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Genetic Networks Activated by Blast Injury to the Eye

Professionally, we are using these data to define biomarkers to predict the severity of the injury and to predict eventual outcomes. We have examined the phenotypic changes in the eye in the BXD strains before and 5 days after a 50psi blast and have observed no strain specific change in either the cornea or the IOP. We have completed the normal retina database containing 222 microarrays from 58 strains of mice. We have also prepared the manuscript describing the database and annotation are complete and will be released to the public upon the acceptance of the manuscript. We have collected 76 retinas from 19 strains 5 days after a 50psi blast. These retinas are being held for a batch RNA isolation and microarray run. Microarrays were run on an additional 27 strains. Next year we will complete the dataset by adding 84 retinas from 21 BXD strains. We have taken a preliminary examination of the blast dataset and found that Sox1 is a relatively good marker for injured retinal ganglion cells. These results are presented in a paper currently under revision for publication in Molecular Vision.
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1. **INTRODUCTION**

In a collaboration with Dr. Tonia Rex we developed a mouse model of blast injury to the eye, which accurately mimics the traumatic blast injury increasingly suffered by warriors under current battlefield conditions (Hines-Beard et al., 2012). Using this mouse model in combination with a powerful combination of systems biology, microarray analysis, expression genetics, and bioinformatics, we are defining the genetic networks activated by the ocular blast injury. At the heart of our approach is a genetic reference panel of mice, the unique resource of BXD recombinant inbred (RI) strain set. The set of RI strains was produced from a genetic cross between the C57BL/6J mouse and the DBA/2J mouse. Using 60 BXD strains provides a new and powerful method to defining elements in the genome regulating the response of the eye to blast injury. This allows us to generate specific, testable hypotheses to define the pathways that regulate the response of the eye to blast injury and reactive responses in the retina. As more diverse gene expression data sets become available, comparison of gene expression and regulation in different biological contexts will help identify the regulatory elements controlling the injury response of the eye and the retina. We are currently identifying genetic networks activated by blast injury and the genomic regions controlling these networks. We are also identifying markers for retinal injury and potential targets for intervention.

2. **KEYWORDS**

Mouse Genomics, Blast Injury, Eye, Retina, Gene Expression, Microarray
3 ACCOMPLISHMENTS

Major Goals:

Task 1) Quantify the strain-to-strain differences in the severity of blast-induced ocular pathologies, using a set of 60 BXD RI mouse strains and map the genomic loci that regulate the response of the eye to blast injury. In this Task we were measuring intraocular pressure (IOP), central corneal thickness (CCT) and visual acuity.

Task 2) Define the genetic networks activated by blast injury in the eye and in the retina, using transcriptome-wide profiling across the BXD RI strain set. We are using the Affymetrix GeneChip Gene 2.0 ST Mouse Array to characterize the changes occurring following a blast injury to the eye in 60 BXD strains. There were several major benefits to using the new Affymetrix array. Specifically, there are probes for 7,000 non-coding RNAs (RNA that is not converted to protein but does affect the functioning of the cell). We are now finding out that many of these non-coding RNAs play extremely important roles in the body. Within these 7,000 probes, 588 encode microRNAs (small RNAs that regulate protein expression). We are creating an entire normal retina dataset using the Affymetrix GeneChip Gene 2.0 Mouse Array and comparing this data set to a dataset from retinas 5 days after a 50psi blast injury to the eye.

Task 3) Define biomarkers that can predict the severity of injury and eventual outcomes.

This portion of our study was to begin in the latter years of the grant (Months 40 to 48). We are using this to characterize the 50-psi blast injury in advance of resuming the blast microarray study on the BXD RI strain set. Immunostaining sections of retina revealed that SOX11 was upregulated in the neurons of the inner retina following blast. SOX11 labeled cells in the ganglion cell layer and the inner nuclear layer. In the ganglion cell layer SOX11 labeled a majority of the cells, indicating that it was labeling most ganglion cells and displaced amacrine cells. Once the datasets are fully implemented, we will be able to accurately define the changes occurring within the injured retina.

Accomplishments Under These Goals:
Task 1:
At the present time we have measured IOP and central corneal thickness on 33 strains of mice before and after a 50psi blast injury to the eye. At the present time there are 117 animals total in the dataset. When we run a student t test on the data there was no significant difference in CCT or IOP before and after blast in the control eyes. This is expected. We also did not see a significant difference in either CCT or IOP 5 days after a 50psi blast to the experimental eye. This is unexpected. We intend to continue measuring CCT and IOP measurements for the next quarter.
Task 2.

A) We have completed the construction of the DoD CDMRP Normal Retinal Dataset. Using the Affymetrix Mouse Gene 2.0 ST array, to interrogate all exons of traditional protein coding genes, non-coding RNAs and microRNAs. These data are presented in a highly interactive database within the GeneNetwork website. In the Normal Retina Database, we quantified mRNA levels of the transcriptome from retinas using the Affymetrix Mouse Gene 2.0 ST array. The Normal Retina Database consists of gene expression data from male and female mice. The dataset includes a total of 55 BXD RI strains, the parental strains (C57Bl/6J and DBA/2J), and a reciprocal cross. In combination with GeneNetwork, the DoD (Department of Defense) CDMRP (Congressionally Directed Medical Research Programs) Normal Retina Database provides a large resource for mapping, graphing, analyzing, and testing complex genetic networks. Protein-coding and non-coding RNAs can be used to map quantitative trait loci (QTLs) that contribute to expression differences among the BXD strains and to establish links between classical ocular phenotypes associated with differences in genomic sequence. With this resource we are able to extract transcriptome signatures for retinal cells and to define genetic networks associated with the maintenance of the normal retina. Ultimately, we will use this database to define changes occurring following blast injury to the retina. The DoD CDMRP Normal Retina Database uses the Affymetrix MouseGene 2.0 ST Array (May 15 2015). The RMA analysis and scaling was conducted by Arthur Centeno. This data set consists of 55 BXD strains, C57BlU6J, DBA/2J, an F1 cross between C57BlU6J and DBA/2J. A total of 58 strains were quantified. There is a total of 222 microarrays. All of the data from each of the microarrays used in this dataset is publically available on GeneNetwork.org.

Mice were killed by rapid cervical dislocation. Retinas were removed immediately and placed in 1 ml of 160 U/ml Ribolock for 1 min at room temperature. The retinas were removed from the eye and placed in Hank's Balanced Salt solution with Ribolock in 50µl Ribolock (Thermo Scientific RiboLock RNase #E00381 40U/µl2500U) and stored in -80°C. The RNA was isolated using a QiaCube. All RNA samples were checked for quality before running microarrays. The samples were analyzed using the Agilent 2100 Bioanalyzer. The RNA integrity values ranged from 7.0 to 10. Our goal was to obtain data for independent biological sample pools from both sexes for most lines of mice. The four batches of arrays included in this final data set collectively represent a reasonably well-balanced sample of males and females, in general without within-strain-by-sex replication.
The data is presented using the Affymetrix Mouse Gene 2.0 ST Array. These expression arrays have been designed with a median of 22 unique probes per transcript. Each unique probe is 25 bases in length, which means that the array measures a median of 550 bases per transcript. The arrays provide comprehensive transcriptome coverage with over 30,000 coding and non-coding transcripts. In addition there is coverage for over 600 microRNAs. For some arrays the RNA was pooled from two retinas and other arrays were run on a single retina. Dr. XiangDi Wang (UTHSC) and Rebecca King (Emory) were involved in the retinal extractions and isolation of RNA. Two different research cores ran the Affymetrix arrays: the Molecular Resource Center at UTHSC (Dr. William Taylor Director) and the Integrated Genomics Core at Emory University by Robert B. Isett (Dr. Michael E. Zwick, Director). In a separate set of experiments we tested a set of arrays from C57BU6J retinas run at each facility to determine if there were batch effects or other confounding differences in the results. We could not detect any significant difference in the arrays run at UTHSC or at Emory University. Thus, we have included both sets of data into the analysis.


