Retinal Cell Responses to Elevated Intraocular Pressure: A Gene Array Comparison between the Whole Retina and Retinal Ganglion Cell Layer

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PURPOSE. To determine and compare gene expression patterns in the whole retina and retinal ganglion cell layer (RGCL) in a rodent glaucoma model.

METHODS. IOP was unilaterally elevated in Brown Norway rats ($N = 26$) by injection of hypertonic saline and monitored for 5 weeks. A cDNA microarray was used on whole retinas from one group of eyes with extensive optic nerve injury and on RGCL isolated by laser capture microdissection (LCM) from another group with comparable injury, to determine the significantly up- or downregulated genes and gene categories in both groups. Expression changes of selected genes were examined by quantitative reverse transcription-PCR (qPCR) to verify microarray results.

RESULTS. Microarray analysis of the whole retina identified 632 genes with significantly changed expression (335 up, 297 down), associated with 9 upregulated and 3 downregulated biological processes. In contrast, the RGCL microarray yielded 3726 genes with significantly changed expression (2003 up, 1723 down), including 60% of those found in whole retina. Thirteen distinct upregulated biological processes were identified in the RGCL, dominated by protein synthesis. Among 11 downregulated processes, axon extension and dendrite morphogenesis and generation of precursor metabolism and energy were uniquely identified in the RGCL. qPCR confirmed significant changes in 6 selected messages in whole retina and 11 in RGCL. Increased *Atf3*, the most upregulated gene in the RGCL, was confirmed by immunohistochemistry of RGCs.

CONCLUSIONS. Isolation of RGCL by LCM allows a more refined detection of gene response to elevated pressure and improves the potential of determining cellular mechanisms in RGCs and their supporting cells that could be targets for enhancing RGC survival. (*Invest Ophthalmol Vis Sci.* 2010;51:3003–3018) DOI:10.1167/iovs.09-4663

In glaucoma, retinal ganglion cells (RGCs) have been shown to die by apoptosis.^{1,2} However, the nature of the initial insult to RGCs has not been identified, and it is not clear at

what point this programmed cell death is triggered. Studies in primates³ and rodents⁴ have described morphologic changes in RGCs, including size reduction of the dendritic field, axon atrophy, and soma shrinkage in experimental glaucoma. This finding implies that RGCs undergo gradual structural and probably functional changes well in advance of their commission to apoptotic death and suggests that there is a time window for neuroprotective intervention that could be used to salvage these RGCs. To take advantage of this opportunity, we must understand the cellular events and molecular mechanisms occurring in RGCs within this time window.

The technique of gene microarrays has been increasingly applied to glaucoma research.^{5–10} As a high-throughput genome analysis tool, gene microarrays provide the possibility of studying global gene expression in pathologic conditions and of identifying underlying molecular mechanisms by examining thousands of genes at one time. Several studies have used gene microarrays to characterize retinal responses in experimental glaucoma and have reported gene expression changes associated with processes like immune response and glia activation.^{5,7–9} However, these studies, performed on whole-retina samples, have generally identified only a limited number of regulated genes, particularly genes and gene categories specifically reflecting RGC dysfunction. Because analysis of the whole retina will reflect gene regulation in retinal layers other than the RGC layer (RGCL), changes specific to the RGC may be masked by this approach.

In this study, we tested the hypothesis that gene microarray applied specifically to tissue from the RGCL will provide a more robust analysis of RGC responses to elevated IOP. Using our rat glaucoma model, we applied cDNA microarray analysis to determine gene expression changes in whole retinas of eyes with extensive pressure-induced optic nerve degeneration and performed an identical analysis on tissue from just the RGCL, isolated by laser capture microdissection (LCM). The comparison of results from these two tissue sources at the message level revealed a striking increase in the number of changed genes and affected gene categories in the RGCL. The results suggest that this approach can be used to advance our knowledge of specific cellular events preceding RGC death and to identify potential RGC-specific molecular mechanisms that could be targeted by new therapeutic strategies.

METHODS

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Oregon Health and Science University (OHSU) for use in all the experiments.

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Glaucoma Model

Eight-month-old Brown Norway rats were housed in constant low-level light.¹¹ Aqueous outflow obstruction was created by injecting hypertonic saline unilaterally into episcleral veins, resulting in sustained elevation of IOP.¹² IOP was measured in conscious animals at least three times a week with a handheld tonometer (Tono-PenXL for the whole-retina study; Medtronic, Minneapolis, MN, and TonoLab for the RGCL study; Tiolat Oy, Helsinki, Finland). A weighted mean IOP elevation was calculated for each injected eye by dividing the cumulative IOP by the number of postinjection days. The cumulative IOP was determined as the area under the curve of the plot of days after injection versus corresponding IOP measurement, subtracting the mean of the corresponding values for control eyes.

Tissues were harvested for further analysis 5 weeks after the injection. Optic nerve injuries resulting from IOP elevation were evaluated as previously described.¹¹ Briefly, optic nerves were removed and postfixed in glutaraldehyde and embedded in plastic, and nerve cross sections were graded from 1 (no injury) to 5 (active degeneration involving the whole nerve area) by five masked observers. Nerves with injury grades between 1.5 and 4 demonstrated focal lesions of increasing size, with 5% to 50% of axons degenerating. In nerves with injury grades greater than 4, more than 50% of the axons were degenerating. Tissues from eyes with injury grades greater than 4.8 were used for these studies ($n = 26$ glaucoma model) along with 32 noninjected control eyes. Previous direct comparisons of injury grade obtained by this system to actual axonal counts by transmission electron microscopy in the same optic nerves of glaucoma model eyes have demonstrated that optic nerves with a grade 5 injury will have an axon count that is approximately 45% of that in a normal optic nerve.¹¹

Laser Capture Microdissection

Whole globes were collected, immediately immersed in 100% ethanol with a volume >10 mL and fixed overnight at 4°C. The cornea was carefully cut open to allow immediate access of the fixative to the inner retina. After fixation, the globes underwent dehydration by two washes in 100% ethanol and four washes in xylene and then paraffin infiltration before embedding. Vertical 10- μ m sections were cut from the central globe to include both superior and inferior portions of the retina and were mounted on regular glass slides (Super Up-rite; Richard-Allen Scientific, Kalamazoo, MI). Caution was taken during the whole sectioning process to avoid RNase contamination.

Air-dried sections were deparaffinized immediately before LCM, which was performed no later than 24 hours after sectioning. The RGCL along the entire length of the section was captured onto LCM caps (Capsure Macro) with an LCM system (Arcturus PixCell II; both from Molecular Devices, Sunnyvale, CA). Typical laser settings were 7.5 μ m for spot diameter, 0.7 to 1.2 ms for duration, and 60 to 80 mW for laser power. The RGCL was easily identified by the morphologic appearance of the retinal layers in unstained tissue sections using the optics of the LCM microscope (Fig. 1). In each globe, the RGCLs were captured from multiple sections (average, 9–12) to maximize RNA yield and were dissolved in a GITC-based, β -mercaptoethanol-containing buffer solution (Arcturus PicoPure RNA Isolation Kit; Molecular Devices) immediately after LCM.

RNA Isolation and Amplification

Total RNA was purified from quick-frozen (–70°C), whole retinas, as previously described,¹³ or from the RGCL (Arcturus PicoPure RNA Isolation Kit; Molecular Devices) and quantitated with a fluorometric assay (RiboQuant; Invitrogen-Molecular Probes, Eugene, OR). All RNA samples were linearly amplified with a kit (MessageAmp aRNA Amplification Kit; Ambion, Austin, TX) that was developed based on the aRNA amplification procedure involving *in vitro* transcription by T7 RNA polymerase.¹⁴ For LCM, two rounds of amplification were needed to provide adequate material for microarray analysis. The integrity of the amplified RNA was assessed on a bioanalyzer (model 2100; Agilent Technologies, Palo Alto, CA).

FIGURE 1. Isolation of RGCL from ethanol-fixed, paraffin-embedded retinal sections by LCM. Distinct retinal layers were discernible without staining, as shown in (A), a retinal section before LCM. (B) Remaining tissue from the same section after LCM. (C) Captured RGCL tissue on the LCM cap. IPL, inner plexiform layer; INL, inner nuclear layer. Scale bar, 50 μ m.

Microarray Experimental Design

Gene expression changes in whole retina and LCM-isolated RGCL were examined by comparing samples from eyes with extensive optic nerve injury (grade 4.9–5.0 and without evidence of gliosis)⁶ to controls using the SMCmou8400A and SMCmou600A cDNA microarrays at the OHSU Gene Microarray Shared Resource Facility. These cDNA microarrays contain more than 15,000 cDNA probes and represent the initial NIA (National Institute of Aging) 15k mouse library (<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>/ National Institutes of Health [NIH], Bethesda, MD). The experimental design for the RGCL study is shown in Figure 2. Each microarray determined expression in RGCL obtained from a single globe. Therefore, each experimental group consisted of independent, biological replicates. Because one of the glaucoma model samples failed to amplify properly, it was excluded from the study, resulting in a final comparison of expression in five glaucoma model RGCLs to that in six control RGCLs. A comparable design was used for

FIGURE 2. Experimental design of the RGCL cDNA microarray study. Gene expressions in six glaucoma model RGCLs (G1–G6) were compared to six control specimens (C1–C6) on 12 separate arrays. Each array compares (*double arrows*) gene expression in the RGCL from a single independent globe to that in the reference standard. The controls were not paired with the glaucoma model eyes. Therefore, each experimental and control group was composed of independent biological samples. All the glaucoma model eyes had extensive optic nerve injury induced by IOP elevation, with injury grade 4.9 to 5.0. The reference standard was constructed by combining aliquots from each of the RNA samples used in the microarray study.

the whole-retina study, with six glaucoma model and six control whole-retina arrays.

