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Differential gene expression profiling of large and small retinal ganglion cells

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Abstract

Different sub-populations of retinal ganglion cells (RGCs) vary in their sensitivity to pathological conditions such as retinal ischemia, diabetic retinopathy and glaucoma. Comparative

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transcriptomic analysis of such groups will likely reveal molecular determinants of differential sensitivity to stress. However, gene expression profiling of primary neuronal sub-populations represent a challenge due to the cellular heterogeneity of retinal tissue. In this manuscript, we report the use of a fluorescent neural tracer to specifically label and selectively isolate RGCs with different soma sizes by fluorescence-activated cell sorting (FACS) for the purpose of differential gene expression profiling. We identified 145 genes that were more active in the large RGCs and 312 genes in the small RGCs. Differential data were validated by quantitative RT-PCR, several corresponding proteins were confirmed by immunohistochemistry. Functional characterization revealed differential activity of genes implicated in synaptic transmission, neurotransmitter secretion, axon guidance, chemotaxis, ion transport and tolerance to stress. An *in silico* reconstruction of cellular networks suggested that differences in pathway activity between the two sub-populations of RGCs are controlled by networks interconnected by SP-1, Erk2(MAPK1), Egr1, Egr2 and, potentially, regulated via transcription factors C/EBPbeta, HSF1, STAT1- and c-Myc. The results show that FACS-aided purification of retrogradely labeled cells can be effectively utilized for transcriptional profiling of adult retinal neurons.

Keywords

retinal ganglion cells; gene expression; soma size; FACS; microarrays; networks

1. Introduction

In the rodent retina, ganglion cells are subdivided into multiple subtypes (or classes) based on their morphology and function in processing visual information (Thanos 1988; Calkins *et al.* 2007; Callaway 2005). Experimental evidence, however, indicates that sensitivity to certain stressors correlates more with soma size rather than morphology or functional specialization of RGCs (Glovinsky *et al.* 1991; Glovinsky *et al.* 1993; Dreyer *et al.* 1994; Sucher *et al.* 1997; Isenmann *et al.* 2003; Feit-Leichman *et al.* 2005; Quigley 2005). Large ganglion cells (LRGCs) with soma sizes up to 20–35µm, were shown to possess an increased sensitivity to stress relative to the small ganglion cells (SRGCs) of 7–12 µm in rat retinas (Glovinsky *et al.* 1991; Glovinsky *et al.* 1993; Sucher *et al.* 1997). Studies modeling the impact on common neurotoxicity pathways by substances like kainate, glutamate and NMDA, showed that higher sensitivity of LRGCs that suffered faster degeneration compared to SRGCs in rat both *vivo* and *in vitro* (Dreyer *et al.* 1994; Sucher *et al.* 1997; Vorwerk *et al.* 1999). A size-dependent sensitivity has been consistently detected in rat models of experimental intraocular pressure-induced glaucoma (Glovinsky *et al.* 1991; Glovinsky *et al.* 1993; Sucher *et al.* 1997; Quigley 2005; Danias *et al.*, 2006); and a similar size-dependent susceptibility to glaucoma has been reported for human RGCs, where a population of LRGCs and corresponding large axons were lost faster than SRGCs and smaller axons (Glovinsky *et al.* 1991; Chaturvedi *et al.* 1993; Glovinsky *et al.* 1993; Sucher *et al.* 1997; Isenmann *et al.* 2003; Quigley 2005).

It is plausible to suggest that the difference in cell susceptibility to environmental and pathological stressors is caused by distinct activity of cellular pathways, which can be captured by microarrays and interpreted using bioinformatics. The use of microarrays for

differential profiling of gene expression in neural retina has been stifled by the high complexity of this tissue composed of a variety of heterogeneous cell types. The vast majority of studies utilized total retina preparation, which results in capturing the transcriptional output of a complex mixture of neuronal, glial, vascular and epithelial cell types, and makes data interpretation extremely challenging. Rapid purification of adult primary neurons that allows preparation of high quality, condition-specific RNA will allow investigators to circumvent this challenge and significantly increase data quality and relevance. Existing methods of RGC purification were developed and used for studies of neonatal and juvenile tissues, required in vitro sub-cultivation (Meyer-Franke *et. al*, 1995; Wang *et. al.*, 2007) and are not directly applicable to adult retinal neurons. In this work, we report the use of fluorescence-activated cell sorting (FACS) for purification of adult rat RGCs, and subsequent isolation of two sub-populations with distinct soma sizes for the purpose of microarray analysis. Differential microarray data were used to screen for candidate stress-susceptibility pathways affecting survival of large size RGCs. The differences in the molecular signatures of the two sub-groups of retinal ganglion cells revealed in this work lay the foundation for future research of differential responses in distinct neuronal subgroups observed in pathology. Paralleled with the rapid development of biochemical and genetic labeling of specific neuronal sub-populations, the method we report in this study will provide opportunities to directly target selected neurons for high-content molecular profiling.

