Iris transillumination defect and its gene modulators do not correlate with intraocular pressure in the BXD family of mice

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Purpose: Intraocular pressure (IOP) is currently the only treatable phenotype associated with primary open angle glaucoma (POAG). Our group has developed the BXD murine panel for identifying genetic modulators of the various endophenotypes of glaucoma, including pigment dispersion, IOP, and retinal ganglion cell (RGC) death. The BXD family consists of the inbred progeny of crosses between the C57BL/6J (B6) strain and the glaucoma-prone DBA/2J (D2) strain that has mutations in Tyrp1 and Gpnmb. The role of these genes in the iris transillumination defect (TID) has been well documented; however, their possible roles in modulating IOP during glaucoma onset and progression are yet not well understood.

Methods: We used the IOP data sets and the Eye M430v2 (Sep08) RMA Database available on GeneNetwork to determine whether mutations in Tyrp1 and Gpnmb or TIDs have a direct role in the elevation of IOP in the BXD family. We also determined whether TIDs and IOP are coregulated.

Results: As expected, Tyrp1 and Gpnmb expression levels showed a high degree of correlation with TIDs. However, there was no correlation between the expression of these genes and IOP. Moreover, unlike TIDs, IOP did not map to either the Tyrp1 or Gpnmb locus. Although the Tyrp1 and Gpnmb mutations in BXD strains are a prerequisite for the development of TID, they are not required for or associated with elevated IOP.

Conclusions: Genetic modulators of IOP thus may be independently identified using the full array of BXD mice without concern for the presence of TIDs or mutations in Tyrp1 and/or Gpnmb.

Glaucoma is a complex, multifactorial, polygenetic disease that is the leading cause of irreversible blindness worldwide [1]. Trends indicate that by 2020, as many as 80 million people will have glaucoma worldwide, with as many as 11 million being blinded [2,3]. The various subtypes of glaucoma share the common clinical pathologies of retinal ganglion cell (RGC) damage, optic nerve axonal destruction, and visual field defects [4]. Several risk factors are known for glaucoma [5-7], and elevated intraocular pressure (IOP) is the major risk factor linked to the development and progression of primary open angle glaucoma (POAG) [3,5,8-10]. The elevation in IOP can be traced back to several causative factors, including faulty fluid dynamics of the aqueous humor within the anterior segment ocular structures. Currently, elevated IOP is the only clinical manifestation of glaucoma that can be treated.

Pigment dispersion glaucoma (PG)—a form of secondary open angle glaucoma—is the second most common form of glaucoma in young adults. In this disease, pigment-containing particles slough off the posterior iris and block the drainage of aqueous humor through the trabecular meshwork. In most cases, the resultant IOP increase leads to glaucoma, yet in some cases, the IOP increase does not, indicating that additional factors, including differentially expressed genes and/or genetic heterogeneity, may influence disease progression [11]. DBA/2J (D2) mice have served as the leading model for PG for more than two decades [12-16]. It has been well documented that the pigmented dispersion syndrome in D2 mice is due to recessive mutations in the Gpnmb and Tyrp1b genes [12,16-22] with other pigmentation genes modulating the phenotype [23]. In addition to iris stromal atrophy and pigment dispersion, D2 mice develop elevated IOP, RGC loss, optic nerve damage, and optic disc cup excavation that is age-related and progressive, yet highly variable in severity with some mice having relatively normal pressure and optic nerves [16,18,20,24].

Recombinant inbred (RI) strains of mice are a valuable resource for identifying genetic sources of variation in disease risk and progression, in this case, the various molecular and clinical phenotypes associated with glaucoma onset and severity [21,22]. The largest panel of these strains—the BXD...
family—consists of approximately 150 inbred strains generated from crosses between the wild-type normotensive B6 strain and the PG-prone D2s train. The BXD strains have been used extensively over the past two decades in genetic and genomic studies of the eye and the primary visual system [21,22,25-28].

In the present study, we determine whether an increase in the severity of iris transillumination defects (TIDs) or mutations in Tyrp1 and Gpmmb modulate IOP levels within the BXD family. The current study was motivated by initial observations that several BXD strains carrying wild-type alleles of both Tyrp1 and Gpmmb (WT/WT) had elevated IOP, while others had low IOP. This range of variation in IOP values suggested that mutations in Tyrp1 and Gpmmb are neither necessary nor sufficient to cause IOP elevation.