Task 3) We have identified a list of potential biomarkers for injury to the retinal ganglion cells. The best marker is SOX11 (manuscript being revised). We are using this to characterize the 50psi blast injury in advance of resuming the blast microarray study on the BXD RI strain set. Immunostaining sections of retina revealed that SOX11 was upregulated in the neurons of the inner retina following blast. SOX11 labeled cells in the ganglion cell layer and the inner nuclear layer. In the ganglion cell layer SOX11 labeled a majority of the cells, indicating that it was labeling most ganglion cells and displaced amacrine cells. Amacrine cells in the inner nuclear layer were also lightly labeled by SOX11. On immunoblots there was approximately a 2-fold increase in the intensity of the SOX11 band. The manuscript describing these results is currently being revised to respond to the editorial suggestions.


Training and Professional Development Opportunities:
Nothing to Report

Dissemination of Results:

Invited Talks:

2014 Genetic Network of Innate Immunity in the Retina: Relevance to CNS Injury and Disease Department of Molecular Physiology and Biophysics University of Iowa.

2014 Genetic Network of Innate Immunity in the Retina: Relevance to CNS Injury and Disease VA Atlanta GA.

ARVO Meeting:

Papers Submitted for Publication:


Plans for Next Reporting Period to Accomplish the Goals:

1) Finish Beta testing and open the DoD CDMRP Normal Retina Database to the public in August 2015.

2) We will complete the DoD Blast Microarray Database. This will entail running several hundred microarrays along with the costs of analysis. We anticipate this to consume a significant amount of the remaining budget.

3) Prepare manuscript describing the Blast database.

4) Release the Blast database to the public.
4 IMPACT

Impact on the Development of the Principal Discipline of the Project:

Once the proposed studies are completed they will provide a comprehensive analysis of the molecular pathways activated in the retina by blast injury to the eye.

Impact on Other Disciplines:

When developing Biomarkers for retinal injury, our microarray dataset will provide a means to determine if any specific biomarker could have originated from the retinal injury itself.

Impact on Society Beyond Science and Technology:

Nothing to Report

5) Changes/Problems

Changes in Approach and Reasons for Change:
None

Actual or Anticipated Problems or Delays and Actions or Plans to Resolve Them:
None

Changes that had Significant Impact on Expenditures:
None

Significant Changes in the Use or Care of Human Subjects Vertebrate Animals Biohazards, or Select Agents:
None

6. PRODUCTS

Publications, conference papers, and presentations:

2014 Genetic Network of Innate Immunity in the Retina: Relevance to CNS Injury and Disease Department of Molecular Physiology and Biophysics University of Iowa.
2014 Genetic Network of Innate Immunity in the Retina: Relevance to CNS Injury and Disease VA Atlanta GA.

ARVO Meeting:

Papers Submitted for Publication:


Website(s) or other Internet site(s):
The DoD CDMRP Retina Affy MoGene 2.0 ST Database and the DoD TATRC Retina Affy MoGene 2.0 ST Exon Level Database are hosted on GeneNetwork.org. This database will be open to the public in August 2015. These datasets describe gene expression in the normal retina of the BXD Strains. Both databases can be found under Mice, BXD, retina and then either DoD CDMRP Retina Affy MoGene 2.0 ST Database or DoD CDMRP Retina Affy MoGene 2.0 ST Exon Level Database.

Technologies or techniques:
None

Inventions, patent applications, and/or licenses:
None

Other products:
None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
At Emory University (7/15/14 to present):

Becky King, Research Technician (50% effort)
Eldon E. Geisert, Principal Investigator (25% effort)

We have been collaborating with Dr. Mike luvone to construct and test a new blast gun. We are currently in the process of writing a manuscript describing the effects of a 50psi blast to the mouse eye.

Has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?

No.

What other organizations have been involved as partners?
None

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

A) Preprint of the DoD CDMRP Normal Retina Database paper.

8) Preprint of the SOX11 (marker of retinal injury) paper.
Gene Expression and Genetic Networks in the Mouse Retina

Rebecca King,¹ Lu Lu,² Robert W. Williams,² and Eldon E. Geisert,¹

¹Department of Ophthalmology and Emory Eye Center, Emory University, Atlanta, GA 30322; ²Department of Anatomy and Neurobiology and Center for Integrative and Translational Genomics, University of Tennessee Health Science Center, Memphis, TN 38163

Abstract

Purpose: Differences in gene expression provide diverse retina phenotypes and may also contribute to susceptibility to injury and disease. The present study defines the transcriptome of the retina in the BXD RI strain set. Using the Affymetrix Mouse Gene 2.0 ST array, to interrogate all exons of traditional protein coding genes, non-coding RNAs and microRNAs. These data are presented in a highly interactive database within the GeneNetwork website.

Methods: In the Normal Retina Database, quantified mRNA levels of the transcriptome from retinas using the Affymetrix Mouse Gene 2.0 ST array. The Normal Retina Database consists of gene expression data from male and female mice. The dataset includes a total of 55 BXD RI strains, the parental strains (C57Bl/6J and DBA/2J), and a reciprocal cross.

Results: In combination with GeneNetwork, the DoD (Department of Defense)
CDMRP (Congressionally Directed Medical Research Programs) Normal Retina
Database provides a large resource for mapping, graphing, analyzing, and
testing complex genetic networks. Protein-coding and non-coding RNAs can be
used to map quantitative trait loci (QTLs) that contribute to expression
differences among the BXD strains and to establish links between classical
ocular phenotypes associated with differences in genomic sequence. With this
resource we are able to extract transcriptome signatures for retinal cells and to
define genetic networks associated with the maintenance of the normal retina.
Ultimately, we will use this database to define changes occurring following blast
injury to the retina.

Conclusions: The high level of variation in mRNA levels found among BXD RI
strains of mice make it possible to identify expression networks underlying
differences in retina structure and function.

INTRODUCTION
Large-scale sequencing initiatives have led to a new era in understanding
gene and genome functions [1-5]. There is now an acute need for powerful
approaches to integrate and analyze massive omics data sets. For vision
research there are many known single gene variants that cause vision loss,
including retinitis pigmentosa [6-9], Usher’s syndrome [10, 11] and some forms of
glaucoma [12]. However, many ocular diseases have a complex genetic basis
with multiple chromosomal loci contributing to differences in susceptibility and
severity of disease. Two prominent examples are glaucoma [13-15] and age-
related macular degeneration [16, 17]. In addition, the response of the eye and
retina to trauma is driven by a host of different genes expressed in a large
number of different cell types.

Until recently, it was extremely difficult to define the genetic and molecular basis
of complex diseases or to adequately monitor the response of eye and retina to
injury. We have use a novel and powerful approach relying on systems biology
and a mouse genetic reference panel, the BXD family of recombinant inbred (RI)
strains. This resource is particularly well suited to define complex genetic
networks that are also active in human diseases. This approach not only allows
us to identify specific gene variants involved in the retinal disease and response
to injury, but also allows us to place corresponding molecular changes in a global
contest in eye and retina.
Our initial efforts of our group explored the genetic diversity of the BXD family of strains to define genetic networks active in the eye (see data sets and refs [18]) and ([19]).

In this study we have created the new mouse retinal database that offers a more complete description of the mouse transcriptome. This resource uses the genetic covariance of expression across a panel of 55 BXD strains to identify cellular signatures and genetic networks within the mouse retina. The array we have used provides expression profiling at the exon level for 26,191 well-established annotated transcripts, as well as 9,049 non-coding RNAs including over 600 microRNAs. Using the bioinformatics tools located within GeneNetwork (genenetwork.org), we examine a cellular signature of RPE cells. We also have analyzed a genetic and molecular network involved in neuronal development and axon growth. In both of these examples we highlight the specific benefits of the new database with a special emphasis on microRNAs, non-coding RNAs and the exon level data available with the Affymetrix MouseGene 2.0 ST array.

