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Haixi Miao  
Northwestern University

Lin Chen  
Northwestern University

Sean M. Riordan  
Northwestern University

Wenjun Li  
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Santiago Juarez  
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See next page for additional authors

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Gene Expression and Functional Studies of the Optic Nerve Head Astrocyte Transcriptome from Normal African Americans and Caucasian Americans Donors

Haixi Miao1, Lin Chen1, Sean M. Riordan1, Wenjun Li1, Santiago Juarez1, Andrea M. Crabb1, Thomas J. Lukas2, Pan Du3, Simon M. Lin3, Alexandria Wise4, Olga A. Agapova5, Ping Yang6, Charles C. Gu7, M. Rosario Hernandez1*

1 Department of Ophthalmology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America, 2 Department of Molecular Pharmacology and Biological Chemistry, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America, 3 Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America, 4 Department of Biostatistics, Washington University School of Medicine, St. Louis, Missouri, United States of America, 5 Department of Pharmacology and Experimental Therapeutics, Washington University School of Medicine, St. Louis, Missouri, United States of America, 6 Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, United States of America, 7 Division of Biostatistics, Washington University School of Medicine, St. Louis, Missouri, United States of America

Abstract

Purpose: To determine whether optic nerve head (ONH) astrocytes, a key cellular component of glaucomatous neuropathy, exhibit differential gene expression in primary cultures of astrocytes from normal African American (AA) donors compared to astrocytes from normal Caucasian American (CA) donors.

Methods: We used oligonucleotide Affymetrix microarray (HG U133A & HG U133A 2.0 chips) to compare gene expression levels in cultured ONH astrocytes from twelve CA and twelve AA normal age matched donor eyes. Chips were normalized with Robust Microarray Analysis (RMA) in R using Bioconductor. Significant differential gene expression levels were detected using mixed effects modeling and Statistical Analysis of Microarray (SAM). Functional analysis and Gene Ontology were used to classify differentially expressed genes. Differential gene expression was validated by quantitative real time RT-PCR. Protein levels were detected by Western blots and ELISA. Cell adhesion and migration assays tested physiological responses.

Results: Multiple analyses selected 87 genes differentially expressed between normal AA and CA (P<0.01). The most relevant genes expressed in AA were categorized by function, including: signal transduction, response to stress, ECM genes, migration and cell adhesion.

Conclusions: These data show that normal astrocytes from AA and CA normal donors display distinct expression profiles that impact astrocyte functions in the ONH. Our data suggests that differences in gene expression in ONH astrocytes may be specific to the development and/or progression of glaucoma in AA.

Introduction

Primary open angle glaucoma (POAG), the most common form of glaucoma, is a blinding disease that affects older adults [1]. POAG in many individuals is associated with elevated intraocular pressure (IOP), a common risk factor [2]. Visual impairment in glaucoma is due to progressive loss of retinal ganglion cells (RGC) that clinically presents loss of visual field and cupping of the optic disc [3]. The site of initial damage to the retinal neurons in glaucoma is thought to be at the level of the lamina cribrosa in the optic nerve head (ONH) [4].

Astrocytes, the major glial cell type in the ONH in humans, provide cellular support function to the axons while interfacing between connective tissue surfaces and surrounding blood vessels [5]. In response to elevated IOP in human POAG and in experimental glaucoma, astrocytes undergo marked phenotypic changes [5]. Changes from the quiescent to the reactive astrocyte phenotype and the onset and progression of various human central nervous system (CNS) diseases are well established [6,7]. In POAG, reactive astrocytes express neurotoxic mediators such as nitric oxide [8] and TNF-α [9] that may damage the axons of RGCs and remodel the extracellular matrix (ECM) of the lamina.
Astrocyte Gene Expression

Results

We established primary cultures of ONH astrocytes from 16 normal African American donors (age 60±11) and 21 normal Caucasian American donors (age 62±12) as described in Material and Methods. For each different assay and determination, astrocytes were cultured under identical conditions until reaching 90% confluence and then processed. ONH astrocytes from the AA and CA populations displayed a uniform polygonal shape in culture and were positive for GFAP and NCAM, which are markers of ONH astrocytes in vivo and in vitro [14,15].

Identification of differentially expressed genes in normal AA and CA ONH astrocytes

There were no significantly different demographic variables between populations of the 24 astrocyte lines used in microarray (Table S1). We analyzed global gene expression differences in mRNA samples from primary cultures of ONH astrocytes from 12 AA normal donors (age 58±12 years) and 12 CA normal donors (age 58±11 years). We used Affymetrix GeneChips HG U133A chips for 3 AA and 3 CA donor samples [GSE9939] and HG U133A 2.0 chips for 9 AA and 9 CA donor samples [GSE9939]. Of the 22,277 gene probes in the Affymetrix GeneChips, there were 16,710 present calls, representing 10,504 genes left for the analysis. Data normalized using RMA and analyzed by SAM [16].

Validation of selected differentially expressed genes in AA and CA astrocytes by real-time qRT-PCR

For each probe, a list of the differentially expressed genes for each modeled population was generated (Table S2). Differences in the mRNA abundance of signaling molecules comparing AA and CA astrocytes indicate the potential for differential activation of these signaling pathways in response to stress.

Regulator of G protein signaling 5 (RGS5). Expression of the RGS5 gene was upregulated in AA astrocytes by microarray and confirmed by qRT-PCR (Figure 2A). The protein product appeared greater in AA astrocytes as shown by immunostaining localized to the cytoplasm and the nucleus (Figure 2B). Western blot detected RGS5 increased protein in cell lysates of AA astrocytes (Figure 2C). Astrocytes in the lamina cribrosa tissue from normal AA donors contained abundant RGS5 compared to CA tissues (Figure 2D). The abundant expression of RGS5 in AA astrocytes suggests an inhibitory role in the regulation of signal transduction in this population.

