Retinal Gene Expression Changes Related to IOP Exposure and Axonal Loss in DBA/2J Mice

Lambros Panagis,[1] *Xiujun Zhao,*[2] *Yongchao Ge,*[3] *Lizhen Ren,*[1] *Thomas W. Mittag,*[2,4] *and John Danias*[1,5]

Purpose. To determine the effects of cumulative IOP exposure and axonal damage on retinal gene expression in DBA/2 mice.

Methods. DBA/2J, DBA/2J^{pe} (pearl), and C57BL/6 mice from 3 to 12 months of age were used. IOP was measured with a rebound tonometer, and optic nerve (ON) damage was determined by grading of ON sections. Retinal RNA was subjected to microarray analysis. Comparisons explored the effects of cumulative IOP exposure (cIOPx) as well as ON damage (ONd) in the DBA/2J animals compared with that in the C57BL/6 and pearl mice. RT-PCR was performed to confirm some of the genes and bioinformatic analysis to identify affected gene networks.

Results. Microarrays revealed that an increasing number of genes were up- or downregulated in 9- and 12-month DBA/2J mice with various degrees of ONd. A smaller number of genes were expressed differentially between eyes with different cIOPx at the same age, from 6 months on. Expression of 1385 and 1133 genes differed between DBA/2J animals and C57BL/6 or pearl mice, respectively, and some them were confirmed by RT-PCR. Bioinformatics analysis identified functional gene networks, including members of the complement system, that appeared to be related to cIOPx, ONd, or both.

Conclusions. Gene expression changes occur in retinas of DBA/2 mice with various amounts of cIOPx as well as ONd. Genes involved, code for proteins with diverse cellular functions and include among others the complement system. cIOPx and ONd affect common as well as unique gene sets. (*Invest Ophthalmol Vis Sci.* 2011;52:7807–7816) DOI:10.1167/iovs.10-7063

Glaucoma is one of the main causes of blindness worldwide.[1] Despite the fact that intraocular pressure (IOP) has been unequivocally linked to both the development[2] and progression[3] of the disease, it is still unclear how resistance to outflow of aqueous humor causes the optic neuropathy that leads to blindness. It has been proposed that elevated IOP has direct mechanical or vascular effects on the optic nerve head (ONH)[4] or that it affects retinal physiology in such a way that it ultimately leads to axonal and RGC loss.[5] However, it is hard to prove such effects in humans.

Animal models of glaucoma have been used to study this relationship. In the past decade, mouse models of the disease have been extensively studied. Although mice do not have a formed lamina cribrosa, their ONH has significant similarities to the human ONH.[6] Mouse models offer the potential of using the power of genetics to answer specific questions regarding the pathophysiology of the disease. The spontaneous DBA/2 mouse has emerged as the most widely used model of glaucoma,[7,8] as it develops a well characterized optic neuropathy with elements that strongly mimic the human disease.

Various investigators have attempted to determine how IOP potentially causes changes in the retina or optic nerve in the DBA/2 mice by investigating gene expression changes that occur in these tissues in response to either high IOP or various amounts of RGC loss.[9–14] Some of the characteristics of glaucoma in these animals, however, make studies on DBA/2 mice challenging:

1. DBA/2 mice develop cataracts at approximately the time that the optic neuropathy starts, making in vivo quantitative evaluation of the ONH impossible.
2. Glaucomatous damage (as in humans) is variable, even in this inbred mouse strain. Such damage is at times dramatically different even between pairs of eyes of the same animal. Ultimately, a portion of the eyes (~10% for female animals) maintain a normal RGC and axonal count late into life.
3. Cumulative elevated IOP exposure shows strong association with RGC and axonal loss (as in humans). However, correlation studies (and similar to human glaucoma) have shown that IOP accounts for roughly 50% of the effect observed (JD, unpublished data; see also Refs. 15, 16). Of course, one can argue that IOP measurements are momentary in nature and that this lack of very high correlation results from the necessary extrapolation from a very limited sample. It is also plausible that some other IOP function (e.g., maximal IOP, IOP fluctuation) may be a better predictor of optic neuropathy. To date, however, no such function has been convincingly identified.

Taking these characteristics into account, we attempted to determine the effects of IOP exposure and various amounts of axonal damage on global retinal gene expression in DBA/2 mice. To achieve that, we used microarrays to analyze independently the effect of IOP elevation and degree of axonal damage. Although similar studies have been performed in the past,[10] this is the first time a genetically more appropriate mouse (i.e., a mouse that is closer genetically to the DBA/2J mouse) has been used as the control, to eliminate age-related changes that may confound these comparisons.

From the Departments of [1]Cell Biology and [5]Ophthalmology, SUNY Downstate, Brooklyn, New York; and the Departments of [2]Ophthalmology, [3]Neurology, and [4]Pharmacology, Mount Sinai School of Medicine, New York, New York.

Supported by National Eye Institute Grant R01-EY15224 and Research to Prevent Blindness.

Submitted for publication December 15, 2010; revised March 6 and May 15, 2011; accepted June 5, 2011.

Disclosure: **L. Panagis**, None; **X. Zhao**, None; **Y. Ge**, None; **L. Ren**, None; **T.W. Mittag**, None; **J. Danias**, None

Corresponding author: John Danias, Department of Ophthalmology, Box 5, SUNY Downstate, 450 Clarkson Ave, Brooklyn, NY 11203; john.danias@downstate.edu.

Materials and Methods

Animals

DBA/2J, DBA/2J^{pc}, and C57BL/6 mice ranging from 3 to 18 months of age were used. DBA/2 and C57BL/6 animals were obtained from Jackson Laboratories (Bar Harbor, ME) at the appropriate age and kept at the Mount Sinai School of Medicine animal care unit for the duration of the experiment. DBA2J^{pc} mice were obtained from cryopreserved stock at Jackson Laboratories and were used to establish a colony at the Mount Sinai School of Medicine. Animals from this colony were used after appropriate aging. The mice were kept in a 12-hour light/12-hour dark cycle and fed ad libitum. All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care Committee.