MATERIALS and METHODS

The DoD (Department of Defense) CDMRP (Congressionally Directed Medical Research Programs) Normal Retina Database uses the Affymetrix MouseGene 2.0 ST Array (May 15 2015). The RMA analysis and scaling was conducted by Arthur Centeno. This data set consists of 55 BXD strains, C57BL/6J, DBA/2J, an F1 cross between C57BL/6J and DBA/2J. A total of 58 strains were quantified. There is a total of 222 microarrays. All of the data from each of the microarrays used in this dataset is publically available on GeneNetwork.org.

This is RMA expression data that has been normalized using what we call a 2z+8 scale, but without special correction for batch effects. The data for each strain was computed as the mean of four samples per strain. Expression values on a log2 scale range from 3.81 to 14.25 (10.26 units), a nominal range of approximately 1,000-fold. After taking the log2 of the original non-logged expression estimates, we convert data within an array to a z-score. We then multiply the z-score by 2. Finally, we add 8 units to ensure that no values are negative. The result is a scale with a mean expression of the probes on the array of 8 units and a standard deviation of 2 units. A two-fold difference in expression is equivalent roughly to 1 unit on this scale. The lowest level of expression is 3.81 (Olfr1186) from DoD CDMRP (Normal Retina Database uses the Affymetrix MouseGene 2.0 ST Array (May 15 2015) The highest level of expression is Rhodopsin for 17462036 (Rho). Highest single value is about 14.25.

About the cases used to generate this set of data:

Almost all animals are young adults between 60 and 100 days of age. We measured expression in conventional inbred strains, BXD recombinant inbred (RI) strains, and reciprocal F1s between C57BL/6J and DBA/2J.
BXD strains:
The first 32 of these strains are from the Taylor series of BXD strains generated at the Jackson Laboratory by Benjamin A. Taylor. BXD1 through BXD32 were started in the late 1970s, whereas BXD33 through 42 were started in the 1990s. BXD43 and higher were bred by Lu Lu, Jeremy Peirce, Lee M. Silver, and Robert W. Williams starting in 1997 using B6D2 generation 10 advanced intercross progeny. This modified breeding protocol doubles the number of recombinations per BXD strain and improves mapping resolution [20]. All of the Taylor series of BXD strains and many of the new BXD strains are available from the Jackson Laboratory. Several strains were specifically excluded from the dataset. For the BXD43 and higher, the DBA/2J parent carried both the Tyrp-1 mutation and the Gpnmb mutation and these two mutations produce pigment dispersion glaucoma. All of the mice carrying these two mutations were not included in the dataset: BXD53, BXD55, BXD62, BXD66, BXD68, BXD74, BXD77, BXD81, BXD88, BXD89, BXD95 and BXD98. In addition BXD24 was omitted, since it developed a spontaneous mutation, rd16 (Cep290) which resulted in retinal degeneration and was renamed BXD24b/TyJ [21]. Several additional strains were excluded due to abnormally high Gfap levels observed in our Full HEI Retina (April 2010) dataset, these include: BXD32, BXD49, BXD70, BXD83 and BXD89.

Tissue preparation protocol.
Mice were killed by rapid cervical dislocation. Retinas were removed immediately and placed in 1 ml of 160 U/ml Ribolock for 1 min at room temperature. The retinas were removed from the eye and placed in Hank’s Balanced Salt solution with Ribolock in 50µl Ribolock (Thermo Scientific Ribolock RNase #EO0381 40U/µl 2500U) and stored in -80°C. The RNA was isolated using a QiaCube and the in column DNase procedure. All RNA samples were checked for quality before running microarrays. The samples were analyzed using the Agilent 2100 Bioanalyzer. The RNA integrity values for ranged from 7.0 to 10. Our goal was to obtain data for independent biological sample pools from both sexes for most lines of mice. The four batches of arrays included in this final data set collectively represent a reasonably well-balanced sample of males and females, in general without within-strain-by-sex replication.

Affymetrix Mouse Gene 2.0 ST Array: These expression arrays have been designed with a median of 22 unique probes per transcript. Each unique probe is 25 bases in length, which means that the array measures a median of 550 bases per transcript. The arrays provide comprehensive transcriptome coverage with over 30,000 coding and non-coding transcripts. In addition there is coverage for over 600 microRNAs. For some arrays the RNA was pooled from two retinas and for other arrays were run on a single retina. Dr. XiangDi Wang (UTHSC) and Rebecca King (Emory) were involved in the retinal extractions and isolation of RNA. The Affymetrix arrays were run by two different research cores: the Molecular Resource Center at UTHSC (Dr. William Taylor Director) and the Integrated Genomics Core at Emory University by Robert B. Isett (Dr. Michael E.
Zwick, Director). In a separate set of experiments we tested a set of arrays from C57BL/6J retinas run at each facility to determine if there were batch effects or other confounding differences in the results. We could not detect any significant difference in the arrays run at UTHSC or at Emory University. Thus, we have included both sets of data into the analysis.

RESULTS

The DoD CDMRP Retina Database presents the retinal transcriptome profiles of 52 BXD RI strains in a highly interactive website, GeneNetwork. There are two separate presentations of the microarray data. The first is at the gene level (DoD CDMRP Retina Affy MoGene 2.0 ST (May15) RMA Gene Level Database) and the same data presented at the exon level (DoD CDMRP Retina Affy MoGene 2.0 ST (May15) RMA Exon Level Database). For the analysis of these dataset there is a suite of bioinformatics tools integrated into the GeneNetwork website. These tools allow for: the identification of genes that vary across the BXD RI strains, the construction of genetic networks controlling the development of the mouse retina, and the identification of genomic loci underlying complex traits in the retina. In the present paper we present these two new datasets and illustrate their use with two examples. The first was to identify genetic signatures of the retinal pigment epithelium (RPE). The second will identify a genetic network associated with roundabout homolog 2 (Robo2) gene and the modulating axonal growth.

Cellular Signature of RPE in the DoD CDMRP Retina Database

The DoD CDMRP Retina Database has a unique signature for RPE cells. When looking at the expression of the RPE marker Rpe65 there was an almost biphasic distribution of expression (Figure 1). Many of the strains expressed relatively low levels of Rpe65 (approximately 7 units on our scale) while other strains had high levels of expression ranging from 2 to 8 fold higher (8 to 11 units). When we examined the dataset for genes with similar expression across the BXD strains, a list of genes uniquely expressed in RPE was observed (Table 1). This cellular signature represents genes that are uniquely expressed within the RPE, including: Rgr (retinal G protein coupled receptor), Lrat (Lecithin-retinol acyltransferase), Rdh5 (retinol dehydrogenase 5), Trf (transferrin) and Rrh (retinal pigment epithelium derived rhodopsin homolog). This signature can also be thought of as the result of genetic networks that drive gene expression within a given cell type. With the new Affymetrix chip we not only have protein-coding genes that correlate with Rpe65, but we also have microRNAs and non-coding RNAs. If we examine the top 500 correlates of Rpe65 (all of which have a correlation higher than 0.8 with Rpe65), there are five microRNAs present: Mir98, Mir666, Mir449a, Mir301b and Mir28b. Using the bioinformatics tools on
TargetScan (Targetscan.org) [22-24] we were able to predict targets for each of
the microRNAs from the top 500 correlates of Rpe65. One microRNA, Mir666
did not appear on the Targetscan website. The remaining 4 microRNAs did
appear on the website. When scanned for targets Mir98 had 29 targets in the
RPE signature, Mir449a had14 targets, Mir301b had 13 targets and Mir28b had 1
target. This type of analysis may be one approach to constructing and
understanding microarray networks within a specific cell type like the RPE of the
mouse.