2. Materials and methods

2.1. Animals and retrograde labeling of RGCs

All experiments were performed in compliance with the animal protocol approved by the University of Miami IACUC. A total of sixteen adult retinas were utilized. To label retinal ganglion cells, each Long Evans rat (250–300 g) was anesthetized by an intramuscular injection of 40 mg/kg of ketamine hydrochloride (Sankyo, Tokyo, Japan) plus 5 mg/kg of xylazine hydrochloride (Bayer, Tokyo, Japan), and fixed into a stereotaxic frame. Two holes were made in a skull with a 2 mm drill (Dremel, Racine, WI), and 2.1 mkl of 5% 4DI-10ASP (Invitrogen, Carlsbad, CA) in dimethylformamide was injected into the superior colliculus using a Hamilton microsyringe. Coordinates for the injection were – 6 mm behind bregma, 1.2 mm lateral from the superior sagittal suture, and 4.2 mm deep to the surface of the skull according to the coordinates of the Rat Brain Atlas. Rats were sacrificed for RGC isolation 10 days after dye injection.

2.2. Isolation and size-sorting of adult RGCs

Rats were euthanized, eyes were enucleated, paired retinas from the same animal were mechanically dissected out on ice within five minutes post-mortem. Four retinas from each group of two animals were pooled and incubated in a digestion solution containing papain (0.7 U/ml; Worthington, Lakewood, NJ) and L-cysteine (0.3 mg/ml; Sigma, St. Louis, MO) in Neurobasal-A medium (Invitrogen, CA) for 30 min (37°C; CO2 incubator), rinsed twice in 5 ml of medium, triturated in 4 ml of the medium containing 1:50 B27 supplement (Invitrogen, CA). In order to block new transcription induced by experimental procedures, we added 5 µg/ml of Actinomycin D into all solutions and media. The DiA-labeled RGCs

were sorted into two size groups with size margins 6–13 μm and 15–25 μm , using a modified protocol of sorting dissociated brain cells by FACS reported previously (Fischer *et al.* 2004). In brief, dissociated retinal cells were passed twice through a 40 μm nylon strainer (BD-Falcon, Bedford, MA), cooled to 4°C, and sorted for size and DiA fluorescence immediately in a FACSVantage SE cell sorter (Becton-Dickinson, San Jose, CA). A 530/30 filter was used to detect DiA-labeled RGCs. We used 6, 10, 15, 25 μm AlignFlow™ flow cytometry alignment beads (Molecular probes, OR), as well as rat erythrocytes and leucocytes as size reference markers to calibrate the instrument and adjust gating parameters. Cells were sorted at a speed of 12,000 objects/sec and collected directly into the vials containing lysis buffer (Absolutely RNA® Nanoprep kit, Stratagene, USA) pre-dried in a Savant SpeedVac concentrator, and proceeded for RNA extraction. Optimal sorting conditions (forward and side scatter: 160 and 280; FITC threshold 1,000) were determined in pilot experiments; typical yield was about 20,000 for large and 40,000 for small RGCs from each pooled sample. The 2-micron size selection gap was set to eliminate an overlap between the two sub-populations of RGCs.

2.3. Efficiency of RGC purification

The efficiency of purification was assessed by two methods: microscopically and by qRT-PCR after the total RNA was extracted (see 2.7 below for details). For microscopy, approximately 2000 FACS-counted cells were loaded on the lysine/laminin –coated cover glass and incubated at 37°C for 30 min in serum-free Neurobasal-A media. After cell attachment occurred, the RGCs were fixed in 4% paraformaldehyde/PBS for 15 minutes, rinsed with PBS, permeabilized for 15 minutes with 0.15% Triton X-100 (Sigma) in PBS, stained with DAPI (Invitrogen) and mounted using Fluoromount. The percentage of cells double-labeled DiA/DAPI positive cells were assessed by counting 5 standard fields (20x objective lens) on Leica TCS SP2 AOBS Confocal Microscopy system.