For each microarray study, gene expression in each individual sample was independently compared to a common reference standard derived by combining aRNA aliquots from all samples in each study. After RNA amplification, the target was generated by reverse transcription and was labeled with Cy3 (standard) or Cy5 (sample). Each probe was represented by two duplicate spots on two identical slides, making four technical replicates for each array. After hybridization, gene expression data were reported as ratios of average signal intensity (local background subtracted) in each sample to that of the reference standard. Data were normalized with a modified Lowess procedure¹⁵ at the OHSU Microarray Facility.

Statistical Analysis of Microarrays

We used the unpaired two-class comparison analysis in the Web tool Significance Analysis of Microarrays (SAM, ver. 2.5; Stanford University, Stanford, CA) to identify genes with significant changes in expression. The parameters for testing significance were set at 1000 permutations with a false-discovery rate of 2%. In addition, to limit the significant genes to those with expression changes likely to be of biological significance, we set a minimum change of 1.3-fold. Genomic locations of human homologues of the significant genes were determined using batch extract function of the gene data unification web tool SOURCE (<http://smd.stanford.edu/cgi-bin/source/sourceSearch>; the Genetics Department, Stanford University, Stanford, CA). The gene ontology web tool DAVID (<http://david.abcc.ncifcrf.gov/>; National Institute of Allergies and Infectious Disease, NIH, Bethesda, MD) was used to determine significantly affected gene categories based on EASE (expression analysis systematic explorer) scores. This tool functionally classifies the affected genes by biological process, cellular component, and molecular function, using the controlled vocabulary of the Gene Ontology Consortium (<http://www.geneontology.org/>).

Real-Time Reverse Transcription-PCR

Message levels of selected genes were examined by qPCR to verify array results. Several genes that are not on the microarray but were of particular interest to us were also examined. Gene-specific primers were designed on computer (Primer Designer 4 or Clone Manager Professional, ver. 9.0; both: Sci.Ed Software, Cary, NC) and are listed in Table 1. For both the whole-retina and RGCL comparisons, each

sample represented mRNA from a single, independent globe, so that experimental groups consisted of independent biological replicates. For whole-retina analysis, 150 ng RNA from each sample (12 glaucoma model and 13 control whole retinas) were reverse transcribed, along with a 17.5- to 1200-ng RNA standard curve of combined retinal RNA from both the control and the experimental eyes.¹³ Expression levels were determined by qPCR using a real-time thermocycler (LightCycler, software 3.5 and DNA Master SYBR Green kit; Roche, Indianapolis, IN) according to the manufacturer's protocol, as previously described.⁶ Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as the housekeeping gene, since array analysis demonstrated that *Gapdh* message levels in whole retinas were unchanged by elevated IOP exposure. *Gapdh* levels were measured in triplicate, and the average values for each sample were used for normalization.

For RGCL analysis, 20 ng amplified RNA was reverse transcribed for each sample (6 glaucoma model and 13 control RGCL) and 2 to 400 ng reference RNA for the standard curve with random primers (250 ng; Invitrogen, Carlsbad, CA) and reverse transcriptase (SuperScript III; Invitrogen). Quantitative PCR was performed according to the same protocol as that for whole-retina analysis. Based on a lack of significant change in ribosomal protein 16 (*Rps16*) in the RGCL microarray study, this gene was used as the housekeeping gene for normalization, rather than *Gapdh*, which showed more change in these samples.¹⁶ All primers were designed to target the mRNA region within 350 bp of its 3' polyadenylation site.

All data were expressed as the change ($\times$ -fold) compared to the mean $\pm$ SEM value obtained in the control eyes. Statistical analysis was performed by comparing the experimental eyes to the control eyes by unpaired *t*-test (Excel; Microsoft, Redmond, WA).

Immunohistochemistry

An additional group of seven grade 5 glaucoma model rats were perfused with buffered paraformaldehyde, the globes were paraffin embedded and sectioned, and the slides were deparaffinized. For antigen retrieval, the slides were immersed in a solution containing 10 mM Tris base (pH 9.0), 1 mM EDTA, and 0.05% Tween 20 and then exposed to heat for 30 minutes in a rice cooker (http://www.ihcworld.com/_protocols/epitope_retrieval/tris_edta.htm; IHCWORLD, a Web-based life sciences information network). Polyclonal rabbit anti-human Atf3 antibodies (0.03 μ g/mL, SC188; Santa Cruz Biotechnology, Santa Cruz) were used for immunolocalization by using the ABC technique and 3,3'-diaminobenzidine chromo-

TABLE 1. Gene-Specific Primers Used in the qPCR Analyses

Target	Forward Primer (5'–3')	Reverse Primer (5'–3')	Size (bp)
<i>A2m</i>	GTGCATGTGAGCCGAACAGAAAG	TTGCTGAACCGTGAAGGACAAG	99
<i>Aif1</i>	CTCCGAGGAGACGTTTCAGTT	CCAGTTGGCTTCTGGTGTTTC	120
<i>Atf3</i>	GACTGGTATTTGAAGCCAGGAGTG	GGACCGCATCTCAAAATAGC	96
<i>Brn3a</i>	CAGGAGTCCCATGTAAGA	ACAGGGAACACTTCTGC	133
<i>Brn3b</i>	CACAGCGACTGGTACCTTCTCTAT	CTTTGGAGAGCCTCAAAATGG	112
<i>Brn3c</i>	ACTCTGCTTTCCTGCCGACT	CAGAAGGGTCCGGTCTGTGGT	96
<i>Clqa*</i>	TGGACAGTGGCTGAAGAT	CCTTTAAGACCTCGGATACC	149
<i>Clqa†</i>	GAAGCCGACAGCATCTTCAGTG	CAACAGGGAGTGCAGGAGATGG	94
<i>Cnd2</i>	CAGCAGGACGAGGAAGTGA	ACAGACTTGGAGCCGTTGTG	173
<i>Cryab</i>	CTGTGAACCTGGACGTGAAG	TGAGGACTCCATCCGATGAC	204
<i>Fbln2</i>	CGCTGTCTGCGCTTCGATTG	GGCCGATGCGGAAGATATGG	175
<i>Gadd45g</i>	TGTTCCGAGCCCAGGACTCT	GCCTTGCTTTCCCTCCTTTTC	154
<i>Gap43</i>	TGTCCACGGAAGCTAGCCTGAA	GGGGGAATAAGGAAGAGAGGAGGA	198
<i>Gapdh</i>	TGCCACTCAGAAGACTGTGGATG	GCCTGCTTCACCACCTTCTTGAT	249
<i>Gjap</i>	TGTACGGAGTATCGCCTAGA	AATACGAAGGCACTCCACAC	96
<i>Gja1</i>	CGTGCCGCAATTACAACAAG	TTGGTCCACGATGGCTAATG	194
<i>Hmox1</i>	CAGCCCCAAATCCTGCAACAGA	CAACATGGACGCCGACTACCAA	92
<i>Jun*</i>	CCAGAAGATGGTGCAGTGTT	GCGCATGCTACTTGATATGG	116
<i>Jun†</i>	AGTCCAGACTGTTGACCAGAAG	AAACGATCACAGCGCATGCTAC	142
<i>Junb</i>	TACTCAGCCTGTGGGGATACTA	GACACGAAGTGCCTGTTTCT	92
<i>Lcn2</i>	AGGAGCGATTTCGTGAGCTTTC	ACGCTCACCGTCTGTTTCAGTTG	107
<i>Lif</i>	TGATCTGGCTGTCTGCATTC	CATTCCCACAGGGTACATTC	155
<i>Lifr</i>	CCCAACTCAAACGACTCT	CAGCGACGTAACATTTCC	98
<i>Mmp9</i>	CCAGGAGTCTGGATAAGTTG	ACTCACACGCCAGAAAGTA	257
<i>Nefb</i>	TGCTGAACGCTCCACGTAACAAAC	GCTCATTCTACTGCGGTACAC	113
<i>Nefr</i>	ATGCTCAGATCTCCGTGGAGATG	GCTTCGAGCTCATTCTCCAGTT	365
<i>Nefl†</i>	CCCAAAATCAGGTCAACCTCATC	GAAAGCCACTCTGCAAGCAAAAC	170
<i>Rps16</i>	AATAAGTGGCTGTGGGGACT	GCACAGCTCTTGGGTTTAAAG	132
<i>Socs3</i>	GGTAGCAGCCATGGAATTACCT	AAAAGCCACCCCAACACACAGC	109
<i>Stat3</i>	CACCCATAGTGAGCCCTTGGAA	CTGTACTGGGAGGCCAAGCAAA	105
<i>Thy1</i>	GATTTCCCAGACAACACAGAG	TGGTGATGGGAGAGGTAGAG	144
<i>Timp1</i>	GTTCCCTGGCATAATCTGAG	GGGATGAGGATCTGATCTGT	155
<i>Tuba1a</i>	CCACCATCAAGACCAAGGCTACC	CATCAGTGAAGTGGACGGCTC	525

* Primers used in whole retina analysis.

† Primers used in RGCL analysis.

gen, as previously described.¹⁷ Purified rabbit IgG was substituted for the primary antibody on control sections.

RESULTS

IOP History and Nerve Damage

With IOP elevation, all the glaucoma model eyes used for microarray analyses demonstrated extensive optic nerve damage, with injury grade 4.88 and higher. The mean IOP of the glaucoma model eyes in the whole-retina study (36.49 ± 3.33 mm Hg, mean $\pm$ SD) was not different from that of the eyes in the RGCL study (36.59 ± 8.66 mm Hg, mean $\pm$ SD). No nerve damage was present in the control eyes in either group.