Example of a functional network in the DoD CDMRP Retina Database

To illustrate the features of the new DoD CDMRP Retina Database, we have
chosen one specific gene, Robo2 (roundabout homolog 2), and will use this gene
to demonstrate the analytical powers of the database and the bioinformatics tools
associated with GeneNetwork. Robo2 is highly expressed in the retina with a
mean value of 10.7 across the BXD strain set. The expression within individual
strains varies from a low of 10.2 to a high of 11.1. This is a log2 scale and
represents approximately a two-fold difference in expression (Figure 2). When
we examine the database for genes with a similar pattern of expression across
the BXD strain set, there is a group of genes that are highly correlated with the
expression pattern of Robo2 (Table 3). One example is the third correlate on the
list, Ncam2 (Figure 3) with a value of 0.926. Even the 100th correlate on the list
(Git1) has a relatively high correlation (r = 0.873) with Robo2 (See Supplemental
Table 1).

To define the regions of the genome modulating the expression of Robo2, we
plotted a genome wide scan for Robo2 (Figure 4). This plot defines regions of
the genome that correlate with the level of Robo2 expression, a quantitative trait
locus (QTL). In this interval map there is one significant QTL on chromosome 16
(notice the peak reaches the red line on the scan, p = 0.5) and there are two
suggestive peaks on Chromosome 1 and Chromosome 17 (above the gray line).
The expression of Robo2 is modulated by genomic elements on Chromosome
16. There are two types of elements that could be affecting the expression of
Robo2; a cis-QTL or a gene with a nonsynonymous SNP. If we examine the
significant QTL on Chromosome 16 (21-27 Mb), we find there are no significant
cis-QTLs at the gene level. With the DoD CDMRP Retina Database it is now
possible to look at the individual probes in exons and introns. When we
interrogate the DoD CDMRP Retina Exon Level Database, we find one probe
(Affy_17329472) that lies within the Leprel1 gene. When we checked the
location of the probe with the Verify function on GeneNetwork, the probe lies in
an intron and may be a non-coding RNA. However when we examined the RNA-
seq data from GeneNetwork it appears that this was detected in an RNA-seq
analysis of the hippocampus and thus this may be part of Lepre1 gene itself.
Nonetheless, this probe marks a candidate for modulating the expression of
Robo2. The second approach is to examine this region for nonsynonymous
SNPs. Using the SNP browser in GeneNetwork, we looked at Chromosome 16
(21-27 Mb) and found four known genes with nonsynonymous SNPs: Kng2,
Kng1, BC106179 and Masp1. This analysis provides us with five candidates for
modulating the expression of Robo2.

To determine whether this highly correlated set of genes in the Robo2
network have functional relationship(s), we examined the top 500 correlates of
Robo2 to determine if there were specific functional transcript enrichments using
correlates of Robo2 was enriched for a number of biological processes (nervous
system development, synaptic transmission and neuron differentiations);
molecular functions (enzyme binding, PDZ domain binding, inorganic cation
transmembrane transporter, and metal ion transmembrane transporter activity);
and cellular components (cell projection part, neuron projection, intracellular part,
and axon genes). This type of analysis plays a critical role in many genetic
networks, defining the functional role of the network. In this specific case the
analysis demonstrates that the Robo2 network is involved in axonal growth and
neuronal development.

DISCUSSION

This paper announces the release of two new BXD retina databases on
GeneNetwork. The first is at the gene level (DoD CDMRP Retina Affy MoGene
2.0 ST (May15) RMA Gene Level Database). The second dataset is exon level
analysis of the same data presented in the first dataset (DoD CDMRP Retina Affy
MoGene 2.0 ST (May15) RMA Exon Level Database). In this paper we attempt to
emphasize some of the special aspects of these two datasets including an exon
level of analysis, the inclusion of microRNAs and many non-coding RNAs. To
illustrate many of these new features we presented two different approaches for
analysis using the datasets.

The first was an examination of a cell signature within the dataset. Within the
DoD CDMRP Retina Database, there is a pronounced RPE signature. Some
strains demonstrate very low levels of expression of RPE65 while other strains
have over 16-fold higher levels of expression. This difference could not be due to
differences in expression within the RPE, for we know that all RPE cells express
this gene at approximately the same level. We believe that this is due to
differences in the time of day the retinas were isolated. The retinal samples at
two different locations. At the University of Tennessee samples were usually
isolated starting at 10:00 AM and lights on in the animal colony occurred at 6:00
AM. Thus, the retinas were isolated at least four hours after lights on. At Emory
University, the retinas were isolated starting at 9:00 AM and lights on occurred at
7:00 AM or starting at 2 hours after lights on in the animal colony. These
differences in the time of day the retinas were isolated may be related to the
number of RPE adhering to the retinal samples [25].

There are a number of bioinformatics tools available to the vision research
community. These include NEI Bank project
(http://neibank.nei.nih.gov/index.shtml), which provides transcriptome profiling of
the tissues of the eye including mouse and human [26]. The Cepko group has
provided the mouse retina serial analysis of gene expression (SAGE) library
(http://itstgp01.med.harvard.edu/retina) that includes gene expression of the
embryonic and postnatal retina [27, 28]. Steve Dager and his group have lists of
mapped loci and cloned genes associated with inherited retinal disease on the
RetNet website (www.sph.uth.tmc.edu/RetNet/). GENSAT (Gene Expression
Nervous System Atlas) now has a section devoted to the Retina Project [29] at
www.gensat.org/retina.jsp. The cell specific labeling in the retina for different
genes is illustrated using BAC transgenic mice. The pattern of labeling in the
retina defines the retinal cells types expressing specific genes. This cellular
localization aids in defining localization of genetic networks in the retina. Finally
we have posted the data from the study of glaucoma by Howell et al.[30] on the
GeneNetwork website under the BXD eye database. These data are very helpful
in understanding the role of specific genetic networks in glaucoma (for example,
see Templeton et al.[31]).

In conclusion, the DoD CDMRP Retina Databases offered on GeneNetwork is
new resource in an expanding variety of bioinformatics tools available to vision
research community. Previously we have offered several BXD microarray
databases on GeneNetwork included to the vision science community: the
transcriptome of the whole eye (Eye M430v2 (Sep08) RMA Database) described
in detail by Geisert et al.[18]; a normal retina database (Normal Retina (April
2010) RankInv Database) described in detail by Freeman et al.[19]; and the
retina 2 days after optic nerve crush (ONC Retina (April 2012) RankInv
Database) described in Templeton et al. [32]. This new website offers a unique
look at expression at the exon level. In addition, there are many non-protein
coding transcripts represented in the dataset. The bioinformatics tools offered on
GeneNetwork and these new databases are a unique resource for the vision
research community.

Acknowledgments

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Advanced Technology (to EEG), NIH Grant R01EY017841 (to EEG), Vision Core
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XiangDi Wang for her technical assistance in the early phases of this work.
REFERENCES


468 Figures

469 Figure 1 Expression of *Rpe65* across the BXD strains in the DoD CDMRP Normal Retina Dataset. The expression levels of *Rpe65* are shown for many of the BXD strains as mean expression and standard error of the mean. The individual strain identifications are shown along the bottom and the scale is log₂. Notice the relatively low levels of *Rpe65* in some stains (DBA/2J, BXD5 BXD12, BXD34, BXD40, BXD48a, BXD60, BXD69, BXD100, BXD101 and BXD102) and 8-fold high levels of expression other strains (BXD16, BXD31, BXD42, BXD43, BXD50, BXD56, BXD75 and BXD85).
Figure 2 Expression of Robo2 across the BXD strains in the DoD CDMRP Normal Retina Dataset. The expression levels of Robo2 are shown for many of the BXD strains as mean expression and standard error of the mean. The individual strain identifications are shown along the bottom of the plot and the scale is log₂. Notice the variability in Robo2 expression across the BXD strains.
Figure 3 The Pearson correlation between Robo2 and Ncam2. Ncam2 was the second highest correlate to Robo2 in the DoD CDMRP. Notice that in strains where Robo2 is highly expressed, Ncam2 is also highly expressed and that in strains where Robo2 is low, Ncam2 is also low.
Figure 4. Genome-wide Interval Map of Robo2. This genome-wide graph displays the quantitative trait loci (QTL) distribution across the DoD CDMRP Normal Retina Dataset. The X-axis plots the locations of the QTLs controlling the transcript expression. Positions are measured in mega-bases from Chromosome 1 to Chromosome X (1-2600 Mb). The y-axis plots Likelihood Ratio Statistic (LRS). The significant levels of individual QTLs are color-coded. The red line represents a genome-wide significance level ($p > 0.05$) and the gray line is suggestive. Notice a significant QTL on Chromosome 16.