Noninvasive IOP Measurement in Mice

IOP was measured in mice noninvasively on approximately a biweekly basis with a rebound tonometer.[17] The animals were held in a custom-made restraint that does not compress the chest or neck while IOP is measured.[18] IOP measurements were performed after application of 0.5% proparacaine topical anesthesia. Five measurements per eye were obtained and averaged. IOP measurements were performed between 10 AM and 12 PM, to minimize the effect of diurnal IOP variation. To determine the effects of IOP on gene expression, the eyes with the upper and lower quartiles of cumulative IOP exposure in the 6-, 9-, and 12-month-old groups of animals were used for RNA extraction for microarray comparison.

Optic Nerve Damage Assessment

In another set of mice, animals were perfused transcardially with 4% paraformaldehyde (PFA) at the time of euthanization and after enucleation (see below). The cranium was opened, and the brain, together with the optic nerves, was removed and immersed in a mixture of 1.2% PFA/0.8% glutaraldehyde for 24 hours at 4°C. The fixative was then washed off, and the optic nerves were osmicated in 2% osmium tetroxide and embedded in epoxy resin (LR White; Electron Microscopy Sciences, Hatfield, PA). Semithin sections were cut with an ultramicrotome, stained with *p*-phenylenediamine, mounted, and observed under a microscope equipped with a 100× oil-immersion lens. Nerves were graded for the amount of axonal damage semiquantitatively on a three-point scale (none–mild, moderate, or severe) based on the presence of abnormal or disorganized myelin staining by two independent observers. Differences between the two observers were resolved by common review. In cases of disagreement during common review, differences were adjudicated by a third observer. To determine the effects of optic nerve damage on gene expression, retinal RNA from eyes with severe ONH damage were compared with those with none–mild damage in the 9- and 12-month-old animals.

Microarrays

Eyes were removed from animals under deep anesthesia before they were euthanized. The eyes were rinsed in diethyl-pyrocarbonate (DEPC)-treated water and placed in RNA stabilizer (RNAlater; Ambion/Invitrogen, Carlsbad CA). Dissection of the retina was immediately performed in the stabilizer on ice. Retinas were homogenized, and total RNA was extracted (TRIzol reagent; Gibco/Invitrogen, Carlsbad CA). RNA was further purified on RNeasy spin columns (Qiagen, Valencia, CA) and subsequent lithium chloride precipitation. Typical yields were 50 to 80 μg RNA from 16 to 20 mg of tissue (~1–2 μg per retina). RNA concentrations were determined with a spectrophotometer (Nanodrop; Thermo Scientific, Wilmington, DE) and the 260/280-nm absorbance ratio calculated to determine RNA purity. Before array hybridization RNA quality was verified using agarose gel electrophoresis and bioanalysis (Bioanalyzer; Agilent Technologies, Santa Clara, CA). Linear amplification, cRNA synthesis, and hybridization to mouse oligonucleotide arrays (GeneChip mouse genome 430_2.0; Affymetrix, Santa Clara, CA) was performed at the Mount Sinai core microarray facility. Signal intensities for approximately 45,000 probe sets (each probe set consists of 11 perfect matches and 11 mismatches) were obtained from each array. Data quality was verified by inspection of various plots of the probe signal intensities, such as histograms, box plots, and RNA degradation plots, as well as different Affymetrix quality metrics such as the percentage of probe sets that are called "present" by the MAS 5.0 algorithm,[19] the average background, the scale factor, and the GAPDH and β-actin 3′:5′ ratios. Arrays failing quality control were replaced with additional arrays.

Probe intensities were processed using the GeneChip Robust Multiarray Average (gcrma)[20] package of Bioconductor.[21] The gcrma package adjusts for background intensities in Affymetrix microarray array data, which include optical noise and nonspecific binding, and converts background-adjusted probe intensities to expression measures after normalization. The summarized data are thus normalized signal intensities that are logarithm base 2 ($\log_2$) transformed. These summarized data were subjected to statistical analysis, as described below.

Statistical and Bioinformatics Analysis

To correlate gene expression changes with the degree of ON damage (and account for age), microarray assays were set up as in Table 1.

Similarly, to correlate gene expression, changes with the degree of exposure to IOP (and account for age), microarray assays were set up as in Table 2.

Several statistical analyses were performed for both of these two datasets:

1. We identified genes that are differentially expressed at 9 months between DBA/2 mice with different amounts of optic nerve damage (none–mild versus severe) and between DBA/2 mice with different (low versus high) IOP exposure. Similarly, we identified genes that are differentially expressed between the same groups at 12 months of age. We also identified genes differentially expressed at 6 months of age in eyes with different (high versus low) IOP exposure. The computation is performed by using the Bioconductor Limma package.[22] For gene j, the comparison is based on a moderated t-statistic, defined to be $(\bar{x}_j - \bar{y}_j)/\sqrt{\tilde{s}_j^2(1/n_1 + 1/n_2)}$. Here, $\bar{x}_j$, $\bar{y}_j$ are, respectively, the average $\log_2$ signal intensities from the gcrma package for the two groups, and $\tilde{s}_j^2$ is the posterior value for the residual SD for the gene j. The empiric Bayes method in the Limma package[22] gives

Table 1. Groups Studied for Gene Expression in Relationship to Degree of ON Damage

Strain	3 Months	6 Months	9 Months	12 Months
C57BL/6	No damage (3)	No damage (3)	No damage (3)	No damage (3)
Pearl	No damage (3)	No damage (3)	No damage (3)	No damage (3)
DBA	None–mild damage (3)	None–mild damage (3)	None–mild damage (3)	None–mild damage (3)
				Moderate damage (3)
			Severe damage (3)	Severe damage (3)

The number in parentheses is the number of mouse eyes analyzed.

TABLE 2. Groups Studied for Gene Expression in Relationship to IOP Exposure

Strain	3 Months	6 Months	9 Months	12 Months
C57BL/6	Normal IOP (3)	Normal IOP (3)	Normal IOP (3)	Normal IOP (3)
Pearl	Normal IOP (4)	Normal IOP (3)	Normal IOP (3)	Normal IOP (3)
DBA	Normal IOP (3)	Low exposure to elevated IOP (3)	Low exposure to elevated IOP (3)	Low exposure to elevated IOP (3)
		High exposure to elevated IOP (3)	High exposure to elevated IOP (3)	High exposure to elevated IOP (3)

Number in parentheses is the number of mouse eyes analyzed.