2.4. RNA extraction and probe preparation

RNA samples were extracted from purified and sorted RGCs using the Absolutely RNA® Nanoprep kit according to manufacturer's protocol, and finally resuspended in 10 μl . A total of four independent biological replicates (multiple pools each representing size-sorted samples derived from four eyes of two animals) were obtained for comparative profiling of large vs. small RGCs. The yield of total RNA varied between 120 and 180 ng for a typical SRGC and 80 and 130 ng for a LRGC sample. RNA purity and RGC-specificity was tested by quantitative RT-PCR using an array of well characterized marker genes for RGCs, as well as for potential contaminating types, i.e. microglia, astrocytes and photoreceptor cells. Following two rounds of linear amplification of mRNA using the Amino Allyl MessageAmp™ Kit (Ambion, TX, USA) that typically yielded 10–15 μg of aRNA, 0.5 μg of each aRNA that passed RNA quality control, were taken for labeling reaction with Cy3 or Cy5 dyes using CyDye Post-labeling Reactive Dye Pack (Amersham, USA). RNA quality was assessed by detecting 28S/18S ribosomal RNA peaks using an Agilent Bioanalyzer 2100. Labeled aRNA from four biological experiments (four pair wise comparisons of large vs. small RGCs) were hybridized with the Agilent Rat Oligo Microarrays (Agilent Technologies) according to the manufacturer's instructions. For each biological replicate we performed two technical replicates (dye-swap) in order to eliminate dye bias. The

comparison of gene expression levels has been performed in cell sub-populations derived from samples pooled from two animals, which allowed us to increase total RGC yield. Pooling retinas from two animals in this experimental design did not challenge the results of differential profiling because transcript abundances in final probes remain normalized for individual genetic variability. Even if the levels of a particular transcript varied between pooled retinas, the ratios of this transcript in sub-populations of LRGs vs. SRGs remained preserved in mixed samples as described earlier (Shannon et. al., 2005).

2.5. Immunohistochemistry and microscopy

Immunohistochemistry (IHC) was performed in slices of rat retinas fixed by blood perfusion with 4% Paraformaldehyde in PBS and post-fixed in the same solution for 20 minutes at 4 degrees C. Agar-embedded samples were sectioned to a thickness of 80 μ m with a Vibratome 1000 (St. Louis, MO) and used directly for IHC. The following commercially available antibodies were used: anti rat IL-1 β (R&D Systems, Minneapolis, MN), anti mouse Ccl2/MCP-1 (BioLegend, San Diego, CA), anti-rat fractalkine/Cx3CR1 (eBioscience, San Diego, CA). Distribution of primary antibodies was visualized by staining with AlexaFluor488 and/or AlexaFluor 543 dye-conjugated secondary antibody (Invitrogen/Molecular Probes). Control sections were incubated with nonimmune rabbit serum instead of primary antibodies. Specific fluorescence labeling in retina slices was visualized by confocal microscopy with Leica TSL AOBS SP5 (Leica Microsystems, MA). To nuclear DNA, fixed and permeabilized slices were incubated with DAPI (1:10,000 dilution in PBS, Molecular Probes) to visualize nuclei.

2.6. Transcriptome profiling and data analysis

Differentially expressed genes were captured by microarrays, each array was normalized for signal intensities across the whole array and locally, using the Lowess normalization. Genes that passed GenePix Pro 5.1 (Axon Instruments at Molecular Devices) standard quality control criteria (detailed in Ivanov *et al.* 2005), one class Significance Analysis of Microarrays (SAM, <http://www-stat.stanford.edu/tibs/SAM>) with false discovery rate (FDR) <1% and minimum fold change of 1.5, were considered differentially expressed and used in the analysis. These genes were analyzed for association (enrichment) with biological categories using over-representation in Gene Ontology (GO) functional folders (<http://vortex.cs.wayne.edu/Projects.html>) as prioritization parameter, and for connectivity using interactions network (interactome) analysis (Elkins *et al.*, 2007). The functional analysis workflow consisted of series of qualitative and quantitative procedures for parsing large datasets into smaller, functionally-meaningful subsets, such as linear signaling and metabolic pathways, and cellular and molecular processes. Multiple functional categories can be scored for each dataset, a procedure referred to as enrichment analysis (Myers et al. 2005). The basic enrichment analysis for GO categories, was performed in the MetaCore™ 4.0 (GeneGo Inc, St. Joseph, MI), a widely used commercial database and analytical software platform, also available as a plugin for the public platform Cytoscape.

2.7. Quantitative RT-PCR

The quality and cell type- specificity of the extracted RNA has been assessed by qRT-PCR for known cell markers including *Thy1*, *Gfap*, *Cd11b*, *Cd68* and *Actb* prior to amplification (see primer sequences and purity test results in Supplement Folder1, S2 and S3). QRT-PCR was also used to validate the microarray gene expression data for a group of randomly selected genes (Table 1). Total RNA was extracted from FACS-purified RGCs, which were sorted directly into RNA extraction buffer pre-dried in Savant SpeedVac concentrator to avoid excessive dilution. Genomic DNA contamination was removed with DNase I, and RNA was reverse-transcribed using the Message Sensor™ RT Kit (Ambion, USA). Primers are listed in Supplement Folder1). Real-time RT-PCR was carried out on Bio-Rad iCycler (Hercules, CA) using the QuantiTect SYBR Green PCR kit (Qiagen, USA). The PCR conditions were as follows: 95 C, 15 min; 45 cycles at 95C, 45 s; 60C, 45 s; 72C, 1 min. Melting curve analysis confirmed that each product was homogeneous and specific. Each cDNA was amplified in triplicate for every gene analyzed. Standard curves were run in parallel by using sequence-validated templates, and mRNA concentration was estimated by using MyiQ Real-Time PCR Detection Software (v1.0). Negative (no template) controls were run in parallel. We used non-amplified RNA for qRT-PCR to avoid potential amplification-induced errors. The measured transcript abundance was normalized to the level of *Actb* (β -actin) for all samples. The size of the amplified PCR product was confirmed by the gel electrophoresis.

