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Methods in eye research

Isolation of intact astrocytes from the optic nerve head of adult mice

Hee Joo Choi, Daniel Sun, Tatjana C. Jakobs*

Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, United States

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ABSTRACT

The astrocytes of the optic nerve head are a specialized subtype of white matter astrocytes that form the direct cellular environment of the unmyelinated ganglion cell axons. Due to their potential involvement in glaucoma, these astrocytes have become a target of research. Due to the heterogeneity of the optic nerve tissue, which also contains other cell types, in some cases it may be desirable to conduct gene expression studies on small numbers of well-characterized astrocytes or even individual cells. Here, we describe a simple method to isolate individual astrocytes. This method permits obtaining astrocytes with intact morphology from the adult mouse optic nerve and reduces contamination of the isolated astrocytes by other cell types. Individual astrocytes can be recognized by their morphology and collected under microscopic control. The whole procedure can be completed in 2–3 h. We also discuss downstream applications like multiplex single-cell PCR and quantitative PCR (qPCR).

1. Introduction

Astrocytes are the most numerous glial cell type in the mammalian brain. Originally, their role was thought to be mainly supportive for the neurons in their vicinity. More recently, however, astrocytes have been shown to be active players in almost all CNS activity both under normal and under pathological conditions (Sofroniew and Vinters, 2010; Sun and Jakobs, 2012). In general terms, astrocytes can be subdivided into two types, the protoplasmic astrocytes that reside in the grey matter of the brain, and the fibrous astrocytes in the white matter. Both types of astrocyte respond to CNS injury of any kind by becoming reactive. This was first described as a morphological change that involves an upregulation of intermediate filaments in the cytoplasm and a hypertrophy of the cells' processes (Eng et al., 2000; Pekny and Nilsson, 2005; Sofroniew and Vinters, 2010). But reactivity also leads to profound changes in gene expression. This is well documented on the transcriptome level for protoplasmic astrocytes that lend themselves to isolation of pure populations due to their high numbers and the availability of transgenic mice with astrocyte-specific expression of GFP (Cahoy et al., 2008; Lovatt et al., 2007; Orre et al., 2014; Zamanian et al., 2012). Whether reactive changes in astrocytes are beneficial or harmful for neuronal survival or recovery after injury is an active topic of research (Karimi-Abdolrezaee and Billakanti, 2012; Sofroniew, 2005).

In glaucomatous optic neuropathy astrocyte reactivity is observed in all stages of the disease, including very early time points before obvious loss of ganglion cells has occurred in the retina (Balaratnasingam et al., 2008; Hernandez, 2000; Lye-Barthel et al., 2013). The optic nerve head, especially the region of the lamina cribrosa in primates and its glial counterpart in rodents, is thought to be the site of first injury to retinal ganglion cell axons (Howell et al., 2007; Jakobs et al., 2005; Quigley et al., 1981, 1983). This has led to considerable interest in studying the cellular components of the optic nerve and their gene expression profiles in response to elevated intraocular pressure and other types of injury, such as nerve crush or transection (Howell et al., 2011a; Jakobs, 2014; Johnson et al., 2011, 2007; Qu and Jakobs, 2013). However, the optic nerve contains astrocytes, microglia, NG2 expressing cells, endothelial cells, and ganglion cell axons, all of which may contribute to the total RNA extracted from the nerve for analysis. It can, therefore, be difficult to determine which cell type is responsible for the differential regulation of genes or pathways. If one wants to study pure astrocytes, it is in principle possible to establish cell cultures from the optic nerve and use them for gene expression profiling or a targeted assay of the regulation of individual genes (Hernandez et al., 2002; Miao et al., 2008; Nikolskaya et al., 2009).

* Corresponding author. Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114, United States.
E-mail address: Tatjana_Jakobs@meei.harvard.edu (T.C. Jakobs).

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Unfortunately, if astrocytes are taken into culture, under most conditions they tend to change in morphology and gene expression, which complicates the analysis of biological effects (Foo et al., 2011). Though culture of pure optic nerve astrocytes remains a powerful technique, in some cases it may be preferable to work on freshly dissociated, individual cells.

Dissociated cells from various parts of the CNS in normal and pathological conditions have been used successfully to assay the expression of individual genes, multiple genes in parallel, and the construction of cDNA libraries for genetic screening or sequencing (Dulac and Axel, 1995; Jakobs et al., 2007; Janssen-Bienhold et al., 2001; Kamphuis et al., 2003; Pannicke et al., 2000; Saliba et al., 2014). A potential drawback of cell dissociation is that it can be difficult to recognize the target cells as the process involves enzymatic and mechanical disruption of the tissue which can lead to the cells losing their distinctive features. In this case, an antibody against an extracellular epitope may be used to identify the cell, if such an epitope is available (Jakobs et al., 2003). However, in many cases optimizing the dissociation protocol results in single cells with well-preserved morphology (Haseleu et al., 2013). Here we describe a method to isolate intact astrocytes from the glial lamina of the murine optic nerve and discuss downstream applications. Analysis of optic nerve head astrocytes on the single-cell level should be of interest not only in glaucoma research but also in studies that aim at understanding the biology of white matter astrocytes under normal and pathological conditions.

