Early Gene Expression Changes in the Retinal Ganglion Cell Layer of a Rat Glaucoma Model

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PURPOSE. To identify patterns of early gene expression changes in the retinal ganglion cell layer (GCL) of a rodent model of chronic glaucoma.

METHODS. Prolonged elevation of intraocular pressure (IOP) was produced in rats by episcleral vein injection of hypertonic saline ($N = 30$). GCLs isolated by laser capture microdissection were grouped by grading of the nerve injury ($<25\%$ axon degeneration for early injury; $>25\%$ for advanced injury). Gene expression was determined by cDNA microarray of independent GCL RNA samples. Quantitative PCR (qPCR) was used to further examine the expression of selected genes.

RESULTS. By array analysis, 533 GCL genes (225 up, 308 down) were significantly regulated in early injury. Compared to only one major upregulated gene class of metabolism regulation, more were downregulated, including mitochondria, ribosome, proteasome, energy pathways, protein synthesis, protein folding, and synaptic transmission. qPCR confirmed an early upregulation of *Atf3*. With advanced injury, 1790 GCL genes were significantly regulated (997 up, 793 down). Altered gene categories included upregulated protein synthesis, immune response, and cell apoptosis and downregulated dendrite morphogenesis and axon extension. Of all the early changed genes, 50% were not present in advanced injury. These uniquely affected genes were mainly associated with upregulated transcription regulation and downregulated protein synthesis.

CONCLUSIONS. Early GCL gene responses to pressure-induced injury are characterized by an upregulation of *Atf3* and extensive downregulation in genes associated with cellular metabolism and neuronal functions. Most likely, these changes represent those specific to RGCs and are thus potentially important for enhancing RGC survival in glaucoma. (*Invest Ophthalmol Vis Sci.* 2011;52:1460–1473) DOI:10.1167/iovs.10-5930

The progressive loss of vision in glaucoma patients has been attributed to degeneration of retinal ganglion cells (RGCs) and their axons and the eventual death of these cells. As intraocular pressure (IOP) remains the best established risk factor for glaucoma, therapy for this blinding disease continues to rely heavily on lowering pressure by medical and surgical

measures. Although pressure reduction has been effective in preventing onset of glaucoma and delaying its progression,^{1,2} some patients do not respond well or are intolerant of these treatments, whereas others continue to experience vision loss despite significant reduction in IOP.

Recently, neuroprotective strategies other than pressure lowering, including supplementation of neurotrophins and prevention of caspase activation and apoptosis, have been proposed and are being investigated as new goals for glaucoma therapy.^{3,4} Effective neuroprotection, aimed at salvaging functional RGCs and their axons before they are committed to die, requires early intervention and targeting of upstream events. These early changes may involve increased susceptibility of RGCs to elevated IOP, which could explain progressive vision loss in patients despite lowered eye pressure. Conversely, they may be protective mechanisms that could be therapeutically enhanced. Identification of the early critical molecular events in RGCs would add to our understanding of the nature of glaucomatous injury and provide potential targets for neuroprotective strategies.

The primary injury in glaucoma is now widely believed to be axonal at the lamina in the optic nerve head.^{5–7} However, the nature of the injury and the initial molecular events occurring in injured RGCs are largely unknown. Studies have described early morphologic changes in RGCs,^{8–10} suggesting that structural and possibly functional changes occur well in advance of their death. Also, pathways involved in RGC soma degeneration, although not well characterized, have been suggested to be distinct from those in axon degeneration in glaucoma.¹¹ To examine global changes in gene expression, several studies have applied genomewide analysis of microarrays to the retina of rodent and primate glaucoma models as well as the DBA/2J mice.^{12–17} These studies have found significant regulation of gene expression in the retina and implicate multiple processes in glaucomatous injury, including neuroinflammation, glia activation, apoptosis, and cytoskeleton-based processes. However, because RGCs represent only a small fraction of the total retinal cell population, in these studies performed on whole retina samples, many responses specific to RGCs may have been masked. In an effort to identify changes more specific to RGCs, Wang et al.¹⁸ studied global gene expressions in individual RGCs captured by laser in experimental glaucoma and reported extensive expression changes in genes associated with multiple signaling pathways and apoptosis. Like the whole-retina studies, however, this study did not specifically target the events of early injury.

Using a rodent glaucoma model in which chronic IOP elevation is achieved by injection of hypertonic saline into episcleral veins,¹⁹ we have recently demonstrated that detection of RGC responses can be greatly enhanced by analyzing just the RGC layer (GCL) compared with the whole retina.²⁰ Successfully isolating the GCL from the whole retina by laser capture microdissection (LCM) and applying cDNA microarray analysis to the GCL mRNA, we identified a significantly larger

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number of genes with altered expression in the GCL compared with those in whole retinas of eyes with comparable injury. That report, which focused on responses in eyes with extensive nerve injury, represents only part of a larger study in which we examined the GCL in eyes with all grades of optic nerve injury. Here, we present the complete analysis, but mainly concentrate on early-injury responses that, as mentioned previously, are likely to be important for understanding the cellular mechanisms of glaucoma and identifying their therapeutic implications.

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.

Glaucoma Model

Eight-month-old Brown Norway rats, housed in constant low-level light,²¹ were injected unilaterally with hypertonic saline through episcleral veins to obstruct aqueous outflow.¹⁹ IOP was measured in awake animals at least three times a week with a handheld tonometer (Tonolab; Icare Finland Oy, Espoo, Finland) and monitored typically for 5 weeks. 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 the corresponding IOP measurement, subtracting the mean of the corresponding values for control eyes.

Pressure-induced optic nerve injuries were evaluated as previously described.²¹ Briefly, optic nerves were removed, 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). Sections were graded by five masked observers and the grades were averaged. We have previously determined that each unit of grade corresponds to an approximate 12% increase in the number of degenerating axons in the optic nerve.²¹ In this study, for the purpose of effective analysis, glaucoma model eyes were grouped by nerve grading: the early-injury group, including those with grade <2.5, or less than 25% axon degeneration, and the advanced injury group, composed of eyes with nerve grades >2.5, or >25% axon degeneration.

Laser Capture Microdissection

GCL was isolated from paraffin-embedded retinal sections, as previously described.²⁰ Briefly, whole globes were collected and postfixed in 100% ethanol, and embedded in paraffin. After the blocks were sectioned and the slices deparaffinized, LCM (Arcturus PixCell II LCM system; Molecular Devices, Sunnyvale, CA) was immediately performed on multiple retinal sections from each globe using typical parameters as follows: 7.5- μ m spot diameters, 0.7- to 1.2-ms duration, and 60- to 80-mW laser power.

RNA Isolation and Amplification

Total RNA was purified from GCL captured on LCM caps (Capsure Macro (Molecular Devices) by using an isolation kit (Arcturus PicoPure RNA; Molecular Devices). RNA amount was quantitated with a fluorometric assay (RiboQuant; Molecular Probes, Eugene, OR). At the OHSU Gene Microarray Shared Resource Facility, all samples of RNA were subjected to two rounds of linear amplification (MessageAmp aRNA Amplification Kit; Ambion, Austin, TX). Yield of the amplified (a)RNA, as well as its integrity, was assessed on a bioanalyzer (model 2100; Agilent Technologies, Palo Alto, CA).

cDNA Microarrays and Data Analysis

A total of 6 control and 24 injected eyes with elevated IOP, including 7 eyes with a shorter term of IOP exposure (<5 weeks), were used in the microarray analysis. Nerve evaluation determined that there were 9 eyes with early nerve injury and 15 with advanced injury. The latter included eyes with extensive nerve injury (grade 5) that had been analyzed separately.²⁰ Amplified RNA was reverse transcribed and dye-labeled before hybridization, to probe sequences of SMCmou8400A and SMCmou6600A cDNA microarrays. These steps, together with slide scanning and data compilation, were all completed at the OHSU Gene Microarray Shared Resource Facility. The cDNA microarrays contain a total of 15,400 cDNA probes and represent the initial NIA (National Institute of Aging, Bethesda, MD) 15k mouse library (<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>). Each probe was represented by two duplicate spots on two identical slides, making four technical duplicates for each array. Gene expression data were reported as ratios of average signal intensity (local background subtracted) in each sample to that of a common reference standard derived by combining aRNA aliquots from all samples. Data were normalized by using a modified Lowess procedure²² at the OHSU Microarray Facility.