Cyclic AMP signaling. Amongst genes differentially regulated in AA astrocytes were several genes that impact upon...
cAMP signaling. β-adrenergic receptor kinase (ADRBK2) is downregulated in AA normal astrocytes (Figure 2A). The AK3L1 gene that regulates the amount of available nucleotides in cells was also downregulated. Two adenylyl cyclases (ADYC3 and ADYC9) were upregulated in normal AA astrocytes (Figure 2A) however there were no differences in basal levels of cAMP amongst normal AA and CA astrocytes (data not shown), suggesting other components of the cAMP pathway are also involved in the regulation of cAMP basal level. Additional upregulated signaling genes were: Phosphodiesterase 4D interacting protein (PDE4DIP) and SOS1, son of sevenless 1 (Table S8).

AA astrocytes exhibit decreased cell adhesion

Comparing AA to CA astrocytes, differentially expressed genes that are associated with cell adhesion were ephrin B2 and GPR56, which were both upregulated, and ITGA6, which was downregulated (Table 1, Figure 3A, and B) Differential expression in AA astrocytes was consistent with differences in the protein products of GPR56, EFNB2 and ITGA6 by immunoblot (Figure 3C). GPR56 was visibly more abundant in AA astrocytes and localized to the cell surface and cell borders in vitro (Figure 3A). We also detected strong staining for GPR56 in astrocytes in the lamina cribrosa of AA normal donor tissue (Figure 3D).

The molecular changes described above suggested to us decreased cell adhesion of AA astrocytes. An assay was used to compare AA astrocytes to CA astrocytes for adhesion to collagen type IV. We found decreased attachment to collagen type IV of AA astrocytes compared to CA astrocytes (Figure 3E, p<0.05).

AA astrocytes exhibit increased migration

mRNA levels of the autocrine motility factor receptor (AMFR), myosin light chain kinase (MYLK) a calcium/calmodulin dependent kinase and PPP1R12B (also referred as MYPT2), a myosin phosphatase were upregulated in AA astrocytes compared to CA astrocytes (Table 1, Figure 4A).

Western blots detected two isoforms of MYLK: 210 kDa and 130 kDa. The 130 kDa isoform was the predominant form in both AA and CA normal astrocytes. The protein level of MYLK 130 kDa was significantly higher in AA astrocytes compared to CA astrocytes (Figure 4B and C). 210 kDa isoform was expressed at very low levels in both AA and CA normal astrocytes and no difference was detected (Figure 4B and C).
### Table 1. Selected functional categories of genes differentially expressed in AA vs. CA.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
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<th>Fold Change</th>
<th>P-value</th>
<th>Chromosome location</th>
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<tr>
<td>PSPH</td>
<td>phosphoserine phosphatase</td>
<td>2.70</td>
<td>0.0000</td>
<td>7q11.2</td>
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<td>PDE4DIP</td>
<td>phosphodiesterase 4D interacting protein</td>
<td>2.50</td>
<td>0.0000</td>
<td>1q12</td>
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<td>RG55</td>
<td>regulator of G-protein signalling 5</td>
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<td>0.0007</td>
<td>1q23.1</td>
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<td>SOS1</td>
<td>son of sevenless homolog 1</td>
<td>1.67</td>
<td>0.0001</td>
<td>2p22-p21</td>
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<tr>
<td>RAB3B</td>
<td>Member RAS oncogene family</td>
<td>1.64</td>
<td>0.0006</td>
<td>1p32-p31</td>
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<td>GPR56</td>
<td>G protein-coupled receptor 56</td>
<td>1.62</td>
<td>0.0051</td>
<td>16q12.2-q21</td>
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<td>PLA2G4C</td>
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<td>1.60</td>
<td>0.0016</td>
<td>19q13.3</td>
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<td>PPP1R12B</td>
<td>protein phosphatase 1, regulatory (inhibitor) subunit 12B (MYPT2)</td>
<td>1.53</td>
<td>0.0026</td>
<td>1q32.1</td>
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<td>MYLK</td>
<td>myosin, light polypeptide kinase</td>
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<td>0.0435</td>
<td>3q21</td>
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<td>CENTG2</td>
<td>centaurin, gamma 2</td>
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<td>0.0103</td>
<td>2p24.3-p24.1</td>
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<td>0.0040</td>
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<td>0.0039</td>
<td>8q24-qter</td>
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<td>ADCY9</td>
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<td>0.0216</td>
<td>16p13.3</td>
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<td>TEK tyrosine kinase</td>
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<td>9p21</td>
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<td>AK3L1 (AK3)</td>
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<td>1p13.3</td>
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<td>SH3 and cysteine rich domain</td>
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<td>adrenergic, beta, receptor kinase 2</td>
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<td>0.001</td>
<td>22q12.1</td>
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<td><strong>Cell Adhesion</strong></td>
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<td>WISP2</td>
<td>WNT1 inducible signaling pathway protein 2</td>
<td>2.00</td>
<td>0.0001</td>
<td>20q12-q13.1</td>
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<td>EFNB2</td>
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<td>0.0038</td>
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<td>NLGN1</td>
<td>neuroligin 1</td>
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<td>EPB41L3</td>
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<td>1.66</td>
<td>0.0020</td>
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<td>ITGA6</td>
<td>integrin, alpha 6</td>
<td>1.64</td>
<td>0.0055</td>
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<td>JUP</td>
<td>junction plakoglobin</td>
<td>1.55</td>
<td>0.0024</td>
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<td>ST3GAL5</td>
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<td>ANTXR1</td>
<td>anthrax toxin receptor 1</td>
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<td>0.0000</td>
<td>2p13.1</td>
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<td>MYLK</td>
<td>myosin, light polypeptide kinase</td>
<td>1.47</td>
<td>0.0435</td>
<td>3q21</td>
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<td>PPP1R12B</td>
<td>protein phosphatase 1, regulatory (inhibitor) subunit 12B</td>
<td>1.53</td>
<td>0.0026</td>
<td>1q32.1</td>
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<td><strong>ECM and related protein</strong></td>
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<td>ELN</td>
<td>elastin</td>
<td>2.20</td>
<td>0.0023</td>
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<td>COL18A1</td>
<td>collagen type XVIII, alpha 1</td>
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<td>LTBP1</td>
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<td>0.0239</td>
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<td>MFAP2</td>
<td>microfibrillar-associated protein 2</td>
<td>1.51</td>
<td>0.0016</td>
<td>1p36.1-p35</td>
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<tr>
<td>MTCP5</td>
<td>membrane-type 1 matrix metalloproteinase cytoplasmic tail binding protein-1</td>
<td>1.43</td>
<td>0.0012</td>
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<tr>
<td>NID2</td>
<td>nidogen 2</td>
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<td>0.0001</td>
<td>14q21-q22</td>
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<td>PLOD2</td>
<td>procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2</td>
<td>1.60</td>
<td>0.0054</td>
<td>3q23-q24</td>
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<td><strong>Cellular detoxification/oxidative stress</strong></td>
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<td>GSTT2</td>
<td>glutathione S-transferase theta 2</td>
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<td>22q11.22; 22q11.23</td>
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<td>22q11.22</td>
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<td>GGT2</td>
<td>gamma-glutamyltransferase 2</td>
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<td>0.0019</td>
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<td>GGT1A4</td>
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<td>glutathione S-transferase M1</td>
<td>1.38</td>
<td>0.0010</td>
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Based on the gene expression and protein data for MYLK, we compared migration in AA and CA astrocytes. Experiments using a chemotaxis model indicated that AA astrocytes migrated faster than CA (Figure 4D). Since AA astrocytes migrated faster and have higher levels of MYLK, we tested ML-7, a known inhibitor of MYLK, on its effect on migration in astrocytes derived from three AA normal donors. The ML-7 treated cells migrated significantly slower, compare to vehicle treated cells (Figure 4E), suggesting increased migration in AA astrocytes is due at least in part to MYLK. In normal AA and CA astrocytes stained with phalloidin, stress fibers run parallel to the major axis of the cells (Figure 4F, 4G). Treatment with with ML-7, a known inhibitor of MYLK, caused a marked loss in actin stress fibers from the center of the cell (Figure 4H, 4I) supporting the role of MYLK in maintenance of the astrocyte cytoskeleton in both AA and CA astrocytes.