$\tilde{s}_j^2 = (d_0 s_0^2 + d_j s_j^2)/(d_0 + d_j)$, where the prior d_0, s_0^2 are estimated from the data of all genes. The moderated *t*-statistic has the advantage that it does not become extremely large just due to an extremely small sample residual variance s_j^2. The genes are selected by the following criteria: The *P* value associated with the moderated *t*-statistic is no greater than 0.05, the maximum group average $\log_2$ signal intensity is no less than 5 and the $\log_2$ signal change $(\bar{x} - \bar{y})$ is no less than 0.5 in absolute value.

2. We identified genes that are associated with optic neuropathy by comparing gene expression in DBA/2 animals with that in the control strains (C57BL/6 and pearl). A two-way ANOVA model with mouse strain and age as factors was used in the Bioconductor Limma package. Gene selection was performed based on the following criteria:
   a. The maximal group average $\log_2$ signal intensity is no less than 5.
   b. The P values in comparing pearl and C57BL/6 mice (the two "control" strains) at 3, 6, 9, and 12 months should be no more than 0.05 and the corresponding $\log_2$-fold change should be less than 2, as we expect both mouse strains to have gene expression that is similarly changing (if at all) as a result of age only.
   c. The *P* values associated with moderated *t*-statistics in comparing the Pearl and DBA/2 mice at both 3 and 6 months should be no more than 0.05, and the corresponding $\log_2$-fold change should be less than 2, as we expect DBA/2 mice before the onset of axonal loss to have gene expression similar to that of pearl mice.
   d. The background subtracted $\log_2$-fold change (defined as the maximum of $\log_2$-fold changes at 9 and 12 months of DBA/2 severe optic neuropathy relative to Pearl, subtracted by the average $\log_2$-fold at the comparison in [c]) should be at least 0.5, and its corresponding *P* value for the moderated *t*-statistic should be less than 0.01 and the false-discovery rate no larger than 0.05.

For the genes that are associated with IOP exposure, the comparison in (c) is only on 3-month-old mice, and the computation in (d) considers the maximum of the $\log_2$-fold changes at 6, 9, and 12 months of DBA/2 high IOP exposures relative to pearl after the subtraction of $\log_2$-fold at the 3 months between DBA/2 and pearl.

Genes selected using these procedures were subjected to bioinformatics analysis using pathway analysis software (Ingenuity Systems, Redwood City, CA) to determine functional gene networks and canonical groups whose expression is differentially affected. To find out whether the lists of genes that are differentially expressed in the process of neurodegeneration and prolonged IOP elevation share some specific transcription factors through activation or repression, we used the *transcription factor targets* (tft) *dataset* in MSigDB ver 3.0 from the Broad Institute (www.broadinstitute.org, Cambridge, MA) and performed hypergeometric tests to identify transcription factors that are significantly overrepresented in the two gene lists.

Real-Time Polymerase Chain Reaction

cDNA was synthesized using random primers and a reverse transcription kit (Quantitect; Qiagen), per the manufacturer's instructions. cDNA was subjected to RT-PCR (QuantiTect SYBR Green RT-PCR Kit; Qiagen) with specific primer sets (SuperArray Biosciences, Frederick MD). The samples were analyzed at the Mount Sinai School of Medicine RT-PCR core facility. The *rps11* gene (encoding for a ribosomal protein) was used as an internal control for each sample for normalization purposes.

RESULTS

Cumulative IOP exposure was used to determine the effects of IOP on gene expression, as shown in Figure 1. To improve statistical comparisons, only eyes in the upper and lower quartiles of IOP exposure of the 6-, 9- and 12-month-old animals were used for microarray analysis to study the effect of IOP. Cumulative IOP exposure was significantly different between the high and low IOP exposure groups at all three time points ($P < 0.005$, 0.0009, and 0.006 for 6, 9, and 12 months, respectively; *t*-test).

ON damage was minimal in the 3- and 6-month-old animals (Fig. 2). Despite the fact that the damage was graded on a three-point scale the majority (25/28) of the eyes of 9-month-old animals showed either none–mild or severe damage. Thus only these two comparison groups were used in the microarray experiments. At the 12-month age point there were enough eyes with moderate ON damage to allow for inclusion of that group in the analysis as a separate group (Fig. 2).

Gene Expression Correlated with ON Damage in DBA/2 Mice

In 9-month-old animals with severe ON damage, 240 genes were detected as upregulated and 395 as downregulated when compared with that in similarly aged animals with none–mild ON damage. For 12-month-old animals, the respective numbers were 321 genes and 756 genes. The 10 genes with the largest change in expression (in both directions) are presented in Tables 3 and 4 for the 9- and 12-month-old animals, respectively (full lists are available in the Supplementary Data, http://

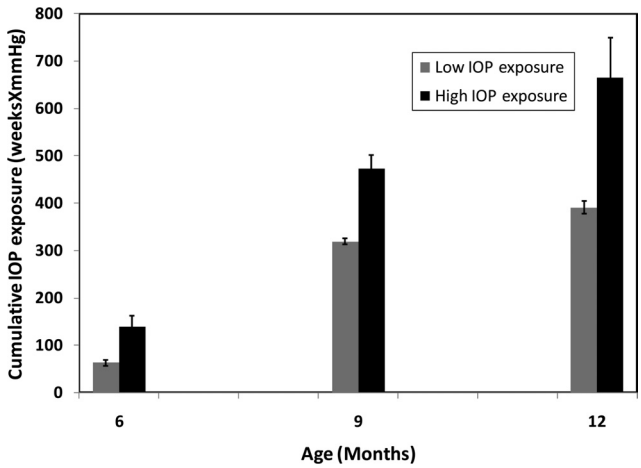

FIGURE 1. Cumulative IOP exposure of the DBA/2 eyes used in microarray analysis (three eyes per group). These eyes represent eyes in the higher and lower quartiles of the cumulative IOP distribution at each age group. All eyes included in the analysis were from different animals.

