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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 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.

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 and Sterling, 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, 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 20–35 μm, 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, 1993; Sucher et al., 1997). Studies modeling the impact on common neurotoxicity pathways by substances like kainate, glutamate and NMDA, showed that LRGCs exhibited faster degeneration compared to SRGCs in rat both in 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, 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 and Hedley-Whyte, 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

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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 16 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 scull with a 2 mm drill (Dremel, Racine, WI), and 2.1 μl of 5% 4DI-10ASP (Invitrogen, Carlsbad, CA) in dimethilformamide 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 5 min 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; CO_2 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/s 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 1000) 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-μm 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 Section 2.7). 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 min, rinsed with PBS, permeabilized for 15 min 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 (20× 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 LRGCs vs. SRGCs 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