Differential expression of genes associated with the extracellular matrix in AA astrocytes

Microarray analysis indicated a significant upregulation of elastin (ELN) mRNA which was confirmed by qRT-PCR and immunoblot in AA astrocytes (Figure 5A, B). LTBP1, a member of the elastin microfibrils that binds TGF-β was also upregulated in AA astrocytes in vitro (Figure 5A, B, C, and D). MIFAP2, the gene encoding for MAGP1, a component of the elastin associated microfibrils, was downregulated in AA astrocytes (Figure 5A). However, ONH tissue immunostaining with ELN antibody did not show differences in ELN between populations (Figure 5E, 5F). COL18A1, a collagen with strong anti-angiogenic properties, was upregulated in AA astrocytes compared to CA astrocytes by qRT-PCR and immunoblot (Figure 5B).

Several genes associated with basement membranes were downregulated in AA astrocytes (Table 1, Table S6); including Nidogen 2 (NID2), type XIII collagen [17], and PLOD2 [18]. Figures 5G and H show decreased immunostaining for collagen type IV, a ubiquitous component of basement membranes, in donor ONH tissue from AA compared to an age matched normal CA donor. AA astrocytes may be attached to an altered ECM.

Upregulation of glutathione metabolic enzymes in AA astrocytes

Genes involved in GSH metabolism, including glutathione S-transferases (GSTs) and gamma-glutamyltransferases (GGTs), are upregulated in AA astrocytes (Table 1, Figure 6A). We measured basal levels of GSH in astrocytes from 10 AA lines and 10 CA lines. Consistent with upregulation of the GSH metabolizing enzymes, AA astrocytes exhibited lower GSH levels compared with CA astrocytes (Figure 6B, p<0.01).

Several chaperones were upregulated in AA astrocytes compared to CA astrocytes, including heat shock protein 70 protein 2 (HSPA2), alpha-crystallin-related heat shock protein B6 (HSPB6), and crystallin-β B2 (CRYBB2) (Table 1). HSP70 protein are more abundant in AA astrocytes compared to CA astrocytes (Figure 6C and D).