Genes with Significantly Altered Expression in Whole Retina

In the whole retina, SAM (2% FDR, 1.3-fold change, and $q < 0.02$) identified 335 significantly upregulated and 297 significantly downregulated genes. Among these, 102 upregulated and 24 downregulated genes had a more than twofold change. Genes that were upregulated more than fivefold were insulin-like growth factor-binding protein 3 (*Igfbp3*), lipocalin 2 (*Lcn2*), α -2-macroglobulin (*A2m*), suppressor of cytokine signaling 3 (*Socs3*), and ADAM metalloproteinase with thrombospondin type 1 motif, 1 (*Adamts1*). Genes that were downregulated more than threefold were crystallin gamma C (*Crygb*), alpha B (*Cryab*), gamma synuclein (*Sncg*), lens epithelial protein (*Lenep*), and myo-inositol 1-phos-

phate synthase A1 (*Isyna1*). The most upregulated and downregulated genes are listed in Tables 2 and 3, respectively. (All accession numbers in gene lists are GenBank; <http://www.ncbi.nlm.nih.gov/Genbank/>, provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD.)

Functional Classes of Significantly Altered Genes in Whole Retina

DAVID analysis of the affected genes in whole retina identified by SAM indicated nine significantly upregulated and three significantly downregulated biological processes (EASE score < 0.05). The upregulated genes were mainly associated with immune response, cell adhesion, signal transduction, cell proliferation, programmed cell death, plasma membrane, extra cellular matrix (ECM), and cytoskeleton (Table 4), whereas the downregulated gene classes included lipid biosynthesis, neurophysiological process (light sensation), cytoskeleton organization and biogenesis, cytoskeleton, and membrane (Table 5).

Significant Genes with Altered Expression in RGCL

With the same microarray platform, the RGCL analysis (SAM, 2% FDR, 1.3-fold change, and $q < 0.02$) yielded 3726 genes with significantly altered expression, including 2003 upregulated and 1723 downregulated. The number of genes detected with significantly changed expression increased more than fivefold compared with the number in the whole

TABLE 2. Genes Most Upregulated in Expression in Whole Retina with IOP-Induced Extensive Nerve Injury

Change (x-fold)	GenBank Accession	Gene Name	Gene Symbol	Gene Ontology	Genomic Location of Human Homologue
8.03	BG088567	Insulin-like growth factor binding protein 3	<i>Igfbp3</i>	Regulation of cell growth and proliferation, vascular endothelial cells	7p13-p12
7.86	BG070106	Lipocalin 2	<i>Lcn2</i>	Protection of MMP-9, iron transport	9q34
6.10	BG076712	Alpha-2-macroglobulin	<i>A2m</i>	Protease inhibitor activity	12p13.3-p12.3
5.23	BG076991	Suppressor of cytokine signaling 3	<i>Socs3</i>	Suppression of cytokine signaling by inhibition of Janus kinases	17q25.3
4.98	BG065692	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1	<i>Adamts1</i>	Metalloproteinase, associated with decreased synaptic density	21q21.2
4.30	BG063821	Ladinin	<i>Lad1</i>	Anchoring filament protein of basement membranes	1q25.1-q32.3
4.27	BG085662	Fibronectin 1	<i fn1<="" i=""></i>	Extracellular matrix	2q34
4.18	BG070892	MAK10 homolog, amino-acid N-acetyltransferase subunit, (S. cerevisiae)	<i>Mak10</i>	Wound healing	9q21.33
4.08	BG072209	Clusterin	<i>Clu</i>	Complement lysis inhibitor, chaperone protein, apoptosis and cell survival	8p21-p12
3.83	BG087241	Mesoderm specific transcript	<i>Mest</i>	Proteolysis and peptidolysis	7q32
3.68	BG077129	Transmembrane BAX inhibitor motif containing 1	<i>Tmbim1</i>	Integral membrane protein	2p24.3-p24.1
3.67	BG084377	Signal transducer and activator of transcription 3	<i>Stat3</i>	JAK-STAT cascade, IL6 family response	17q21.31
3.27	BG072156	Interferon induced transmembrane protein 3	<i>Ifitm3</i>	Negative regulation of cell proliferation	11p15.5
3.24	BG067364	Activating transcription factor 3	<i>Atf3</i>	CREB family transcription factor associated with axonal regeneration	1q32.3
3.22	BG068359	Ceruloplasmin	<i>Cp</i>	CNS iron homeostasis and neuroprotection	3q23-q25
3.14	BG064176	Lectin, galactose binding, soluble 3	<i>Lgals3</i>	Phagocytosis of apoptotic cells, endothelial cell migration	14q21-q22
3.13	BG075854	AXL receptor tyrosine kinase	<i>Axl</i>	Stimulates cell proliferation	19q13.1
3.01	BG075211	Adipose differentiation related protein	<i>Adfp</i>	Plasma membrane, lipid storage	9p22.1
2.97	BG087322	Ribosomal protein S6 kinase, polypeptide 2	<i>Rps6ka2</i>	Growth-factor and stress induced activation of the transcription factor Creb	6q27
2.91	BG079463	Leukotriene B4 12-hydroxydehydrogenase	<i>Ltb4db</i>	Inactivation of leukotriene B4	9q31.3
2.85	AA408197	Signal transducer and activator of transcription 1	<i>Stat1</i>	IFN-gamma and EGF response, transcription regulation	2q32.2
2.82	BG067419	Growth arrest and DNA-damage-inducible 45 gamma	<i>Gadd45g</i>	Activation of the p38/JNK pathway in response to environmental stress	9q22.1-q22.2
2.79	BG077550	Transgelin 2	<i>Tagln2</i>	Calponin-like actin-binding, muscle development	1q21-q25
2.71	BG085219	Transmembrane protein 176B	<i>Tmem176b</i>	Integral to membrane	7q36.1
2.69	BG063994	Beta-2 microglobulin	<i>B2m</i>	MHC class I receptor activity, involved in synaptic plasticity of neurons	15q21-q22.2
2.69	BG074325	Cyclin D2	<i>Ccnd2</i>	Regulation of cell cycle, initiation of g1/s (start) transition	12p13
2.66	BG074675	Aprataxin and PNKP like factor	<i>Aplf</i>	Nucleus hypothetical protein	2p13.3
2.65	BG071182	Transmembrane protein 176A	<i>Tmem176a</i>	Integral membrane protein	7q36.1
2.63	BG063694	Vinculin	<i>Vcl</i>	Actin binding, adherens junction, cell adhesion	10q22.2
2.62	BG073167	Cd63 antigen	<i>Cd63</i>	Lysosomal protein, integral to membrane	12q12-q13
2.62	BG064105	Zinc finger protein 36, C3H type-like 2	<i>Zfp3612</i>	Response to growth factors, mRNA stability	2p22.3-p21
2.62	BG085029	Carnitine O-octanoyltransferase	<i>Crot</i>	Fatty acid metabolism and transport	7q21.1
2.56	BG067456	Lysozyme 2	<i>Lyz2</i>	Cell wall catabolism, cytolysis	12q15

retina. Nearly 25% of these genes had a more than twofold expression change, with 777 upregulated and 195 downregulated. The most affected genes are listed in Tables 6 (upregulated) and 7 (downregulated). Three of the genes had a more than 15-fold upregulation: activating transcription factor 3 (*Atf3*), lipocalin 2 (*Lcn2*), and OTU containing domain 4 (*Otd4*). Four genes were downregulated more than fourfold: ELAV-like 2 (*Elavl2*), which codes for an RNA binding protein; ephrin A5 (*Efn5*), which functions in RGC axon guidance^{18,19}; potassium voltage-gated channel Shal-

related family, member 2 (*Kcnd2*); and sortilin-related receptor, LDLR class A repeats-containing (*Sor11*), which is involved in lipid metabolism and transport.

Functional Classes of Significantly Altered Genes in the RGCL

Consistently, more functional classes of affected genes were identified by DAVID analysis (EASE score < 0.05) in the RGCL than in the whole retina, especially the downregu-

TABLE 3. Genes Most Downregulated in Expression in Whole Retina with IOP-Induced Extensive Nerve Injury