Genes with significantly different expression between injury groups (>1.3 fold) were identified by using multiclass comparison analysis in the web tool Significant Analysis of Microarrays (SAM, version 2.5; <http://www-stat.stanford.edu/~tibs/SAM>; provided in the public domain by Stanford University, Stanford, CA) with 2% FDR and followed by ANOVA and Tukey-Kramer posttest ($P < 0.05$ corrected for multiple comparisons). DAVID Bioinformatics Resources 2008 (<http://david.abcc.ncifcrf.gov/>; National Institute of Allergies and Infectious Disease, Bethesda, MD) was used to determine significantly regulated functional classes of the affected genes based on EASE scores, using the controlled vocabulary of the gene ontology consortium (<http://www.geneontology.org/>).

Quantitative Real-Time Reverse Transcription PCR (qPCR)

To expand the sample size, we combined an additional group of animals with those in microarray analysis, making a total of 13 control and 30 glaucoma model eyes (14 in early and 16 in advanced injury group) for qPCR analysis. Gene-specific primers were designed (Primer Designer 4 or Clone Manager Professional, ver. 9.0; both from Science Ed Software, Cary NC) and are listed in Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5930/-/DCSupplemental>. All primers were designed to target the mRNA region within 350 bp of its 3' polyadenylation site. Using random primers (250 ng; Invitrogen, Carlsbad, CA) and reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA), we reverse transcribed 20 ng aRNA for each sample and 2 to 400 ng reference aRNA for the standard curve. Expression levels were determined by qPCR on a real-time PCR system (LightCycler, software 3.5, and DNA Master SYBR Green 1 kit; Roche, Indianapolis, IN) according to the manufacturer's protocol. Based on a lack of significant change in ribosomal protein 16 (*Rps16*) in the microarray study, this gene was used as the housekeeping gene for normalization,²³ rather than glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which showed more change in these samples.

All data were expressed as a percentage of the control values. Statistical analysis was performed by comparing the glaucoma model eyes to the controls by using an unpaired *t*-test (Excel; Microsoft, Redmond, WA), and a value of $P < 0.05$ was considered significant.

RESULTS

IOP History of Injury Groups in Microarray

The cumulative IOP exposure of the early-injury group averaged 69.8 ± 94.4 mm Hg (mean $\pm$ SD). The average grading for nerve injury in this group was 1.5 ± 0.4 (mean $\pm$ SD). For the group with advanced injury, the average cumulative IOP expo-

TABLE 1. IOP Histories of Early and Advanced Injury Groups in Microarray Analysis

Group	Eyes (n)	Injury Grade*	Cumulative Elevated IOP* (mm Hg × d)	Mean IOP* (mm Hg)
Controls	6	1.0 ± 0.0		21.6 ± 1.4
Early injury	9	1.5 ± 0.4	69.8 ± 94.4	23.3 ± 2.3
Advanced injury	15	3.9 ± 0.8	314.4 ± 261.0	30.8 ± 7.1

* Mean ± SD.

sure was 314.4 ± 261.0 mm Hg, and the nerve injury grades averaged 3.9 ± 0.8 . The results are summarized in Table 1.

Significant GCL Genes Identified in Early Injury

Comparison of the early injury to the controls identified a total of 533 genes significantly altered in expression, with 225 upregulated and 308 downregulated (complete list in Supplementary Table S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5930/-/DCSupplemental>). Listed in Tables 2 and 3 are the 30 genes with the most increased or reduced expression, respectively. The most upregulated three genes were activating transcription factor 3 (*Atf3*; 3.4-fold), a member of the ATF/CREB family of transcription factors, and the acute and stress response genes lipocalin 2 (*Lcn2*; 2.98-fold), and heme oxygenase 1 (*Hmox1*; 2.95-fold). The three most downregulated genes included: cold shock domain containing E1, RNA binding (*Csde1*; -2.25-fold), a cytoplasmic RNA-binding protein involved in transcription regulation; glycolytic enzyme triosephosphate isomerase 1 (*Tpi1*; -2.22-fold); and Dickkopf homolog 3 (*Xenopus laevis*; *Dkk3*; -2.18-

fold), a divergent member of the Wnt signaling regulator Dkk family protein with an antiapoptotic and cytoprotective role in the retina.^{24–26}

Functional Gene Categories Regulated by Early Injury

As summarized in Table 4, with early injury, two biological processes were significantly upregulated: regulation of metabolism and regulation of translational initiation. The single affected cell component associated with the significantly upregulated genes was the nucleus. In contrast, 12 biological processes were downregulated by early injury (Table 5), including generation of precursor metabolites and energy (energy pathways), glycolysis, tricarboxylic acid cycle (the TCA cycle), protein biosynthesis, oxidative phosphorylation and electron transport, protein folding and catabolism, and nerve-nerve synaptic transmission. Accordingly, cell components and molecular functions associated with mitochondria, ribosome, proteasome, oxidoreductase activity, and unfolded protein binding were downregulated.

Significant GCL Genes Identified in Advanced Injury

With advanced injury, 1790 genes with significantly altered expression were identified, including 997 up- and 793 downregulated genes (complete list in Supplementary Table S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5930/-/DCSupplemental>). The most changed 30 genes are listed in Tables 6 (up) and 7 (down). The most upregulated three genes in the early-injury group, *Atf3*, *Lcn2*, and *Hmox1*, showed even greater increases with the advanced injury by 23.84-, 18.63-,