Table 1. cont.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Fold Change</th>
<th>P-value</th>
<th>Chromosome location</th>
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<td>glutathione S-transferase M3</td>
<td>1.31</td>
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<td>1p13.3</td>
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<td>GSTM2</td>
<td>glutathione S-transferase M2</td>
<td>1.30</td>
<td>0.0021</td>
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<td>IGFBP3</td>
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<td>FGF9</td>
<td>fibroblast growth factor 9 (glia-activating factor)</td>
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<td>GFRα1</td>
<td>GDNF family receptor alpha 1</td>
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<td>VEGF</td>
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<td>HBEGF</td>
<td>heparin-binding EGF-like growth factor</td>
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doi:10.1371/journal.pone.0002847.t001
athy in AA, in whom the disease occurs more frequently, has a
contribute to the pathophysiology of glaucomatous optic neurop-
differential susceptibility of the ONH to elevated IOP and
Population based differences in astrocyte functions may produce
demonstrated higher levels of RGS5 protein in AA astrocytes, compared
to CA astrocytes. Adenelyl cyclase activity is modulated by two G
protein subunits: Gs to increase and Gi to decrease activity.
G protein signaling 5 (RGS5) promotes GTP
Regulator of G protein signaling 5 (RGS5) promotes GTP
hydrolysis [24] and therefore is a negative regulator of G-
protein-mediated signaling. It interacts with Gi-, Go-, and Gq
classes of G-proteins, but not with Gs. Therefore, the increased
expression of RGS5 in AA astrocytes inhibit Gi activity, but not Gs
activity. This may further enhance activation of adenylate cyclase
in AA astrocytes and increase cAMP accumulation. These findings
suggest increased responsiveness to β-adrenergic receptor stimu-
lation in AA astrocytes. Similar finding has been reported for the
cardiovascular system in AA [25].

Signal transduction in AA astrocytes
Amongst genes differentially regulated in AA astrocytes were
several genes that impact cAMP signaling. β-adrenergic receptor
kinase (ADRBK2), which is downregulated in AA normal
astrocytes, specifically phosphorylates the agonist-occupied form
of the β-adrenergic receptor and promotes its desensitization and
internalization [23]. Thus, a decrease in ADRBK2 will likely slow
β-adrenergic receptor endocytosis and prolong its activation at the
membrane. In addition, two adenylate cyclases (ADCY3 and
ADCY9) that are coupled to the Gs (stimulatory) GTPases
activated through the β-adrenergic receptor were upregulated in
AA astrocytes. Adenyl cyclase activity is modulated by two G
protein subunits: Gs to increase and Gi to decrease activity.
Regulator of G protein signaling 5 (RGS5) promotes GTP
hydrolysis [24] and therefore is a negative regulator of G-
protein-mediated signaling. It interacts with Gi-, Go-, and Gq
classes of G-proteins, but not with Gs. Therefore, the increased
expression of RGS5 in AA astrocytes inhibit Gi activity, but not Gs
activity. This may further enhance activation of adenylate cyclase
in AA astrocytes and increase cAMP accumulation. These findings
suggest increased responsiveness to β-adrenergic receptor stimu-
lation in AA astrocytes. Similar finding has been reported for the
cardiovascular system in AA [25].

Cell adhesion/migration in AA astrocytes
AA astrocytes exhibited differential expression of genes
associated with cell adhesion and cell migration. Cell adhesion
genes which were downregulated in AA astrocytes included:
t integrin β-6 (TGA6), which connects these cells with laminin
family members [26,27]. Basement membrane components
nido-2 (NID2) and type XIII collagen were also downregulated
in AA astrocytes.
Upregulated genes in AA astrocytes that are involved in cell
adhesion of astrocytes included: GPR56, that activates pathways
that inhibit cell adhesion [28] and interacts with transglutaminase
2 (TGM2), an enzyme that cross-links a various ECM proteins and
ephrin B2, a cell membrane ligand that mediates repulsive forces
between neighboring cells [29]. These results plus those of the
adhesion assay suggest that cell-surface adhesion in the normal
ONH in vivo by AA astrocytes may differ than that of CA
astrocytes.

Cell migration involves changes in adhesion and cytoskeletal
proteins and changes in cell shape during movement via
reorganization of actin filament networks in the cell periphery
[30]. ONH astrocytes change the distribution of the actin
microarray results, differential expression of elastin, ephrin-2B,
VEGF-C, PDGF-A and collagen XVIII (endostatin) has recently
been demonstrated in human astrocytes in ONH tissue [20–22].
Immunohistochemistry indicates that many gene products detect-
ed by microarray in cultured astrocytes are present in astrocytes in
the ONH in situ. We believe that the findings presented here using
in vitro techniques identify functional parameters that should be
investigated in vivo.

Population based differences in astrocyte functions may produce
differential susceptibility of the ONH to elevated IOP and
contribute to the pathophysiology of glaucomatous optic neurop-
athy in AA, in whom the disease occurs more frequently, has a
dent enzyme which phosphorylates myosin regulatory light chains to facilitate myosin interaction with actin filaments, producing contractile activity [34]; upregulation of AMFR, a cell surface receptor that initiates migration via activation of inositol phosphate, tyrosine kinase and protein kinase C [35] and has been characterized in glia during normal cell migration in wound healing and embryogenesis [36]; myosin phosphatase target subunit 2 (MYPT2), which dephosphorylates the phosphorylated myosin light chain to modulate the levels of contractility in cells and is uniquely abundant in brain and in the heart [37].