Change (x-fold)*	GenBank Accession	Gene Name	Gene Symbol	Gene Ontology	Genomic Location of Human Homologue
-25.00	BG080972	Crystallin, alpha B	<i>Cryab</i>	Structural constituent of eye lens	11q22.3-q23.1
-4.95	BG075084	Crystallin, gamma B	<i>Crygb</i>	Structural constituent of eye lens, chaperone.	2q33-q35
-4.74	BG079620	Synuclein, gamma	<i>Sneg</i>	Neurofilament network integrity, increases NFH susceptibility to proteolysis	10q23.2-q23.3
-4.06	BG069202	Lens epithelial protein	<i>Lenep</i>	Lens epithelial cell differentiation.	1q22
-3.06	BG066302	Mvo-inositol 1-phosphate synthase A1	<i>Isvnal</i>	Phospholipid biosynthesis	19p13.11
-2.94	BG087831	Potassium voltage-gated channel, Shal-related family, member 2	<i>Kcnd2</i>	Repolarization of membrane in action potential	7q31
-2.88	BG074349	RIKEN cDNA 4930562C15 gene	4930562C15Rik		16p13.3
-2.85	AU043877	Neurofilament, light polypeptide	<i>Nefl</i>	Axonal intermediate filament cytoskeleton	8p21
-2.70	BG086212	Phosducin-like 3	<i>Pdel3</i>	Modulates the activation of caspases during apoptosis	2q11.2
-2.41	BG069739	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	<i>Hmgcs1</i>	Sterol synthesis	5p14-p13
-2.36	BG080868	Guanine nucleotide binding protein (G protein), gamma 3 subunit	<i>Gng3</i>	Activation of MAPK, highly expressed in brain	11p11
-2.28	C81465	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	<i>Slc6a6</i>	Sodium and chloride dependent taurine transporter, osmoregulation	3p25-p24
-2.22	BG072148	Tubulin polymerization-promoting protein family member 3	<i>Tppp3</i>	p25 family protein	16q22.1
-2.17	BG067910	Ubiquitin carboxy-terminal hydrolase L1 (brain form)	<i>Uchl1</i>	Ubiquitin-dependent protein catabolism	4p14
-2.17	BG081044	Protocadherin 7	<i>Pcdh7</i>	Expressed in brain and heart, Ca++ dependent cell adhesion	4p15
-2.13	BG074458	24-dehydrocholesterol reductase	<i>Dher24</i>	Cholesterol biosynthesis, inactivation of MAPK	1p33-p31.1
-2.13	BG069578	Cyclin G1	<i>Ceng1</i>	Cell cycle, g2/m phase. P53 inhibition of cell proliferation	5q32-q34
-2.13	AW544616	ATPase, Na+/K+ transporting, beta 1 polypeptide	<i>Atp1b1</i>	Potassium ion transport	1q24
-2.08	BG076766	Transthyretin	<i>Ttr</i>	Retinol and thyroxine transport	18q12.1
-2.08	BG064943	Carbonic anhydrase 14	<i>Car14</i>	Integral to membrane, pH regulation and ion transport	1q21
-2.08	BG077733	ATPase, Na+/K+ transporting, beta 1 polypeptide	<i>Atp1b1</i>	Noncatalytic component of the active enzyme, regulates sodium pump transport to the plasma membrane.	1q24
-2.04	BG069211	Farnesyl diphosphate farnesyl transferase 1	<i>Fdft1</i>	Squalene synthase, cholesterol synthesis	8p23.1-p22
-2.04	BG070367	Transferrin receptor	<i>Tfrc</i>	Iron uptake	3q29
-2.00	BG069999	HnRNP-associated with lethal yellow	<i>Raly</i>	RNA binding	20q11.21-q11.23
-2.00	BG086722	Ubiquitin carboxy-terminal hydrolase L1	<i>Uchl1</i>	Protein deubiquitination, axonogenesis and axonal transport of mitochondrion	4p14
-2.00	BG063103	Myosin IB	<i>Myo1b</i>	Cytoskeleton organization and biogenesis	2q12-q34
-2.00	BG065012	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	<i>Ywbab</i>	Cytoskeleton regulation during apoptosis and cell division	22q12.3

* Calculated by dividing the mean value in experimental whole retinas by the mean value in control specimens. If this number (the relative quantity) was <1, the (negative) reciprocal was listed (e.g., 0.5, or a decrease of 50% compared with the control, is reported as a -2-fold change).

lated genes. Thirteen upregulated biological processes were identified (Table 8), dominated by protein synthesis. These included all four gene categories identified in the whole

retina: immune response, cell adhesion, signal transduction, and regulation of cell proliferation, but with a greater number of genes in each category. In contrast to only 3 down-

TABLE 4. Significantly Upregulated Gene Categories in Whole Retina Identified by DAVID Analysis

GO Term	Genes (n)	EASE Score*
Biological Processes		
Immune response	27	7.30E-09
Cell adhesion	24	3.80E-05
Signal transduction	59	6.30E-05
Regulation of cell proliferation	14	1.70E-03
Programmed cell death	22	2.90E-03
Cytokine biosynthesis	6	3.50E-03
Cell motility	12	1.90E-02
Hemopoiesis	8	2.10E-02
Ion transport	17	2.90E-02
Cell Component		
Plasma membrane	49	5.50E-08
Extracellular region and ECM	56	5.40E-05
Cytoskeleton	32	2.60E-04
MHC protein complex	3	6.90E-03
Cell junction	9	2.50E-02
Golgi-associated vesicle membrane	4	4.00E-02
Molecular Function		
Cytoskeletal protein binding	20	1.70E-04
MHC class I receptor activity	4	9.10E-04
Calcium-dependent phospholipid binding	4	1.60E-02
Transmembrane receptor activity	12	3.80E-02
Anion transporter activity	5	3.80E-02

Gene categories are listed in descending order of probability.

* EASE score <0.05

regulated biological processes identified in the whole retina, 11 distinct processes were found to be significantly downregulated in the RGCL, including axon extension and dendrite morphogenesis (Table 9).

Common Genes in Retinal and RGCL Microarrays with Significantly Altered Expression

A comparison of the specific genes between the whole-retina and RGCL analyses showed that of all the significant genes identified in whole retina, 394 (approximately 60%) were confirmed to have significant expression changes in RGCL. Of these common genes, 317 changed in the same direction (186 up and 131 down). The most up- or downregulated common genes are listed in Tables 10 (upregulated) and 11 (downregulated). For a majority of these most changed genes, a much greater change in expression was found in RGCL compared with the whole retina. For example, *Atf3* was upregulated only 3.24-fold in whole retina, whereas in RGCL, it became the highest upregulated gene, with expression increased by 28.38-fold. *Lcn2* was the second most upregulated gene in both analyses, with a 7.86-fold change in whole retina and a 20.36-fold change in the RGCL. *Igfbp3* was one of the few genes that had a comparable percentage change increase in both whole retina (8.03) and RGCL (8.72).

Verification of Whole Retina and RGCL Gene Expression Changes

qPCR, used to examine selected messages in whole retina and RGCL to provide confirmation of our microarray results, indicated a good agreement between the two methods of analysis (Table 12). Changes in expression of all these genes were found to be in the same direction by qPCR as well as microarray, with a generally comparable extent of change.

In addition, we extended the qPCR analysis to study several other genes not present on the array but with potential contributing roles in the glaucomatous injury process.

qPCR Analysis of Selected Genes in Whole Retina. As shown in Figure 3A, cell cycle gene cyclin D2 (*Ccnd2*) was upregulated 4.82-fold ($P < 0.001$). We have identified upregulation of cyclin D1 in the optic nerve head of eyes with early injury.⁶ Complement component *C1qa* was upregulated 2.79-fold ($P < 0.001$). We noted that the ECM protein fibulin 2 (*Fbln2*) was also upregulated 3.20-fold ($P < 0.001$). All these changes are comparable to those on the microarray (Table 12). TIMP metalloproteinase inhibitor 1 (*Timp1*), another ECM protein not on the microarray, was greatly upregulated (34.76-fold; $P < 0.001$).

A prominent downregulation of RGC neurofilament light chain (*Nefl*) to 0.29 ($P < 0.001$) was detected by qPCR (Fig. 3B), confirming a similar reduction on the microarray (Table 12). Of note, matrix metalloproteinase 9 (*Mmp9*, gelatinase B; not on the microarray), the principle target of *Timp1*, was also greatly reduced to 0.39 ($P < 0.001$). *Cryab* was one of the most downregulated genes on the microarray. However, its reduction in the qPCR analysis was not statistically significant due to high variability in gene expression within groups. α -Tubulin (*Tuba1a*), unchanged on the microarray, also showed no change in qPCR. No change was found for connexin 43 (*Gja1*), a gap junction component involved in cell-cell communication, or for growth associated protein 43 (*Gap43*), a calmodulin-binding protein that has been suggested to play a role in such processes as axon guidance and neurite outgrowth.

qPCR Analysis of Selected Genes in RGCL. These results are illustrated in Figure 4. *Atf3*, the most upregulated gene on the microarray, demonstrated an even more striking increase (94.81-fold; $P < 0.001$) in qPCR (Fig. 4A). The mRNA level for *Jun* was upregulated 5.11-fold ($P < 0.001$), an increase similar to that on the microarray (Table 12). Both microarray and qPCR changes of *Jun* were greater than those in the whole retina. No significant change was found in *Junb*, which is not on the array. Messages in JAK/Stat pathway mediated LIF signaling were also measured. We have identified an early upregulation of *Lif* in rat optic nerve head in response to pressure elevation (Dyck JA, et al. *IOVS*

TABLE 5. Significantly Downregulated Gene Categories in Whole Retina Identified by DAVID Analysis

GO Term	Genes (n)	EASE Score*
Biological Process		
Lipid biosynthesis	13	8.00E-04
Neurophysiological process (light sensation)	11	2.90E-03
Cytoskeleton organization and biogenesis	16	1.80E-02
Cell Component		
Cytoskeleton	24	1.30E-02
Membrane	90	3.20E-02
Cytoplasm	90	4.60E-02
Molecular Function		
Calmodulin binding	9	5.10E-04
Vitamin binding	5	1.50E-02
Calcium ion binding	19	1.70E-02
Carboxylic ester hydrolase activity	5	4.00E-02
ATP binding	36	4.10E-02
Cytoskeletal protein binding	13	4.30E-02
Translation initiation factor activity	7	4.90E-02

Gene categories are listed in descending order of probability.

* EASE score <0.05.