TABLE 2. The Thirty Most Upregulated Genes in the GCL with Early Nerve Injury

GenBank Accession	Entrez Gene ID	Gene Name	Gene Symbol	Change (x-Fold)
BG067364	11910	Activating transcription factor 3	<i>Atf3</i>	3.40
BG070106	16819	Lipocalin 2	<i>Lcn2</i>	2.98
BG077732	15368	Heme oxygenase (decycling) 1	<i>Hmox1</i>	2.95
BG070357	320184	Leucine rich repeat containing 58	<i>Lrrc58</i>	2.42
BG081601	235493	cDNA sequence BC031353	<i>BC031353</i>	2.42
BG064262	18607	3-phosphoinositide dependent protein kinase-1	<i>Pdk1</i>	2.33
BG078388	225876	F-box and leucine-rich repeat protein 11	<i>Fbxl11</i>	2.27
BG069041	216965	TAO kinase 1	<i>Taok1</i>	2.27
BG077145		Transcribed locus, strongly similar to NP_904337.1 NADH dehydrogenase subunit 4		2.26
BG070105	105387	Aldo-keto reductase family 1, member C14	<i>Akr1c14</i>	2.26
BG077271	53333	Translocase of outer mitochondrial membrane 40 homolog (yeast)	<i>Tomm40</i>	2.25
BG067861	78785	CAP-GLY domain containing linker protein family, member 4	<i>Clp4</i>	2.23
BG063282	223435	Triple functional domain (PTPRF interacting)	<i>Trio</i>	2.19
BG070342	67579	Cytoplasmic polyadenylation element binding protein 4	<i>Cpeb4</i>	2.15
BG077059	66631	Hippocampus abundant transcript-like 1	<i>Hiatl1</i>	2.14
BG084377	20848	Signal transducer and activator of transcription 3	<i>Stat3</i>	2.09
BG070137	11855	Rho GTPase activating protein 5	<i>Arhgap5</i>	2.08
BG078469	18970	Polymerase (DNA directed), beta	<i>Polb</i>	2.06
BG068032	229055	Zinc finger and BTB domain containing 10	<i>Zbtb10</i>	2.05
BG080829	140780	BMP2 inducible kinase	<i>Bmp2k</i>	2.01
BG073457	106064	Expressed sequence AW549877	<i>AW549877</i>	1.98
BG074359	76205	STARD3 N-terminal like	<i>Stard3nl</i>	1.98
BG071123	98823	Expressed sequence AA763515	<i>AA763515</i>	1.96
BG063173	109711	Actinin, alpha 1	<i>Actn1</i>	1.96
AA410046	76857	RIKEN cDNA 4921517N04 gene	<i>4921517N04Rik</i>	1.95
BG067684	108829	Jumonji domain containing 1C	<i>Jmjd1c</i>	1.94
AW536733	17101	Lysosomal trafficking regulator	<i>Lyst</i>	1.93
BG082399	56738	Molybdenum cofactor synthesis 1	<i>Mocs1</i>	1.90
BG082419	109019	Oligonucleotide/oligosaccharide-binding fold containing 2A	<i>Obfc2a</i>	1.89
BG066534	52615	Suppressor of zeste 12 homolog (Drosophila)	<i>Suz12</i>	1.89

TABLE 3. The 30 Most Downregulated Genes in the GCL with Early Nerve Injury

GenBank Accession	Entrez Gene ID	Gene Name	Gene Symbol	Change (x-fold)
BG064909	229663	Cold shock domain containing E1, RNA binding	<i>Csde1</i>	-2.25
BG075608	21991	Triosephosphate isomerase 1	<i>Tpi1</i>	-2.22
BG088107	50781	Dickkopf homolog 3 (<i>Xenopus laevis</i>)	<i>Dkk3</i>	-2.18
BG074818	320480	RIKEN cDNA 6430537K16 gene	<i>6430537K16Rik</i>	-2.12
BG064838	22143	Tubulin, alpha 1B	<i>Tuba1b</i>	-2.09
BG082031	81702	Ankyrin repeat domain 17	<i>Ankrd17</i>	-1.99
BG077276	29812	N-myc downstream regulated gene 3	<i>Ndr3</i>	-1.97
BG065155	67134	Nucleolar protein 5A	<i>Nol5a</i>	-1.95
BG077488	19172	Proteasome (prosome, macropain) subunit, beta type 4	<i>Psm4</i>	-1.92
BG080372		2 days neonate thymus thymic cells cDNA		-1.91
BG071068	14688	Guanine nucleotide binding protein, beta 1	<i>Gnb1</i>	-1.91
BG064815	16002	Insulin-like growth factor 2	<i>Igf2</i>	-1.91
BG076877	12709	Creatine kinase, brain	<i>Ckb</i>	-1.90
BG088231	57320	Parkinson disease (autosomal recessive, early onset) 7	<i>Park7</i>	-1.90
BG070222	69684	Alanyl-tRNA synthetase domain containing 1	<i>Aarsd1</i>	-1.89
BG065964	14688	Guanine nucleotide binding protein, beta 1	<i>Gnb1</i>	-1.89
BG065432	27406	ATP-binding cassette, sub-family F (GCN20), member 3	<i>Abcf3</i>	-1.89
C87546	20869	Serine/threonine kinase 11	<i>Stk11</i>	-1.89
BG063860		Transcribed locus		-1.88
BG064831	227613	Tubulin, beta 2c	<i>Tubb2c</i>	-1.88
BG074355	12874	Carboxypeptidase D	<i>Cpd</i>	-1.86
BG064832	227613	Tubulin, beta 2c	<i>Tubb2c</i>	-1.86
BG069067	74026	RIKEN cDNA 4121402D02 gene	<i>4121402D02Rik</i>	-1.85
BG069637		Transcribed locus		-1.84
BG065478	22273	Ubiquinol-cytochrome c reductase core protein 1	<i>Uqcrc1</i>	-1.83
BG073254	19656	RNA binding motif protein, X chromosome retrogene	<i>Rbmxt</i>	-1.83
BG077791	66537	Proteasome maturation protein	<i>Pomp</i>	-1.82
BG074917	14886	General transcription factor II I	<i>Gtf2i</i>	-1.82
BG078478	14734	Glypican 3	<i>Gpc3</i>	-1.82
BG086020	16499	Potassium voltage-gated channel, shaker-related subfamily, beta member 3	<i>Kcnab3</i>	-1.82

and 8.59-fold, respectively. Growth arrest and DNA damage-inducible 45 gamma (*Gadd45g*), a member of the DNA damage-inducible gene family that inhibits cell growth and induces apoptosis in response to stress, was upregulated 9.56-fold. The most downregulated genes in advanced injury included ELAV (embryonic lethal, abnormal vision)-like 2 (*Elavl2*; -4.87-fold) and 14-3-3 family member tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (*Ywhab*; -3.13-fold). *Elavl2* encodes an RNA-binding protein, and its transcript has been found to be highly enriched in GCL of human retina.²⁷ The downregulation of *Ywhab* was consistent with previous findings in individual RGCs exposed to elevated IOP, which showed a similar magnitude of reduction in *Ywhab* by microarray.¹⁸ In advanced injury, *Dkk3* remained downregulated (-3.13-fold).

TABLE 4. Significantly Upregulated Gene Categories Identified by DAVID Analysis in the GCL with Early Nerve Injury

Gene Categories	Genes (n)	EASE Score
Biological Process		
Regulation of metabolism	36	0.039
Regulation of translational initiation	3	0.043
Cell Component		
Nucleus	63	0.017
Molecular Function		
Zinc ion binding	29	0.031
Nucleic acid binding	48	0.040
Protein dimerization activity	6	0.042
Transition metal ion binding	33	0.044

Functional Gene Categories Regulated by Advanced Injury

Eyes with advanced injury showed many more biological processes that were significantly upregulated than those with early injury. These included immune response, cell adhesion, protein metabolism, cytoskeleton regulation, and apoptotic program (Table 8). The gene categories of ubiquitin cycle, microtubule-based process, lipid metabolism, and axon and dendrite extension were downregulated by advanced injury, in addition to energy pathways, mitochondria, and synaptic transmission, which were also downregulated in early injury (Table 9).

Significant Genes and Gene Categories Specifically Regulated by Early Injury

A comparison of the significant genes in both injury groups identified 261 genes (120 upregulated and 141 downregulated) not present in the advanced-injury group (Supplementary Table S4, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10.5930/-DCSupplemental>), approximately 50% of the early changed genes. These are considered genes specifically regulated by early injury. By DAVID analysis, several significantly changed biological processes and cell components associated with these genes were identified, including upregulation of transcription and downregulation of protein synthesis and ribosomal genes (Table 10). The analysis also identified significant changes in the insulin signaling pathway (EASE score = 0.0089), with seven altered genes in the pathway including 3-phosphoinositide dependent protein kinase-1 (*Pdpk1*, 2.33-fold; Supplementary Table S5, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10.5930/-DCSupplemental>).