Table 1. Correlates of Rpe65 in the DoD CDMRP Normal Retina Dataset.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Location (Chr: Mb)</th>
<th>Mean Expr</th>
<th>Sample r</th>
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<tbody>
<tr>
<td>A65</td>
<td>Retinal pigment epithelium 65</td>
<td>Chr3: 159,260,243</td>
<td>8.637</td>
<td>1.320</td>
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<td>Rgr</td>
<td>Retinal G protein-coupled receptor</td>
<td>Chr14: 37,860,076</td>
<td>10.680</td>
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<tr>
<td>Ptk1</td>
<td>Retinoid X receptor 1</td>
<td>Chr5: 5,118,059</td>
<td>7.608</td>
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<tr>
<td>Trn</td>
<td>Transferrin</td>
<td>Chr16: 20,327,561</td>
<td>10.858</td>
<td>0.846</td>
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<tr>
<td>Ernm</td>
<td>ERM-binding protein</td>
<td>Chr2: 57,639,526</td>
<td>7.713</td>
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<tr>
<td>Lcat</td>
<td>Leucine-rich acyltransferase 1</td>
<td>Chr3: 82,690,500</td>
<td>9.150</td>
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<tr>
<td>Rdh5</td>
<td>Retinol dehydrogenase 5</td>
<td>Chr10: 128,350,845</td>
<td>8.763</td>
<td>0.924</td>
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<tr>
<td>Scol2a</td>
<td>Soluble carrier family 5, member 20</td>
<td>Chr9: 123,345,240</td>
<td>8.364</td>
<td>0.916</td>
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<tr>
<td>Tcf7</td>
<td>T-cell factor 7</td>
<td>Chr3: 103,100,531</td>
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<td>Scl2a7</td>
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<td>Car12</td>
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<tr>
<td>Cw1n1</td>
<td>Cortyloseptin 1</td>
<td>Chr9: 148,360,577</td>
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<tr>
<td>Car12b</td>
<td>Calcium</td>
<td>Chr11: 5,666,465</td>
<td>8.982</td>
<td>0.978</td>
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<td>Ptk1</td>
<td>Pyruvate kinase, muscle</td>
<td>Chr12: 59,504,170</td>
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<td>Thbd</td>
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<td>Abl1</td>
<td>C-terminal Src kinase</td>
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<td>8.015</td>
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<td>Igsf4</td>
<td>Integrin beta 8</td>
<td>Chr12: 120,394,955</td>
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<td>Rtn</td>
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<td>Turn1</td>
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<tr>
<td>Chond2</td>
<td>Chondrocyte-derived 1</td>
<td>Chr16: 32,420,736</td>
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<td>Gm2565</td>
<td>Predicted gene</td>
<td>Chr15: 160,326</td>
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<td>Hexp5</td>
<td>Haptoglobin-related protein 5</td>
<td>Chr11: 31,909,138</td>
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<td>Cthn2</td>
<td>Cthn 2</td>
<td>Chr1: 134,436,029</td>
<td>8.845</td>
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<tr>
<td>Olfr175</td>
<td>Odorant receptor 175</td>
<td>Chr9: 37,560,222</td>
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<td>Gm86522</td>
<td>Predicted gene</td>
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<td>Ssef2</td>
<td>Selenoprotein 2</td>
<td>Chr3: 134,060,499</td>
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<tr>
<td>Nrnx2</td>
<td>Neurexin II</td>
<td>Chr19: 6,419,371</td>
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<tr>
<td>Olfr175</td>
<td>Odorant receptor 176</td>
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<td>Suz2a</td>
<td>Synaptic vesicle glycoprotein 2a</td>
<td>Chr9: 95,056,576</td>
<td>12.192</td>
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<td>Temt181b</td>
<td>Transmembrane protein 181B</td>
<td>Chr1: 24,36,190</td>
<td>10.916</td>
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<td>Ptdet5</td>
<td>Programmed cell death 5</td>
<td>Chr1: 191,101,817</td>
<td>11.281</td>
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<tr>
<td>Fap</td>
<td>Fibroblastic activation protein</td>
<td>Chr2: 62,339,900</td>
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<td>Caspa9g</td>
<td>Calcium channel, voltage-dependent</td>
<td>Chr11: 3,14,070,705</td>
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<td>Rhoqf3</td>
<td>Ras guanyl-nucleotide exchange factor (GEF) 3</td>
<td>Chr2: 29,745,940</td>
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<td>Nofc2</td>
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<td>Chr6: 80,263,073</td>
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<td>Use2b</td>
<td>Ubiquitin-conjugating enzyme E2B</td>
<td>Chr2: 51,796,898</td>
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<td>Acea1</td>
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<td>Chr2: 125,366,129</td>
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<tr>
<td>Acya3</td>
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<td>Sic1a7</td>
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<td>Sno12d</td>
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<td>Lpdpd4</td>
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<td>Vasa</td>
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<td>Scl12a5</td>
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<td>Cad9</td>
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<td>Chr1: 24,207,996</td>
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<td>Cdh1</td>
<td>Complement component D3</td>
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<td>Dgla</td>
<td>Diacylglycerol alpha</td>
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<td>Pcdh4cp</td>
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<td>Chr10: 7,943,251</td>
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<tr>
<td>Akin1</td>
<td>Amyloid beta (A4) precursor protein binding, A1, membrane</td>
<td>Chr2: 24,085,316</td>
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<tr>
<td>Zincf14a</td>
<td>Zinc finger, CCCH domain-containing 14</td>
<td>Chr14: 12,628,33</td>
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<td>Sst1</td>
<td>Signal sequence receptor, membrane</td>
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<td>Mfcr6t1</td>
<td>MetAP domain containing 1</td>
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<tr>
<td>Apob1c</td>
<td>Amyloid beta (A4) precursor protein binding, A2, membrane</td>
<td>Chr12: 23,533,508</td>
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<tr>
<td>Acod13</td>
<td>Acyl-CoA dehydrogenase</td>
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<tr>
<td>Rb1</td>
<td>Retinoblastoma-like 1 (Rb107)</td>
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<td>Cpxn2</td>
<td>Carboxypeptidase E2 (M4 family)</td>
<td>Chr7: 139,23,4493</td>
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<td>Ret</td>
<td>Retinoic acid receptor</td>
<td>Chr5: 118,10,755</td>
<td>10.226</td>
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<tr>
<td>Mossd1</td>
<td>Motile sperm domain containing 1</td>
<td>ChrX: 50,605,815</td>
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<tr>
<td>Lpl</td>
<td>Low density lipoprotein receptor-related protein 3</td>
<td>Chr5: 35,094,980</td>
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</table>
Table 2. MicroRNAs in the Rpe65 signature.

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<th>Mir98</th>
<th>Mir449a</th>
<th>mir301b</th>
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<td>Zcchc14</td>
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Table 3. Top 20 correlates of Robo2.

<table>
<thead>
<tr>
<th>Symbol</th>
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<th>Mean Expr</th>
<th>Sample r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robo2</td>
<td>roundabout homolog 2 (Drosophila)</td>
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<td>Gsk3</td>
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<td>Lamin5</td>
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<td>Thbr1</td>
<td>thrombin 1</td>
<td>Chr12:974065</td>
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<td>0.98</td>
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Upregulation of SOX11 in Retinal Ganglion Cells Following Injury

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Abstract

Purpose: The present study was designed to identify early changes associated with injury of retinal ganglion cells.