2.8. Meta-analysis by interactome reconstruction

Interactome analysis was used to identify and prioritize functional “parts” of neuronal cellular machinery that were differentially activated in the two sub-populations of RGCs. This analysis was performed using the Metacore™ software coupled to the MetaBase™ interactions database (free trial available at www.genego.com) and enabled with network building capabilities described earlier (Ekins *et al.* 2007). Basic principles and algorithms of such meta-analysis of gene expression data are described elsewhere (Ekins *et al.* 2006). In brief, a subset of differentially expressed genes is screened by the software using several algorithms to recover and link into a network those genes (nodes on the network) that encode products with known “binary” interactions, shown as network “links. Each network is, therefore, unique for the data set at the level of specific differentially activated nodes and binary interactions. This software automatically maps gene expression data on the resultant interactions networks. Some, more advanced algorithms (description in Supplement Folder 1), may also connect nodes via shortest paths via regulatory molecules that show below-threshold changes in expression, which is rather common for transcription factors and other molecules regulated post-transcriptionally. The network analysis process does not require clustering or statistical analysis other than when defining the probability (p-value) of the assembly of a network of a certain size out of randomly selected relevant nodes. The networks can be characterized by a variety of parameters, which depict network topology, distribution of nodes and hubs (defines as the top 25% of nodes by the number of interactions), transcription factors, surface receptors etc (Lukashin et al. 2003). Typically, hubs on human networks encompass transcription factors or membrane receptors, each forming a cluster (module) of direct targets and upstream regulators. A detailed description

of the network building algorithms and network legend are provided in the Supplement Folder 1, and described in the literature (Ekins *et al.* 2006).

2.9

The enrichment analysis of differentially expressed genes for transcription factor binding sites has been performed using the Transcription Element Listening System software (at www.telis.ucla.edu/index.htm). The hierarchical clustering of differentially expressed genes according to Log2 changes in expression level has been performed in the TM4 software package for microarray data management and analysis (<http://www.tm4.org/>).

For the description of standard technique of leukocytes isolation from peripheral blood, the list of PCR primers, for algorithms of interactome meta-analysis and network detailed legend see Supplement Folder 1.

3. Results

3.1. Quality of cell purification

Isolation of RGCs with intact RNA and minimal contamination by other retinal cell types was critical for obtaining the gene expression profiles specific to the sub-populations of SRGC and LRGC. To achieve the required degree of purification, we used intracranial injections with 4DI-10ASP dye (DiA) into the superior colliculus, a technique which is known to produce highly specific fluorescent labeling of up to 99% of RGCs (in the rat) projecting into this region (Linden R, Perry, 1983). The new transcription that might be triggered by cell purification procedures was blocked by the addition of 5 µg/ml of Actinomycin D into all solutions, as described by Huusko *et al.* 2004. The combination of 4DI-10ASP labeling with flow cytometry has been successfully used for RGC isolation and gene expression profiling (Fischer *et al.* 2004). Analysis of the FACS results showed a 2:1 stoichiometry in the yields of small (6–13 µm) vs large (15–25 µm) cells, which is consistent with the ratios reported by other investigators (Kashiwagi *et al.* 2000). Relatively high yields of undegraded, high-quality RNA from both sub-populations were consistently obtained, for which the quality and cell type-specificity of the extracted RNA were determined by Bio-Analyzer and quantitative RT-PCR (qRT-PCR) amplification of several single copy genes, including *Thy1*, *Gfap*, *Cd11b*, *Cd68* as described earlier (Ivanov *et al.* 2006).