TABLE 6. Genes Most Upregulated in Expression in RGCL with IOP-Induced Extensive Nerve Injury

Change (x-fold)	GenBank Accession	Gene Name	Gene Symbol	Gene Ontology	Genomic Location of Human Homologue
28.38	BG067364	Activating transcription factor 3	<i>Atf3</i>	Regulation of transcription	1q32.3
20.36	BG070106	Lipocalin 2	<i>Lcn2</i>	Transporter activity	9q34
15.82	C79058	OTU domain containing 4	<i>Otd4</i>	Protein binding	4q31.21
13.18	AA410137	WNK lysine deficient protein kinase 1	<i>Wnk1</i>	Protein kinase activity, ion transport	12p13.3
13.14	C75991	Chromobox homolog 5 (Drosophila HP1a)	<i>Cbx5</i>	Chromatin assembly or disassembly	12q13.13
12.94	BG067419	Growth arrest and DNA-damage-inducible 45 gamma	<i>Gadd45g</i>	Apoptosis, cell differentiation, activation of MAPKK	9q22.1-q22.2
12.41	BG068326	Glycine decarboxylase	<i>Gldc</i>	Glycine metabolism	9p22
12.29	BG067456	Lysozyme 2	<i>Lyz2</i>	Cytolysis, cell wall catabolism	12q15
12.27	BG063921	Solute carrier family 43, member 2	<i>Slc43a2</i>	Integral to membrane	17p13.3
12.01	AU021372	FUS interacting protein (scrine-arginine rich) 1	<i>Fusip1</i>	Nuclear mRNA splicing	1p36.11
11.49	BG073735	Procollagen, type I, alpha 2	<i>Col1a2</i>	Cell adhesion, ECM structural constituent	7q22.1
11.47	AU045725	Ring finger protein 121	<i>Rnf121</i>	Ubiquitin conjugation	11q13.4
11.18	BG072750	Transforming growth factor, beta induced	<i>Tgfb1</i>	Cell adhesion	5q31
11.06	BG077076	Caseinolytic peptidase, ATP-dependent, proteolytic subunit homolog (E. coli)	<i>Clpp</i>	Proteolysis and peptidolysis	19p13.3
10.74	BG077732	Heme oxygenase (decycling) 1	<i>Hmox1</i>	Heme oxidation, response to stimulus	22q12
10.58	BG085576	Procollagen, type III, alpha I	<i>Co13a1</i>	Cell adhesion, ECM structural constituent	2q31
10.45	AU041770	EMI domain containing 2	<i>Emid2</i>	Phosphate transport, protein binding	7q22.1
10.28	BG077473	RIKEN cDNA 0610031J06 gene	<i>0610031J06Rik</i>	Integral membrane protein of the lysosome	1q22
9.95	BG064176	Lgals3 lectin, galactose binding, soluble 3	<i>Lgals3</i>	IgE binding	14q21-q22
9.80	BG083135	Tropomodulin 3	<i>Tmod3</i>	Barbed-end actin filament capping	15q21.1-q21.2
9.61	AW539348	Fucosidase, alpha-L-2, plasma	<i>Fuca2</i>	Carbohydrate metabolism	6q24
9.50	BG077087	Parathyroid hormone-like peptide	<i>Pthlb</i>	Epithelial cell differentiation	12p12.1-p11.2
9.45	AW538113	Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	<i>Slc25a19</i>	Mitochondrial inner membrane, transporter protein	17q25.3
9.44	AU045395	Spermatogenesis associated 7	<i>Spata7</i>	β -hydroxysteroid dehydrogenase	14q31.3
9.41	AU020524	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	<i>Hmgcs1</i>	Lipid synthesis, regulation of transcription	5p14-p13
9.22	BG076966	Transglutaminase 1, K polypeptide	<i>Tgm1</i>	Cell-cell adherens junction	14q11.2
9.15	BG063357	Metaxin 3	<i>Mtx3</i>	Mitochondria membrane protein	5q14.1
9.09	BG066681	IQ motif containing GTPase activating protein 1	<i>Iqgap1</i>	Ras GTPase activator, signal transduction	15q26.1
8.95	AW537264	Nuclear mitotic apparatus protein 1	<i>Numa1</i>	Tubulin binding	11q13
8.94	BG075075	Ubiquitin specific peptidase 48	<i>Usp48</i>	Ubiquitin cycle	1p36.12
8.85	AW540988	DnaJ (Hsp40) homolog, subfamily A, member 3	<i>Dnaja3</i>	Apoptosis, heat shock protein binding, small GTPase	16p13.3
8.85	BG077117	Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	<i>Malat1</i>	Myoblast differentiation	11q13.1
8.78	BG078028	Lectin, galactose binding, soluble 1	<i>Lgals1</i>	Negative growth factor regulating cell proliferation	22q13.1
8.72	BG088567	Insulin-like growth factor binding protein 3	<i>Igfbp3</i>	Regulation of cell growth	7p13-p12

2008;49:ARVO E-Abstract 3694). In RGCL, qPCR detected a substantial increase (41.69-fold $P < 0.001$) in the message level for the cytokine LIF. A significant upregulation (3.58-fold; $P < 0.01$) of LIF receptor (*Lifr*) mRNA was detected as well. Also, the mRNA level of signal transducer and activator of transcription 3 (*Stat3*) was upregulated 2.68-fold ($P < 0.05$), comparable to its increase on the microarray (Table 12). Concomitantly, a prominent increase of *Socs3* mRNA

(18.34-fold; $P < 0.001$), a feedback inhibitor of LIF signaling via the JAK/Stat pathway, was detected in RGCL.

qPCR analysis of other stress response genes (Fig. 4B) identified a 5.33-fold upregulation in *C1qa* mRNA ($P < 0.001$). *Lcn2* and *Gadd45g* mRNAs were greatly upregulated (12.80-fold; $P < 0.001$, and 10.59-fold, $P < 0.001$, respectively). *Hmox1*, an oxidative stress-responding gene noted to increase in response to hydrostatic pressure in cultured

TABLE 7. Genes Most Downregulated in Expression in RGCL with IOP-Induced Extensive Nerve Injury

Change (α -fold)*	GenBank Accession	Gene Name	Gene Symbol	Gene Ontology	Genomic Location of Human Homologue
-5.64	BG082125	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	<i>Elavl2</i>	mRNA processing, nuclear mRNA splicing	9p21
-5.45	BG074818	Ephrin A5	<i>Efn5</i>	Axon pathfinding and fasciculation	5q21
-4.48	BG087831	Potassium voltage-gated channel, Shal-related family, member 2	<i>Kcnd2</i>	Potassium ion channel	7q31
-4.05	BG075389	Sortilin-related receptor, LDLR class A repeats-containing	<i>Sor11</i>	Lipid metabolism and transport	11q23.2-q24.2
-3.72	BG077733	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	<i>Atp1b1</i>	Sodium:potassium-exchanging ATPase activity	1q24
-3.72	BG065461	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	<i>Psmd1</i>	Proteasome subunit	2q37.1
-3.67	BG077833	Translin	<i>Tsn</i>	DNA-binding protein	2q21.1
-3.64	BG077573	Nuclear receptor subfamily 6, group A, member 1	<i>Nr6a1</i>	Negative regulation of transcription from RNA polymerase II promoter	9q33-q34.1
-3.63	BG066372	Microtubule-associated protein tau	<i>Mapt</i>	Negative regulation of microtubule depolymerization, maintenance of neuronal polarity	17q21.1
-3.59	BG072849	Roundabout homolog 2 (Drosophila)	<i>Robo2</i>	Axon guidance; eurogenesis; sensory perception	3p12.3
-3.55	BG069654	Receptor accessory protein 5	<i>Reep5</i>	Receptor expression enhancing protein 5	5q22-q23
-3.46	BG080731	Ngfi-A binding protein 1	<i>Nab1</i>	Negative regulation of transcription	2q32.3-q33
-3.31	AW558903	Sortilin-related VPS10 domain containing receptor 3	<i>Sorcs3</i>	Intracellular protein transport	10q23-q25
-3.27	BG078945	N-ethylmaleimide sensitive fusion protein	<i>Nsf</i>	Delivery of cargo proteins to all compartments of the Golgi stack	17q21
-3.24	BG083786	General transcription factor IIIC, polypeptide 4	<i>Gtf3c4</i>	Essential for RNA polymerase III to make a number of small nuclear and cytoplasmic RNAs	9q34.13
-3.22	BG086020	Potassium voltage-gated channel, shaker-related subfamily, beta member 3	<i>Kcnab3</i>	Potassium ion channel	17p13.1
-3.20	BG078404	Apolipoprotein B	<i>Apob</i>	Lipid metabolism	2p24-p23
-3.18	BG075191	Fraser syndrome 1 homolog (human)	<i>Fras1</i>	Cell communication	4q21.21
-3.14	BG064386	Neuropilin (NRP) and tolloid (TLL)-like 2	<i>Neto2</i>	Development and/or maintenance of neuronal circuitry	16q11
-3.05	BG072085	Runt-related transcription factor 1; translocated to, 1 (cyclin	<i>Runx1t1</i>	Regulation of transcription, DNA-dependent	8q22
-3.05	BG071667	Phosphoglucosylase 2-like 1	<i>Pgm2l1</i>	The phosphohexose mutase family protein, carbohydrate metabolism	11q13.4
-3.03	BG073378	T-complex protein 1	<i>Tcp1</i>	Molecular chaperone, folding of actin and tubulin	6q25.3-q26
-3.02	BG064745	Phosphoglycerate kinase 1	<i>Pgk1</i>	Glycolysis	Xq13
-2.96	BG069624	N-myc downstream regulated gene 4	<i>Ndr4</i>	Multicellular organismal development	16q21-q22.1
-2.93	BG065113	Branched chain aminotransferase 1, cytosolic	<i>Bcat1</i>	Amino acid biosynthesis	12p12.1
-2.92	AW544616	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	<i>Atp1b1</i>	Sodium:potassium-exchanging ATPase activity	1q24
-2.92	BG085811	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	<i>Ywbab</i>	Adapter protein	20q13.1
-2.90	BG088107	Dickkopf homolog 3 (Xenopus laevis)	<i>Dkk3</i>	Negative regulation of Wnt signaling pathway	11p15.2
-2.87	BG083129	WW domain containing adaptor with coiled-coil	<i>Wac</i>	Spliceosome complex	10p12.1
-2.87	BG065114	Uridine phosphorylase 1	<i>Upp1</i>	Nucleoside metabolism	7p12.3
-2.86	BG085840	Olfactomedin 1	<i>Olfm1</i>	Latrotoxin receptor activity	9q34.3
-2.85	AU043877	Neurofilament, light polypeptide	<i>Nefl</i>	Axonal intermediate filament cytoskeleton	8p21

* Calculated as explained in Table 3.