TABLE 5. Significantly Downregulated Gene Categories Identified by DAVID Analysis in GCL with Early Nerve Injury

Gene Categories	Genes (n)	EASE Score
Biological Process		
Generation of precursor metabolites and energy	33	0.0000
Cofactor metabolism	19	0.0000
Glycolysis	8	0.0001
Tricarboxylic acid cycle	7	0.0003
Protein biosynthesis	31	0.0004
Oxidative phosphorylation	9	0.0005
Electron transport	16	0.0054
Protein folding	13	0.0120
Protein catabolism	13	0.0140
ATP biosynthesis	6	0.0140
Proton transport	6	0.0230
Nerve-nerve synaptic transmission	3	0.0490
Cell Component		
Mitochondrion	49	0.0000
Ribosome	19	0.0000
Proteasome complex (sensu eukaryota)	6	0.0056
Molecular Function		
Oxidoreductase activity	29	0.0000
Structural constituent of ribosome	19	0.0000
Unfolded protein binding	13	0.0003
Ion transporter activity	21	0.0007
Electron transporter activity	12	0.0027
Nucleoside-triphosphatase activity	21	0.0054
Hydrolase activity, acting on acid anhydrides	21	0.0110
Proteasome endopeptidase activity	4	0.0170
RNA binding	24	0.0170
NADH dehydrogenase activity	5	0.0180
Iron-sulfur cluster binding	4	0.0400

qPCR Analysis of Early-Injury GCL

To further identify gene expression changes in GCL and verify array results, we used qPCR to examine expressions of selected early messages identified in microarray analysis as well as a number of others that are not present on the microarrays but may be implicated in glaucomatous injury process. Since we targeted to identify the early events, the following qPCR results are focused on those of the early-injury group, while some of the advanced injury changes are also included.

Messages for Cell Type Markers

We first examined expressions of several common RGC marker proteins: POU4f1, POU4f2, and POU4f3, three members of the POU family transcription factors, as well as Thy-1 cell surface antigen (Thy1; Fig. 1A). With early injury, the only change in these messages was a significant reduction in POU4f1 (to $52\% \pm 7\%$; $P < 0.01$). For axonal mRNAs, the heavy and light polypeptide of neurofilament (Nefh and Nefl) appeared decreased, but the reduction was not enough to reach statistical significance. In contrast, axonal membrane protein growth associated protein 43 (Gap43) had a more than 200% increase in expression at the message level ($211\% \pm 63\%$; $P < 0.05$).

In addition to RGC, amacrine cells are abundant in the GCL, representing approximately 50% of the neurons.²⁸ While a universal marker for GCL amacrine cells is lacking and many amacrine subclass markers were not on our arrays,²⁹ other markers (*Caln1*, *Calb1*, *Ndr4*, *Atp11a*, *Gas7*, *Dab1*, and *Gabra3*) were not significantly affected by our array analysis. Synaptic vesicle protein, *Stx1a* (*Hpc-1*, syntaxin 1a) mRNA is

produced by both amacrine and retinal ganglion cells.³⁰ By qPCR, we found that *Stx1a* mRNA levels were reduced in the early-injury group ($45\% \pm 6\%$; Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5930/-/DCSupplemental>).

As shown in Figure 1B, although there was some variation in levels, no significant change of mRNA expression was detected in any microglial or astrocytic marker proteins examined in the early-injury group. The former included allograft inflammatory factor (Aif1, aka IBA1), a calcium binding protein associated with microglial activation in the central nervous system (CNS), colony stimulating factor 1 receptor (Csf1r) which plays a central role in microglial activation, and purinergic receptor P2Y12 (P2ry12), an adenine nucleotide receptor which is dramatically downregulated after microglial activation. The astrocytic markers included glial fibrillary acidic protein (Gfap), an intermediate filament protein, and a common marker for active astrocyte, aquaporin 4 (Aqp4), the main water channel in the CNS, and vimentin, a marker for proliferating astrocytes. Only with the advanced injury, were most of these glial marker messages significantly changed (Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5930/-/DCSupplemental>).

Messages for Neurotrophins (NTs) and NT Receptors

Despite a lack of direct evidence, deprivation of retrograde transported NT and NT receptors has been hypothesized to result in RGC death in glaucoma. Our previous work in the whole retina suggested a more complex response of the NT system to elevated IOP.³¹ In GCL with early injury, the qPCR found a $262\% \pm 51\%$ ($P < 0.0001$) and $222\% \pm 46\%$ ($P < 0.05$) increase in brain derived neurotrophin factor (Bdnf) and NT3 (Ntf3) mRNA levels, respectively (Fig. 2). Concomitantly, TrkB (Ntrk2) was found to decrease to $49\% \pm 7\%$ ($P < 0.001$) of control values and p75NTR(Ngfr) was reduced to $48\% \pm 13\%$ ($P = 0.01$). No significant change was found in message levels for Ngf, TrkA (Ntrk1), or TrkC (Ntrk3). NT4/5 (Ntf4) mRNA was not detected in the GCL.

Messages for Transcription Factors

The most upregulated gene identified by microarray in early injury was *Atf3* (3.4-fold, Table 2), a member of the ATF/CREB family of transcription factors that is inducible by a wide variety of stress signals and upregulated in the whole retina in both experimental glaucoma and after optic nerve transection.^{16,20,32} Using qPCR (with more samples), we found a much more dramatic increase in the mRNA level of *Atf3* ($1492\% \pm 949\%$; $P < 0.01$) in GCL with early injury (Fig. 3). As the nerve injury advances, *Atf3* mRNA increases in a pattern linear to the injury grades ($r^2 = 0.25$; $P < 0.001$). Jun family proteins and activator protein 1 (AP-1) components c-Jun and Junb, two early stress responding transcription factors that can dimerize with *Atf3*,^{33,34} showed no change at the mRNA level in the early-injury group. Signal transducers and activators of transcription 3 (Stat3), another early response transcription factor commonly upregulated after nerve injury, including isolated glaucoma model RGC¹⁸ and whole retinas after optic nerve transection,^{35–37,16,32} and shown to act together with *Atf3* and c-Jun in damaged neurons,³⁸ was not changed either. With advanced injury, however, c-Jun ($445\% \pm 67\%$; $P = 0.0001$), Junb ($305\% \pm 99\%$; $P < 0.05$), and Stat3 ($304\% \pm 33\%$; $P < 0.0001$) mRNAs were all significantly upregulated in GCL, confirming findings in whole retinas and laser-captured RGCs.^{16,18} In early-injury GCL, we did not detect any change in the message level for transcription factor hypoxia-inducible factor 1, alpha subunit (Hif1 α) which mediates cellular responses to hypoxic stimuli. Also, no change was detected in