Isolated cells were tested for the size, presence of specific DiA labeling and contaminating cell types using microscopy (Fig. 1), as well as by qRT-PCR to detect cell type-specific marker gene transcripts. Fluorescence microscopy revealed that the minimum of 90% of FACS-purified cells of the expected soma size range possessed specific DiA labeling. Provided that the remaining 10% might have lost the DiA labeling during sample processing and permeabilization with Triton X-100, we used qRT-PCR to validate the abundance of neuronal marker and to determine the level of contamination by non-neuronal cells like glia and macrophages. We assessed the relative abundance of the marker genes for RGCs (*Thy1*), astrocytes (*Gfap*), macrophage (*CD68*) and microglia (*CD11β*) cells in non-amplified RGC-enriched samples. qRT-PCR did not detect contamination with astroglia (*Gfap*), microglia (*Cd11β*), and macrophages were only detected in SRGCs, with relative abundance of *Cd68/Thy1* marker genes mRNA ranging between 1–7% (Supplemental Folder 1, S2). The

calculated efficiency of RGC immunoaffinity purification was, therefore, over 99% for LRGCs and 93–99% for SRGS.

3.2. Differential microarray analysis of RGCs

We used sensitive two-color Agilent Rat Genomic Oligo Arrays and the dye swap design of microarray experiments to compare gene expression profiles of the two cell sub-populations of RGCs in the normal retina. The data from four independent biological experiments performed in two technical replicates were analyzed using the SAM algorithm with $FDR < 1\%$ to identify statistically significant expression differences. All primary microarray data are available at the GEO web site (<http://www.ncbi.nih.gov/geo/>; series GSE11468). Using a 1.5 fold cut-off level, we identified 145 genes that were preferentially expressed in the LRGCs and 312 genes - in the SRGCs (Supplementary Table 1). Among these genes only 30 differed in excess of 2-fold, including 9 representing uncharacterized ESTs (Table 1). The differential expression was verified by qRT-PCR and showed a good correlation with microarray data for a group of randomly selected genes (Table 2). To further validate the gene expression data, we performed immunohistochemistry in fixed retinal slices. These data showed the presence of *Ccl2*, fractalkine/*Cx3cl1* and *IL-1 β* proteins in adult RGCs at the levels easily detectable by IHC (Fig. 2). The IHC data, however, did not show significant differences in protein accumulation between RGCs of different size, which is common for small-scale differences in gene expression. In addition, we performed cross-comparison between the list of differentially activated gene identified in this study and neuron- specific gene expression profiles obtained in other studies that utilized alternative methods: the array profiling of immunoaffinity-purified RGCs (Ivanov et. al., 2006) and EST library sequencing data generated from juvenile rat RGCs (Farkas et. al., 2004). This analysis showed, correspondingly, a 95% and 73% overlap providing additional evidence of high specificity of the RGC purification.

3.3. Enrichment analysis and interactome reconstruction

Functional classification of a subset of transcripts differentially enriched in the SRGCs revealed many neuron-specific GO processes including synaptic transmission, sensory perception, learning and memory and nervous system development (Table 3). Significant differences in other categories related to cell adhesion, signal transduction, cation transport and blood pressure regulation, all of which were more active in SRGCs. The only top-scored gene category relatively activated in LRGCs included GO process “chemotaxis and inflammation”. LRGCs possessed consistently higher levels of genes encoding CC chemokines, including *Ccl2*, *Ccl3*, *Ccl4*, CC receptor *Ccr5*, the cytokine *Il1 β* and a family of early growth response genes *Egr1* (Table 2), *Egr2*, and *Egr3* (regulators of pro-inflammatory gene expression) relative to SRGCs (Tables 1 and 2). Consistently, interactome analysis revealed a low p-value network interlinking chemokines and the STAT1- mediated pathway of *Il1 β* activation (Fig. 3). While most pro-inflammatory genes were relatively over-activated in LRGCs, the gene encoding neuronal chemokine *Cx3cl1* (fractalkine) that was more active in SRGCs (Table 1). Fractalkine is known to be expressed in the CNS neurons and has been implicated in Ca^{2+} -dependent postsynaptic excitatory activity, neuron-glia interaction, adhesion and neuronal survival (Harrison et al. 1998; Meucci et al. 2000; Nishiyori et al. 1998). Although a significant body of published data is

available to independently validate endogenous expression of *Ccl2*, *Ccl3*, *Ccl4* chemokines, *Ccr5* and *Il1 β* in neurons (Rock *et. al.*, 2006; Sun *et. al.*, 2006; Guo *et. al.*, 2003; Tixier *et. al.*, 2006, and also GEO records # GDS 956, GDS1799, GDS1398, GDS1076 at <http://www.ncbi.nlm.nih.gov/geo/>), we tested our RNA samples for the presence and relative abundance of glia-specific transcripts *CD68*, *GFAP* and *CD11 β* . In RNA samples extracted from either SRGC or LRGC these transcripts were very scarce (detectable only after 45 cycles, a 1000-fold difference *vs. Thy1* transcript), providing the assurance that glia-derived transcripts had not affected the RGC-specific transcriptomic profile. In accord with these results, the signals from all three control transcripts were below detection level of Agilent arrays. The IHC data also validated presence of Ccl2, fractalkine/Cx3cl1 and IL-1 β proteins in adult RGCs and their axons (Fig. 2).