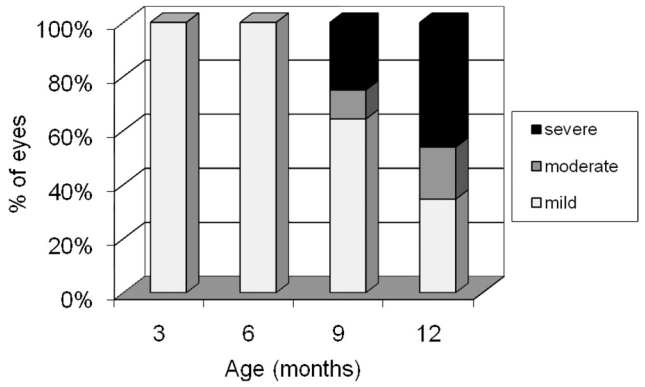

FIGURE 2. Amount of ON damage in DBA/2 animals studied in the various age groups. Three eyes in each group were selected for microarray analysis. All eyes included in the analysis were from different animals. When contralateral eyes had different degrees of ON damage, only the most severely affected eye from that animal was included in the analysis.

www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-7063/-/DCSupplemental).

Gene Expression Correlated with Cumulative IOP Exposure in DBA/2 Mice

Similarly, in 9-month-old animals with high IOP exposure, 144 genes were detected as upregulated and 58 as downregulated when compared with animals with low IOP exposure. For 12-month-old animals, the respective numbers were 26 and 39 genes. The 10 genes with the largest change in expression (in both directions) are presented in Tables 5 and 6 for the 9- and 12-month-old animals, respectively (full lists are available in the Supplementary Data, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-7063/-/DCSupplemental).

TABLE 4. Top 10 Up- and Top 10 Downregulated Genes in 12-Month-Old DBA/2J Retinas with Severe Axonal ON Damage Compared with Retinas from Similarly Aged Animals with None–mild ON Damage

ID*	Log2 Difference	*P*	Public ID	Gene Symbol
Upregulated Genes				
1457483_at	2.584272	0.004575	BM214036	*Pdgfc*
1441829_s_at	2.450934	0.040125	BB314595	—
1442709_at	2.351295	0.043117	BF147024	*Cebpd*
1441326_at	2.207975	0.028057	AV381752	—
1457666_s_at	2.141777	0.039087	AV229143	—
1444722_at	2.088037	0.037627	BG075584	*Ifi202b*
1459957_at	2.083325	0.019254	BG073884	*Cp*
1423233_at	1.964315	0.022318	BB831146	—
1439220_at	1.808861	0.02837	BB306426	*Akap10*
1449351_s_at	1.771766	0.017837	NM_019971	—
Downregulated Genes				
1428393_at	−6.46918	1.85E-05	AK003046	*Nrn1*
1428379_at	−6.07293	5.13E-06	BQ180367	*Slc17a6*
1418610_at	−5.7995	3.43E-05	NM_080853	*Slc17a6*
1437588_at	−5.2749	0.00016	BB547375	*Pou4f2*
1436520_at	−4.99152	0.000862	BB378317	*Ahnak2*
1416286_at	−4.95001	0.000379	NM_009062	*Rgs4*
1417788_at	−4.75056	5.21E-05	NM_011430	*Sncg*
1422520_at	−4.69464	0.000101	NM_008691	*Nefm*
1426255_at	−4.62596	0.000248	M20480	*Nefl*
1442077_at	−4.55995	0.000305	BB197581	*2310076G05Rik*

* Affymetrix ID, Santa Clara, CA.

Gene Expression of DBA/2 Mice with ON Damage Compared with Control Mice (C57BL/6 and Pearl)

When comparing gene expression of DBA/2 mice with various amounts of ON damage with that in C57BL/6 and pearl mice and using the above-described criteria (see the Methods sec-

TABLE 3. Top 10 Up- and Top 10 Downregulated Genes in 9-Month-Old DBA/2J Retinas with Severe Axonal ON Damage Compared with Retinas from Similarly Aged Animals with None–mild ON Damage

ID*	Log_2 difference	*P*	Public ID	Gene Symbol
Upregulated Genes				
1420511_at	3.189386659	0.00092004	NM_008938	*Prph2*
1460197_a_at	3.1573267	0.001197008	NM_054098	*Steap4*
1424254_at	2.678278647	0.048744359	BC027285	*Ifitm1*
1421551_s_at	2.441105986	0.014967518	NM_011940	*Ifi202b*
1449161_at	2.111220861	0.04529975	NM_007902	*Edn2*
1458945_at	2.035268495	6.39E-05	BG070195	*AU015148*
1431739_at	1.786135957	0.023212618	AK017573	*Mto1*
1459578_at	1.655340939	0.036218056	BG063140	*AA407175*
1418240_at	1.637179211	0.049042504	NM_010260	*Gbp2*
1440123_at	1.546095611	0.015365036	BM221049	—
Downregulated Genes				
1428077_at	−2.781693359	0.012301953	AK011522	*LOC100047091///Tmem163*
1439228_at	−2.60777538	0.021397414	AV346852	—
1417788_at	−2.239868648	0.043425902	NM_011430	*Sncg*
1455936_a_at	−2.094999667	0.026368354	BG069460	*Rbpms*
1417653_at	−1.996950434	0.047689841	NM_013645	*Pvalb*
1422834_at	−1.938054623	0.017046987	BB051684	*Kcnd2*
1439609_at	−1.912868602	0.032288782	BB536410	—
1438059_at	−1.778338273	0.032408776	BM116248	*Ctxn3*
1416713_at	−1.720998158	0.023842196	NM_026481	*Tppp3*
1423183_at	−1.705752147	0.030282071	BB314774	*Gm3888///Lgi1*

* Affymetrix ID, Santa Clara, CA.

Table 5. Top 10 Up- and Top 10 Downregulated Genes in 9-Month-Old DBA/2J Retinas in the Highest Quartile of IOP Exposure Compared with Retinas from Similarly Aged Animals with IOP Exposure in the Lowest Quartile

