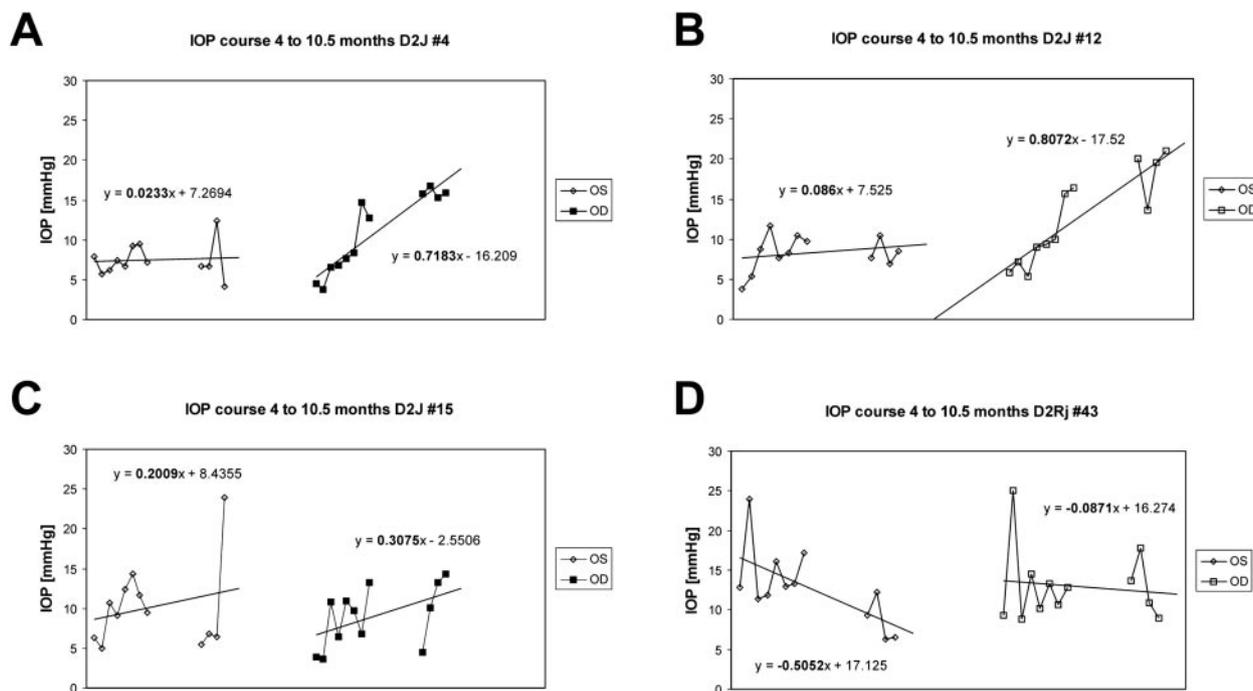
Individual optic nerve degeneration of B6, D2J, and D2Rj mice at different ages was classified in two stages: ONS 1 and -2, which was based on the density and number of optic nerve

axons (Fig. 4). To correlate the individual IOP progression with glaucomatous changes in the corresponding optic nerves, we investigated 10 individuals each in the D2J and D2Rj cohorts (Fig. 5). These 10 mice from each D2 cohort were randomly selected to reflect the IOP progression of each cohort from 4 to 10.5 months.

In the investigated D2J individuals we found in 50% of the eyes an elevated IOP progression up to 10.5 months, together with an ONS 2 (Fig. 5A; mice 4/OD, 5/OS, 7/OS/OD, and 9/OD). The other 50% of eyes with an elevated IOP progression in the D2J mice showed no degenerative changes in the optic nerve (Fig. 5A; mice 1/OS, 5/OD, 11/OD, 12/OD, and 13/OS). Moreover, we found in two D2J eyes a steady IOP course in the observed period in combination with a severe ONS 2 (Fig. 5A; mice 9/OS and 15/OD). These results demonstrate no stringent association between elevated IOP and



**FIGURE 2.** IOP measurements in all eyes in both the D2J and D2Rj cohorts at 4, 6, 7, and 10.5 months of age. IOPs of the B6 eyes were measured only at 4 months, to validate the noninvasive IOP measurement with the rebound tonometer and to gain a control distribution of IOPs at the beginning of the IOP study. Dashes: the average IOP in each group at the corresponding age.