RGCs and the mouse retina,²⁰ was also significantly upregulated (8.20-fold; $P < 0.001$). All these expression changes confirmed the microarray results (Table 12). *A2m* mRNA, shown by others to mediate RGC death in a rat model of

glaucoma,²¹ but not present on our microarray, increased 3.37-fold ($P < 0.01$). A large increase (20.37-fold, $P < 0.001$) was also found in message level for the Mmp inhibitor *Timpl*.

TABLE 8. Significantly Upregulated Gene Categories in RGCL Identified by DAVID Analysis

GO Term	Genes (n)	EASE Score*
Biological Process		
Protein biosynthesis and metabolism	133	6.40E-07
Cell adhesion	81	8.40E-04
Signal transduction	242	3.00E-02
Immune response	55	3.20E-02
Phosphate transport	16	9.90E-03
Cytoskeleton organization and biogenesis	70	1.20E-02
Sequestering of lipid	5	1.70E-02
Oligosaccharide metabolism	5	1.70E-02
Regulation of cell size	20	1.70E-02
Coagulation	10	3.20E-02
Ribosome biogenesis and assembly	31	3.70E-02
Regulation of cell cycle	64	4.80E-02
Regulation of body fluids	10	4.50E-02
Cell Component		
Ribonucleoprotein complex	103	2.40E-06
Cytoskeleton	128	5.40E-06
Extracellular matrix	46	2.70E-04
Plasma membrane	165	4.20E-04
Extracellular region	236	5.40E-04
Cell junction	30	2.70E-02
Cytoplasmic membrane-bound vesicle	37	3.20E-02
Molecular Function		
Structural constituent of ribosome	71	2.90E-10
Cytoskeletal protein binding	72	4.10E-06
Extracellular matrix structural constituent	16	3.50E-03
Transferase activity, transferring glycosyl groups	35	1.90E-02
Magnesium ion binding	47	1.70E-02
Prenylated protein tyrosine phosphatase activity	13	4.60E-02
Oxygen binding	4	4.90E-02
beta-N-Acetylhexosaminidase activity	4	4.90E-02

Gene categories are listed in descending order of probability.

* EASE score < 0.05.

Among the RGCL cell type markers examined by qPCR, both *Aif1* (for microglia²² and *Gfap* (for astrocytes and Müller cells) were significantly upregulated (13.01-fold for *Aif1*, $P < 0.001$; 4.05-fold for *Gfap*, $P < 0.01$; Fig. 4C). In contrast, all RGC marker messages (*Thy1* and *Brn3* family proteins) and the RGC axonal marker messages *Nefl* and *Nefb* were reduced in glaucoma model RGCL with extensive nerve injury. The *Nefl*, *Nefb*, and *Brn3a*, *-b*, and *-c* mRNAs dropped to less than 0.20 of the control eye values; and *Thy1* mRNA was reduced to 0.29 (Fig. 4D). By qPCR, we found no significant change in mRNA expression in the experimental group for the amacrine cell markers calbindin (*Calb1*; 1.28 ± 0.28 , $P = 0.35$) or syntaxin 1 (*Stx1a*) (0.76 ± 0.25 , $P = 0.5$). By gene array, only one of the three probes present for *Calb1* showed a significant downregulation (to 0.75), and none of the other amacrine cell markers on our arrays, including tyrosine hydroxylase (*Th*), calneuron 1 (*Caln1*), and myeloid ecotropic viral integration site-related gene 1 (*Mrg1*), were significantly changed in our RGCL analysis.

Atf3 Immunohistochemistry. Immunohistochemistry for Atf3 confirmed a substantial increase in its protein level in RGCL with extensive nerve injury. As illustrated in Figure 5, the transcription factor was localized primarily to neuronal nuclei within this layer.

DISCUSSION

Previous gene array studies of retinal responses to elevated IOP have been performed in rat and monkey models of experimental glaucoma as well as in the spontaneous DBA/2J glaucoma mouse model.^{5,7-9,23} Although findings have been based on different species and methods of elevating IOP, changes in expression of specific genes and gene categories have been identified across studies, including upregulation of genes involved in neuroinflammation, apoptosis, glial activation, and tissue remodeling, along with downregulation of crystallins and cytoskeleton-related genes, particularly neurofilaments.

However, in these studies, all of which were performed on tissue from whole retinas, the overall number of changed genes was relatively modest, ranging from 1% to 2% of the total genes studied. Because the initial injury in glaucoma and animal models of glaucoma is to RGC axons within the optic nerve head, primary retinal injury would be expected to affect RGCs and their associated cells within the inner ganglion cell layer of the retina. Because of this, dilution by genes from the large number of cells in the other retinal layers may cause an analysis of tissue from whole retina to overlook gene changes due to glaucomatous and IOP-induced injury. For this reason, we performed a gene array analysis of just the RGCL in a model of chronic experimental IOP elevation, to see whether isolating this most vulnerable portion of the retina would improve detection of the number of changed genes and provide further insights into RGC responses in this model.

We chose to analyze material isolated from the RGCL by LCM in our rat model of experimental glaucoma 5 weeks after

TABLE 9. Significantly Downregulated Gene Categories in RGCL Identified by DAVID Analysis

GO Term	Genes (n)	EASE Score*
Biological Process		
Generation of precursor metabolites and energy	92	2.50E-06
Coenzyme metabolism	39	4.70E-04
Cellular carbohydrate metabolism and glycolysis	43	2.10E-03
Protein catabolism	41	1.30E-02
Intracellular transport	99	2.30E-02
Sterol metabolism	14	2.20E-02
Protein folding	39	2.40E-02
mRNA splice site selection	6	2.50E-02
Nucleotide biosynthesis	23	3.30E-02
Axon extension and dendrite morphogenesis	10	4.00E-02
Ubiquitin cycle	74	4.40E-02
Cell Component		
Cytoplasm	457	4.80E-04
Proteasome complex	12	1.20E-02
Golgi trans face	8	4.90E-02
Molecular Function		
Oxidoreductase activity, acting on NADH or NADPH	16	2.10E-03
Hydrogen ion transporter activity	29	4.90E-03
Monovalent inorganic cation transporter activity	29	5.90E-03
Unfolded and heat shock protein binding	31	7.60E-03
Calmodulin binding	19	1.10E-02
Oxidoreductase activity, acting on CH—OH group of donors	19	2.50E-02
Antioxidant activity	9	4.30E-02

Gene categories are listed in descending order of probability.

* EASE score < 0.05.

TABLE 10. Most Upregulated Common Genes Identified in Both Whole Retina and RGCL by Microarray Analysis

GenBank Accession	Gene Name	Gene Symbol	RGCL (x-fold)	Whole Retina (x-fold)	Genomic Location of Human Homologue
BG067364	Activating transcription factor 3	<i>Atf3</i>	28.38	3.24	1q32.3
BG070106	Lipocalin 2	<i>Lcn2</i>	20.36	7.86	9q34
C75991	Chromobox homolog 5 (Drosophila HP1a)	<i>Cbx5</i>	13.14	2.19	12q13.13
BG067419	Growth arrest and DNA-damage-inducible 45 gamma	<i>Gadd45g</i>	12.94	2.82	9q22.1-q22.2
BG067456	Lysozyme 2	<i>Ly2</i>	12.29	2.56	12q15
BG072750	Transforming growth factor, beta induced	<i>Tgfb1</i>	11.18	1.49	5q31
BG072990	Hemoglobin, beta adult major chain	<i>Hbb-b1</i>	11.00	2.23	11p15.5
BG077732	Heme oxygenase (decycling) 1	<i>Hmox1</i>	10.74	2.21	22q12
BG064176	Igals3 lectin, galactose binding, soluble 3	<i>Igals3</i>	9.95	3.14	14q21-q22
BG088567	Insulin-like growth factor binding protein 3	<i>Igfbp3</i>	8.72	8.03	7p13-p12
BG072760	Hemoglobin alpha, adult chain 1	<i>Hba-a1</i>	8.44	2.10	16p13.3
AW544771	Folliculin interacting protein 2	<i>Fnip2</i>	8.27	1.30	4q32.1
BG074327	Procollagen, type III, alpha 1	<i>Col3a1</i>	7.84	1.35	2q31
BI076713	MutS homolog 4 (E. coli)	<i>Msb4</i>	7.75	2.40	1p31
BG077553	Arginyl aminopeptidase (aminopeptidase B)	<i>Rnpep</i>	7.31	1.39	1q32
AW550998	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	<i>Mthfd11</i>	7.06	1.88	6q25.1
BG064802	Secreted acidic cysteine rich glycoprotein	<i>Sparc</i>	6.33	1.49	5q31.3-q32
AW547306	Complement component 1, q subcomponent, C chain	<i>Clqc</i>	6.05	1.78	1p36.11
BG076991	Suppressor of cytokine signaling 3	<i>Socs3</i>	5.90	5.23	17q25.3
BG080700	Tumor necrosis factor receptor superfamily, member 12a	<i>Tnfrsf12a</i>	5.61	1.56	16p13.3
BG064382	Cathepsin 8	<i>Cts8</i>	5.59	2.03	n/a
BG074248	Kinesin family member 22	<i>Kif22</i>	5.20	1.66	16p11.2
BG085662	Fibronectin 1	<i fn1<="" i=""></i>	5.17	4.27	2q34
BG064126	Solute carrier family 2 (facilitated glucose transporter), member 1	<i>Slc2a1</i>	5.09	1.72	1p35-p31.3
AU041598	Histocompatibility 2, K1, K region	<i>H2-K1</i>	5.05	2.34	6p21.3
BG077883	TRIO and F-actin binding protein	<i>Triobp</i>	5.04	1.36	22q13.1
BG066632	Moesin	<i>Msn</i>	4.90	2.43	Xq11.2-q12
BG069465	Cd63 antigen	<i>Cd63</i>	4.83	2.78	12q12-q13
BG082776	Lysosomal-associated protein transmembrane 5	<i>Laptm5</i>	4.78	1.37	1p34
BG084377	Signal transducer and activator of transcription 3	<i>Stat3</i>	4.44	3.67	17q21.31
BG085219	Transmembrane protein 176B	<i>Tmem176b</i>	4.41	2.71	7q36.1
BG076072	UDP glucuronosyltransferase 1 family, polypeptide A7C	<i>Ugt1a7c</i>	4.38	1.87	2q37
BG083794	Ribose 5-phosphate isomerase A	<i>Rpia</i>	4.31	2.30	2p11.2
BG073116	Chloride intracellular channel 1	<i>Clc1</i>	4.31	2.23	6p22.1-p21.2
BG077793	Tax 1 (human T-cell leukemia virus type 1) binding protein 3	<i>Tax1bp3</i>	4.29	1.32	17p13
BG080846	Jun oncogene	<i>Jun</i>	4.10	1.33	1p32-p31
BG075377	Melanoma cell adhesion molecule	<i>Mcam</i>	4.06	1.72	11q23.3
BG085499	Spermidine/spermine N1-acetyl transferase 1	<i>Sat1</i>	4.02	1.81	Xp22.1
BG074325	Cyclin D2	<i>Ccnd2</i>	4.01	3.15	12p13