Methods: The normal retina database and optic nerve crush (ONC) database on GeneNetwork (www.genenetwork.org) were used to identify markers of retinal injury. One gene, Sox11, was examined further using two neuronal injury paradigms, ONC and blast injury to the eye of C57BL/6 mice. The distribution of SOX11 was determined using indirect immunohistochemical methods and the levels of SOX11 protein expression were defined by semi-quantitative immunoblot methods. In situ hybridization was performed using the Affymetrix 2-plex Quantigene View RNA In Situ Hybridization Tissue Assay System.

Results: SOX11 was dramatically upregulated in the retina following ONC and blast injury. The level of Sox11 message increased by approximately 8-fold 2 days after ONC. In the normal retina, there was only light immunostaining for SOX11 in retinal ganglion cells and cells in the inner nuclear layer. After ONC or blast injury, the staining intensity increased dramatically in both layers. In situ hybridization demonstrated a similar distribution of message for Sox11 in the normal retina as well as a profound increase in Sox11 message within the ganglion cells following ONC.

Conclusion: Taken together, these data indicate that Sox11 may be involved in the initial response of the retina to injury, playing a role in the early attempts of the ganglion cells to survive.
Introduction:

Advances in our ability to monitor molecular changes in neurons have led to an increased understanding of the events that transpire following neuronal injury [1-5]. After axonal damage, the initial response of a central nervous system (CNS) neuron may be similar to that of a neuron in the peripheral nervous system (PNS) [6]. Ultimately, neurons in the PNS will regenerate their axons and survive, while those in the CNS do not regenerate their axons and the cell bodies die. The initial response of the CNS neurons to regenerate and eventual failure of this regenerative response was described by Ramón y Cajal and termed “abortive regeneration” [7]. When examining the initial response of neurons to injury, there appear to be some common responses in the CNS and PNS. One transcription factor activated in both the CNS and PNS after injury is Sox11 [8]. There is strong evidence that this gene is part of the transcriptional network activated by injury and involved in axonal regeneration in the PNS [9, 10].

Sox11 is a member of the SRY-related box group C (SoxC) gene family of transcription factors [11, 12]. SOX11 along with SOX4 play a critical role in the normal development of neurons and specifically retinal ganglion cells [13-15]. SOX11 is expressed in retinal progenitor cells as part of the process leading progenitor cells to become neuroblasts [16, 17]. In knock-downs of either Sox11 or Sox4, there is a moderate reduction in retinal ganglion cell number; however when both Sox11 and Sox4 are knocked-down, there is a complete loss of ganglion cell development [13]. During eye development, Sox11 is also required to maintain proper levels of hedgehog signaling, and mutations have been associated with coloboma due to improper optic fissure closure [18, 19]. Furthermore, SOX11 is critical for axonal growth, driving the expression of axon growth-related proteins such as class III beta tubulin and MAP2 [20]. SOX11 also plays a similar role in adult neurogenesis. High levels of SOX11 are found in the cells within the subventricular zone, the rostral migratory stream and within the
neuroprogenitor zone of the dentate gyrus [15, 16, 21]. These studies underline the importance of SOX11 in terminal differentiation of progenitor cells to neurons and axon extension.

In addition to functioning in neuronal differentiation, SOX11 has a prominent role in the response of neurons to injury. After peripheral nerve injury, SOX11 is immediately upregulated in the neuronal cell bodies as the axon is regenerating [9, 22]. Decreasing levels of SOX11 in the neuronal cell body results in slower axonal regeneration of peripheral nerves [9]. Similar results are observed in tissue culture. When Sox11 is knocked down in cultured peripheral neurons, there is also a reduction in neurite growth and an increase in apoptosis [23]. Conversely, overexpressing Sox11 in cultured dorsal root ganglion cells produces an increase in neurite growth, and in vivo overexpression of Sox11 accelerates the growth of regenerating axons [10]. One intriguing anatomical experimental model is the dorsal root ganglion, where the central projection of the dorsal root ganglion enters the spinal cord (CNS) and the peripheral projection extends out into a peripheral nerve that is myelinated by Schwann cells. When the central rootlet is severed, there is a modest (51%) increase in Sox11 expression in the ganglion even when the central portion will not regenerate back into the spinal cord. However, when the peripheral root is damaged, a relatively massive (1004%) increase in Sox11 is seen as the axons regenerate down the peripheral nerve.

In the present study, we examine the role of SOX11 in the retina following injuries to the axons of the optic nerve. We propose that the upregulation of SOX11 after injury is an attempt of neurons to regenerate, but ultimately results in abortive regeneration and cell death.
Materials and Methods

Optic Nerve Crush. The Optic Nerve Crush procedure was performed as previously described in Templeton et al [24]. Briefly, C57BL/6 (n = 5) mice were deeply anesthetized with 13 mg/kg Rompum and 87 mg/kg Ketalar for the surgery. An incision was made into the lateral aspect of the conjunctiva, and the eye was rotated nasally to expose the optic nerve. The optic nerve was then grasped for 10 seconds with Dumont cross-clamp #7 forceps (Roboz, cat. #RS=5027, Gaithersburg, MD), using only the spring action of the instrument to crush the nerve. The instrument was then removed and the eye was allowed to rotate back into place. The animals were allowed to recuperate from surgery on a water-heated warming pad. The Institutional Animal Care and Use Committee approved all procedures for the surgery and handling of mice (at the University of Tennessee Health Science Center and Emory University).

Blast Injury to the Eye. Blast injuries to the eye were produced as previously reported in Hines-Beard et al [25]. Briefly, the mice were deeply anesthetized and secured in a PVC pipe. A 50psi overpressure wave was delivered selectively to the right eye of C57BL/6 mice (n = 5). The output pressure of the blast apparatus was measured immediately before and after the procedure, ensuring an accurate and precise injury. The animals were sacrificed 5 days after the blast injury. The Institutional Animal Care and Use Committee approved all procedures for the surgery and handling of mice (at the University of Tennessee Health Science Center and Emory University).

Immunoblot Analysis. Protein samples from 12 retinas were examined to determine the relative levels of SOX11. Animals were anesthetized with a mixture of xylazine (13 mg/kg, Rompum) and ketamine (87 mg/kg, Ketalar) and following a cervical dislocation the retinas were removed. The dissected retinas were placed in 1X reducing sample buffer (2% SDS and 10% glycerol in 0.05 M Tris-HCl buffer, pH 6.8). Equal amounts of protein were loaded onto each lane of
an SDS PAGE gel. The balance of the proteins was determined by coomassie blue staining and subsequent quantification of total protein in each lane. To analyze the concentrations of SOX11 in the retina, the proteins were transferred to PVD membranes. The blots were blocked with 2% non-fat dry milk in phosphate buffer (pH 7.4) and probed overnight with the rabbit anti-SOX11 primary antibody (Santa Cruz Biotechnology, Inc. California). We then rinsed the blots and probed them with the HRP-labeled donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania). The blots were rinsed with 0.5 M Tris buffer (pH 7.4) and reacted with 0.05% DAB and hydrogen peroxide. The blots were washed and visualized using an ECL detection kit (Thermo Fisher Scientific Inc. Rockford, Illinois) and Kodak 4000 MM image station. To define the amount of protein loaded onto each lane the blot was stripped and restained for actin using a mouse anti-B-Actin antibody (A5441, Sigma Aldrich, St Louis MO) followed by an HRP-labeled Donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania). The blots were then rescanned.