ID*	Log2 difference	*P*	Public ID	Gene Symbol
Upregulated Genes				
1460302_at	1.630409542	0.01034354	AI385532	*Tbbs1*
1455663_at	1.411435588	0.0202076	BB055163	*Olfml1*
1448395_at	1.325167643	0.00225075	BI658627	*Sfrp1*
1423858_a_at	1.299618868	0.02112739	BC014714	*Hmgcs2*
1447402_at	1.204494852	0.00797482	AI849052	—
1460250_at	1.178346469	0.02558453	BC021458	*Sostdc1*
1423691_x_at	1.156654905	0.02577492	M21836	*Krt8*
1425191_at	1.120195883	0.00842907	BC019407	*Ocel1*
1460470_at	1.071436729	0.01147122	AK004775	*Acoxl*
1420647_a_at	1.056411226	0.02939853	NM_031170	*Krt8*
Downregulated Genes				
1427747_a_at	−3.641328308	0.01456463	X14607	*Lcn2*
1423954_at	−3.009353974	0.01911834	K02782	*C3*
1460197_a_at	−2.956302494	0.0110985	NM_054098	*Steap4*
1423547_at	−2.316127285	0.01041586	AW208566	*Lyz2*
1436996_x_at	−2.124877663	0.00047648	AV066625	*Lyz1*
1439426_x_at	−1.739863166	0.00245869	AV058500	*Lyz1*
1451537_at	−1.686808785	0.0491064	BC005611	*Chi3l1*
1437726_x_at	−1.515392698	0.02381945	BB111335	*C1qb*
1455393_at	−1.406335574	0.03531675	BB009037	*Cp*
1448620_at	−1.269035702	0.03699339	NM_010188	*Fcgr3*

* Affymetrix ID, Santa Clara, CA.

tion), we identified 1523 IDs (AffyID; Affymetrix) that mapped to 1385 genes with different expression. Similarly, when comparing gene expression of DBA/2 mice with various amounts of IOP exposure to that in C57BL/6 and pearl mice, we identified 1308 IDs, which mapped to 1133 genes with different expression levels. The top 20 genes (in terms of maximum absolute log-fold difference) of each of these comparisons are presented in Tables 7 and 8 (full lists are available in the Supplementary Data, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-7063/-/DCSupplemental).

RT-PCR was performed to confirm a number of the unique as well as the common genes identified. Table 9 lists the genes tested by RT-PCR. Of all the pair-wise comparisons among all adjacent time points in all groups between microarray and RT-PCR results, concordance was present 72.4% of the time, whereas only 1.7% of the comparisons were in the opposite direction.

Bioinformatic analysis of the dataset of genes identified as changing in the retina of DBA/2 mice with extensive ON damage, revealed that of these genes, 947 (68%) can be categorized in functional groups and canonical pathways. The top molecular and cellular functional groups were Protein Synthesis (71 genes), Cellular Assembly and Organization (58 genes), Carbohydrate Metabolism (52 genes), Nucleic Acid Metabolism (28 genes), and Small Molecule Biochemistry (74 genes). The top canonical pathways involved were Oxidative Phosphorylation (24/165 genes), Mitochondrial Dysfunction (22/169 genes), Pyrimidine Metabolism (21/231 genes), Ubiquinone Biosynthesis (11/119 genes), and Androgen Signaling (17/144 genes). Of the genes identified, 1069 (77%) could be also categorized into at least 50 well-characterized gene networks (with 35 members each). The percentage of genes changing within each one of these networks varied between 88.6% and 42.9%.

Bioinformatic analysis of the dataset of genes identified as changing in the retina of DBA/2 mice with high IOP exposure, revealed that, of these genes, 779 (69%) can be categorized in functional groups and canonical pathways. The top molecular and cellular functional groups were Cellular Function and Maintenance (60 genes), Cell Morphology (76 genes), Cell-To-Cell Signaling and Interaction (52 genes), Molecular Transport (67 genes), and Protein Trafficking (43 genes). The top canonical pathways involved were PI3K/AKT Signaling (15/139 genes) NRF2-mediated Oxidative Stress Response (19/185 genes), 14-3-3-mediated Signaling (13/116 genes), Thrombin Signaling (20/206 genes), and Aminoacyl-tRNA biosynthesis (6/

Table 6. Top 10 Up- and Top 10 Downregulated Genes in 12-Month-Old DBA/2J Retinas in the Highest Quartile of IOP Exposure Compared with Retinas from Similarly Aged Animals with IOP Exposure in the Lowest Quartile

Affy ID	Log_2 Difference	*P*	Public ID	Gene Symbol
Upregulated Genes				
1452376_at	1.080785207	0.028182732	BE951951	*Zfp444*
1451737_at	0.74861225	0.003724397	M60651	*Pik3r1*
1434381_at	0.733570935	0.030845057	AV287602	*Atmin*
1438398_at	0.707698967	0.049289886	BB203348	—
1440645_at	0.678472184	0.009891672	BB277291	*BB114814*
1452195_s_at	0.67780985	0.025431476	AW744519	*Sfi1*
1446899_at	0.666791739	0.033087344	BB165801	—
1443066_at	0.664377982	0.030246618	BB280150	—
1446758_at	0.643470816	0.015747625	BB437647	*Bre*
1435592_at	0.620957486	0.036630776	BB149997	*Eif5b*
Downregulated Genes				
1455714_at	−0.89084705	0.02302684	BQ174072	*Vstm2l*
1441263_a_at	−0.851786338	0.025824527	AV009179	*A930005H10Rik*
1424433_at	−0.839718215	0.048375725	BC021619	*Msrb2*
1441971_at	−0.834117956	0.015419475	AW543723	—
1429105_at	−0.820543731	0.006039066	AK016873	*Dlgap1*
1426464_at	−0.793865631	0.043577511	W13191	*Nr1d1*
1419470_at	−0.729778533	0.048241221	BI713933	*Gnb4*
1443437_at	−0.674048729	0.030052498	BB016042	—
1458640_at	−0.671461033	0.003868581	BB284122	—
1455271_at	−0.668828007	0.002980242	BB560177	*Gm13889*

Table 7. Top 20 Genes with Different Expression in DBA/2J Animals with Severe ON Damage Compared to C57BL/6 and Pearl Animals Ranked According to Maximum (Absolute) Difference Observed