injection of hypertonic saline. LCM has been successfully used to generate an RGC-enriched sample for a microarray in normal human retinas.¹⁰ However, ours represents the first microarray study of just the RGCL in an animal model of glaucoma. To demonstrate the feasibility of this approach and the impact of analyzing material thus enriched, we compared gene array results in RGC-enriched samples from eyes with extensive optic nerve injury obtained by LCM with those in whole-retina samples from animals with comparable injury grade, using an identical cDNA microarray platform and statistical analysis tools.

In this analysis, each retina was used as an individual array and independently compared to the reference standard, providing a large enough number of independent biological samples to allow analysis of statistical significance of changes. The material used herein represents a portion of a larger study covering the entire spectrum of pressure damage. A detailed analysis of these results, with emphasis on potential RGC gene changes characteristic of early injury compared to later injury, will be presented in a subsequent report.

Miyahara et al.⁸ reported the first retinal gene array analysis in an animal glaucoma model in 2003. In monkeys with experimental glaucoma, they found that only 0.7% of the genes examined had a 1.7-fold or greater change. Ahmed et al.,⁵

working with the same model used in this report, noted 81 genes that demonstrated a twofold or greater change in retinas with elevated IOP, representing approximately 1% of the genes on the arrays. A subsequent array study in the DBA/2J mouse model of chronic glaucoma revealed a comparable number of changed genes (84 total), using a 1.8-fold cutoff.⁷ In rats with laser-induced IOP elevation, Yang et al.,⁹ identified 543 retinal genes that were regulated by at least twofold, representing approximately 2% of the genes evaluated.

These results are generally comparable to the whole-retina analysis results of the present study. We found 632 significantly changed genes (4.2% of total tested), with a false-discovery rate of 2% and a 1.3-fold difference. Of these, 125 genes (0.8% of total) demonstrated a twofold or greater change. By contrast, our LCM analysis of RNA obtained specifically from the RGCL showed a nearly 6-fold increase in the number of genes that were changed by 1.3-fold (3726; 24.5% of total) and a more than 8-fold increase in genes changed by 2-fold or greater (972; 6.5% of total). In addition to this increase in the total number of significantly changed genes, we found that, of the genes changed in common with whole retina analysis, the extent of change in those in the RGCL was uniformly greater than in those in whole retina (Tables 10, 11).

TABLE 11. Most Downregulated Common Genes Identified in Both Whole Retina and RGCL by Microarray Analysis

GenBank Accession	Gene Name	Gene Symbol	RGCL (x-fold*)	Whole Retina (x-fold*)	Genomic Location of Human Homologue
BG082125	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	<i>Elavl2</i>	-5.64	-1.56	9p21
BG087831	Potassium voltage-gated channel, Shal-related family, member 2	<i>Kcnd2</i>	-4.48	-2.94	7q31
BG077733	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	<i>Atp1b1</i>	-3.72	-2.06	1q24
BG077833	Translin	<i>Tsn</i>	-3.67	-1.45	2q21.1
BG072849	Roundabout homolog 2 (Drosophila)	<i>Robo2</i>	-3.59	-1.39	3p12.3
BG069654	Receptor accessory protein 5	<i>Reep5</i>	-3.55	-1.34	5q22-q23
BG086020	Potassium voltage-gated channel, shaker-related subfamily, beta member 3	<i>Kcnab3</i>	-3.22	-1.84	17p13.1
BG075191	Fraser syndrome 1 homolog (human)	<i>Fras1</i>	-3.18	-1.68	4q21.21
BG064386	Neuropilin (NRP) and tolloid (TLL)-like 2	<i>Neto2</i>	-3.14	-1.57	16q11
BG073378	T-complex protein 1	<i>Tcp1</i>	-3.03	-1.54	6q25.3-q26
BG069624	N-myc downstream regulated gene 4	<i>Ndr4</i>	-2.96	-1.77	16q21-q22.1
AW544616	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	<i>Atp1b1</i>	-2.92	-2.13	1q24
BG085840	Olfactomedin 1	<i>Olfm1</i>	-2.86	-1.64	9q34.3
AU043877	Neurofilament, light polypeptide	<i>Nefl</i>	-2.85	-2.85	8p21
BG071373	Ependymin related protein 1 (zebrafish)	<i>Epdr1</i>	-2.83	-1.36	7p14.1
BG073871	Eukaryotic translation initiation factor 5A2	<i>Eif5a2</i>	-2.78	-1.42	3q26.2
BG065012	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	<i>Yubab</i>	-2.64	-1.99	22q12.3
BG088504	Heparan sulfate 6-O-sulfotransferase 2	<i>Hs6st2</i>	-2.57	-1.63	Xq26.2
BG080229	Aldolase 3, C isoform	<i>Aldoc</i>	-2.56	-1.58	17cen-q12
BG074398	SPARC-like 1 (mast9, hevin)	<i>Sparcl1</i>	-2.52	-1.41	4q22.1
C85845	Ganglioside-induced differentiation-associated-protein 1	<i>Gdap1</i>	-2.49	-1.54	8q21.11
BG088948	Phosphofructokinase, muscle	<i>Pfkfb</i>	-2.45	-1.46	12q13.3
BG070656	SRX-box containing gene 4	<i>Sox4</i>	-2.41	-1.32	6p22.3
BG065752	Polybromo 1	<i>Pbrm1</i>	-2.36	-1.49	3p21
BG081951	Praja 1, RING-H2 motif containing	<i>Pja1</i>	-2.32	-1.32	Xq13.1
BG079634	Necdin	<i>Ndn</i>	-2.24	-1.45	15q11.2-q12
BG087233	Ubiquitin-conjugating enzyme E2E 2 (UBC4/5 homolog, yeast)	<i>Ube2e2</i>	-2.23	-1.37	3p24.2
BG075594	Fibronectin type III domain containing 5	<i>Fndc5</i>	-2.22	-1.71	1p35.1
BG075958	Tao kinase 1	<i>Taok1</i>	-2.21	-1.39	17q11.2
BG069211	Farnesyl diphosphate farnesyl transferase 1	<i>Fdft1</i>	-2.19	-2.04	8p23.1-p22
BG069739	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	<i>Hmgcs1</i>	-2.19	-2.41	5p14-p13
BG087239	Rho guanine nucleotide exchange factor (GEF) 4	<i>Arhgef4</i>	-2.16	-1.34	2q22
BG077950	Squalene epoxidase	<i>Sqle</i>	-2.14	-1.42	8q24.1
BG079988	Creatine kinase, mitochondrial 1, ubiquitous	<i>Ckmt1</i>	-2.13	-1.45	15q15
BG081044	Protocadherin 7	<i>Pcdh7</i>	-2.10	-2.17	4p15
BG063138	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2	<i>Spock2</i>	-2.10	-1.65	10pter-q25.3

* Calculated as described in Table 3.

In this comparison, we used specimens from the same model with comparable degrees of optic nerve damage, and we used identical statistical analysis tools. Therefore, we attribute the differences primarily to the source of tissue and the use of a sample enriched in RGCs, most likely because of the reduced dilution of the genes of interest by the highly cellular outer layers of the retina. Because an improved detection rate would be expected in a condition that affects primarily the RGC and inner retina, our findings further support the fidelity of this model for reproducing glaucomatous optic nerve damage.^{5,6,11,13,17,22,24-26}

Our DAVID analysis of changed gene categories showed that, in addition to enhancing the detection and extent of significantly changed genes, sampling the RGCL by LCM also refined the analysis of the individual cell processes most affected by elevated IOP in this layer of the retina. In all prior whole-retina gene array studies, genes related to inflammation and neuroinflammation and increased glial activity have been noted.^{5,7-9} Yang et al.,⁹ in addition to immune response genes, found by qPCR increases in cell cycle genes and those involved in stress response, including *Atf3*, which demonstrated just

over a onefold increase, 1 day after pressure elevation, and then normalized by 2 weeks. Additional gene classes noted in these studies included upregulation of ECM and cell adhesion components. Notable downregulated gene classes included cytoskeletal genes, crystallins, metabolic enzymes, and neurofilament protein.