We then used the list of 111 differentially expressed genes (1.5 fold change, genes with known or predicted function from Supplementary Table1) for *in silico* reconstruction of cellular networks that possess differential activity in the two sub-populations of retinal neurons using MetaCore analytical software. The largest network revealed multiple pathways differentially activated in SRGCs and interconnected by SP-1, c-Myc, Egr1, Erk2 (MAPK1) positioned as highly connected “hubs” (Supplementary Fig. 2). Less connected minor hubs included Erk (MAPK1/3) and CCR5 relatively activated in LRGCs. The genes selectively activated in LRGCs (marked with red dots) formed a compact “local” network showing that increased signaling from Egr1, Egr2 transcription factors and chemokines in this sub-population is transduced to IL-1 β via STAT1, and from Egr1 to NFD1/NeuroD via Erk (Fig. 3A). Cellular processes enriched in genes that were relatively activated in SRGCs included receptor signaling via s-Src (see local network on Fig. 3B), and Ca²⁺ transport, with Ca²⁺ channels and calmodulin as major hubs on the “global” network (Supplementary Fig. 2). The topology of these networks suggests that differences in transcriptomes of the two sub-populations of RGCs are broadly controlled by a group of regulatory factors including SP-1, Erk2(MAPK1), Egr1, Egr2 and, potentially, by signaling via STAT1 and c-Myc. To validate whether several transcription factors revealed by connectivity criteria on the network are, indeed, capable of regulating differentially activated subset of genes, we analyzed over-representation of transcription factor binding sites among these genes in Transcription Element Listening System (at www.telis.ucla.edu/index.htm). These complementary approaches validated SP-1, C/EBP β and HSF1 as the most likely regulators of differentially activated genes in the two sub-populations of RGCs (Table 4).

4. Discussion

In this study we showed that adult primary neurons can be isolated, distinct sub-populations of these neurons can be separated and profiled for gene expression using whole genome microarrays. We have modified a FACS-based cell purification technique and successfully utilized it for isolation of the RGCs from pooled rat retinas. A similar approach has been also successfully utilized for the analysis of distinct subtypes of brain neurons (Fischer *et al.* 2004; Yao *et. al.*, 2005; Sugino *et al.* 2006) and astrocytes (Cahoy *et. al.*, 2008). The combination of sorting cells for both specific, retrogradely delivered fluorescence, and for soma size allowed for high degree enrichment in RGC using a one-step procedure. Efficient sorting, size-separation and up to 99% purity of RGC sample allowed us to isolate a

significant amount of high quality RNA and perform differential microarray profiling of SRGCs vs. LRGCs. The abundance of neuron-specific cellular processes in microarray data and results of cross-comparison with neuron-specific profiles obtained in an independent study confirmed sufficient purity of RGC samples. The relative activation of genes corresponding to the GO category “chemotaxis and inflammation” in LRGCs is rather perplexing, given this category is typically associated with glial and macrophage cell function in the CNS. One possible explanation could be contamination of purified neurons with microglial cells that became fluorescent by phagocytosis of dying retrogradely-labeled RGCs, as reported earlier (Higashide *et al.* 2006; Naskar *et al.* 2002). However, such phagocytosis-induced co-labeling can be excluded, given it has only been observed during massive RGC death. In contrast, we used only normal retrogradely-labeled retinas, a technique known for highly specific RGC labeling (Supplementary Fig. 1), which is retained by RGCs without passing to neighboring cells for up to several months in our own and other laboratories (Thanos *et al.*, 2000). Our control tests also showed no significant contamination of RGC-specific mRNA samples with glia- and microglia-specific transcripts (Supplementary Folder 1, S2). Finally, the factor of contamination can also be dismissed because relative increase of pro-inflammatory transcripts has been consistently detected only in the RNA from LRGCs, rather than SRGCs, while the latter have the soma size range more comparable to that of glia and would have been more prone to co-sorting by FACS. Combined, the results of our control experiments presented convincing evidence that microarray data indeed reflect the differential activity of several cellular pathways in LRGCs vs SRGCs.

In general, when compared to transcript abundances in LRGCs, significantly more genes were over-represented in SRGCs. Our enrichment analysis showed that several neuron-specific processes, such as synaptic transmission, sensory perception, learning and memory and nervous system development, were among those exhibiting the greatest enrichment in the samples obtained from SRGCs. In addition, SRGCs demonstrated relatively higher activity of genes related to cell adhesion, signal transduction, cation transport and blood pressure regulation. Increased activity in these functional categories is suggestive of functionally more dynamic phenotype of smaller RGCs, contrasted by a higher level of specialization in the larger neurons. This may potentially reflect the fact that “LRGCs” sub-population included Type I RGCs that are analogous to alpha-cells, the key functional component of the visual system in many mammals that are involved in basic form vision (Perry, 1979; Thanos *et al.*, 1988).