**FIGURE 3.** Example data sheets of IOP progression from 4 to 10.5 months in the left and right eyes from individuals 4 (A), 12 (B), and 15 (C) of the D2J cohort and individual 43 of the D2Rj group (D). Slopes of the regression line of IOP progression in each mouse are shown. *Open symbols:* unaffected optic nerve morphology (ONS 1). *Filled symbols:* glaucomatous damage within the corresponding optic nerve (ONS 2). IOP data of D2J mouse 4 showed a steady IOP course and ONS 1 in the left eye, whereas increased IOP progression in the right eye led to severe optic neuropathy (A). In contrast to this, the right eye IOP course of mouse 12 also showed an increased progression, but no glaucomatous changes (B, ONS 1 in both optic nerves). Even in D2J mice with a steady IOP course up to 10.5 months, severe ONS 2 was observed in one eye (C, OD), whereas the other eye remained unaffected (C, OS). In none of the investigated D2Rj eyes were glaucomatous changes found, although IOP peaks over 21 mmHg were detectable at early ages (D, ONS 1 in both optic nerves).

the development of an optic neuropathy in the eyes of D2J mice.

In the D2Rj mice we observed a large interindividual variability of IOPs between 4 and 10.5 months (Fig. 2). None of these eyes showed signs of damage to the optic nerve (Fig. 5B).

**Anterior Eye Morphology**

To evaluate whether there are morphologic predispositions in D2Rj mice that could explain the early IOP peaks in some of these animals, we conducted a morphologic investigation of the semithin sections of the eye globes of 12 B6, 8 D2Rj, and 11 D2J mice between the ages of 1 and 4 months. In comparison to the B6 control (Fig. 6A) the ciliary body was anteriorly displaced in the D2Rj (Fig. 6B) and the D2J mice (Fig. 6C). Quantitatively, we observed that the distance between the anterior end of Schlemm’s canal and the deepest point of the chamber angle at the transition zone between the pectinate ligament and iris root (sulcus of the chamber angle) was shorter or even negative compared with those in the B6 mice (Fig. 6D).

After 10.5 months, we measured the corneal thickness of all investigated D2J and D2Rj eyes in this study to evaluate inter-strain differences in the eye tissue. Neither the central, nor the peripheral thicknesses showed significant differences in both

D2 mouse strains, although the central corneal average appeared higher in the D2J than in the D2Rj corneae (Fig. 7).

**DISCUSSION**

D2J mice are still regarded as a model of hypertension glaucoma with pigment dispersion.<sup>6,9</sup> Point mutations affecting the *Gpnmb* and *Tyrp1* genes have been described to be causative of a severe iris phenotype with pigment dispersion, stromal atrophy, synechia between iris and cornea/lens, and slight inflammatory processes in D2J eyes. This phenotype leads to increased IOP, which in turn induces glaucomatous damage in the retina and optic nerve.<sup>5,8</sup> In this study, we showed by genotyping and immunohistochemical analyses of D2Rj and D2J mice that no *Gpnmb* mutation and therefore no pigment dispersion or iris synechia phenotype are present in D2Rj animals. Because of the *Tyrp1* mutation detectable in both D2 strains, the phenotype of iris stroma atrophy was found in the D2Rj and in D2J mice.

