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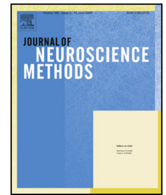

Properties of human central nervous system neurons in a glia-depleted (isolated) culture system

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HIGHLIGHTS

- MHCI (Major Histocompatibility Complex Class I) as a biomarker for direct isolation of human primary neurons.
- MHCI(−) isolation significantly increased the purity of human neurons compared to existing methods.
- We established a defined condition for improved culture of human primary neurons.
- Our method improves maturation and visualization of human neurons in culture.
- We provide an effective system to study human neuron biology in dissociation and in co-cultures.

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ABSTRACT

Background: Current methods for studying human neurons depend on a feeder layer of astroglia supplemented with animal serum to support the growing neurons. These requirements undermine many of the advantages provided by in vitro cell culture approaches when compared with more complex in vivo techniques.

New Method: Here, we identified a reliable marker (MHCI) that allows for direct isolation of primary neurons from fetal human brain. We utilized a magnetic labeling and isolation technique to separate neurons from other neural cells. We established a defined condition, omitting the astroglial supports that could maintain the human neurons for varying amounts of time.

Results: We showed that the new method significantly improved the purity of human neurons in culture without the need for further chemical/mechanical enrichment. We demonstrated the suitability of these neurons for functional studies including Rho-kinase dependent regulation of neurite outgrowth and ensheathment in co-cultures with oligodendrocyte progenitor cells derived from fetal human brain.

Comparison with existing methods: The accountability for neuron-only seeding and the controllable density allows for better neuronal maturation and better visualization of the different neuronal compartments. The higher purity culture constitutes an effective system to study and screen for compounds that impact neuron biology without potential confounding effects from glial crowding.

Conclusions: High purity human neurons generated using the improved method will enable enhanced reliability in the discovery and development of drugs with neuroregenerative and neuroprotective activity.

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1. Introduction

Primary cultures of neurons derived from the embryonic rodent central nervous system (CNS) have long been used to study fundamental principles of their cell biology, define mechanisms underlying disease development and screen potential neuroprotective therapeutic agents. Disease directed studies include mechanisms underlying immune mediated neuronal injury in multiple sclerosis and autoimmune encephalomyelitis and intracellular aggregation of hyper-phosphorylated tau protein and extracellular deposits of amyloid beta (Aβ) protein in Alzheimer's disease (Kiryu-Seo et al., 2010; Jin et al., 2011; Trapp et al., 2007), see

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reviews (Lassmann, 2009; Mohamed et al., 2013). Rodent based culture systems are defined in which such neurons are maintained as a highly enriched population or in the presence of other neural cell populations such as co-culture technique.

The capacity to culture primary neurons harvested from the developing human brain was demonstrated more than a decade ago (Chiu et al., 1998; Kerkovich et al., 1999; Sah, 1995; Chiu et al., 1994). The prevailing method from that time has been to rely on a feeder layer of astrocytes in the presence of animal serum to support the growing neurons. As part of the method, additional mechanical shake-off and chemical refinement by means of anti-mitotic agents such as L-leucine methyl ester (LME), cytosine β-D arabinofuranoside (Arac-C) or 5′-deoxy-5-fluorouridine (DfU) are needed to enrich for the growing neurons and to reduce the growth of the feeder astroglia and reduce presence of microglia before experiments are carried out (Kerkovich et al., 1999; Filipovic and Zecevic, 2008; Saikali et al., 2007; LeBlanc, 1995; Darlington et al., 2008; Giuliani et al., 2003; Haile et al., 2011). Fetal neurons can be enriched to ~85% using this method (Darlington et al., 2008). However, long-term culture of the neurons post-enrichment is limited due to the sub-optimal neuronal purity and re-population by the astroglial cells. Nevertheless, this sub-optimal protocol continues to be used for culturing human fetal neurons, in large part due to the lack of neuron-specific surface biomarkers for isolating these cells. Surface Thy-1 has to date been used only for isolating rodent retinal ganglion cells (Barres et al., 1988; Meyer-Franke et al., 1995). Although an indirect method to deplete non-neuronal cells in rodents using a combination of cell surface markers such as astrocyte cell surface antigen (ACSA)-1 and -2 to select out astrocytes and CD11b for microglia has been proposed, this has not yet been successfully applied to human cortical neurons. Surface MHCI was reported on microglia, astrocytes, oligodendrocytes, and endothelial cells but not neurons in adult human brains (Grenier et al., 1989; Lampson, 1995; Tooyama et al., 1990). However, inducible neuronal expressions have been observed in certain circumstances such as in Rasmussen's encephalitis, focal cortical dysplasia and ganglioglioma (Bien et al., 2002; Prabowo et al., 2013). More recently, specific subsets of neurons such as the substantia nigra dopaminergic and locus coeruleus norepinephrinergic neurons have been reported to express MHCI (Cebrian et al., 2014).