One major advantage provided by neuron-specific (same cell type) vs. whole retina-derived (diverse cell types) data is the compatibility with methodology of an *in silico* reconstruction of differentially activated cellular pathways. Interactome analysis, performed in order to reconstruct differences in regulation of genes and pathways functionally related to stress response, revealed relative activation of signaling via ERK1/2, Egr1 and Ccr5 in LRGCs, whereas transcription factors SP1, C/EBPbeta, HSF1, STAT1, c-Myc, and Ca²⁺ ion transport regulation were more active in SRGCs (Supplementary Fig. 2). Among transcription factors potentially controlling differential gene expression in two RGC subgroups, our analysis suggested SP1, C/EBPbeta and HSF1. Synergy between activation

of MAPK-ERK signaling and Egr1-mediated transcriptional activation in LRGCs (Fig. 3) is of a particular significance. In homeostatic CNS, this crosstalk has been shown to be essential for normal neuronal activity, such as synaptic transmission, learning and memory (Li *et al.*, 2007a). This corroborates well with topologically proximal Ccr5-interlinked endogenous chemokines on the network, which are now being increasingly suggested to play physiological roles in synaptic function and plasticity (Tonelli *et al.*, 2005) and in hippocampal-dependent memory and long-term potentiation (Avital *et al.*, 2003; Ross *et al.*, 2003). In pathology, however, Egr1 has been shown to play a key role in cellular responses to ischemia and in chemokine activation (Yan *et al.*, 2000), suggesting that higher constitutive activity of this transcription factor may play a role in decreased resistance of LRGCs to stress. Thus, the ERK-dependent increase in expression of Egr-1, which leads to increased expression of cdk5, has been implicated in p53-dependent neuronal death following oxidative DNA damage (Lee and Kim, 2007). Provided that oxidative stress is very common in many retinal degenerations, the constitutively increased activity of MAPK-ERK –EGR1 network may represent potential mechanism of increased vulnerability of LRGCs to stress. In parallel to this mechanism, relatively decreased activity of calcium transport system in LRGC can selectively affect survival of this sub-population during calcium overload typically observed in stress (Nakamura *et al.* 1999; Li *et al.* 2007). Although we detected relatively subtle differences in transcriptome activity between the two subgroups of RGCs, it is conceivable that greater differences and contrast responses will be revealed upon exposure of the retina to stress. While further experiments are required to confirm differential activation of major interactome hubs in the two RGC groups and test their effect on LRGC susceptibility to stress, this study provides a robust method and the baseline data, which will serve as a foundation for identification of molecular pathways of differential vulnerability.

To conclude, the present paper demonstrates the feasibility of using FACS-based purification of retrogradely labeled sub-populations of adult retinal neurons for the purpose of global transcriptome profiling. High quality of RNA and adequate cell type-specificity of purified samples, enabled us to characterize differences in a global pattern of gene expression and to perform meta-analysis of differential data in order to screen for candidate stress-susceptibility pathways affecting survival of large size RGCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Scheme depicting methodology of RGC purification using FACS from dissociated rat retinas retrogradely labeled with 4DI-10ASP dye. **A**, RGCs of all sizes were labeled with similar efficiency (green). **B**, Sorting cells for both RGC-specific fluorescence and size resulted in separation of small (6–13 μm, P1 region) and large (15–25 μm, P2 region) RGCs using FACS. **C**, Efficiency of sorting was controlled by fluorescence microscopy, where soma size and percentage of cells double-labeled with DiA (green) and DAPI (blue) was assessed. Bar is 20 μm.

Figure 2.

Immunohistochemistry for the Cx3cl1(fractalkine), Ccl2 and IL-1 β proteins was performed in fixed retinal slices to validate gene expression data at the level of protein accumulation. Neuronal bodies in the retinal ganglion cell layer of the retina were stained for NeuN (green), target proteins recognized by antibodies specific to Cx3cl1, IL-1 β or Ccl2 antibodies are shown in red; nuclei were labeled with DAPI (cyan). Representative image of control staining, performed with primary anti-target protein antibodies omitted, is shown in the bottom panels. Bar is 20 μ m.

Figure 3.

The highest scored “local” interactions networks reconstructed from the list of genes (1.5 fold change) differentially activated in either LRGCs (A) or SRGCs (B). Functional clustering of genes shown on the networks is based on experimentally confirmed interactions (arrows) and suggests that difference in gene expression profiles between the two sub-populations of RGCs is controlled by transcription factors Egr1, Egr2, Ndf1, and signaling via STAT1 in LRGCs. In SRGCs, activated signaling to c-Src is facilitated via Fgfr2, Pdgf-A, Ephrin B, Srbp1, and adenosine receptor A1. Relative gene activation in LRGCs is indicated by red dots, in SRGCs-by blue dots next to the network symbols.