We compared general IOP progression in D2J and D2Rj mice. Our findings showed that in the D2J mice with gene mutations in *Gpnmb* and *Tyrp1*, a steadily increasing average IOP was detectable within the study range from 4 to 10.5 months. At 4 months of age, the individual IOPs achieved in

**TABLE 3.** IOP Progression Slopes of D2J Eyes

	Mouse Number																		
	1	4	5	7	9	11	12	13	14	15	16	17	18	19	20	21	23	24	
OS	0.42	0.02	0.47	0.45	0.33	0.36	0.09	0.60	0.34	0.16	0.06	0.37	0.07	-0.06	0.14	0.59	-0.20	0.31	
OD	0.10	0.72	0.71	0.58	0.93	0.66	0.81	0.36	0.29	0.31	0.60	0.80	0.16	0.40	0.40	0.57	0.47	0.23	

TABLE 4. IOP Progression Slopes of Left and Right D2Rj Eyes

	D2Rj Mouse										D2Rj Mouse														
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
OS	-0.06	0.01	-0.44	0.05	0.08	-0.18	-0.59	-0.36	-0.22	-0.84	-0.39	-0.34	-1.12	OS	-0.02	-0.33	-0.51	-0.39	-0.51	-0.65	-0.24	-0.28	-0.86	-0.87	-0.49
OD	-0.19	-0.10	-0.11	-0.19	0.20	0.12	-0.24	-0.38	-0.20	-0.57	-0.01	-0.09	-0.30	OD	-0.78	-0.44	-0.46	-0.40	-0.60	-0.59	-0.52	-0.34	-0.58	-0.44	-0.50