2. Materials and supplies

2.1. Animals

All animal were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures were approved by the Institutional Animal Care and Use Committee at Schepens Eye Research Institute. Four mouse strains were used in this study: (1) wild-type C57BL/6 (Charles River Laboratories, Wilmington, MA). (2) A transgenic mouse line (B6.hGFAPpr-EGFP) produced by backcrossing hGFAPpr-EGFP mice that express GFP under the control of the promoter of human glial fibrillary acidic protein (GFAP) onto the C57BL/6 background. (3) B6.129P-Cx3cr1^{tm1Litt}/J mice expressing EGFP in immune cells such as microglia, monocytes, and NK cells (the Jackson laboratory, Bar Harbor, ME, strain #005582). (4) A transgenic mouse line in which astrocytes in the brain and optic nerve express red fluorescent protein. The transgenic hGFAPpr-EGFP mice on the FVB/N background (Nolte et al., 2001) were obtained from the laboratory of Helmut Kettenmann (Max Delbruck Center, Berlin, Germany). This mouse strain displays bright expression of enhanced green fluorescent protein (EGFP) in many, but not all astrocytes, thus the complete morphology of individual astrocytes can be observed directly (Sun et al., 2009, 2013, 2010). We crossed the hGFAPpr-EGFP mice with C57BL/6 mice for at least six generations to remove the retinal degeneration mutation in the FVB/N-derived transgenic mice. To generate mice that express a red fluorescent protein in astrocytes under the control of the GFAP promoter, we crossed B6.Cg-Tg(Gfap-cre)73.12Mvs/J with the reporter strain B6; 129S6-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J (both from the Jackson laboratory, Bar Harbor, ME, strain numbers 012886 and 007905, respectively). This strain was used as an additional method to ensure that the isolated cells were astrocytes. Mice were housed under a 12 h light/dark cycle with free access to food and water.

2.2. Reagents

Reagents used in cell dissociation and single cell collection were: 10× phosphate-buffered saline (PBS; USB, Cleveland, OH), Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (HBSS, Lonza, Walkersville, MD), Papain (Worthington Cat No. 3126, Lakewood, NJ), and Normal horse serum (Jackson Immuno Research, West Grove, PA). DNAse (Cat No. D-5025, 15,000 U/ml), BSA (Cat No. A-2153), and L-Cysteine (Cat No. C-1276) were purchased from Sigma–Aldrich (St. Louis, MO). For single-cell reverse-transcription polymerase chain reaction (RT-PCR) in 3.3 the Access RT-PCR system (Promega Cat No. A1250, Madison, WI) was used for reverse transcription and the first round of PCR, and the AmpliTaq Gold® DNA Polymerase (Applied Biosystems Cat No. 4311814, Foster City, CA) was used for the second round of PCR. For qPCR in 3.4 the Arcturus Pico Pure RNA isolation kit (Applied Biosystems, Cat No. 12204-01) was used to extract total RNA from 12 astrocytes and the RNA was transcribed and amplified using an Ovation qPCR system kit (NuGen, Cat No. 2210-24, San Carlos, CA).

2.3. Equipment

Transverse sections of B6.hGFAPpr-EGFP and B6.129P-Cx3cr1^{tm1Litt}/J mice were made on a TPI Vibratome 1000 (Technical Products International, Inc., St. Louis, MO), and the images were taken using a Leica SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL).

Collecting single astrocytes was performed using glass micropipettes (World Precision Instruments Cat No. TW150-4) attached to manual linear stages (Newport M-423, Franklin, MA) under a Zeiss Axiovert 200 with Hoffman optics (Fig. 1A). The glass micropipettes were pulled with a two-stage puller (Narishige PP-83, Tokyo, Japan) to produce ~20 μm tips before use. Gold Seal microscope slides were used to hold the cell suspension and the washing buffer (Gold Seal Fluorescent Antibody Micro Slides with 2 etched rings, Gold Seal Products Cat No. 3032, Portsmouth, NH).

Single-cell RT-PCR in 3.3 and qPCR in 3.4 were performed in the GeneAmp PCR System 9700 (Applied Biosystems) and in the StepOnePlus qPCR thermocycler (Applied Biosystems), respectively.

3. Detailed methods

3.1. Isolation of single astrocytes from the optic nerve head

Before the start of the experiment, the enzymatic cell dissociation solution is freshly prepared by adding 9 μl papain suspension (the concentration of papain is ~35 mg protein per ml, with the exact concentration dependent on the lot) and 2.5 μl L-cystein solution (10 mg/ml in HBSS) to 500 μl HBSS. The cell dissociation solution is incubated at 37 °C for 15 min to activate the papain. During that time, the mouse is euthanized with CO_2, and the mouse eyes are pulled out using a pair of curved forceps and placed in prechilled PBS. The extraocular muscles, meningeal sheath, cornea, lens, and sclera are removed, and then the optic nerve head is dissected from the retina and the myelinated region of the optic nerve. Due to the small amount of tissue, two optic nerve heads are used for cell dissociation. The optic nerve heads are transferred into the papain solution and incubated for 15–25 min. The incubation time should be adjusted depending on the age of mice (older mice generally needing longer incubation times). After enzymatic treatment, digested optic nerve heads are centrifuged at 2000 rpm for 5 min at room temperature to remove the papain solution. The optic nerve heads are resuspended in 200 μl HBSS with 10% normal horse serum to stop the enzymatic reaction and mechanically dissociated by gentle trituration 3–5 times using a heat-polished Pasteur pipette (the lumen diameter at the tip of the pipet should be approximately 300 μm). The cell suspension and undissociated tissue are centrifuged again at 2000 rpm for 5 min at room