FEBS Letters 580 (2006) 331–335

Microarray analysis of gene expression in adult retinal ganglion cells

Dmitry Ivanov[a,b,*], Galina Dvoriantchikova[a], Lubov Nathanson[c], Stuart J. McKinnon[d], Valery I. Shestopalov[a,e,*]

[a] *Bascom Palmer Eye Institute Department of Ophthalmology, University of Miami Miller School of Medicine, 163 NW 10th Avenue, Miami, FL 33136, USA*
[b] *Vavilov Institute of General Genetics RAS, Moscow, Russia*
[c] *Department of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, Miami, FL, USA*
[d] *Departments of Ophthalmology and Neurobiology, Duke University Medical Center, Durham, NC, USA*
[e] *Department of Cell Biology and Anatomy, University of Miami Miller School of Medicine, Miami, FL, USA*

Received 5 November 2005; revised 25 November 2005; accepted 6 December 2005

Available online 19 December 2005

Edited by Lukas Huber

Abstract **Retinal ganglion cells (RGCs) transfer visual information to the brain and are known to be susceptible to selective degeneration in various neuropathies such as glaucoma. This selective vulnerability suggests that these highly specialized neurons possess a distinct gene expression profile that becomes altered by neuropathy-associated stresses, which lead to the RGC death. In this study, to identify genes expressed predominantly in adult RGCs, a global transcriptional profile of purified primary RGCs has been compared to that of the whole retina. To avoid alterations of the original gene expression profile by cell culture conditions, we isolated RNA directly from adult RGCs purified by immunopanning without prior sub-cultivation. Genes expressed predominantly in RGCs included: *Nrg1*, *Rgn*, *14-3-3* family (*Ywhah*, *Ywhaz*, *Ywhab*), *Nrn1*, *Gap43*, *Vsnl1*, *Rgs4*. Some of these genes may serve as novel markers for these neurons. Our analysis revealed enrichment in genes controlling the pro-survival pathways in RGCs as compared to other retinal cells.**

Keywords: Retina; Retinal ganglion cells; Immunopanning; RNA amplification; Gene expression; Cell markers

1. Introduction

Mammalian retina is a complex tissue composed of neuronal, glial, and vascular cell types among which retinal ganglion cells (RGCs) are the only type of output neurons that send their axons to the visual cortex of the brain [1]. It is now well established that the crosstalk between RGCs and different retinal cell types is critical for RGC survival [2–4]. In various neuropathies like glaucoma known to progress with aging, RGCs die, while other retinal cells remain mostly unaffected [5,6]. As an essential step to understanding the molecular basis of such selective vulnerability, it is important to characterize the molecular profile of the fully differentiated adult RGCs and determine how it differs from other retinal cells. Microarrays have been effectively implemented in such studies. Recent work reported successful isolation of juvenile RGCs by immunopanning followed by in vitro cultivation for the purposes of EST analysis [7]. However, this approach relied on in vitro sub-cultivation, which is unlikely to work with adult neurons due to their low survival rate in vitro [8]. Recent advances in RNA amplification have made it possible to study gene expression in ultra-small samples, and even detect differences between individual cell types within the same tissue [9–11]. This approach allows gene expression profiling of a small number of purified adult RGCs without an in vitro cultivation, and eliminates potential concerns over cell culture-induced alterations of the gene expression profile. In this work we performed microarray analysis using amplified RNA extracted both from the retina and adult RGCs purified by immunopanning without in vitro sub-cultivation. The analysis of genes enriched in RGCs allowed us to identify novel RGC markers and genes essential for survival and homeostasis in these cells.

2. Materials and methods

2.1. Isolation of RGCs

All experiments were performed in compliance with the ARVO statement for use of animals in ophthalmic and vision research. Brown Norway rats (250–300 g) were euthanized according to the IACUC approved protocol. Eyes were enucleated and retinas were mechanically dissected out. RGCs were isolated according to the two-step immunopanning method reported earlier to yield 95–99% enrichment for RGCs [12]. In order to preserve RNA and limit potential gene expression changes we modified the procedure to shorten the total treatment time to 4 h. Briefly, the whole retinas were incubated in papain solution (16.5 U/ml) for 30 min. To analyze similarly treated samples, whole retina suspensions were sampled for RNA extraction only at this point. In the next step macrophage and endothelial cells were removed from the cell suspension by panning with the anti-macrophage antiserum (Axell Accurate Chemical Corp., Westbury, NY). RGCs were specifically bound to the panning plates containing anti-Thy1.1 antibody, and unbound retinal cells were removed by washing with DPBS. Purified RGCs were released by trypsin incubation and immediately processed for RNA extraction. To eliminate the effects of genetic variability and reduce the animal group size, the comparison of the RGC expression profile to that of a whole retina was performed using samples derived from the same animal.

*Corresponding authors: Fax: +1 305 547 3658.
E-mail addresses: divanov@med.miami.edu (D. Ivanov), vshestopalov@med.miami.edu (V.I. Shestopalov).

Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PRL, photoreceptor layer

0014-5793/$32.00
doi:10.1016/j.febslet.2005.12.017

2.2. RNA extraction

RNA from whole retina and RGCs was extracted from three different biological samples. Processed individual samples from the same animal contained about 100 000 cells in each. Cells were collected, treated with the lysis buffer supplied with the Absolutely RNA® Nanoprep kit (Stratagene, USA), and RNA was purified using this kit according to the manufacturer's protocol. Prior to amplification, the RNA quality was tested in each sample by qRT-PCR for *Thy1*, *Vim*, *GFAP*, *S100b*, *CD11b*, *CD34* and *Actb* (Supplement 1).