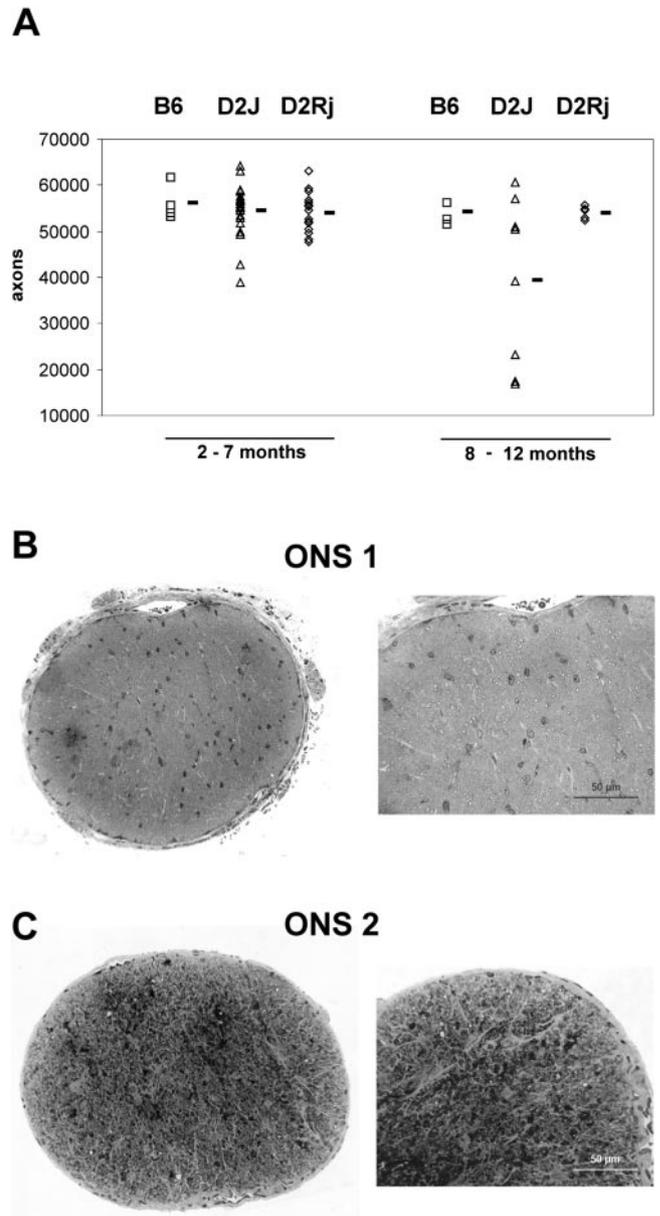
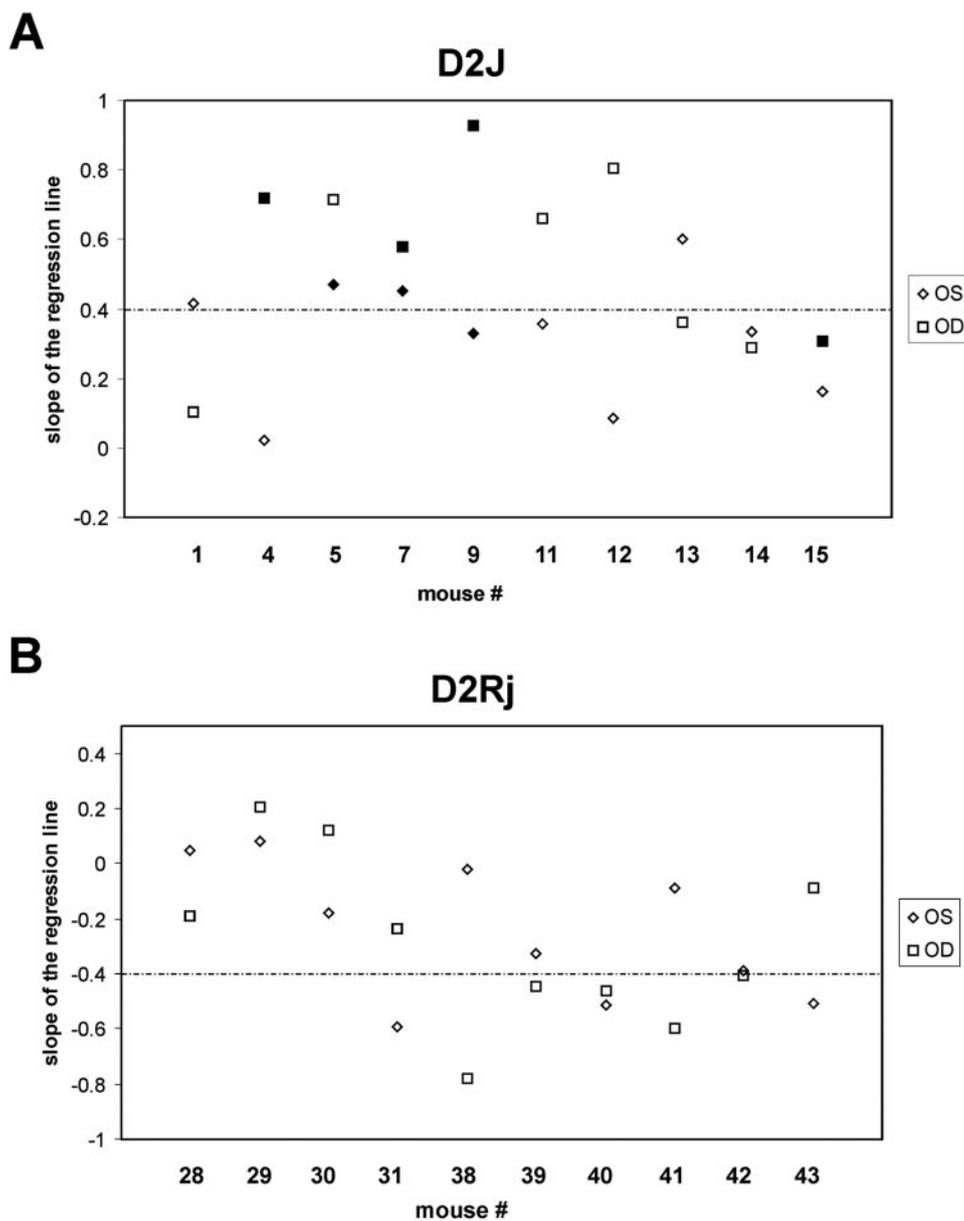


FIGURE 4. (A) Quantitative comparison of axon counts in the optic nerves of 4 B6, 23 D2J, and 15 D2Rj optic nerve sections of animals of ages 2 to 7 months and 4 B6, 9 D2J, and 7 D2Rj optic nerve sections of animals of ages 8 to 12 months. In older B6 and D2Rj animals, no differences in the number of axons were observed, compared with the data of younger animals in these strains. In older D2J animals, a significant decrease in the number of axons was found in approximately 50% of the optic nerves of the older age group, if compared with the data of D2J mice in the group from 2 to 7 months. *Dasbes*: average number of axons in each strain. Optic nerve sections of D2J mice with nearly unaffected morphology (B; 10 months; 62,482 axons in toto) and severe optic neuropathy (C; 11 months; 17,488 axons in toto). Based on this quantitative approach we classified the optic nerve states (ONS) 1 and 2, respectively.

the D2J eyes were in the same range as in the B6 control group at this time. In the D2Rj animals, very high individual IOPs, higher than the glaucoma-relevant 21 mm Hg, were already detectable at 4 months of age. Considered in total, the D2Rj cohort showed a decreasing IOP course with age.