ID*	Max Absolute Log_2 Difference	*P*	Public ID	Gene Symbol
1428393_at	7.447345	9.85E−09	AK003046	*Nrn1*
1428379_at	6.742584	5.93E−08	BQ180367	*Slc17a6*
1417788_at	6.725845	3.74E−13	NM_011430	*Sncg*
1437588_at	6.236556	4.31E−08	BB547375	*Pou4f2*
1436520_at	5.72866	4.87E−09	BB378317	*Abnak2*
1454672_at	5.191901	2.22E−09	BE952212	—
1426255_at	4.954822	1.10E−08	M20480	*Nefl*
1415978_at	4.455029	1.01E−14	NM_023279	*Tubb3*
1423840_at	4.432079	1.08E−08	BC026206	*Ccdc56*
1418610_at	4.219421	2.22E−06	NM_080853	*Slc17a6*
1416713_at	4.218574	2.11E−10	NM_026481	*Tppp3*
1424212_at	4.204713	7.40E−11	BC016463	*9430023L20Rik*
1417916_a_at	4.176493	2.11E−09	NM_019502	*Fxc1*
1444545_at	4.097369	3.51E−06	BB639371	—
1416287_at	4.052694	1.23E−08	NM_009062	*Rgs4*
1451846_at	4.051343	1.67E−08	BC025863	*Nebl*
1421085_at	4.012388	2.00E−09	NM_011302	*Rs1*
1428077_at	3.82122	9.71E−07	AK011522	*LOC100047091/// Tmem163*
1416762_at	3.810754	2.29E−12	NM_009112	*S100a10*
1451800_at	3.781458	4.05E−10	BC027339	*Gcc2*

* Affymetrix, Santa Clara, CA.

84 genes). Of the genes identified 873 (77%) could be also categorized into at least 50 well-characterized gene networks (with 35 members each). Percentage of genes changing within each one of these networks varied between 91.4% and 51.4%.

Of the genes with changing expression in DBA/2J animals, 1223 were identified in eyes with severe ON damage *only*, 1008 in eyes with high IOP exposure *only*, and 300 in *both* eyes with high IOP exposure, as well as in eyes with severe ON damage. The top molecular and cellular functional groups for the genes associated with ON damage *only* were Protein Synthesis (56 genes), Nucleic Acid Metabolism (23 genes), Small Molecule Biochemistry (50 genes), Molecular Transport (27 genes), and Cellular Assembly and Organization (36 genes). The top canonical pathways involved were Oxidative Phosphorylation (23/165 genes), Mitochondrial Dysfunction (21/169 genes), Pyrimidine Metabolism (19/231 genes), Ubiquinone Biosynthesis (10/119 genes) and Purine Metabolism (30/439 genes).

The top molecular networks and cellular functional groups for the genes associated with high IOP exposure *only* were Cellular Function and Maintenance (63 genes), Cell-to-Cell Signaling and Interaction (38 genes), Molecular Transport (53

Table 8. Top 20 Genes with Different Expression in DBA/2J Animals in the Highest Quartile of IOP Exposure Compared to C57BL/6 and Pearl Animals Ranked According to Maximum (Absolute) Difference Observed

ID*	Max Absolute Log_2 Difference	*P*	Public ID	Gene Symbol
1428393_at	5.235747	0.000921	AK003046	*Nrn1*
1437588_at	5.215857	0.000792	BB547375	*Pou4f2*
1447415_at	4.63426	8.13E-06	BB799439	*Bdp1*
1428364_at	4.534631	3.72E-06	AK013948	*Scnm1*
1429667_at	4.519533	0.000264	BB465134	*Pou4f1*
1428379_at	4.063704	0.003367	BQ180367	*Slc17a6*
1460314_s_at	3.883367	0.004771	NM_019469	*Hist1h3a-i/Hist2h3b/Hist2h3c1-2*
1424254_at	3.788744	0.000709	BC027285	*Ifitm1*
1422520_at	3.586509	0.001239	NM_008691	*Nefm*
1431233_at	3.566105	0.00135	AK017367	*Cnnm4*
1436520_at	3.563183	0.002537	BB378317	*Abnak2*
1458426_at	3.549331	5.22E-08	BM941075	—
1428077_at	3.467014	0.002766	AK011522	*LOC100047091///Tmem163*
1442077_at	3.460935	0.000598	BB197581	*2310076G05Rik*
1445427_at	3.451781	0.000204	BB636266	—
1420175_at	3.415516	7.89E-06	C85320	*Tax1bp1*
1417788_at	3.360333	0.00132	NM_011430	*Sncg*
1445065_at	3.279633	4.88E-05	BM237637	—
1454672_at	3.240591	0.002525	BE952212	—
1425180_at	3.23815	0.001052	AV344708	*Sgip1*

* Affymetrix, Santa Clara, CA.

TABLE 9. Genes Subjected to RT-PCR to Confirm MA Results

Genes Changing with IOP Exposure Only (Not Affected by ON Damage)	Genes Changing with ON Damage Only (Not Affected by IOP Exposure)	Genes Changing with Both IOP Exposure and ON Damage
tax1bp1	*nebl*	*atp6v1f*
gjb6	*tusc5*	*cacna2d4*
cask	*ifi202b*	*clta*
tubgcp5	*ifitm1*	*cp*
trf	*hspb8*	*elavl2*
epn2	*edf1*	*nr6a1*
ddn	*c1qb*	*nrn1*
pou4f1	*cryab*	*pou4f2*
fliih	*prdx4*	*rgs4*
marcks		*sncg*
caln1		*syn2*
		gcn1/1
		pgea1/cby1
		tm2d2
		vps25

genes), Protein Trafficking (35 genes), and Cell Morphology (66 genes). The top canonical pathways involved were PI3K/AKT Signaling (15/139 genes), Aminoacyl-tRNA Biosynthesis (6/84 gens), 14-3-3-mediated Signaling (12/116 genes), Rac Signaling (11/124 genes), and Chemokine Signaling (9/75 genes).

Finally the top molecular networks and cellular functional groups for the genes associated with *both* severe ON damage as well as high IOP exposure were Cellular Assembly and Organization (35 genes), Gene Expression (7 genes), Cellular Function and Maintenance (12 genes), Cell Morphology (16 genes), and Molecular Transport (24 genes). The top canonical pathways involved were Pentose Phosphate (3/ 89 genes) and Tumoricidal Function of Hepatic Natural Killer Cells (2/24 genes).

Since genes that change early in the disease process are more likely to be involved in the pathogenesis of the disease, we also subjected to bioinformatic analysis the genes with differential expression in 6-month-old DBA/2J eyes with high versus low IOP. Of the 94 IDs changing, 83 could be mapped to genes. Only four of these (*atad2b*, *dock3*, *aatk*, and *gjb6*) were network eligible.