METHODS
The BXD family of strains: Mice were handled in accordance with the Guide for the Care and Use of Laboratory Animals. Studies involving all mice were approved the Animal Care and Use review board of the AAALAC-accredited University of Tennessee Health Science Center and followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research in addition to the guidelines for laboratory animal experiments (Institute of Laboratory Animal Resources, Public Health Service Policy on Humane Care and Use of Laboratory Animals). Mice were housed as previously reported [23] and were euthanized by cervical dislocation.

The health of all mice was monitored a minimum of twice weekly. Lights were maintained on a 12 h:12 h light-dark lighting cycle with light onset occurring at 6 AM. A total of 3,856 mice were used in this study and were distributed as follows: 3,548 mice from 73 BXD strains (268 for microarray studies and 3280 for phenotyping studies), 226 mice from parental strains (16 for microarray studies and 210 for phenotyping studies). The BXD family—consists of approximately 150 inbred strains generated from crosses between the wild-type normotensive B6 strain and the PG-prone D2s train. The BXD strains have been used extensively over the past two decades in genetic and genomic studies of the eye and the primary visual system [21,22,25-28].

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BXD genotypes database and mapping algorithm: The genotypes that we used have been described previously [23,30,31]. Briefly, the BXD genotype file was created using classical microsatellite marker maps and high-density single nucleotide polymorphism (SNP) genotypes generated using Affymetrix and Mega Mouse Universal Genotyping Arrays (MegaMUGA). The final genotype file consists of approximately 3,795 markers. This genotype file includes all markers, both SNPs and microsatellites, with unique strain distribution patterns (SDPs), as well as pairs of markers for the SDPs represented by two or more markers. The latest smoothed BXD genotype data file can be downloaded by ftp from GeneNetwork [23].

IOP measurements: The IOP of both eyes of all mice were measured using an induction-impact tonometer (Tonolab, Colonial Medical Supply, Franconia, NH) between 9:00 am and 6:00 pm during the light cycle. Mice were lightly anesthesitized with ketamine and xylazine (25 mg/kg and 5 mg/kg; Butler Schein, Dublin, OH) and IOP measurements were taken as previously described [32]. To determine whether IOP has a diurnal rhythm, IOP was measured in both eyes of the B6 mice every 2 h from 8 AM until 6 pm (i.e., 8 am, 10 am, 12 pm, 2 pm, 4 pm and 6 pm) for five consecutive days (n=5 mice). These data are presented as mean±SEM and were analyzed using one-way ANOVAs followed by Tukey’s HSD test for differences between means ( AnalystSoftStatPlus:mac software, Version 5.8.3.8; Walnut, CA). p<0.05 was considered significant.

To determine whether IOP was influenced by sidedness, we measured and recorded individually the IOP values of the left and right eyes of all mice. Previous reports have shown sex differences in measurements of IOP in various mice strains [19,20]. To assess the possibility of such differences in the BXD mice, we analyzed the IOP of each sex separately within each age group. To determine whether the IOP values of the left and right eyes of the mice differed among mice of each age group or if IOP was influenced by sex of the mouse, two-tailed Student’s t-tests were performed on data within each age group ( AnalystSoftStatPlus:mac software, Version 5.8.3.8; Walnut, CA). p<0.05 was considered significant.

TID grading: The grading of the TID phenotype is described in detail in our previously published paper [23]. Briefly, the iris phenotype of each mouse was photodocumented using a slit-lamp biomicroscope using narrow and wide beam illumination. A grade from 0 (lowest) to 4 (highest) was assigned to each mouse based upon the degree of iris transillumination. The specifics of the grading system are presented in the Results section of [23].
Heritability calculation: The IOP values (mmHg) and the TID grades were plotted in separate panels along with their respective heritability values across the five age groups. Heritability of both the IOP and the TID phenotypes were calculated using the formula: 
\[
h^2 = \frac{0.5V_g}{0.5V_g + Ve}
\]
which accounts for genetic and environmental variance. The factor of 0.5 was included to adjust for the twofold increase in additive genetic variance among the inbred strains relative to the outbred populations [21,22].

Stratification of IOP and TID by genotype and Pearson correlation: Because the mean IOP was highest in the 6- to 9-month-old cohort, in subsequent analyses we focused our attention on this age group. To determine the effect of the mutations in \( \text{Tyrp1} \) and \( \text{Gpnmb} \) on the IOP values and TID scores, we stratified the BXD strains based on their genotype. Using GeneNetwork, we plotted a bar graph (by rank) for the IOP and organized the TID graph to match the strain order of the IOP graph to enable comparisons. The strains were further represented using different colors based on their genotypes at \( \text{Tyrp1/Gpnmb} \) (WT/WT = green, WT/MUT = orange, MUT/WT = blue, MUT/MUT = red, and the parents = black). We also plotted the IOP and TID values for the 6- to 9-month-old cohort and evaluated whether the IOP and TID phenotypes segregate based on the BXD genotypes for the genes \( \text{Tyrp1} \) and \( \text{Gpnmb} \).