Figure 3. AA astrocytes exhibit decreased cell adhesion compared to CA astrocytes. A. Cellular localization of G protein-coupled receptor 56 (GPR56), ephrin-B2 (EFNB2) and integrin α 6 (ITGA6) in primary cultures of ONH astrocytes. Nuclei stained with DAPI (blue). Magnification bar: 25 μm. Upper: Double immunofluorescence for GFAP (green), an intermediate filament characteristic of astrocytes and GPR56 (red). Note granular staining for GPR56 (red) is more abundant in the cytoplasm of AA astrocytes compared to CA astrocytes. Middle: Immunofluorescence showed that EFNB2 is more abundant in the cytoplasm of AA astrocytes compared to CA astrocytes. Lower: Immunofluorescence showed that Integrin α 6 is less abundant in the cytoplasm of AA astrocytes compared to CA astrocytes. B. Confirmation of three differentially expressed adhesion genes by qRT-PCR in human normal ONH astrocytes: GPR56, EFNB2 and ITGA6. Genes were normalized to 18S. Graphical representation of the relative mRNA levels in AA and CA astrocytes (n = 8, respectively, *indicates p < 0.05 in two-tailed t-test). C. Representative Western blots of astrocyte cell lysates with GPR56, EFNB2 and ITGA6 antibodies. β-actin was used as a loading control. Note that AA astrocytes express more GPR56 and EFNB2, less ITGA6 than CA astrocytes. D. Representative immunohistochemistry showed more abundant granular staining of GPR56 (red) in astrocytes in the lamina cribrosa from AA donors compared to CA donors. E. AA astrocytes adhered to collagen IV 26.5% less than CA astrocytes did ( * indicates p < 0.05 in two-tailed t-test). Values represent mean optical density (OD) ± standard deviation of triplicate experiments using primary astrocyte cultures of four AA donors and six CA donors.

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MYLK genetic variants confer increased risk of sepsis and sepsis-associated with acute lung injury and a more severe asthma phenotype in individuals of African ancestry [38,39]. Therefore it is possible that the effects of increased expression of MYLK in AA astrocytes may be further modified by genetic polymorphisms.

In vivo, quiescent astrocytes are terminally differentiated cells that exhibit strong and stable attachments to the ECM and neighboring astrocytes. Under conditions of stress, injury and disease, reactive astrocytes become migratory and detach from the underlying ECM. Our microarray data plus those of the migration assay obtained from primary astrocyte cultures strongly suggest that normal AA astrocytes in situ have the potential of becoming migratory cells in response to stress such elevated IOP as in glaucoma.

Extracellular matrix in AA astrocytes

Our microarray data suggests that alterations in elastic fibers and associated microfibrils, a major component of the ECM in the lamina cribrosa, may be a susceptibility factor predisposing the remodeling of the ONH in response to elevated IOP in glaucoma in the AA population. Microarray analysis indicated a significant upregulation in AA astrocytes of elastin (ELN), LTBP1, a member of the elastin microfibrils that binds TGF-β, and COL18A1, a collagen with strong anti-angiogenic properties. MFAP2, the gene encoding for MAGP1, a component of the elastin associated microfibrils, and the gene encoding for LOXL2, a lysyl oxidase that participates in maturation of elastic fiber, were downregulated in AA astrocytes. We previously reported that normal AA astrocytes expressed high levels of elastin mRNA and protein, and decreased levels of LOXL2 [22]. CA patients of Scandinavian ancestry with pseudoexfoliation glaucoma exhibit variations in the LOXL1 gene sequence that may predispose this group to elevated IOP and glaucomatous optic neuropathy due to accumulation of pseudoexfoliation material [40]. We previously published marked elastosis in the ONHs of patients with pseudoexfoliation glaucoma [41]. The downregulation of LOXL2 in AA astrocytes may confer a similar susceptibility to elevated IOP.

LTBP1 is an ECM glycoprotein that plays a major role in storage of latent TGF-β in the ECM and regulate its availability [42]. Most studies suggest that LTBP2s are secreted together with TGF-β as part of the TGF-β large latent complex [43,44]. LTBP1 is upregulated in AA astrocytes. The expression of ONH astrocyte-specific genes appears to be controlled by TGF-β activity [45,46] in particular synthesis and degradation of ECM in the optic nerve in glaucoma [46]. Higher levels of TGF-β may be available and affect homeostasis of the ECM in the ONH in the AA population.

Additional ECM related genes that were downregulated included: nidogen 2 (NID2), a linker protein that joins laminin...
Cribriform plates (CP) and extend processes into the nerve bundle (NB). Astrocytes cell bodies are located in the tissue in AA and CA donors. ELN is located in the cribriform plates and (red) and GFAP (green) in representative cross sectional of the ONH (blue). Magnification Bar: 25 μm.

Note that there are no apparent differences in ELN staining between AA and CA samples. E is from a 75 year-old AA male donor and F is from a 74 year-old CA female donor. Colocalization between ELN and GFAP in the AA tissue is microscopic effect showing the overlap between ELN and astrocytes processes. Magnification bar: 25 μm. G, H. Double immunostaining of collagen type IV (red) and GFAP (green) in representative sagittal sections of ONH tissues from AA and CA donors. Collagen type IV and GFAP follow the lamellar structure of the astrocytic basement membranes in the human lamina cribrosa. Note that staining for collagen type IV is more intense and abundant in the CA donor than in the AA donor. G is from a 65 year-old AA male donor and H is from a 57 year-old CA male donor. V: blood vessel, Magnification bar: 35 μm.

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and collagen IV networks in basement membranes [47]; type XIII collagen, a type II transmembrane protein found at many sites of cell adhesion in tissues [48], and PLOD2, an enzyme that catalyzes glucosylation of type IV collagen which is essential for the formation of functional basement membranes [18]. Immunohistochemistry for collagen type IV suggested diminished basement membranes in situ for AA astrocytes.

Taken together, population based differences in important components of the ECM that provide elasticity and resiliency to the lamina cribrosa, and in basement membranes that participate in tissue homeostasis and adhesion of astrocytes in the ONH, may render the tissue more susceptible to the elevation of IOP that occurs in glaucoma.