The genes that are differentially expressed in the process of neurodegeneration and prolonged IOP elevation can be the result of activation (or repression) of specific transcription factors. We performed hypergeometric tests to identify such transcription factors that are significantly overrepresented in the gene list. Of the 614 in the *transcription factor targets* (tft) *dataset* in MSigDB (ver. 3.0), 16 and 15 transcription factors respectively were significantly overrepresented ($P < 0.01$) for the differentially expressed genes in retinas either exposed to high IOP or having severe ON damage.

When compiling the data from all microarray studies, we identified several complement genes with altered expression at some time point during glaucomatous pathology development or that are associated with elevated IOP. These genes are shaded in Figure 3. Since some of these gene changes are biphasic, they are presented as changes without direction.

DISCUSSION

DBA/2 mice are a valuable rodent glaucoma model. The glaucoma-like neurodegeneration that develops resembles human pathology in many respects. Specifically, it appears that it is the result of a period of elevated IOP, it develops (at least initially) in a localized pattern, and there is a large interanimal and intereye variability.[8] Since the pathology localizes in the retinal ganglion cell and its axon, we reasoned that changes within the retina and ON would be preceded (or at least accompanied) by changes in gene expression within these cells as well as cells within the retina that interact either directly or indirectly with the RGCs. We decided to use microarrays to study gene expression, because it makes no a priori assumptions about which genes are changing. Although microarray data are undoubtedly "noisy" because of the very large number of genes concurrently analyzed,[23,24] it has been successfully used to identify novel pathways that are operational in a variety of conditions. One of the strategies to minimize the inherent noise in microarray analysis would be to select a pure cellular population to study (in this case most probably the RGCs). Although such a strategy has been used in the past,[25] it does make the assumption that all relevant gene changes are happening in the RGCs only. Focusing on gene and protein expression only in RGCs (using for example cell cultures or laser capture microdissection) may result in missing key genes involved in the development or progression of glaucoma. On the other hand, because of the increased sensitivity when analyzing a pure cell population, it can admittedly identify a larger number of genes with expression changing in RGCs.[26] Given the current state of our understanding of glaucomatous retinal pathology, studying *total* retinal gene expression has specific advantages. Such a strategy makes no assumptions about which other cells (e.g., Müller cells, astrocytes, amacrine cells) may be contributing to the development of glaucoma. In fact, preliminary evidence from both our laboratory[27] as well as other laboratories[28,29] indicate that the sequence of events leading to RGC death may originate or depend on other cells in the retina.

An approach similar to the one we used in this study has been reported.[10] Our data supplement and expand the results of that study. Specifically, MA analysis of the DBA/2 retina had only been performed on groups of animals up to 8 months of age, an age when DBA animals develop early to moderate damage.[30] By extending our experiments to 12 months, we attempt to identify gene expression changes that occur as a result of or contribute to more advanced damage. Although direct comparison with published studies is not possible because of differences in design, methodology, and analysis, it is interesting to note that we were able to identify a much larger number of genes with changing expression, while confirming some of the genes identified in earlier studies. Despite identi-

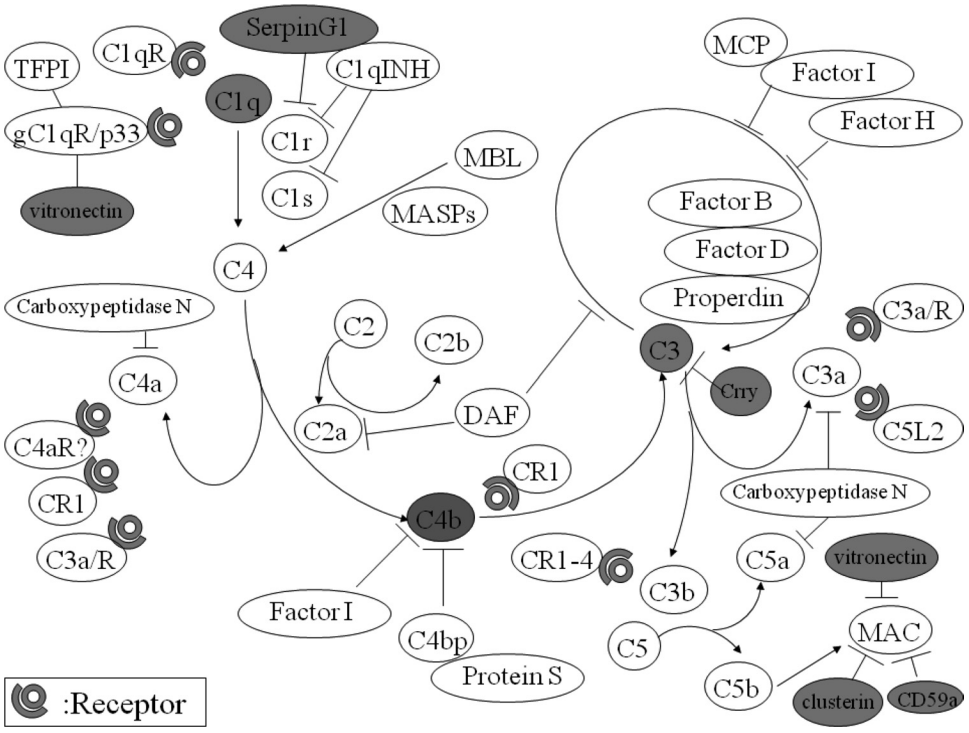

FIGURE 3. Diagram of complement genes affected in DBA/2 retinas subjected to either prolonged IOP elevation or with severe ON damage when compared with nonglaucomatous similarly aged C57BL/6 or DBA/2^{pc} (pearl) animals. *TFPI*, tissue factor pathway inhibitor; *C1qR*, C1q receptor (CD93); *gC1qR/p33*, complement component 1, q subcomponent binding protein; *C1qINH*: C1q inhibitor; *C4aR*, C4a receptor; *C3aR*, C3a receptor; *DAF*, decay-associated factor (CD55); *CR1–4*: complement receptors 1–4; *MAC*, membrane attack complex; *MCP*: membrane cofactor protein, CD59.

fication of a larger number of relevant genes, the asynchronous RGC and axonal loss that occurs in the DBA/2 mice and the uncertain timing of pathology in individual animals makes understanding the temporal sequence of gene expression changes difficult. Nevertheless, publication of the full dataset of gene expression changes identified in this study will allow other investigators to compare their gene(s) of interest across studies and, with the help of other publicly available datasets, will enhance our understanding of glaucoma pathophysiology.