2.3. RNA amplification, labeling and array hybridization

Amplification was performed using the Amino Allyl MessageAmp™ Kit (Ambion, USA), as described previously [11]. Amplified Rat Universal Reference RNA was labeled with Cy-3; experimental aRNAs from retina and RGCs were labeled with Cy-5 dye (Amersham, USA). Equivalent amounts of reference and experimental aRNAs were hybridized with the Agilent Rat Oligo Microarrays (Agilent, USA) according to the manufacturer's instructions.

2.4. Microarray image and data analysis

Each array was normalized for signal intensities across the whole array and locally, using the Lowess normalization [13]. We analyzed only those genes that passed quality control criteria described previously [11]. To compare the gene expression levels in RGCs with those in the retina, the "RGC/reference RNA" ratios were divided by the ratios of "retina/reference RNA" obtained from the same animal. One class SAM (http://www-stat.stanford.edu/tibs/SAM) with FDR <1% and fold change of 2.0 was used to determine genes with consistent differences in expression [14]. To verify the "RGC/reference RNA" microarray dataset for specificity to RGCs we compared our data with the data from UniGene library (ID.14630) for RGCs [7]. Our dataset was obtained as the result of the application of one class SAM (FDR <1%) to identify genes with consistent ratios of "RGC/reference RNA". The second dataset contained genes from the UniGene library, which are present on the Agilent Rat Oligo microarray (1137 out of 1792 in the UniGene library). Selected genes were classified according to Gene Ontology category "biological process" using Onto-Express (http://vortex.cs.wayne.edu/Projects.html) [15].

2.5. qRT-PCR, in situ hybridization, immunocytochemistry

For qRT-PCR, in situ hybridization and immunocytochemistry we applied standard techniques; brief protocols including the table of primers are included in Supplement 1.

3. Results

3.1. RGC and retina specific gene expression

We compared both RGC and retina specific gene expression profiles using 2.0-fold change as a threshold and identified 326 genes enriched in RGCs, and 330 genes enriched in the whole retina (Supplements 2 and 3; Table 1). Relative transcript enrichment detected by microarrays was confirmed by qRT-PCR for 21 genes randomly selected from both the RGC and retina gene groups (Table 2). Functional classification of a subset of RGC-enriched transcripts is shown in Table 1. Several genes, like *Amhr2* and *Rgn*, were under-represented in the retina and for this reason were not detected by microarrays with retina samples. For those genes we performed qRT-PCR and used the data to calculate the RGC/retina ratios (included in Table 2). Analysis of the main gene ontology (GO) categories revealed that genes vital for neurogenesis and survival were enriched in RGCs; conversely, genes essential for visual perception were enriched in the whole retina. Notably, about two thirds of the differentially enriched transcripts belonged to ESTs, some of which potentially representing novel genes.

Table 1
Functional annotation for the selected top genes enriched in RGCs

Gene	RGCs/retina	FDR	Function
Nrn1; neuritin	6.18	0.088	Promotes neurite outgrowth
Prph1; peripherin 1	5.35	0.088	Cytoskeleton organization
Gap43; growth associated protein 43	5.22	0.088	Neurite outgrowth and nerve regeneration
Vsnl1; visinin-like 1	5.01	0.088	Calcium dependent signalling
Rgs4; regulator of G-protein signaling 4	4.85	0.287	G-protein coupled receptor signaling
Nef3; neurofilament 3, medium	4.32	0.088	Cytoskeleton organization
Ywhah; 14-3-3 family, eta polypeptide	4.08	0.088	Negative regulation of apoptosis
Ywhaz; 14-3-3 family, zeta polypeptide	3.91	0.088	Mitochondrial import stimulation factor
Sema6b; semaphorin 6B	3.82	0.238	Neurogenesis and axon guidance
Nrp; neuropilin 1	3.59	0.088	Neuronal development
Rnd1; Rho family GTPase 1	3.57	0.895	Neuritic process formation
Plcb1; phospholipase C, beta 1	3.52	0.287	Production of the second messenger molecules
Gsto1; glutathione *S*-transferase omega 1	3.40	0.446	Metabolism
NCAML1; neural cell adhesion molecule L1	3.39	0.088	Neural and glial cell adhesion
Nfl; neurofilament, light polypeptide	3.34	0.088	Cytoskeleton organization
Ptpn5; protein tyrosine phosphatase, non-receptor type 5	3.24	0.088	Regulating the duration of ERK activation
Inpp4a; inositol polyphosphate-4-phosphatase, type 1	3.19	0.088	Phosphatidylinositol-3,4-bisphosphate 4-phosphatase activity
Stmn2; stathmin-like 2	3.12	0.895	Microtubule depolymerization and synaptic plasticity
Ret; Ret proto-oncogene	3.07	0.355	Neurogenesis
Nrg1; neuregulin 1	2.86	0.789	Neural and organ development
Rgs3; regulator of G-protein signalling 3	2.76	0.355	G-protein coupled receptor signaling
Map2k1; mitogen activated protein kinase kinase 1	2.73	0.088	Signal transduction
Pik3r1; phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1	2.66	0.088	Negative regulation of apoptosis
Coch; coagulation factor C homolog, cochlin	2.41	0.789	Perception of sound
Ywhab; 14-3-3 family, beta polypeptide	2.34	0.088	Signal transduction
Fbxo2; F-box only protein 2	2.30	0.355	Maintaining neurons in a postmitotic state
Nell2; Nel-like 2 homolog	2.22	0.287	Growth and differentiation of neural cells
Calb2; Calbindin 2 (Calretinin)	2.22	0.713	Calcium ion binding
Ywhag; 14-3-3 family, gamma polypeptide	2.17	0.713	Signal transduction
Grb2; growth factor receptor bound protein 2	2.15	0.088	Signal transduction