Simple interval mapping: Simple interval mapping using 5,000 permutation tests was performed [21-23,30] to identify quantitative trait loci (QTLs) that modulate IOP (Record ID 16339, \( n=71 \) strains) and TID (Record ID 12324, \( n=73 \) strains) in mice aged 6-9 months. Logarithm of odds (LOD) values can be obtained by dividing the likelihood ratio statistic (LRS) by 4.61. The IOP simple interval map was examined for the presence of significant or suggestive expression QTLs (eQTLs) at the locations of \( \text{Tyrp1} \) (Chr4 at ~80.5 Mb) and \( \text{Gpnmb} \) (Chr6 at ~49 Mb), as well as any other eQTLs.

RESULTS AND DISCUSSION

IOP in BXD mice is not influenced by time during the light cycle, sidedness, or sex: To determine whether the time of the day has any influence on IOP measurements, we selected B6 mice as the representative strain and measured IOP at six different time points during the light cycle. The IOP values of the B6 mice ranged between 15.1±0.3 and 15.5±0.2 mmHg in each age group and did not differ significantly throughout the day (Figure 1A, \( p>0.05 \)). Other groups have shown a distinct circadian fluctuation of IOP over 24 h that included a dark period [34,35]; however, the present study measured IOP only during normal working hours (8 AM to 6 PM). During that time interval, we detected no statistically significant change in IOP, although there was a trend toward a reduction in IOP during the middle to the latter part of the light cycle, in agreement with published reports [34,35]. It is also possible that the use of anesthesia blunted larger IOP differences that may have been present, as has been described previously [36]. Nonetheless, the study measurements detected no statistically significant IOP variation from 8 AM to 6 PM, and therefore, we pooled the IOP data from the different age groups in each BXD strain into a single data set that was stratified only by age.

Within each age cohort, no significant difference was observed between the right and left eyes in each age group (Figure 1B, \( p>0.05 \)). Likewise, no significant differences were observed between females and males of both eyes within each age group (Figure 1C, \( p>0.05 \)). We have also shown in our previous work that the TID phenotype showed no significant differences between females and males or between right and left eyes [23]. Although there may be individual strains of BXD mice in which the IOP segregates with the genotypes at \( \text{Tyrp1} \) and/or \( \text{Gpnmb} \), on average, there is no association in this genetic reference panel. Although some investigations using D2 mice have demonstrated that there may be sex differences in basal IOP [16], other studies from the same group have illustrated that the data to support a universal discrepancy in IOP based upon sex is inconsistent, especially when working with genetically diverse mice [37]. Consistent with this conclusion, we did not observe a disparity between sexes regarding IOP or TID values in the BXD mice, thus allowing us to average the phenotypic data for each age group.

IOP and TID phenotypes differ in age of peak expression: If IOP and TID are linked by shared genetic determinants, we would expect a similar age of peak expression in both phenotypes. To test this hypothesis, we plotted the IOP and the TID across the five age cohorts in separate panels along with the respective heritability values. The mean IOP values ranged between 14.31±0.36 (the >13-month-old cohort) and 16.456±0.28 mmHg (the 6- to 9-month-old cohort) across the BXD mice (Figure 2A). IOP increased progressively with age until 6-9 months, followed by a reduction in IOP with age (Figure 2A), which is likely due to ciliary body atrophy, as well as the changes in the plasticity and viscoelastic properties of the mouse eye. These findings are similar to those of John and colleagues [20]. In previously published work [23], we demonstrated that the TID phenotype develops in a progressive fashion within this BXD family. We measured a progressive increase in the TID among the five age cohorts, in which the average TID grade within the BXD mice ranged from 0.71±0.10 (the 1- to
Figure 1. Elevated IOP phenotype in BXD mice is independent of circadian rhythms. A: The intraocular pressure (IOP) values in the B6 mice ranged between 15.1±0.3 and 15.5±0.2 mmHg and did not differ significantly between 8 AM and 6 PM (p>0.05; n = 5 mice at each time of day). B: The elevated IOP phenotype in the BXD mice is independent of sidedness. Within each age cohort, no significant difference was observed between the right and left eyes (p>0.05; the n values, superimposed on each bar of the graph, represent the number of mice). C: The elevated IOP phenotype in BXD mice is independent of gender. Within each age group, no significant differences were observed between female and male mice (p>0.05; the n values, superimposed on each bar of the graph, represent the number of mice).
2-month-old cohort) to 1.29±0.15 (the >13-month-old cohort; Figure 2B). The TID average grade of the D2 and B6 parents was significantly different at all ages. The B6 parents from all cohorts presented a grade of 0. In contrast, the D2 parents had defects that averaged a grade of 2.02±0.21 as early as 1–2 months of age and increased to a maximum of 3.45±0.13 in the mice older than 13 months of age [23].