Since the synergistic action of both mutations in *Gpnmb* and *Tyrp1* has been pointed out to be necessary for IOP elevation in D2J and B6 mice,<sup>2,9</sup> the same cannot be true of



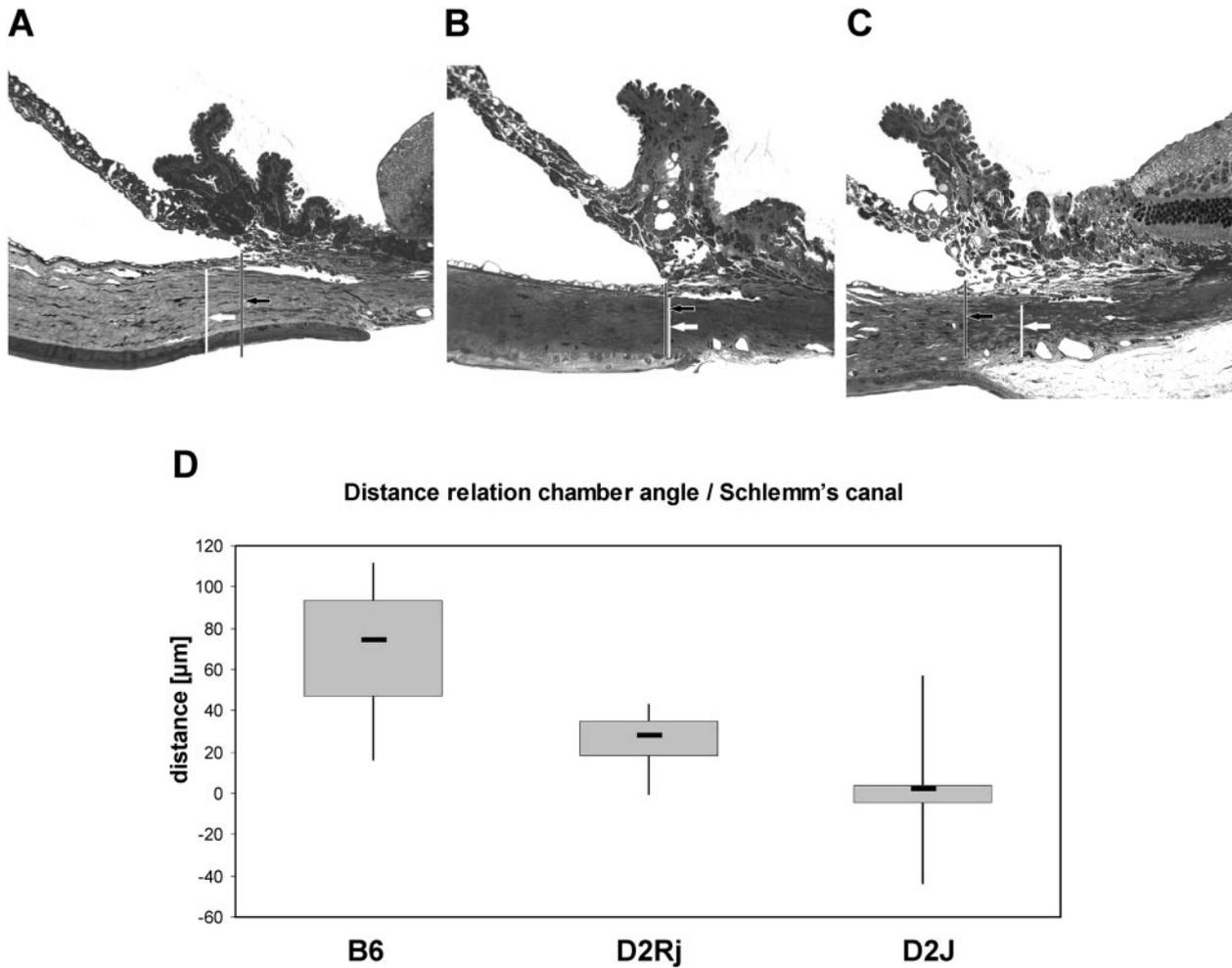
**FIGURE 5.** Individual IOP progression slopes from 4 to 10.5 months of age and the optic nerve degeneration states in 10 representatively selected D2J (**A**) and D2Rj (**B**) mice. IOP with a slope over 0.4 was determined to be progressing quickly and IOP with a slope less than  $-0.4$ , more slowly. *Filled symbols:* glaucomatous optic nerve damage (ONS 2). *Open symbols:* eyes with unaffected optic nerves (ONS 1).