Growth factors and receptors in AA astrocytes

In normal AA astrocytes the GDNF family receptor α 1 (GDRA1) was upregulated. GDRA1 is the receptor for Glial - derived neurotrophic factor (GDNF) and neurturin (NTN) which are two potent neurotrophic factors that play key roles in the control of neuron survival and differentiation [49]. As a glycosylphosphatidylinositol (GPI)-linked cell surface receptor for both GDNF and NTN, GDRA1 mediates activation of the RET tyrosine kinase receptor and is found, with its ligands in ONH astrocytes in culture [50].

Two important members of the IGF axis, the insulin like growth factor binding protein 5 and 3 (IGFBP5 and IGFBP3) were upregulated in normal AA astrocytes. IGFBP5 interacts with heparin containing glycosaminoglycans (GAGs) in the ECM and facilitates migration, an important differentiated function of normal AA astrocytes compared to CA [51]. Among the many functions of IGFBP5 is to maintain a pool of IGF in the vicinity of cells carrying the IGF receptor thus regulating availability.

The membrane associated heparin binding EGF (HBEGF) was downregulated in AA astrocytes. Several ligands and receptors of the EGF family are known to be expressed in ONH astrocytes including HBEGF [52]. Binding of HBEGF to the EGF receptor under compressive stress is thought to be a mechanical signal that gets translated into biochemical responses by cells [33,53]. The marked decrease in expression of HBEGF suggests a protective mechanism that normal AA astrocytes use to dampen IOP-related mechanical signals in the ONH.

Differential expression of genes related to the immune response

AA astrocytes exhibit downregulation of expression of IL-6, a key cytokine upregulated in reactive astrocytes that promotes glial scar formation and is an impediment to axon regeneration and neuronal survival in the CNS [54]. Recent work indicated that elevated hydrostatic pressure caused a transient decrease in IL-6.
levels in retinal glial cultures [55] and in human brain astrocytes exposed to hypoxia in vitro [56]. Decreased IL-6 in AA astrocytes may limit the transition to a reactive phenotype.

Major histocompatibility complex (MHC) Class I genes (HLA-A, HLA-B, HLA-F and HLA-G) and other molecules in the Class I antigen presentation pathway, such as the immunoproteasome (PSMB9, PSMB8), were marginally up-regulated in AA astrocytes. HLA-A and HLA-B belong to the classical class I genes and are expressed in astrocytes in the CNS in disease [57]. HLA-F and HLA-G belong to the non classical class I type genes and their function is under study. Current studies of high-resolution HLA allele and haplotype frequency data are being carried out for typing and use in population-based disease studies [58]. The function of the immunoproteasome (PSMB9, PSMB8) is to process class I MHC peptides for degradation. Finally, the endoplasmic reticulum enzyme, leukocyte-derived arginine aminopeptidase (LRAP) which processes antigenic peptides presented to class I molecules was also upregulated in AA astrocytes. Expression of LRAP was reported in human brain astrocytes in response to hypoxia.

Astrocytes in the CNS participate in the innate immune response by modulating local reactions to endogenous or exogenous antigens, and by modulating astroglialis through release of cytokines and by isolating areas of inflammation [59]. Differential expression of immune genes in AA astrocytes may have an impact on astrocyte reactivation in response to stress in glaucoma.

Figure 6. Upregulation of glutathione (GSH) metabolic enzymes in AA astrocytes. A. Confirmation of two differentially expressed glutathione metabolic enzyme genes by qRT-PCR in human normal ONH astrocytes: glutathione S-transferase theta 2 (GSTT2) and gamma-glutamyltransferase 1 (GGT1). Genes were normalized to 18S. Graphical representation of the relative mRNA levels in AA and CA astrocytes (n = 8, respectively, * indicate p<0.05 in two-tailed t-test). B. AA astrocytes have significantly lower level of intracellular GSH in vitro, compared to CA astrocytes (n = 10, ** indicated p<0.01 in two-tailed t-test). C. Representative Western blots of astrocyte cell lysates with heat shock 70 kDa protein (HSP70) antibody. β-actin was used as a loading control. HSP70 levels are higher in AA astrocytes compared to CA astrocytes. D. Immunofluorescent staining for HSP70 shows that AA astrocytes exhibit more abundant intracellular HSP70 staining compared to CA astrocytes. Magnification bar: 25 μm.

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Oxidative stress in normal AA ONH astrocytes

The antioxidant glutathione (GSH) is vital for cellular defense against oxidative stress in astrocytes and neurons [60]. Microarray analysis revealed upregulation of seven genes involved in GSH metabolism in AA astrocytes, including glutathione S-transferases (GSTs) and gamma-glutamyltransferases (GGTs). GSTs are a superfamily of enzymes that catalyze the conjugation of GSH to a variety of electrophilic and hydrophobic compounds. Polymorphisms in the GSTM1 and GSTT1 genes may be associated with risk of POAG [61–63]. GGTs initiate extracellular GSH breakdown, thus generating substrates for intracellular GSH synthesis [64] and allow a continuous ‘GSH cycling’ to occur across the plasma membrane.

Upregulation of GSTs in AA astrocytes may indicate active detoxification activity by GSH metabolizing enzymes, resulting lower levels of GSH as we demonstrated in vitro and perhaps in vivo. Upregulation of GGTs leads to increase in GSH cycling and thus GSH synthesis. The fact that GSH level is significantly lower in AA astrocytes, despite higher level of GGTs, suggesting there may be a compromised oxidation-reduction system or a deficient antioxidant response. Oxidative stress may be an important process in glaucomatous optic neuropathy [65]. Perhaps consistent with decreased ability to respond to oxidative stress, AA astrocytes have upregulated transcription of an array of cytoprotective genes responsible for stabilizing the cytoskeleton. In this study, several chaperones were upregulated in AA compared to CA astrocytes, including: heat shock protein 70 protein 2 (HSPA2), alpha-crystallin-related heat shock protein B6 (HSPB6), and crystallin-β B2 (CRYBB2). Upregulation of various heat shock proteins occur
in the retina and ONH in response to oxidative stress and in glaucoma [65].