TABLE 6. The 30 Most Upregulated Genes in the GCL with Advanced Nerve Injury

GenBank Accession	Entrez Gene ID	Gene Name	Gene Symbol	Change (x-fold)
BG067364	11910	Activating transcription factor 3	<i>Atf3</i>	23.84
BG070106	16819	Lipocalin 2	<i>Lcn2</i>	18.63
BG067419	23882	Growth arrest and DNA-damage-inducible 45 gamma	<i>Gadd45g</i>	9.56
BG077732	15368	Heme oxygenase (decycling 1)	<i>Hmox1</i>	8.59
BG085576	12825	Procollagen, type III, alpha 1	<i>Col3a1</i>	7.79
BG070105	105387	Aldo-keto reductase family 1, member C14	<i>Akr1c14</i>	7.15
AU045725	75212	Ring finger protein 121	<i>Rnf121</i>	6.42
AW550999	59013	Heterogeneous nuclear ribonucleoprotein H1	<i>Hnrpb1</i>	6.23
BG088567	16009	Insulin-like growth factor binding protein 3	<i>Igfbp3</i>	6.03
BG068326	104174	Glycine decarboxylase	<i>Gldc</i>	5.76
AU021372	14105	FUS interacting protein (serine-arginine rich) 1	<i>Fusip1</i>	5.65
BG077473	56700	RIKEN cDNA 0610031J06 gene	<i>0610031J06Rik</i>	5.63
BG077076	53895	Caseinolytic peptidase, ATP-dependent, proteolytic subunit homolog (E. coli)	<i>Clpp</i>	5.62
C79058	73945	OTU domain containing 4	<i>Otud4</i>	5.37
AW556719	52040	Protein phosphatase 1, regulatory subunit 10	<i>Ppp1r10</i>	5.36
AW550998		Transcribed locus		5.26
AW538113	67283	Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	<i>Slc25a19</i>	5.15
AU020524	208715	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	<i>Hmgcs1</i>	5.13
AW549620		Transcribed locus		5.13
BG080700	27279	Tumor necrosis factor receptor superfamily, member 12a	<i>Tnfrsf12a</i>	5.11
AW556082	223921	Achalasia, adrenocortical insufficiency, alacrimia	<i>Aaas</i>	5.07
BG076966	21816	Transglutaminase 1, K polypeptide	<i>Tgm1</i>	5.06
BG064802	20692	Secreted acidic cysteine rich glycoprotein	<i>Sparc</i>	4.96
BG063357	382793	Metaxin 3	<i>Mtx3</i>	4.95
AW539348	66848	Fucosidase, alpha-L-2, plasma	<i>Fuca2</i>	4.90
BG063033	66101	Peptidyl prolyl isomerase H	<i>Ppib</i>	4.89
BG077017	14969	Histocompatibility 2, class II antigen E beta	<i>H2-Eb1</i>	4.86
BG074327	12825	Procollagen, type III, alpha 1	<i>Col3a1</i>	4.83
BG088348	233912	Armadillo repeat containing 5	<i>Armc5</i>	4.79
BG085864	114679	Selenoprotein M	<i>Selm</i>	4.79

the mRNA level of erythropoietin (*Epo*), a target gene of Hif1 α and marker for hypoxia. Neither Hif1 α nor *Epo* was increased in the advanced injury group.

Messages for Bcl2 Family Members and Other Prosurvival Genes

The Bcl2 gene family has been suggested to play a critical role in RGC death in glaucoma.³⁹ We examined the expression of three Bcl2 family genes at the transcriptional level by qPCR: the proapoptotic gene *Bax* and the antiapoptotic genes *Bcl2* and *BclxL*. Message level for tumor suppressor p53 (Tp53), the Bax gene transcription factor,⁴⁰ was also examined. As summarized in Figure 4A, Bcl2 appeared depressed, but this change was not statistically significant. No significant change was found in the other messages in the GCL with early injury. In the advanced-injury group, mRNA levels of Bax, Bcl2, and BclxL were not changed but Tp53 increased to 251% $\pm$ 45% of control ($P = 0.0003$).

Our microarray analysis identified *Pdpk1* as one of the significant insulin pathway genes (Table 2). Acting downstream to PI3K, *Pdpk1* also mediates effects of other growth factors such as NTs to promote cell survival.^{41–43} Consistent with the microarray results, the qPCR analysis showed an early upregulation of *Pdpk1* mRNA in GCL (to 210% $\pm$ 30% of control; $P < 0.01$; Fig. 4B), which returned to the control level in advanced injury. On the contrary, neuregulin 1 (*Nrg1*), a prosurvival gene that regulates cell–cell communication in the nervous system^{44,45} and is reported to be downregulated in RGCs with advanced nerve injury,¹⁸ was significantly reduced to 63% $\pm$ 8% of control values ($P = 0.01$) in early-injury GCL. *Dkk3*, one of the most downregulated early genes on microar-

ray, was decreased (to 66% $\pm$ 9% of control values; $P = 0.02$), according to qPCR. The qPCR analysis also identified a significant reduction in *Gapdh* mRNA in early-injury GCL (to 44% $\pm$ 7%; $P < 0.001$).

Messages for Stress and Immune Response Genes

As shown in Figure 5, heme oxygenase 1 (*Hmox1*), the inducible isoform of heme oxygenase responding to a variety of stress stimuli, was upregulated to 347% $\pm$ 112% ($P < 0.05$) in early-injury GCL. Lipocalin 2 (*Lcn2*) and cytokine leukemia inhibitory factor (*Lif*) appeared upregulated, but the increases turned out to be nonsignificant. However, both *Lif* and *Lcn2* increased linearly to the injury grades (for *Lif*, $r^2 = 0.49$, $P < 0.0001$; for *Lcn2*, $r^2 = 0.53$, $P < 0.0001$) and were significantly upregulated in the advanced-injury group (Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5930/-/DCSupplemental>). In early injury, complement component 1 q sub-component alpha chain (*C1qa*) was unchanged. Tumor necrosis factor (*Tnf*), suggested to mediate RGC death in glaucoma,^{46–48} seemed to be elevated in early-injury GCL but this increase did not reach statistical significance. Interleukin 6 (IL6) mRNA was not detected in the GCL.

DISCUSSION

Previously, we have analyzed the GCL with extensive nerve injury by microarray and compared these results to those of whole retinas with comparable nerve damage and found a significantly increased number of gene and gene categories with altered expression in the GCL.²⁰ In this report, using this

TABLE 7. The 30 Most Downregulated Genes in the GCL with Advanced Nerve Injury

GenBank Accession	Entrez Gene ID	Gene Name	Gene Symbol	Change (x-fold)
BG082125	15569	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)	<i>Elavl2</i>	-4.87
BG074818	320480	RIKEN cDNA 6430537K16 gene	<i>6430537K16Rik</i>	-3.75
BG075389	20660	Sortilin-related receptor, LDLR class A repeats-containing	<i>Sorl1</i>	-3.54
BG069654	13476	Receptor accessory protein 5	<i>Reep5</i>	-3.35
BG073453	18186	Neuropilin 1	<i>Nrp1</i>	-3.26
BG077833	22099	Translin	<i>Tsn</i>	-3.19
BG066372	17762	Microtubule-associated protein tau	<i>Mapt</i>	-3.17
BG085811	54401	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	<i>Ywbab</i>	-3.13
BG077733	11931	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	<i>Atp1b1</i>	-3.13
BG088107	50781	Dickkopf homolog 3 (<i>Xenopus laevis</i>)	<i>Dkk3</i>	-3.13
BG065461	70247	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	<i>Psmc1</i>	-3.06
BG085933	108155	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	<i>Ogt</i>	-2.89
BG087831	16508	Potassium voltage-gated channel, Shal-related family, member 2	<i>Kcnd2</i>	-2.86
BG073378	21454	T-complex protein 1	<i>Tcp1</i>	-2.76
BG080731	17936	Ngfi-A binding protein 1	<i>Nab1</i>	-2.72
BG078404	238055	Apolipoprotein B	<i>Apob</i>	-2.70
AW558903	66673	Sortilin-related VPS10 domain containing receptor 3	<i>Sorcs3</i>	-2.69
BG083786	269252	General transcription factor IIIC, polypeptide 4	<i>Gtf3c4</i>	-2.69
BG064745	18655	Phosphoglycerate kinase 1	<i>Pgk1</i>	-2.65
BG078945	18195	N-ethylmaleimide sensitive fusion protein	<i>Nsf</i>	-2.63
BG067952	18516	Pre B-cell leukemia transcription factor 3	<i>Pbx3</i>	-2.63
BG067301	140904	Calneuron 1	<i>Caln1</i>	-2.62
BG076847	12314	Calmodulin 2	<i>Calm2</i>	-2.59
BG075191	231470	Fraser syndrome 1 homolog (human)	<i>Fras1</i>	-2.58
BG086020	16499	Potassium voltage-gated channel, shaker-related subfamily, beta member 3	<i>Kcnab3</i>	-2.54
BG065113	12035	Branched chain aminotransferase 1, cytosolic	<i>Bcat1</i>	-2.53
AW544616	11931	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	<i>Atp1b1</i>	-2.53
BG071667	70974	Phosphoglucomutase 2-like 1	<i>Pgm2l1</i>	-2.50
BG074398	13602	SPARC-like 1 (mast9, hevin)	<i>Sparcl1</i>	-2.50
BG077573	14536	Nuclear receptor subfamily 6, group A, member 1	<i>Nr6a1</i>	-2.50