Our approach is also novel, in that we use two control strains. Traditionally, the C57BL/6 animals have been used as a control for DBA/2 experiments (see also the recommendation by Jackson Laboratories[31]). However, it has become apparent that the genetic background plays an important role in the development of glaucomatous pathology. The pathogenic mutations in *Tyrp1* and *Gpnmb* that cause glaucoma in DBA/2 mice[32] are not sufficient to cause the full spectrum of pathology in C57BL/6 mice.[33] We used the DBA/2^{pc} (pearl) strain as an additional control strain. Pearl mice carry the two pathogenic mutations and are on the DBA/2 background. However, because of an additional mutation in the *Ap3b1* gene,[34,35] they do not develop the ocular pathology associated with DBA/2 mice.[32] Inclusion of pearl mice as controls allows better differentiation of strain-specific changes in gene expression and allows focusing on pathology relevant genes. Since the design and initiation of these experiments an additional control has become available (the DBA/2 *gpnmb* wt).[33] Inclusion of this additional control would be ideal, but it was not available to us at the time that this study was performed.

The experimental design takes into account the three factors that can potentially affect gene expression: age, IOP exposure, and axonal loss. Age as discussed above is controlled by comparing DBA/2 mice with the two control strains. For IOP, we elected to compare the eyes with the highest 25% exposure to eyes with the lowest 25% cumulative IOP exposure within our cohort. We measured IOP noninvasively with rebound tonometry, and averaged the results over time to determine total IOP exposure. Cumulative IOP exposure is more appropriate in long-term experiments and has been correlated with IOP damage in other experimental models.[36] Despite the significant variability in IOP over the length of the experiment, we were surprised to see that eyes could clearly be categorized to distinct groups in terms of cumulative IOP exposure. These groups appear to have differential gene expression as early as 6 months of age, a time when ON damage is not yet detectable by the methods used.

The effect of ON damage was independently determined by comparing eyes with severe axonal damage with eyes with none–mild axonal damage at each particular age. Axonal damage was used as a surrogate of RGC loss, since retinas were used for gene expression instead of determining RGC loss directly. During the course of this study, DBA/2 animals expressing the GFP protein under a Thy1 promoter in their RGCs has been reported.[37] Use of these animals may allow a direct assessment of the number of RGCs (and thus of retinal damage). However, the use of ON axonal damage to grade glaucomatous pathology is also appropriate, as axons seem to degenerate slightly before but in close temporal proximity to when RGCs are lost.[38,39] We have previously reported on the regional gene expression changes within the retina of 11- to 13-month-old DBA/2 animals with focal RGC loss.[14] These differences in gene expression between areas of focal loss and relative RGC preservation involve genes that code for proteins with diverse cellular functions. The current experiments expand on that study by providing a temporal aspect to gene expression changes related to RGC loss and especially by looking at earlier time points during the development of the degeneration.

Because IOP is one of the major factors responsible for axonal and RGC loss in glaucoma it is not surprising that there is considerable overlap of genes with expression affected by both high IOP exposure and ON damage. These genes probably contain the pathways that are responsible for RGC loss as a result of IOP elevation. Conversely, genes identified exclusively in the high-IOP exposure group of eyes are probably related to changes in retinal pathophysiology that do not necessarily contribute directly to RGC loss. Similarly, genes identified exclusively in the group of eyes with severe ON damage

are probably secondary changes that occur in the retina as a result of the development of glaucomatous pathology (which includes RGC loss). Some of these changes in gene expression may be responsible for what has been termed "secondary degeneration" in glaucoma.[40]

The number of genes affected by cumulative IOP exposure increased as the DBA/2J mice aged from 6 to 9 months. It thus appears that IOP exposure triggers secondary changes in gene expression that increase progressively with time and are further compounded by RGC loss that begins to occur after the eighth month of age. As more and more of the DBA/2J eyes develop significant RGC loss, the effect of IOP exposure decreases in terms of differences in gene expression and the number of genes detected decreases from 9 to 12 months of age. Conversely, the number of genes affected in DBA/2J mice that are associated with ON damage increases with age from 9 to 12 months. Thus, it appears that age-related changes compound the effect caused by ON damage on gene expression.

Detailed examination of the small number of genes with differential expression because of different cumulative IOP exposure at the 6-month time point (when no significant RGC loss can be detected) may provide important clues as to the initial response of the retina to elevated IOP. However, most of the genes affected do not map to well-characterized gene networks. This prompted us to identify transcription factors that regulate genes that are overrepresented in our gene sets. This and other bioinformatic analyses of the genes identified is important, as genes act together in networks. The extent that some of these gene networks are affected illustrates their involvement rather than that they are pathogenetic. In a complex tissue like the retina, it is also probable that different gene networks are changing within different cellular populations. Follow-up studies using in situ hybridization and immunohistochemistry are necessary to better begin to understand how specific genes in specific cell populations lead to pathologic changes in glaucoma.

We decided to further explore the role of complement in glaucomatous pathophysiology by determining which of the genes among the 35 or so that make up this complex system change in expression. Rather than label genes as up- or down-regulated, we elected to report them as changing. This method of reporting better reflects the experimental design, as changes in some of the genes are not necessarily monotonic (for example an RGC specific gene that is involved in the disease development may go up early in the disease process, leading to RGC loss, which as a result will decrease its expression within the retina). In fact, a significant number of all the genes identified by microarray analysis do not display monotonic behavior with time. With this in mind, it appears that genes in the classic activation pathway of complement as well as C3 activation (which can participate in both the classic and alternative pathways) are clearly affected in the DBA/2 mice. Not surprisingly, genes involved in the membrane attack complex (MAC) are not affected, despite previous reports in other induced glaucoma models that MAC may lead to RGC loss. DBA/2 mice are C5 deficient and cannot thus form MAC.[41]

Whether complement upregulation (and probable activation) plays a protective or destructive role in the disease process is still an open question.[42] It is interesting to note that in human glaucoma (and using a somewhat similar bioinformatic approach) the same genes appear to be involved.[43]

In summary, this study provides a comprehensive survey of gene expression in the DBA/2 mice and explores in depth the effects of age, IOP exposure, and axonal damage on this expression. This wealth of data is provided to other investigators in an effort to improve our collective understanding of the basic mechanisms underlying this potentially blinding disease.

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