Our study raises many questions that will require future investigations. Our data indicates that the microenvironment supported by astrocytes in the normal ONH in AA has important differences that may impact susceptibility to glaucomatous optic neuropathy. An important question that remains is whether the observed differential gene expressions in AA astrocytes compared to CA astrocytes are the result of continued exposure to stress signals in vivo or represent subtle genetic changes in the AA population. Thus, differential gene expression amongst AA and CA astrocytes may provide a basis for higher risk for developing glaucoma in AA. Current studies in ONH astrocytes from glaucomatous donors further supports our hypothesis that the gene expression profile of normal AA astrocytes anticipates the changes seen in reactive astrocytes in glaucomatous optic neuropathy.

Materials and Methods

Human eyes

Twenty one human eyes from 21 normal age-matched Caucasian American (CA) donors (age 62±12) and 16 human eyes from 16 normal African Americans (AA) (age 60±11) were used in this study to generate primary cultures of optic nerve head (ONH) astrocytes (Table S1).

Donors did not have history of eye disease, diabetes, or chronic CNS disease. Eyes were obtained from the local eye banks and from the National Disease Research Interchange (NDRI) (Table S1). Eyes were enucleated shortly after death and maintained at 4°C. Optic nerve heads were dissected within 24 hr of death and processed to generate ONH astrocytes [14,15]. In order to determine whether the eyes in this study did not have hidden optic nerve disease, samples of the myelinated nerves were fixed in 4% paraformaldehyde, post-fixed in osmium, embedded in epoxy resin and stained with paraphenylenediamine to detect axon degeneration [66,67].

Astrocyte Cultures

Cultures of human ONH astrocytes were generated as previously described [15]. Briefly, four explants from each lamina cribrosa were dissected and placed into 25-cm² Primaria tissue culture flasks (Falcon, Lincoln Park, NJ). Explants were maintained in DMEM/F-12 supplemented with 10% FBS and 100 μg/ml amphoter- cin B; Gibco/BRL, Gaithersburg, MD). Cells were kept in a 37°C, 5% CO₂ incubator. After 2–4 weeks, primary cultures were purified by using modified immunopanning procedure described by Mi and Barres (1999) [68]. Purified cells were expanded after characterization by immunostaining for astrocyte markers GFAP by Mi and Barres (1999) [68]. Purified cells were expanded after characterization by immunostaining for astrocyte markers GFAP by Mi and Barres (1999) [68]. Purified cells were expanded after characterization by immunostaining for astrocyte markers GFAP by Mi and Barres (1999) [68]. Purified cells were expanded after characterization by immunostaining for astrocyte markers GFAP by Mi and Barres (1999) [68].

Data Analysis

Pretreatment of Data. The first step in the analysis of the microarray data was to determine which genes were to be considered “present” or “absent.” We estimated the probe-set present/absent calls by using the Wilcoxon signed rank-based algorithm [69]. In order to reduce false positives, we removed the “absent” probe-sets from all samples.

Comparison between ONH astrocytes from normal AA and CA donors. We used RNA and Bioconductor in R [70] for background correction and normalization [71,72]. Genes with significant differential expression levels were detected by SAM (significance analysis of microarray data) and ANOVA. The false discovery rate (FDR) was set at ≤5%. A total of 54 chips of non-pooled RNAs represented 2 or 3 technical replicates for each of the 24 biological samples. Of the 54 chips, 18 were Human Genome HG U133A chips (6 biological samples) and 36 were Human Genome HG U133A 2.0 chips (18 biological samples). Expression values on the slightly different 2 Affymetrix platforms were first matched and merged in R. In addition, we also applied routines implemented in Limma of Bioconductor [73] to fit linear models to identify differentially expressed genes between both populations. A mixed effects model was used to account for the effect of technical replicates. The results are generally consistent between both methods.

Gene Ontology Analysis. To assess the biological significance of the gene list derived above we classified differentially expressed genes in normal AA and CA by manual separation by function using available data from public databases such as UniGene, OMIM and Entrez PubMed and the gene lists from RMA/SAM.

In addition, we used GOstats Bioconductor package [70] for the gene ontology (GO) analysis. The identification process of differentially expressed gene was similar as described in previous section. In order to get longer gene lists, the p-value threshold was set at 0.05 without FDR adjustment. The Hypergeometric test was used to identify the over-represented GO categories based on the identified gene list. Patterns of gene expression within the differentially expressed genes in each group were assessed for over-representation in the context of molecular function, biological process, and cellular component.

Real-time quantitative RT-PCR

Independent confirmation of differential expression was conducted using 17 astrocyte cultures from CA and 13 astrocyte cultures from AA age-matched donors obtained as described earlier in Methods. Cytoplasmic RNA was isolated from cultured ONH astrocytes (passage 3) as previously described [74]. Primers sequences are described in Table S7. cDNA was synthesized using
The cells were then washed 4 times with 250 μl of sterile serum-free DMEM/F12 as a negative control. Adhesion was measured in six CA samples and four AA samples using CytoSelect™ 24-well cell adhesion assay (Cell Biolabs). Assay was performed according to the manufacturer’s protocol. Five hundred μl of media supplemented with 10% fetal bovine serum was added to the bottom wells. Fifty thousand cells resuspended in 300 μl serum-free medium were added to the inserts. The plate was then incubated at 37°C, 5% CO2 for 24 hours. After removing the media from the inserts, non-migratory cells from the interior of the inserts were removed with cotton-tipped swabs. The inserts were stained with 400 μl of cell staining solution and washed three times with water before transfered to a clean well with 200 μl of extraction solution. The absorbance of the extracted samples was measured at 560 nm by a Thermo Multiskan Spectrum plate reader. The means from AA and CA groups were considered significantly different if p<0.05 (unpaired t-test). Three AA samples were used in the assay with ML-7 inhibition. The means from treated and untreated were considered significantly different if p<0.05 (unpaired t-test).