same method, we have focused on early injury, anticipating that we would detect early responses more specific to RGCs. With this minimal pressure-induced injury (average nerve grading = 1.5) and thus limited extent of affected retina, mild responses could be overlooked even by targeting GCL. But by the same token, those identified to be significant are much more likely to be of even greater importance. As shown in the results, our microarray analysis identified a total of 532 such significant genes in early injury, approximately 3.5% of the total genes studied. These significant genes are associated with a wide variety of biological processes and multiple cell components. This suggests that significant GCL gene responses and possibly functional changes have already occurred, while IOP-induced optic nerve damage is rather limited (<25% degenerating axons). As far as we are aware, this is the first report in which patterns of GCL gene expression changes are examined in early pressure-induced injury. The exceptionally large sample sizes in both the microarray and qPCR analyses allowed us to perform effective statistical analysis of biological variations and obtain reliable results.

Although hundreds of significant genes with altered expression were identified in early injury, we did not find supportive evidence for extensive RGC loss at this level of injury. By qPCR, no significant change was found in the mRNA levels for the RGC marker proteins POU4f family members and Thy1, except for a selective decrease in POU4f1. The significance of this selective decrease is not clear, but it may represent some specific gene downregulation, rather than loss of a particular subset of cells considering the substantial overlapping expression of

POU4f1, POU4f2, and POU4f3 in RGCs.⁴⁹ In addition, members of the Bcl2 gene family, including *Bcl2*, *BclxL*, and *Bax*, which play important roles regulating RGC death in glaucoma,³⁹ were not changed at the message level. Neither was *Tp53*, another apoptotic gene which has been associated with RGC death.⁵⁰ Overall, cell apoptosis was not detected as a significant gene category in early injury by microarray, suggesting that it is not a prominent process. As anticipated, it stands out as one of the significantly upregulated biological processes in advanced injury (Table 8).

In addition, our qPCR results found no upregulation of glial cell marker expression in early injury (Fig. 1B), suggesting that glial activation is not yet widespread. Immune response, considered evidence for glial activation and identified as one component of early changes in glaucomatous injury in whole retinas with induced elevated IOP,^{12,16} was only identified in advanced-injury GCL in our microarray analysis, suggesting that it is probably a later event in GCL. This notion was further confirmed by qPCR, showing no change in messages for *C1qa*, *Cp*, *Lcn2*, and *Timp1* in early injury (Fig. 5 and Supplementary Table S6, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10.5930/-/DCSupplemental>). These seemingly contrary results may be attributed to distinct methods of elevated IOP induction as well as the differences in the tissues analyzed between the studies. The IL6 type cytokine genes (including *IL6*, *Lif*, *CNTF*), which play an important role in regulation of glial cell activities through Jak-Stat pathways and have previously been found to be upregulated in the early optic nerve head injury (Dyck JA, et al. *IOVS* 2008;49:ARVO E-Abstract 3684; Johnson EC, et al.

TABLE 8. Significantly Upregulated Gene Categories Identified by DAVID Analysis in the GCL with Advanced Nerve Injury

Gene Categories	Genes (<i>n</i>)	EASE Score
Biological Process		
Protein biosynthesis	90	0.0000
Immune response	39	0.0003
Cell adhesion	45	0.0008
Protein metabolism	241	0.0026
Inorganic anion transport	15	0.0026
Protein kinase cascade	26	0.0040
Response to stress	63	0.0050
Cytoskeleton organization and biogenesis	39	0.0220
Apoptotic program	9	0.0260
Coagulation	7	0.0270
Cytoplasm organization and biogenesis	22	0.0310
Cell Component		
Ribosome	48	0.0000
Extracellular region	133	0.0000
Extracellular matrix	28	0.0002
Cytoskeleton	70	0.0002
Contractile fiber	8	0.0320
Plasma membrane	76	0.0340
Molecular Function		
Structural constituent of ribosome	52	0.0000
Cytoskeletal protein binding	43	0.0000
UDP-glycosyltransferase activity	11	0.0056
Transferase activity, transferring glycosyl groups	21	0.0099
RNA splicing factor activity, transesterification mechanism	6	0.0150
Enzyme regulator activity	43	0.0280
Insulin-like growth factor binding	4	0.0350
Extracellular matrix structural constituent	9	0.0400

IOVS 2009;50:ARVO E-Abstract 2750) were not changed either. In agreement with a previous study,⁵¹ we also found relatively little obvious effect on amacrine cells, which comprise approximately 50% of GCL neurons, since nearly all the amacrine subclass markers on our arrays were unchanged in these early-injury tissues. While *Stx1a*, the one possible exception to this, was significantly downregulated, it is reported to be produced by RGCs as well as amacrine cells.³⁰ Although the relative lack of change in these specific cell messages could be a reflection of the limited amount of involved retina in early injury, our findings, taken together, indicate that the gene expression changes in early-injury GCL are more representative of those specifically occurring in RGCs and may indicate corresponding functional changes in these cells.

Studies using hypertonic saline injection⁵² and limbal laser photocoagulation¹⁶ rat models of induced glaucoma as well as the genetic model in DBA/2J mice⁹ have suggested the downregulation of certain RGC genes occurs while the cells are still viable but responding to axonal injury in the optic nerve. In our microarray analysis, the number of downregulated genes in early injury is slightly more than that of upregulated ones (307 vs. 225). Since there was no extensive RGC loss in the early-injury specimens, this downregulation is more likely a reflection of specific changes in GCL gene expression than a result of cell loss. Interestingly, a much greater variety of functional gene classes were changed in the direction of downregulation in early injury compared with those that were upregulated. As revealed by DAVID analysis, only one functional class of metabolism regulation was associated with the upregulated genes (Table 4). In strong contrast, a total of 12 functional gene

TABLE 9. Significantly Downregulated Gene Categories Identified by DAVID Analysis in the GCL with Advanced Nerve Injury