Figure 2 also highlights the heritability of both phenotypes. As seen in Figure 2A, heritability of the IOP phenotype ranged between 28.07% and 39.87%, with the highest values obtained in the oldest cohort. The TID phenotype, however (Figure 2B), ranged between 88.59% and 97.22%, with the highest values obtained in the 10- to 13-month-old cohort. The lower heritability values of the IOP phenotype compared to the TID phenotype also likely explain the more complex
polygenetic nature of IOP as a phenotype compared to the TID phenotype. The lower values also indicate the extent of the influence of genetic and environmental factors on the phenotypic variations in IOP and TID across the BXD mice population used in these studies.

Interestingly, in the BXD mice, the TID grade increased with age similar to what has been observed by various groups for the D2 iris disease [14,16,18,19,38], highlighting the importance of the mutations in Tyrp1 and Gpnmb for the existence of iris disease in the BXD family. It is expected that the mutations would affect the function of the gene product. We have previously demonstrated that mutations in Tyrp1 and Gpnmb alter the functional categories of the transcripts with which the mutations are correlated. Specifically, the wild-type alleles of both genes are correlated with melanin synthesis. In contrast, the mutant alleles of both genes lose many of those...
associations and correlate with transcripts that have little or no functional relationship to pigmentation, including glycoprotein metabolism and amino acid phosphorylation [21,22]. These functional categories have no apparent influence on IOP, and thus, it can be postulated that the genetic regulatory networks of IOP and TIDs are composed of distinct groups of gene products that are active in functions within the eye.

*Tyrp1* and *Gpnmb* genotypes correlate with the TID phenotype but not IOP: To test for possible genetic correlations between the IOP and TID phenotypes, we examined the influence of mutations in *Tyrp1* and *Gpnmb* on the IOP values and TID grades in the 6- to 9-month-old cohort. After the IOP and TID bar graphs were stratified and color-coded based on the genotypes at *Tyrp1* or *Gpnmb*, important differences were observed (Figure 3A,B). The BXD strains carrying wild-type alleles of *Tyrp1* and *Gpnmb* (WT/WT) had IOPs that varied greatly with some as high as 22.5±2.7 mmHg in some strains (e.g., BXD66), while others had IOP values as low as 12.0±1.1 mmHg (e.g., BXD42). If IOP were influenced only by the *Tyrp1* and *Gpnmb* genotype, we would expect to see higher IOP values in strains that have mutations in both *Tyrp1* and *Gpnmb* (Mut/Mut) compared to what was seen in the IOP values of these strains (contrast Figure 3A,B). Similarly, we observed high TID values for many BXD strains carrying mutant versions of *Tyrp1* and wild-type versions of *Gpnmb* (MUT/WT) in the 6- to 9-month-old mice, yet the IOP values did not follow a similar correlation. For example, the BXD21 mice had a high TID value of 2.00, yet the IOP was 13.50 mmHg. Based on these findings, we conclude that the genetic modulators of these two phenotypes are likely separate and distinct. These findings also lend strong support to our hypotheses that neither TID nor *Tyrp1/Gpnmb* correlate with IOP. Moreover, this finding also suggests that other genetic modulators or factors may be responsible for the variation in IOP across all BXD strains that may not have a direct role in modulation of TID.

*TID does not influence IOP in the BXD murine strain stratified by the *Tyrp1/Gpnmb* genotype.* To further understand the relationship between IOP and TIDs, we plotted IOP versus TIDs for each strain in the 6- to 9-month-old cohort and color-coded the strains by genotype (Figure 4). Some strains that are wild-type at *Tyrp1* and *Gpnmb* developed high IOP (about 20 mmHg) but showed negligible TID. In contrast, some strains that are mutant at *Tyrp1* and *Gpnmb* had low IOP values (about 12 mmHg) and high TID grades. However, consistently elevated TID grades were observed only in the BXD strains that harbored the mutant alleles of