**Immunohistochemistry.** For the immunohistochemical analysis of the distribution of SOX11, ten C57BL/6 mice (2 control mice and 2 mice of each group at both 2 and 5 days postoperatively) were used. The mice were deeply anesthetized with 2,2,2-tribromoethanol and then perfused transcardially with saline followed by 4% paraformaldehyde using a peristaltic pump (Cole Parmer Instruments, Chicago, IL). After an hour of post-fixation in paraformaldehyde at room temperature, retinas were rinsed in 0.1M phosphate-buffered saline (PBS), embedded in 4% low-melt agarose, and cut on a vibratome into 50 µm thick slices. Sections were then blocked in 4% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS with 0.1% Triton X-100 (Sigma) on a rotating platform for 1 hour, washed 3 times and incubated with primary antibody at 4°C overnight. The next day, sections were rinsed again and then incubated in Alexa Fluor-conjugated secondary antibody (Life Technologies). After that, sections were rinsed in PBS, mounted with Fluoromount (Southern Biotech, Birmingham, AL).
and coverslipped. The following primary antibodies and dilutions were used: anti-SOX11 (US Biological S5364-40C, 1:500), anti-TUJ1 (Tuj1, a gift from Anthony Franfurter [26]), and anti-PKC alpha (abcam ab32376, 1:250). The following anti-SOX11 antibodies were tried for Immunohistochemistry but did not work: Santa Cruz sc-20096, abcam ab170916, biorbyt 101332, and LifeSpan Biosciences LS-C10306.

**Confocal Microscopy.** The sections were scanned and 10 µm thick z-stacks were acquired on a Eclipse Ti confocal microscope (Nikon Inc., Melville, NY) equipped with Nikon C1 software. Scans were taken using a 40x Plan Fluor 40x/1.30/0.20 Oil objective. Laser power and gain were kept constant for all pictures. Images were loaded into Fiji [27] and average intensity projections were created. The final composition of the images was made using Photoshop CS 6 (Adobe Systems Inc., San Jose, CA).

**In Situ Hybridization.** *In situ* hybridization was performed using the 2-plex Quantigene View RNA ISH Tissue Assay kit (Affymetrix, Inc., Santa Clara, California). Assays were performed as per the manufacturer's instructions with stock solutions. Eyes were isolated from C57BL/6 mice (n = 6), both control and two days after optic nerve crush, and drop-fixed in 4% Paraformaldehyde for 24 hours. Mid-way through the 24-hour fixation, a 26-gauge needle was used to create a hole to the vitreous humor, assisting with the fixation. Immediately after the 24-hour period, the eyes were serially dehydrated in ethanol and embedded in paraffin. Blocks were sectioned on an American Optical series 1000 microtome (American Optical Co. Buffalo, New York) to a thickness of 5 µm and mounted on Surgipath X-tra micro slides (Leica Biosystems Richmond Inc., Richmond, Illinois). Before beginning the hybridization protocol, the slides were baked at 60°C for 30 minutes to increase tissue adhesion. As per the manufacturer's protocol, the paraffin was removed from the slides with xylene before being boiled in a pretreatment solution (Affymetrix) for 10 minutes and incubated with Protein
Kinase K [Affymetrix Santa Clara, CA] at 40°C for 10 minutes. Custom probes for Sox11 and ChrmA6 [Affymetrix Santa Clara, CA] were then hybridized to the tissue. Signal amplification was accomplished by hybridizing Type 1 and Type 2 specific pre-amp oligonucleotides, amp oligonucleotides and label oligonucleotides sequentially, achieving a 400-fold signal amplification from each mRNA molecule. Sections were viewed with an Olympus BX51 microscope (Olympus America Inc., Melville, New York).
Results

We first compared Sox11 mRNA levels in normal mice to Sox11 levels following ONC using the bioinformatic tools on GeneNetwork (genenetwork.org) and two preexisting databases generated by us: Normal HEI Retina (April 2010) and ONC HEI Retina (April. 2012) [4, 28]. Sox11 was one of the genes with the largest change in expression two days after optic nerve crush. In the normal retinal dataset, the mean expression for Sox11 (detected by Illumina probe ILMN_1235647) across the BXD RI strains was 8.4 on a 2 Z + 8 Log_2 scale (this is just above the mean detection level of mRNA on the array, which is set to 8). In the C57BL/6 parental strain, the expression level in the normal retina was 8.59 and for the DBA/2J strain the mean expression level was 8.54. Two days after ONC, there was a dramatic increase in the level of Sox11 expression (Figure 1), with the mean expression across the BXD strains being 11.03, which corresponded to an approximately 8-fold increase. The same increase was observed in individual strains. The C57BL/6 strain had an expression level of 11.33 after ONC and the expression in the DBA/2J strain increased to 11.44. These data indicate that Sox11 is dramatically upregulated after a specific injury to the ganglion cell axons within the optic nerve.

In situ hybridization was used to identify the cells expressing Sox11 after injury to the retina. For the in situ hybridization, we examined retinas two days after optic nerve crush (Fig. 2B) and compared it to uninjured control retinas (Fig. 2A). Using the Affymetrix 2-plex Quantigene View RNA ISH Tissue Assay kit, we labeled cells expressing the retinal ganglion cell marker Chrna6 blue and Sox11 red (Fig. 2) [3]. In the control retina, many of the cells in the ganglion cell layer were heavily labeled for Chrna6, and a few of the cells expressed low levels of Sox11. Two days after optic nerve crush there was a dramatic decrease in the labeling for Chrna6 and a substantial increase in the amount of labeling for Sox11. This was similar to the changes in message levels observed in our microarray databases on GeneNetwork.org. In the normal retina database, Chrna6 (probe ILMN_2732438) was expressed at relatively high levels (mean
value of 11.04) and two days following optic nerve crush, the expression
decreased two-fold (mean value of 9.96). These data from the in situ
hybridization mirror our microarray expression analysis results.

Indirect immunohistochemistry was used to define the distribution of SOX11
protein in the retina. To identify the cell types expressing SOX11 after retinal
injury, we stained the retina for SOX11 and for class III beta tubulin, a known
ganglion cell marker [26]. In these double-labeled sections, staining for SOX11
was colocalized with class III beta tubulin in retinal ganglion cells (Fig. 3),
indicating that they were also positive for SOX11. Both markers labeled the same
population of cells in the ganglion cell layer and the labeling extended out into
dendritic processes. The staining pattern also indicated that SOX11 was present
in the cytoplasm and the nucleus. Even though nuclear labeling was light in
comparison, there were many cases where a negative spot (presumably the
nucleolus) could be observed. These data demonstrate that SOX11 is present in
injured retinal ganglion cells.

Using immunohistochemistry, we also examined the change in expression of
SOX11 following optic nerve crush (Fig 4B and 4C) and a blast injury to the eye
(Fig. 4E and 4F). In both cases, there was a noticeable increase in ganglion cell
staining relative to that observed in the uninjured control section (Fig. 4A). The
staining was primarily cytosolic and extended out into the dendritic processes of
the cells. In the normal retina, we could identify light labeling of cells within the
inner nuclear layer. The intensity of labeling within the retinal ganglion cells
increased dramatically in the injured retinas (Fig. 4B, 4C, 4E and 4F), and there
was also an increase in staining of cells within the inner nuclear layer, which
colocalized partly to bipolar cells (Fig. 5).

The upregulation of SOX11 following ONC was confirmed with Immunoblotting.
The intensity of the SOX11 band from retinas after nerve crush (Fig. 6, lanes A
and C) is higher than in the control retina samples (Fig. 6 lanes B and D). Thus,
there is a dramatic increase in the expression of both Sox11 mRNA and SOX11 protein following retinal injury.
In the present study, we demonstrate that SOX11 is upregulated following retinal injury. For retinal ganglion cells, there are many known markers, such as: Chrna6, Pou4f1, Tubb3, Thy1, and Sncg [3, 13, 29-33]. Using these marker genes, we performed a meta-analysis of their expression in injured and uninjured retinas (data not shown). Our injury paradigms included controlled optic nerve crush and selective blast injury to the eye, procedures that are known to induce retinal ganglion cell apoptosis [24, 25]. As expected, the expression of these retinal ganglion cell markers decreases as the ganglion cells die. Our data indicate an overall reduction in expression of these markers, both 2 and 5 days after retinal insult [34]. Specifically, a 3-fold reduction in Sncg expression is observed, Chrna6 and Pou4f1 are down regulated 2-fold, and, while not as robust, Thy1 and Tubb3 also show a decrease in expression. Collectively, these data demonstrate that known retinal ganglion cell markers decrease as the optic nerve degenerates.