Gene Categories	Genes (<i>n</i>)	EASE Score
Biological Process		
Generation of precursor metabolites and energy	61	0.0000
Oxidative phosphorylation	17	0.0000
Cofactor metabolism	27	0.0003
Monovalent inorganic cation transport	19	0.0037
Nucleotide biosynthesis	16	0.0050
Cellular carbohydrate metabolism	24	0.0055
Electron transport	28	0.0110
mRNA splice site selection	5	0.0110
Dendrite morphogenesis	5	0.0180
Ubiquitin cycle	42	0.0200
Tricarboxylic acid cycle	7	0.0220
Microtubule-based process	17	0.0230
Synaptic transmission	9	0.0230
Axon extension	4	0.0330
Response to heat	5	0.0350
Cellular lipid metabolism	30	0.0370
Protein catabolism	22	0.0370
Protein polymerization	6	0.0400
Cell Component		
Mitochondrion	72	0.0003
Organelle membrane	49	0.0023
Proton-transporting two-sector ATPase complex	10	0.0050
Molecular Function		
Oxidoreductase activity	52	0.0001
Ion transporter activity	41	0.0003
Hydrolase activity, acting on acid	44	0.0043
Anhydrides, in phosphorus-containing nucleoside-triphosphatase activity	42	0.0043
Lactate dehydrogenase activity	4	0.0047
Electron transporter activity	20	0.0067
NADH dehydrogenase activity	8	0.0120
ATPase activity, coupled to transmembrane movement of substances	14	0.0330
GTPase activity	13	0.0200
Unfolded protein binding	17	0.0240
Iron-sulfur cluster binding	6	0.0390
Heat shock protein binding	7	0.0470
GTP binding	25	0.0500

classes including energy pathways, glycolysis, protein metabolism, and synaptic transmission were downregulated (Table 5). This result appears to suggest that a general suppression in cellular functions dominates the early responses of RGCs and may reflect the degenerative nature of glaucomatous injury.

TABLE 10. Gene Categories Specifically Associated with the Early Injury

Gene Categories	Genes (<i>n</i>)	EASE Score
Up		
Regulation of transcription	19	0.0190
Nucleus	36	0.0170
Down		
Protein biosynthesis	17	0.0000
Coenzyme metabolism	5	0.0220
Ribosome	11	0.0000

FIGURE 1. Expression of GCL cell type markers, detected by qPCR in early pressure-induced injury. **(A)** Among the RGC marker messages, including members of the POU transcription factor family and Thy1, only POU4f1 was significantly reduced ($P < 0.01$). The Axonal mRNAs Nefh and Nefl were decreased, but the reductions were not statistically significant. Gap43 was moderately upregulated ($P < 0.05$). **(B)** None of the glial activation markers, including Aif1, P2ry12, and Csf1r (for microglia); Gfap (for Müller cells and astrocytes); and Aqp4 and Vim (for astrocytes), were significantly changed in early injury. * $P < 0.05$, † $P < 0.01$.

One striking feature of the gene expression changes in early injury is the absolute domination of downregulated processes that are associated with cellular metabolism. The most down-

regulated biological process, identified by DAVID analysis, was the generation of precursor metabolites and energy (energy pathways). Among other significantly downregulated pro-

FIGURE 2. Expression of GCL NT and NT receptors, detected by qPCR in early pressure-induced injury by qPCR. Both BDNF and NT3 were moderately upregulated ($P < 0.001$ and $P < 0.05$, respectively). Concomitantly, TrkB was significantly reduced ($P < 0.01$) and so was p75NTR ($P < 0.05$). No change was found in message levels for Ngf, TrkA, and TrkC. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

FIGURE 3. Expression of a group of transcription factors, detected by qPCR in early-injury GCL. A marked upregulation was found in *Atf3* expression ($P < 0.01$) in early injury. No change was found in mRNA levels of *Jun*, *Junb*, or *Stat3*. *Hif1 α* mRNA, upregulated in response to hypoxic stimuli, was not changed in early-injury GCL, nor was *Epo*, a target gene of *Hif1 α* . † $P < 0.01$.

cesses, five are associated with energy production, including glycolysis, the TCA cycle, oxidative phosphorylation, electron transport, and ATP synthesis. Accordingly, mitochondrion, the major site for energy production, was the most affected cell

component. This confirms affected mitochondrial function previously reported in retinas of a mouse model of glaucoma⁵³ and supports the possible link between mitochondrial dysfunction and pathogenesis of glaucoma.^{54,55} Accompanying the

FIGURE 4. Expression of Bcl2 family genes and other prosurvival genes, detected by qPCR in early-injury GCL. (A) Three Bcl2 family members Bax, Bcl2, and BclxL were examined and no change was detected in Bax or BclxL in early injury. Bcl2 mRNA appears decreased but statistics showed no significant difference. *Tp53*, another apoptotic gene which regulates transcription of Bax, was not changed either. (B) Pdpk1, a key kinase in the PI3K pathway which promotes cell survival, was significantly upregulated in early-injury GCL ($P < 0.01$). In contrast, prosurvival genes *Nrg1* and *Dkk3* were both significantly downregulated in the early-injury group ($P < 0.05$ for both). Also, a marked reduction to less than half of control values was found in *Gapdh* mRNA ($P < 0.001$). * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

FIGURE 5. Expression of stress and immune response genes, detected by qPCR in early-injury GCL. Stress responding gene *Hmox1* was significantly upregulated at the message level in early injury ($P < 0.05$). *Tnf*, *Lif*, and *Lcn2* all appear upregulated in the early-injury group, but these changes are not statistically significant. No change was detected in *C1qa* expression in early-injury GCL. * $P < 0.05$.

downregulated energy production is a decrease in protein synthesis, which is unique to the early injury and possibly secondary to reduced energy production. *Gapdh*, a major glycolytic enzyme with diverse functions, such as apoptosis induction,^{56,57} is significantly reduced at the message level in early injury by both microarray and qPCR. In another study using the same rat model of glaucoma, *Gapdh* protein was shown to be oxidatively modified in RGCs with 12 weeks of IOP elevation and approximately 90% axon loss in the optic nerve.⁵⁸ Since glucose is the major energy source for neurons,⁵⁹ the reduced production of energy is likely an indication of reduced glucose uptake in these cells. It is noteworthy that one of the processes uniquely affected by early injury is altered insulin pathway, which controls a set of metabolic enzymes that regulate glucose uptake. The homeostasis of both glucose and protein is essential for normal function and survival of neurons, and its disruption could be highly detrimental. In fact, the reduced glucose uptake and the early decrease in protein synthesis are consistent with the metabolic changes observed in other types of neurons before they undergo apoptosis.^{60–62}

One potential explanation of these metabolic changes could be deprivation of retrograde transported NT and NT receptors that are hypothesized to cause RGC death in glaucoma.^{63–65} In neurons, which are deprived of growth factors and eventually committed to apoptosis, similar changes in metabolic parameters have been reported.⁶⁶ However, it is not clear yet whether the metabolic changes themselves act as a trigger of apoptosis or just represent an early common pathway as part of the apoptotic program. In our previous study of the whole retina,³¹ no significant changes were found in the mRNA levels for any of the NT family members at any level of injury, except for a reduction in *TrkB* and *TrkC* messages in those with rather extensive nerve damage. In contrast, our analysis of the GCL, with its improved sensitivity in detecting specific GCL changes, found an increase in message levels for both BDNF and NT3 and a decrease in *TrkB* message (to approximately 50% of normal level) in early injury. The increase in the endogenous production of BDNF and NT3 could represent a compensatory response to lack of neurotrophic support in GCL. A similar upregulation has been observed in the GCL after optic nerve transection.⁶⁷ This response, together with upregulation of *Pdprk1* which is required for BDNF-mediated neuronal survival,⁴³ may represent an endogenous protective mechanism. If *TrkB* downregulation implies decreased production of *TrkB* receptor protein, then the effect of such mechanisms as well as RGC-salvaging strategies such as supplementing BDNF alone, could be limited and

ultimately ineffective. Moreover, exogenous BDNF has been shown to downregulate retinal expression of *TrkB* receptor,⁶⁸ further limiting its effectiveness especially in long-term RGC rescue. In whole retinas, we have shown increased p75NTR mRNA in both mild and more advanced injury.²⁰ Interestingly, in early-injury GCL, the p75NTR mRNA level was greatly reduced (to ~50% of the normal level). While retinal p75NTR is primarily expressed in Müller cells,^{69–71} whether it is present in adult RGCs remains controversial. Our results in the GCL seem to be consistent with those studies that have reported expression of p75NTR in adult RGCs,^{72–75} and we have attributed the contradictory results in whole retinas and GCL to the difference in source materials and thus in the activities of distinct cell populations.