In contrast, as we reported previously [23], TIDs are dependent upon mutations in *Tyrp1* and, to a more limited degree, *Gpnmb*. High TID values were observed consistently in the BXD strains carrying mutant alleles of both *Tyrp1* and *Gpnmb* (MUT/MUT), as opposed to what was seen in the IOP values of these strains (contrast Figure 3A,B). Similarly, we observed high TID values for many BXD strains carrying mutant versions of *Tyrp1* and wild-type versions of *Gpnmb* (MUT/WT) in the 6- to 9-month-old mice, yet the IOP values did not follow a similar correlation. For example, the BXD21 mice had a high TID value of 2.00, yet the IOP was 13.50 mmHg. Based on these findings, we conclude that the genetic modulators of these two phenotypes are likely separate and distinct. These findings also lend strong support to our hypotheses that neither TID nor *Tyrp1/Gpnmb* correlate with IOP. Moreover, this finding also suggests that other genetic modulators or factors may be responsible for the variation in IOP across all BXD strains that may not have a direct role in modulation of TID.
Tyrp1 and Gpnmb. Moreover, in strains that are MUT/WT, we observed an inverse relationship, in which higher TID values were present in the strains that had lower IOP values. These results suggest that TIDs do not influence IOP directly, and likely the main genetic modulators Tyrp1 and Gpnmb have no direct roles in modulating IOP in the BXD strains.

Unlike TID, IOP does not map to Tyrp1 or Gpnmb: To further assess the possible relationship of IOP with TIDs, we performed simple interval mapping for IOP and TID for all BXD mice in the 6- to 9-month-old cohort. The interval maps for TIDs and IOP were distinct, and each contained unique peaks. Importantly, the IOP map does not show a statistically suggestive or statistically significant peak at the chromosomal locations of Tyrp1 (Figure 5, left vertical box) or Gpnmb (Figure 5, right vertical box), suggesting that these genes do not directly modulate IOP in BXD mice (Figure 5A). In contrast, the TIDs had a large QTL at the location of Tyrp1 in the 6- to 9-month-old BXD strains (Figure 5B). We had previously reported that TIDs also map to the location of Gpnmb in the older than 13-month-old cohort of BXD mice. These data demonstrate that neither TIDs nor these two genes modulate IOP. Moreover, we have demonstrated conclusively that Tyrp1 and Gpnmb influence the TID phenotype in a significant manner [23].

The role of Tyrp1 and Gpnmb in TIDs is well established in the D2 and BXD strains of mice [13,16,18,23]. Based on the premise that mutations in Tyrp1 and Gpnmb that are present in D2 mice are responsible for the development of glaucoma, Anderson and colleagues introduced the D2-derived Tyrp1b and GpnmbR150X mutations on the widely used B6 background to generate several lines of B6 double-congenic mice [17]. These strains were used in genetic epistasis experiments and compared with D2 regarding clinical indices of iris disease, IOP elevation, and optic nerve damage. The researchers

![Figure 5. TID and IOP interval maps are unique and contain non-overlapping peaks. A: Intraocular pressure (IOP) does not show statistically suggestive or statistically significant peaks at the locations of Tyrp1 (left vertical box) or Gpnmb (right vertical box), suggesting that these genes do not modulate IOP in BXD mice. B: In contrast, the transillumination defect (TID) mapped with a high likelihood ratio statistic (LRS) score to Tyrp1 in the 6- to 9-month-old cohort of the BXD strains.](image-url)
discovered that similar to D2 mice, B6 double-congenic mice develop a severe depigmentation iris disease leading to massive pigment dispersion. To their surprise, in comparison to D2, the B6 double-congenic mice were much less likely to develop increased IOP in response to this pigment dispersing disease. These results indicated that initiation (iris disease) and progression (high IOP) of glaucomatous phenotypes in D2 mice are genetically separable and that additional genetic factors relevant to glaucoma remain to be characterized. These data also suggest that additional important allelic variants specific to the B6 strain may mitigate elevations in IOP. The identification of susceptibility and resistance loci and alleles in the mouse has tremendous potential to enhance our understanding of glaucoma and may help define corresponding loci and genes in human populations. 

Conclusions: We evaluated the relationship of TIDs and IOP and further studied the influence of Tyrp1 and Gpnmb on these traits in the BXD murine panel. We conclude that the elevated IOP phenotype in BXD mice is independent of sidedness and sex. We also observed that high IOP and the most severe TID phenotypes differed in the age of peak expression. The Tyrp1 and Gpnmb gene expression levels correlated with the TID phenotype but not with IOP. Remarkably, the severity of TIDs did not correlate with IOP in any age group. We conclude that the presence of mutations in Tyrp1 and Gpnmb are a prerequisite for TIDs but not for elevated IOP in the BXD family of mice.

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