Migration assay

Migratory properties of five CA samples and six AA samples were measured by CytoSelect™ 24-well cell migration assay (Cell Biolabs). Assay was performed according to the manufacturer’s protocol. Five hundred μl of media supplemented with 10% fetal bovine serum was added to the bottom wells. Fifty thousand cells resuspended in 300 μl serum-free medium were added to the inserts. The plate was then incubated at 37°C, 5% CO2 for 24 hours. After removing the media from the inserts, non-migratory cells from the interior of the inserts were removed with cotton-tipped swabs. The inserts were stained with 400 μl of cell staining solution and washed three times with water before transfered to a clean well with 200 μl of extraction solution. The absorbance of the extracted samples was measured at 560 nm by a Thermo Multiskan Spectrum plate reader. The means from AA and CA groups were considered significantly different if p<0.05 (unpaired t-test). Three AA samples were used in the assay with ML-7 inhibition. The means from treated and untreated were considered significantly different if p<0.05 (unpaired t-test).

Glutathione (GSH) assay

Total GSH content was quantified in astrocyte cell lysates based on previously described methods using the Glutathione Assay Kit from Cayman Chemical (Ann Arbor, MI) [56]. Cells were grown to confluence in 100 mm dishes and collected in 1.0 ml sterile PBS using a disposable cell lifter. Cell suspension were centrifuged, supernatant was removed, and 100 μl of 50 mM phosphate buffer were added to the cell pellet and sonicated in icy bath for 30 min. Samples were then centrifuged for 15 min at 4°C, the supernatant was collected, and 5 μl of the supernatant were used to measure the protein concentration using the Pierce Protein Assay Kit (BCA method). The remaining supernatant was deproteinized as described in the assay protocol. Standards and samples were then aliquoted in a 96-well plate. Freshly prepared assay cocktail was added to each well, then the plate was incubated in the dark on an orbital shaker for 25 min. Absorbance was measured at 405 nm using a plate reader. Total GSH content for each sample was calculated based on the standard curve, and results were expressed as total GSH content (nmol) per mg protein. Ten AA and 10 CA normal donor samples were measured and the means of the content were considered significantly different if p<0.05 (Unpaired t-test).

Immunohistochemistry (IHC)

Six eyes from normal Caucasian donors (CA) and six eyes from normal age-matched African American (AA) donors were used. Detail methods are described in Text S1.

Immunocytochemistry

Primary ONH astrocytes from six normal AA donors and from six normal CA donors were used. Detail methods are described in Text S1.
Supporting Information

Table S1 Demographic information of normal donors. Demographic information of CA and AA normal donor eyes used to generate primary cultures of ONH astrocytes. Found at: doi:10.1371/journal.pone.0002847.s001 (0.09 MB DOC)

Table S2 Genes differentially expressed in ONH astrocytes from normal AA donors compared to their caucasian counterpart using RMA-SAM. List of genes differentially expressed in AA ONH astrocytes compared to CA ONH astrocytes, obtained by RMA-SAM analysis. Found at: doi:10.1371/journal.pone.0002847.s002 (0.11 MB DOC)

Table S3 Genes differentially expressed in normal AA ONH astrocytes compared to their CA counterpart using Lima. List of genes differentially expressed in AA ONH astrocytes compared to CA ONH astrocytes, obtained by using Limma package in Bioconductor. Found at: doi:10.1371/journal.pone.0002847.s003 (0.06 MB DOC)

Table S4 Functional analysis of genes differentially expressed in AA vs. CA. Functional classification of genes differentially expressed in AA ONH astrocytes compared to and CA ONH astrocytes. Found at: doi:10.1371/journal.pone.0002847.s004 (0.06 MB DOC)

Table S5 Selected Gene ontology for AA-CA comparison. Gene Ontology for genes differentially expressed in AA ONH astrocytes compared to and CA ONH astrocytes. Found at: doi:10.1371/journal.pone.0002847.s005 (0.04 MB DOC)

Table S6 Real-Time PCR validation of microarray expression analysis of normal Caucasian American and African American ONH astrocytes. Real-Time PCR validation of microarray expression analysis of normal CA and AA ONH astrocytes. Found at: doi:10.1371/journal.pone.0002847.s006 (0.08 MB DOC)

Table S7 Quantitative RT-PCR primer information. RT-PCR primer information. Found at: doi:10.1371/journal.pone.0002847.s007 (0.10 MB DOC)

Table S8 Primary Antibodies Used in this study. Information on the primary antibodies used in this study. Found at: doi:10.1371/journal.pone.0002847.s008 (0.04 MB DOC)

Text S1 Supplemental Methods. Detailed methods for Western Blots, immunohistochemistry and immunocytochemistry. Found at: doi:10.1371/journal.pone.0002847.s009 (0.05 MB DOC)

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Author Contributions

Conceived and designed the experiments: MRH. Performed the experiments: HM LC SMR WL SJ AMC AW OAA PY. Analyzed the data: PD SML CG. Wrote the paper: HM TL MRH.

References