With advanced injury, the energy production pathways continue to decrease while the protein synthesis and ribosome genes became the most upregulated classes. The increased protein synthesis is consistent with previous reports in whole retina.¹⁶ This may not be surprising, since apoptosis, an active process that requires expression of certain genes, has become significantly active by this point. In our microarray analysis of GCL from eyes with 7-day nerve transection (data not shown) in which apoptosis is an active on-going process, we found similar changes including upregulated ribosomal genes and protein synthesis but downregulated glucose metabolism. However, we cannot exclude the possibility that increased protein synthesis represents activities of GCL cell populations other than RGCs, especially in those with extensive injury and active tissue remodeling.

The most upregulated gene in early-injury GCL was the transcription factor *Atf3*. This increase of *Atf3* mRNA (1492% by qPCR) confirms the previous findings^{16,20} and further establishes *Atf3* upregulation as one of the earliest responses in glaucomatous injury. Responding specifically to cellular injury,^{76,77} *Atf3* has been proposed as a possible indicator of nerve injury, especially in sensory neurons.^{77–80} In this case, *Atf3* expression would be expected to occur specifically in injured RGCs. Our previous immunohistochemistry study showed that this increase is associated with presumably RGC nuclei in the GCL.²⁰ These findings lead to speculation that, in our model of glaucoma, the *Atf3* induction could represent a response of RGC somata to axonal injury at the optic nerve head. This idea is consistent with the notion that the optic nerve head is the initial injury site in glaucoma and the axonal damage signals secondary changes in RGC somata. The upregulation of *Gap43*, a marker protein of axonal regeneration whose expression also

increases in response to nerve injury,^{81,82} was observed in early injury, further supporting this hypothesis.

The biological role of Atf3 is not clear, and whether its effects are protective or detrimental in glaucomatous injury remains an unanswered question. In general, Atf3 plays a pleiotropic role in determining cell fate in stress response, depending on the cellular context.^{34,83} However, Atf3 is known to repress transcription as a homodimer and activate as a heterodimer.⁸³ It has been proposed to play a central role in regulating stress response by interacting with other transcription factors and thus mediating the transcription of multiple genes, which is required in cell responses to injury or stress.⁸⁴ The comparison of the two injury groups in our analysis showed upregulated transcription to be a specific component of the early response. When overexpressed in the liver of mice, Atf3 has been shown to reduce expression of genes encoding gluconeogenic enzymes,^{85,86} providing one potential explanation for the disruption of glucose homeostasis observed in our study. Also, Atf3 has been demonstrated to repress expression of the cytokine IL6⁸⁴ which has been shown to be upregulated in neurons after injury.^{87–89} This finding may explain the undetectable message level of IL6 in the GCL. Taken together, these intriguing data suggest that Atf3 may play a key role in regulating early RGC responses to axonal injury in glaucoma. Its specific roles and physiological significance in glaucomatous injury deserve further investigation.

Induction of *Hmox1*, another stress-response gene, also known as heat shock protein 32, has been considered a general response to cellular stress, since it responds to a broad spectrum of stress stimuli.^{90,91} Increased Hmox1 protein has been shown in RGCs exposed to hydrostatic pressure in vitro as well as mouse retinas exposed to acute elevated IOP.⁹² Among the most common stress signals that induce Hmox1 expression is oxidative stress, which has been suggested to play a role in signaling RGC death in glaucoma.⁹³ In our microarray analysis, the downregulation of gene classes associated with mitochondrial functions, oxidative phosphorylation, electron transport, and oxidoreductase activity in early injury suggests that the presence of oxidative stress is highly possible in the GCL as an early event and may have led to increased expression of Hmox1. This oxidative stress does not seem to result from hypoxia, but from the lack of transcriptional response of Hif1 α and Epo. Because of the generality of Hmox1 induction, stress signals other than oxidative stress may play a role as well. Regardless, there is overwhelming evidence that Hmox1 confers cytoprotective and antiapoptotic effects via its metabolic products, including iron, ferritin, and carbon monoxide,^{90,91} suggesting a potential neuroprotective role for Hmox1 in early injury of glaucoma.

There have been several studies of whole retinal responses shortly after optic nerve transection or crush.^{16,32,94} Agudo et al.⁹⁴ found more than a thousand genes that are commonly regulated within a week of either crush or transection, with apoptosis as the most significant cluster. Among these, they identified *Lcn2* and *Stat3* as genes significantly and consistently upregulated, similar to our findings in early glaucoma model injury. Templeton et al.,³² studying early retinal responses after optic nerve crush, found approximately 700 significantly affected genes at 2 and 5 days postlesion. While nearly half of these were on our arrays, only 34 were significantly affected in early glaucoma injury. Approximately two thirds of these, including *Stat3* and *Atf3*, were changed in the same direction by nerve crush. Yang et al.¹⁶ compared early retinal responses produced by transection and by modeling glaucoma using limbal laser photocoagulation. Of the genes they identified as regulated in at least one of these injuries, 40 were regulated in our early-injury group. Of the 21 genes found by Yang et al. to be regulated by glaucoma only or by both

glaucoma and transection, 13 were similarly regulated in our early-injury group, including upregulation of *Hmox1*, *Stat3*, *Igfr1*, *Atf3*, and *Lcn2*. We also found that 10 of the 19 genes identified as regulated by transection only were similarly regulated in the glaucoma model of early injury in our study. Since all these studies were of whole retina, it is difficult to interpret the significance of differences between them and the present study, in which we concentrated only on the GCL.

In addition to the above, Dieterich et al.⁹⁵ used RNA from the inner retina collected at 1 week after crush by combining en face sectioning of retinal wholemounts with subtractive hybridization to illustrate that three upregulated genes, *Fhl1*, *Klcl1*, and *Csda*, could be identified. These three were also found to be upregulated in our study, but only in the advanced injury group (Supplementary Table S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5930/-/DCSupplemental>).

In summary, our microarray and qPCR analyses of gene expressions indicate that overall GCL gene responses were rather subtle in eyes with mild pressure-induced optic nerve injury in our rat glaucoma model. However, these expression changes, dominated by an extensive downregulation of genes associated with glucose metabolism and protein synthesis, reflect the degenerative nature of glaucoma injury and are important in understanding the cellular mechanisms underlying RGC injury and death. The findings suggest impaired RGC functions and profound metabolic changes in these cells in early glaucomatous injury, possibly in response to the axonal damage in the optic nerve head. As one of the most upregulated genes, transcription factor *Atf3* may play a key regulatory role in the early injury, and unraveling its biological functions in RGCs may advance our knowledge of pathogenesis and progression of glaucoma.

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