Table 1

Genes differentially expressed in excess of 2-fold in either SRGCs or LRGs.

GeneBank ID	LRGCs/SRGCs expression ratio	Unigene ID	Gene/Transcript
XM_344511	-3.05		similar to ubiquitin A-52 residue ribosomal protein fusion product 1
XM_346231	-2.86		similar to 60S ribosomal protein L7a (Surfeit locus protein 3) (PLA-X polypeptide)
BF550484	-2.68	Rn.17896	Ubiquitin protein ligase E3A (predicted)
NM_133289	-2.54	Rn.88082	sodium channel, voltage-gated, type 9, alpha polypeptide
AW917664	-2.52		EST
TC497198	-2.41		EST
XM_347134	-2.27		similar to nidogen 2 protein
AB014883	-2.25		EST
XM_345292	-2.18	Rn.43989	similar to RIKEN cDNA 4933425K02 (predicted)
NM_053750	-2.16	Rn.10637	natriuretic peptide precursor type C
TC485183	-2.146		EST
NM_031686	-2.14	Rn.54541	sodium channel, voltage-gated, type 6, alpha polypeptide
NM_012666	-2.10	Rn.1920	tachykinin 1
XM_347134	-2.06		similar to nidogen 2 protein
XM_215247	-2.06	Rn.106138	Rho GTPase activating protein 18 (predicted)
TC486100	-2.06		EST
XM_343740	-2.03	Rn.79028	similar to GTPase activating protein testicular GAP1
NM_134455	-2	Rn.107266	chemokine (C-X3-C motif) ligand 1, fractalkine
NM_053633	2	Rn.89235	early growth response 2, Egr2
NM_031530	2	Rn.4772	chemokine (C-C motif) ligand 2, Ccl2
BF564914	2.02	Rn.49201	EST
XM_340964	2.05	Rn.137224	EST
NM_053858	2.06	Rn.37880	small inducible cytokine A4, Ccl4
TC482297	2.09		EST
TC495030	2.10		EST
AW143863	2.14	Rn.126047	Gnb1 guanine nucleotide binding protein, beta 1
AW914944	2.18	Rn.16547	similar to hypothetical protein FLJ20481 (predicted)
NM_031512	2.29	Rn.9869	interleukin 1 beta
NM_013025	2.33	Rn.10139	chemokine (C-C motif) ligand 3, Ccl3
NM_021751	2.44	Rn.76668	prominin 1
TC495758	2.90		early growth response 3, Egr3

Table 2

Quantitative RT-PCR validation (LRGC/SRGS ratios) of the microarray data for 18 genes with significantly different levels in either sub-population of RGCs

Gene	LRGC/SRGS expression ratios by qRT-PCR	LRGC/SRGS expression ratios by array	Confirmed by real-time PCR
<i>Ccl2</i>	2.96	1.99	Yes
<i>Ccl3</i>	1.44	2.33	Yes
<i>Ccl4</i>	3.63	2.06	Yes
<i>Cntn2</i>	-1.93	-1.96	Yes
<i>Egr2</i>	3.41	1.97	Yes
<i>Egr3</i>	2.48	2.90	Yes
<i>Ephb1</i>	-2.58	-1.66	Yes
<i>Gpr51</i>	-1.44	-1.49	Yes
<i>Gria2</i>	-1.79	-1.58	Yes
<i>Il1b</i>	1.92	2.29	Yes
<i>Nppc</i>	-4.10	-2.16	Yes
<i>Panx1</i>	-2.20	-1.72	Yes
<i>RGD1305087</i>	-3.95	-2.17	Yes
<i>RGD1311599</i>	3.95	2.18	Yes
<i>Scn6a</i>	-2.15	-2.14	Yes
<i>Scn9a</i>	-1.61	-2.54	Yes
<i>Tac1</i>	-3.32	-2.10	Yes
<i>Tnfrsf12a</i>	-1.14	-1.78	Yes

Table 3

Top GO processes differentially enriched in large vs small RGCs. Processes were scored according their p-values, calculated using the Metacore™ analytical software.

Table 4

Top transcription factors revealed by network analysis and by TELIS.

Major transcription factors revealed by network and TELIS analysis	
<i>Networks</i>	<i>TELIS</i>
SP1	SP1
C/EBPbeta	C/EBPbeta
HSF1	HSF1
(c-Myc)	(SRY)
(EGR1)	(SRF)
(STAT1)	(CEBP)

* Bolded factors were revealed by both analyses, bracketed- by a single type.