D2Rj mice. In this strain without the *Gpnmb* mutation, additional factors must be causative for elevated IOPs. The examination of the anterior eye morphology of B6 and both D2 mouse strains provided evidence for a common feature in D2 eyes in contrast to the B6 control eyes. The D2J and D2Rj eyes showed a significantly anteriorly displaced ciliary muscle and therefore a decreased distance between the chamber angle and the anterior border of Schlemm's canal. This morphologic peculiarity may resemble a prerequisite for temporary angle closures resulting in large IOPs. It is tempting to speculate that the steady increase in the average IOP in the D2J cohort, may be the result of the present pigment dispersion phenotype in these animals which could contribute to a higher frequency of spontaneous angle closure and finally toward a chronic situation. Considered under these aspects, D2J mice may indeed serve as a hypertension model with an initial acute angle-closure syndrome transforming to a chronic angle closure over time. In contrast, D2Rj animals do not pass beyond the acute stage because of the absence of pigment dispersion. Latest studies showed a relation of elevated IOP and corneal thickness in D2J mice.<sup>3</sup> We cannot confirm such a relationship,

however. The central corneal thickness was even smaller in the D2Rj cornea compared with the D2J, although the average IOP in the D2Rj mice at 4 months of age was nearly twice as high as in the D2J.

As only 42% of the investigated D2J mice showed a constant increase in IOP between 4 and 10.5 months of age, we searched for a correlation between IOP increase and axon loss in the corresponding optic nerves as a sign of glaucoma. Recent investigations on the relationship between axon loss and elevated IOP in D2J mice demonstrated an increase in IOP with age in most but not all D2J animals.<sup>3</sup> It was found that there was a correlation between different IOPs and differences in axonal density in age-matched D2J mice. Furthermore, young D2J mice with elevated IOPs showed an axon loss similar to that found in older animals with similar IOP elevation.

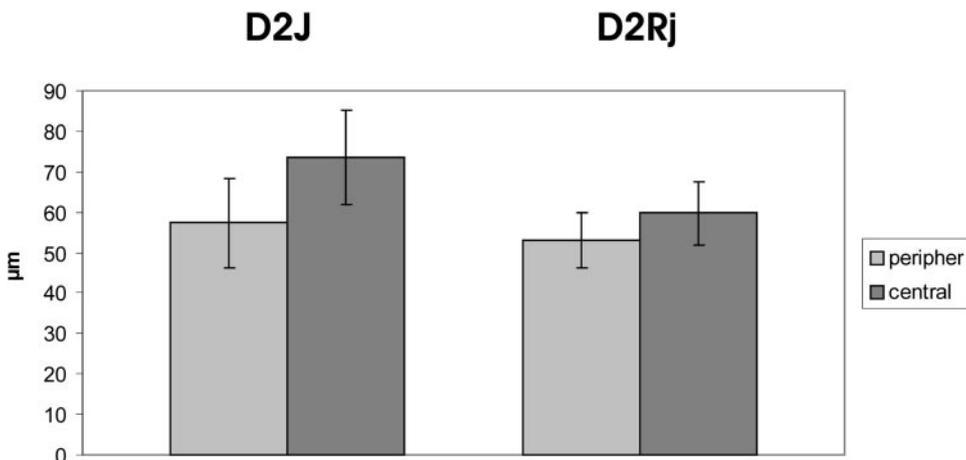
In this study, we found that in D2J eyes with elevated IOP progression up to 10.5 months, only half of the cases were accompanied by severe optic nerve degeneration. Even within the same individual, one eye with IOP increase was detected, whereas the other eye remained unaffected. In agreement with