Unlike these markers for retinal ganglion cells, Sox11 levels increase substantially following injury to the retina and optic nerve. Two days after ONC, there was an almost 8-fold upregulation of Sox11. We wondered if this increase in expression was specific to the severity of retinal injury, and queried a publicly available microarray dataset of glaucomatous mice presented by Howell and colleagues as an independent test on the role of Sox11 in the response of the retina to injury [1]. In this DBA/2J mouse model of pigment dispersion glaucoma, there were relatively low levels of Sox11 in the control mice and in mice that did not have detectable levels of ganglion cell loss (Fig. 7). However, in mice with moderate ganglion cell loss, levels of Sox11 were approximately four-fold higher than in control animals. In animals with severe glaucoma, the level of Sox11 decreased to near control levels. These data suggest that the levels of Sox11 decrease as the ganglion cells die. This transient upregulation of Sox11 in moderate cases demonstrates the exquisite ability of Sox11 to mark injured,
potentially dying neurons. As such, our data indicate that Sox11 is a novel marker for injured neurons, specifically retinal ganglion cells.

What is the potential role of SOX11 following injury to the axons of the retinal ganglion cell? One hint comes from studies on the response of dorsal root ganglion neurons to peripheral nerve injury. Sox11 is dramatically upregulated in the dorsal root ganglion following injury to the peripheral nerve and plays a pivotal role in axonal regeneration [9]. Three days after the transection of the sciatic nerve in the rat, there is a 1004% increase in Sox11 in the dorsal root ganglion and these levels remain elevated for at least the next 4 days [9]. By 4 weeks after transection, Sox11 levels have returned to baseline. This upregulation and sustained expression of Sox11 is critical to the survival of the dorsal root ganglion neurons and the regeneration of peripheral axons along the injured nerve. When the levels of Sox11 were knocked down by delivery of siRNA, there was an increase in apoptosis in cultured neurons as well as a decrease in neurite outgrowth in culture [23]. A similar decrease in axonal regeneration occurred in vivo after Sox11 knockdown in the dorsal root ganglion [9]. Thus, the upregulation of Sox11 appears to be necessary for the normal regeneration of axons in the peripheral nerve. Interestingly, Sox11 was also upregulated when the central projection of the dorsal root was severed; however, the degree of upregulation was lower, only 51% [9, 23]. Just like the neurons in the retina, the dorsal root ganglia did not regenerate their central projections into the adjacent spinal cord [35, 36]. This begs the question, why is Sox11 upregulated in neurons of the retina and the dorsal root ganglion, when the axons will not successfully regenerate? The most parsimonious explanation is that these neurons are attempting to survive using the same program that is successful in peripheral nerve injury; however, other influences derail the regenerative program causing the cell to abort the regenerative process and, in many cases, cause subsequent neuronal death [37-40]. Recent evidence indicates that these inhibitory influences can at least be partially averted by stimulating the ganglion cells to regrow their axons [41-47]. Given that
upregulation of Sox11 is among the initial responses of the neuron to injury and that this response is intact in retinal ganglion cells, it may be possible to identify where the transcriptional cascade leading to axon regeneration and cell survival in the CNS differs from that in peripheral nerve injury.
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References


Figure 1. **Sox11 Expression in the Normal Retina and After Optic Nerve Crush.** The ordinate represents mRNA levels from microarrays, expressed in a log2 scale with the mean set to 8. The mice used to generate these data were C57BL/6J, DBA/2J, their respective F1 crosses, BALB/cByJ, and members of the BXD recombinant inbred strain line. The top graph denotes the expression levels of Sox11 under normal conditions, while the bottom graph denotes the expression of Sox11 two days after the optic nerve crush. Note the dramatic increase in expression following injury, from a normal mean expression value of 8.4 to a mean expression value of 11.0 after nerve crush.
Figure 2. In Situ Hybridization for Sox11 and Chrna6. The distribution of Sox11 and Chrna6 mRNA was defined using two-color in situ hybridization for a section of control retina (A) and a section of a retina 2 days after optic nerve crush (B). The probe for Chrna6 is shown in blue and that for Sox11 in red. Note the extensive staining for Chrna6 within the ganglion cell layer in the control section (Arrow in A). Two days after optic nerve crush there is already a substantial decrease in Chrna6 expression. Very low levels of Sox11 are seen in the control retina. There is a dramatic increase in Sox11 staining after nerve crush within the retinal ganglion cells (Arrow in B). A and B are taken at the same magnification and the scale bar in B = 25 µm.
Figure 3. SOX11 Expression in Retinal Ganglion Cells. A section of retina, two days after optic nerve crush, was double stained for SOX11 (A) and class III beta tubulin (B), using double label indirect immunohistochemistry. Class III beta tubulin is a marker for retinal ganglion cells. In the merged image (C), retinal ganglion cells are double labeled (arrowhead) with SOX11 and Class III beta tubulin. Scale bar in C = 20 µm.
Figure 4. Upregulation of SOX11 After Retinal Injury.

Retinal sections were stained with an antibody directed against SOX11 in the following conditions: normal retina (A), retina two (B) and five (C) days after optic nerve crush, and retina two (E) and five (F) days after blast injury. The negative control without primary antibody is shown in (D). After injury to the retina, there is a dramatic increase in the intensity of staining within the cell bodies of retinal ganglion cells, and some cells in the INL. The layers of the retina are labeled: ONL (outer nuclear layer), INL (inner nuclear layer) and GCL (ganglion cell layer). All photomicrographs are presented at the same magnification and the scale bar in F = 20 µm.
Figure 5. Upregulation of SOX11 in the Inner Nuclear Layer Following Injury.

Retinas were stained with antibodies directed against PKC alpha to label bipolar cells (A) and Sox11 (B) two days after optic nerve crush. In the merged image (C), about half of the bipolar cells are double-labeled, and they are mostly localized to the inner part of the INL (arrowheads).

The layers of the retina are labeled: ONL (outer nuclear layer), INL (inner nuclear layer) and GCL (ganglion cell layer). All photomicrographs are presented at the same magnification and the scale bar in C = 20 µm.
Figure 6. Upregulation of SOX11 protein after optic nerve crush.

Immunoblots of protein samples from normal retina (B and D) and from retinas 2 days after optic nerve crush (A and C) were probed with antibodies directed against SOX11. There was increased staining of the bands from the optic nerve crush samples relative to the normal retinal samples. Loading control in E-H: beta-actin.
Figure 7. **Sox11 mRNA Levels in Pigmentary Glaucoma.** Sox11 levels in a meta-analysis of a DBA/2J mouse pigmentary glaucoma dataset from Howell et al. (2011). The ordinate represents the Sox11 mRNA level from microarrays, expressed in log$_2$, scaled with the mean set to 8. The mice were classified as wild type Gpnmb$^{+/+}$ controls (Gpnmb+ Controls), no detectable glaucoma 1 (NOE 1), no detectable glaucoma 2 (NOE2), moderate glaucoma (Moderate), and severe glaucoma (Severe). Other than the wild type group, all the groups were Gpnmb$^{-/-}$. Notice that there is a dramatic increase in Sox11 expression during early phases of glaucoma in this mouse model. As the ganglion cells die in severe glaucoma, the expression levels of Sox11 appear to decrease. These data suggest that Sox11 is an early marker for retinal injury. This general expression pattern was observed for the following 5 of the 6 Affymetrix probe sets targeting Sox11: 1429051_s_at (illustrated above), 1453002_at, 1429372_at, 1453125_at and 1431225_at.