In our array analysis of tissue from the whole retina, the most upregulated biological processes were also led by those related to immune processes, cell adhesion, signal transduction, regulation of cell proliferation, and programmed cell death (Table 4). The most downregulated processes were lipid biosynthesis, neurophysiological processes, and cytoskeletal organization and biogenesis (Table 5). Specifically, our finding of an upregulation of *C1qa* is in agreement with prior work showing increased retinal *C1qa* after elevated IOP in human and rat eyes²⁷ and in mouse and primate glaucoma models, as well as some human glaucomatous eyes.²⁸ Similar findings in the DBA/2J mouse glaucoma model have been reported,²⁹ along with evidence that the complement cascade may mediate dendritic remodeling, which has been described in RGCs in experimental glau-

TABLE 12. Comparison of Gene Changes between qPCR and Microarray Analyses in Whole Retina and RGCL

Genes	qPCR (x-fold*)	Microarray (x-fold*)
Whole Retina		
<i>Ccnd2</i>	4.82	2.69
<i>Clqa</i>	2.79	2.38
<i>Cryab</i>	-2.44	-25.00
<i>Fbln2</i>	3.20	1.36
<i>Jun</i>	1.42	1.33
<i>Nefl</i>	-3.43	-2.85
RGCL		
<i>A2m</i>	3.37	2.29
<i>Atf3</i>	94.80	28.38
<i>Clq</i>	5.33	3.63
<i>Gadd45g</i>	10.59	12.94
<i>Hmox1</i>	8.20	10.74
<i>Jun</i>	5.11	4.10
<i>Lcn2</i>	12.80	20.36
<i>Lif</i>	41.69	1.87
<i>Nef1</i>	-8.35	-2.85
<i>Socs3</i>	18.34	5.90
<i>Stat3</i>	2.68	4.44

* Calculated as described in Table 3.

coma models.^{4,30} Our finding of upregulation of the ECM messages *Fbln2* and *Timp1* confirms other investigators' findings of elevated retinal *Timp1* mRNA and protein after chronic IOP elevation in rats.^{5,23} On the other hand, we found that *Mmp9* was reduced. This contrasts with previous observations of increased retinal *Mmp9* protein by immunohistochemistry in a similar model.²³

By contrast, our analysis of tissue from the RGCL found that immune response was not the category most likely to be upregulated in response to pressure; instead, protein biosynthesis and metabolism, cell adhesion, and signal transduction were the leading categories (Table 8). Yang et al.⁹ also noted overrepresentation of genes leading to protein synthesis in eyes with late-phase injury. Their finding agrees with our current result in eyes with extensive optic nerve injury, which we interpret to reflect, in part, increased ECM production.

We also found activation of several stress-responsive genes in the RGCL. Most prominent of these was *Atf3*. First reported in whole retinas shortly after IOP elevation,⁹ our findings in eyes with more chronic injury further establish that retinal *Atf3* is increased after elevated IOP.³¹ The upregulation in *Atf3* appeared to be heavily influenced by changes in the RGCL, in which microarray analysis showed a 28.40-fold increase and qPCR a nearly 95-fold increase. By immunohistochemistry, this increase appears to be associated exclusively with neuronal nuclei in the RCCL (Fig. 5). Although we cannot rule out the possibility that some of these are amacrine cells, the label is confined to the RGCL, and the pattern is similar to that noted previously in RGCs labeled with phosphorylated Jun (pJun) antibodies in rat models of elevated IOP.³¹⁻³³ Further, *Atf3* is known to dimerize with Jun (which we also found to be upregulated in both whole retina and RGCL),³⁴ and has been co-localized with pJun in RGCs after optic nerve crush.³⁵ Although the role of *Atf3* in RGC death in our model is not yet well understood, it may contribute to RGC protection, possibly via interaction with heat shock protein 27 (Hsp27).^{9,36}

Perhaps of equal interest is that gene array analysis of the RGCL revealed a strikingly greater number of significantly downregulated biological processes than did the whole ret-

ina (Table 9 vs. Table 5). In particular, downregulation of several metabolic processes (including energy pathways, lipid metabolism, and protein catabolism), protein folding, and nucleotide biosynthesis accompanied a reduction in genes related to axon extension and dendrite morphogenesis. This finding is consistent with the notion that neurons downregulate metabolic processes before the initiation of apoptosis.¹³ The uniform, striking downregulation of several RGC markers from our qPCR study (Fig. 4D), including *Brn3a*, *-b*, and *-c* as well as *Nefl*, and *Nefb*, and *Thy1*, strongly suggests that these are specific changes within the RGCs themselves. Many of these changes appear to correlate with reductions in RGC dendritic patterns in experimental glaucoma,^{3,30,37} suggesting that these changes are part of the RGC response to elevated IOP and are not solely due to the loss of these cells. The gene SPARC-like 1 (*Sparcl1*), also highly expressed in RGCs and considered a candidate gene that induces RGC death after optic nerve lesion,³⁸ was downregulated in both whole retina (-1.41-fold) and RGCL (-2.52-fold; Table 11). Retinal *Sparcl1* has been reported to be downregulated in a rat glaucoma model.⁹

Although we did not enhance yet similar responses of many genes in the RGCL compared with the whole retina (Tables 10, 11), there were several genes that were changed

FIGURE 3. qPCR analysis of selected genes in glaucoma model whole retina. (A) Genes with significantly increased mRNA levels in whole retinas with extensive nerve injury. A large increase of nearly 35-fold was found in *Timp1* mRNA. (B) With IOP elevation and extensive nerve injury, *Nefl* and *Mmp9* mRNA levels were significantly reduced in the whole retina. No change was found in *Cryab*, *Gja1*, *Gap43*, or *Tuba1a*. $\dagger P < 0.001$.

FIGURE 4. qPCR analysis of selected genes in glaucoma model RGCL. (A) Expression changes of transcription factors and JAK/Stat pathway genes in the RGCL with extensive nerve injury. *Atf3* mRNA demonstrated a striking 94.81-fold increase in qPCR. Message level for *jun* was moderately increased and *Junb* mRNA was unchanged. *Lif* and *Socs3* mRNAs were highly upregulated, whereas a small but significant increase was found in message levels for *Lifr* and *Stat3*. (B) Upregulation of other stress response genes. Message levels for *Lcn2*, *Gadd45g*, and *Hmox1* were increased by nearly 10-fold. *Timp1* was highly upregulated (20.37-fold). (C) Significantly upregulated message level for glial cell marker proteins. *Aif1* was used for microglia and *Gfap* for astrocytes and Müller cells. No significant change was found in amacrine cell markers *Stx1a* and *Calb1*. (D) Prominent reduction of mRNA levels for RGC marker proteins and RGC axonal markers *Nefl* and *Nefb*. Neurofilaments *Nefl* and *Nefb* mRNAs were both reduced to approximately 0.10. Message levels for all three *Brn3* family proteins were reduced to a comparable level. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

FIGURE 5. Increased level of *Atf3* protein in RGCL with extensive nerve injury. Typical immunohistochemistry images indicated no specific staining in the control eyes (A) but intense, specific *Atf3* staining (dark brown) localized to neuronal nuclei (arrowheads) within an RGCL with grade 5 nerve injury (B). Scale bar, 50 μ m.

in the RGCL, but in the opposite direction. These included *Cryab*, which in whole retina was found to be downregulated 25-fold, similar to data reported by Ahmed et al.⁵ Belonging to the small heat shock protein family, α -crystallins can serve as molecular chaperones, leading to the speculation that their downregulation could contribute to RGC damage in glaucoma. However, although downregulation of several crystallins has also been reported in the DBA/2J mouse glaucoma model,⁷ and reduced α A-crystallin was noted by proteomic analysis in a rat glaucoma model,³⁹ Hsp 27, another small heat shock protein, has been reported to be elevated by several groups in retinas of eyes with experimental glaucoma^{40,41} and in human glaucomatous eyes.⁴² Other researchers have obtained more variable results, with a suggestion that retinal contamination may play a role.⁹ Of interest, our LCM study showed that *Cryab* in the RGCL in fact increased by 1.83-fold. Because this tissue was harvested from paraffin-embedded sections prepared without globe dissection, contamination is unlikely. Our results strongly suggest that regulation of *Cryab* in the RGCL and perhaps that of other crystallin genes, indeed differs from that in the whole retina, which may explain some of the conflicting results previously reported.

Although LCM of the RGCL provides a sample enriched in RGCs, the gene changes noted herein cannot be exclusively attributed to RGCs. Our qPCR analyses provide evidence that cell marker genes for astrocytes and Müller cells (*Gfap*) as well as microglia (*Aif1*) are upregulated in these eyes with extensive injury. Although amacrine cell marker gene expression changes in the RGCL in our model were minimal (Fig. 4C), our study does not rule out the possibility that a portion of the significantly changed genes found in the RGCL may reflect

changes that are occurring in amacrine cells. The most clearcut evidence of change is found in the RGCs themselves. Our qPCR analysis of RGC markers show that the Brn3 family and neurofilament protein mRNAs are reduced to less than 0.20. Since some of this reduction is due to the downregulation of RGC-specific proteins,¹³ it is likely that these numbers are an overestimate of RGC loss. These data, along with previous findings that optic nerves with this extent of injury correspond to an approximately 55% axon loss,¹¹ suggest that the number of RGCs that remain in the retina is approximately 20% to 40% of normal.

Finally, it must be emphasized that this report is a head-to-head comparison of eyes with extensive damage. It does not address processes that may be specific to early injury or to responses that may occur with progression of pressure-induced optic nerve damage. Our recent work on retinal genes relevant to the neurotrophin hypothesis and Trk signaling in our model suggests that many changes found in eyes with extensive optic nerve injury can also be seen in eyes with minimal injury, just 10 days after the onset of IOP elevation, although to a generally lesser degree.³¹ However, early, subtle responses potentially important for RGC protection may still be present but overlooked. The results of this study strongly indicate that application of the LCM technique to retinas with early injury, currently in progress, should provide a more sensitive understanding of early RGC responses to pressure-induced optic nerve damage.

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