**FIGURE 6.** Sagittal sections of anterior eye segments of 4-month-old B6 (A), D2Rj (B), and D2J (C) mice. In both D2 strains a predisposition of an anteriorly displaced ciliary body was observed. *White arrows*: the anterior beginning of Schlemm's canal; *black arrows*: sulcus of the chamber angle. (D) The box-and-whiskers plot shows the average distance from the anterior beginning of Schlemm's canal to the chamber angle in the examined B6 versus D2Rj and D2J. *Dashes*: the median for each strain.

this finding, D2J mice older than 12 months with no detectable glaucomatous changes have recently been described.<sup>10</sup> However, those authors assumed that a lack or insufficient increase in IOP was responsible for their findings. Our results do not support this conclusion, because we also found D2J individuals with elevated IOP progression, but without optic nerve

changes up to 10.5 months of age. Moreover, we also found D2J individuals with severe glaucomatous optic nerve changes, but without an elevated IOP course up to 10.5 months of age. These results indicate that IOP elevation cannot be regarded as a general prerequisite for the development of glaucoma in D2J mice. This was confirmed by the findings of no detectable



**FIGURE 7.** Corneal thickness evaluation of the D2J and D2Rj eyes at 10.5 months. Although the peripheral and central corneal thicknesses in the D2Rj eyes were lower than in the D2J eyes, no significant differences between the strains were found.

glaucomatous damage in the D2Rj optic nerves. Corroborating results were also found in a new mouse strain with a *Gpnmb* gene of normal function and with a D2J genetic background (D2-*Gpnmb*<sup>+</sup> mice).<sup>20</sup> In these animals, similar to D2Rj mice, no glaucomatous changes were found. As opposed to the D2Rj mice, D2-*Gpnmb*<sup>+</sup> mice did not show IOP elevation. Therefore, the D2Rj mice can be used as an additional interstrain control for D2J mice with IOP elevation but without glaucomatous changes within the optic nerve.

In previous studies it has been described that not only melanosomes but also dendritic cells express GPNMB. The expression of a mutated nonfunctional GPNMB protein in these cells, however, may influence a proper immune response to the progressing inflammatory changes in D2J eyes.<sup>9</sup> Furthermore, it has been demonstrated that high-dose  $\gamma$ -irradiation together with syngeneic bone marrow reconstitution results in an almost complete protection against glaucoma in D2J mice.<sup>26</sup> Even if ocular immune responses are not universally changed by this treatment, as is argued by Anderson et al.,<sup>26</sup> it is obvious that in consequence of this treatment, a lack of mature T-cell population can be expected. It is tempting to speculate that this indeed could contribute to changes in autoimmunity signaling, which is an important mediator of protection against various neurodegenerative stress situations.<sup>27,28</sup> The latest microarray analysis of D2J retinas from animals 3 and 8 months of age revealed differences in gene expression patterns. Although the authors correlated their data only with IOP elevation, the retinas of older D2J mice displayed several upregulated genes that are associated with immune response and glial signaling.<sup>29</sup> Findings of upregulated complement component 1Q expression in glaucomatous D2J retinas, which preceded the extensive retinal ganglion cell death in these animals, suggests an additional important role of complement activation in this model.<sup>12</sup> Our present studies support the hypothesis that in the genetic background of D2J mice with the synergistic effect of *Gpnmb* and *Tyrp1* mutations, additional and still unknown factor(s) besides a negligible effect of IOP elevation as a prerequisite for glaucomatous damage have to be present to cause glaucomatous damage in these mice.

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