More recently, embryonic stem cell (ESC) or induced pluripotent stem cell (iPSC) generated neurons are being used for studying neurodevelopment and disease, and for drug discovery applications. Current methods for generation of iPSC-neurons requires multiple sequential steps, starting from somatic cell harvesting, pluripotency reprogramming, iPSC colony selection, stem cell expansion, and directed differentiation (Nicholas et al., 2013; Maroof et al., 2013; Zhang et al., 2001; Yan et al., 2005; Hu et al., 2010; Zare et al., 2014; Nakamura et al., 2014), see review (Heilker et al., 2014). These processes are labor-intensive and require long-term in vitro cultures, usually taking 1–2 months to generate prior to starting any experiment or drug testing. In addition, consistency of reprogramming and reproducibility of the protocol, as well as the number of the induced neurons and purity of culture can each have an impact on the outcome of any experiment or study.

Here, we described a direct isolation method to culture human primary neurons without the need of astroglial feeder layer. We have established an improved protocol that yields high purity of human neurons that can be cultured in defined medium for varying amounts of time. In addition to describing the features of these cells in dissociated cell culture, we present results of using these neurons in a co-culture paradigm with oligodendrocyte progenitor cells (OPCs) also derived from the fetal human brain.

2. Materials and methods

2.1. Ethics statement

All procedures with human fetal brain tissues were approved by the Montreal Neurological Institute and Hospital (MNI/H) Neurosciences Research Ethics Board (NEU REB) #ANTJ1988/3) and the Albert Einstein College of Medicine Institutional Review Board ((IRB) approval ID #1993-042). Experiments using animals were approved by the McGill Faculty of Medicine Animal Care Committee and performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

2.2. Human fetal neurons

Human brain tissue (16–18 gestation weeks) was acquired from the Human Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY) and was prepared based on published protocol (Leong et al., 2014). Tissue was first dissociated in 0.25% trypsin (Invitrogen, Burlington, Canada) and 25 μg/ml DNase I (Roche Diagnostics, Laval, Canada) for 15 min at 37 °C and then passed through a 140 μm nylon mesh. For culture of human neurons following the prevailing method, dissociated neural cells was plated at 3×10^6 cells per ml in Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum (FCS), Glutamax and penicillin-streptomycin (all from Invitrogen) on poly-L-lysine coated coverglass. The culture was subjected to three cycles of 1 mM 5′-deoxy-5-fluorouridine (DfU) (Sigma, Oakville, Canada) every three days to reduce glial proliferation and to enrich for neurons.

For MHCI(−) isolated neuron cultures, dissociated neural cells were first incubated with allophycocyanin- (APC-)conjugated anti-MHCI (BD Biosciences, Mississauga, ON) for 30 min at 4 °C, followed by incubation with anti-APC microbeads (Miltenyi Biotech, Auburn, CA) for 15 min at 4 °C. The labelled cell suspension was then applied to a MACS® column placed in a MACS® separator (Miltenyi Biotech). The MHCI(−) neurons were collected from the flow-through of the MACS® column. The isolated neurons were plated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with MACS® NeuroBrew-21 (Miltenyi Biotech) and penicillin-streptomycin on poly-L-lysine coated coverglass.

2.3. Flow cytometry analysis

Cells were labeled in PBS containing 1% FCS and 0.1% sodium azide, either with phycoerythrin (PE) conjugated anti-polysialylated neuronal cell adhesion molecule (PSA-NCAM) (Miltenyi Biotech) and APC-conjugated anti-MHCI (BD Biosciences), or matched isotype controls before fixation with 1% formaldehyde-phosphate-buffered saline (PBS). For intracellular labeling, cells were first permeabilized with 4% paraformaldehyde (PFA)-0.1% saponin followed by either Alexa488-conjugated anti-βIII tubulin or anti-glial fibrillary acidic protein (GFAP) (both from Invitrogen). Immuno-labeled cells were analyzed for expression of the markers on BD FACSAria™ II (equipped with 488 nm and 633 nm lasers) using BD FACSDiva™ 6.1.3 software.

2.4. RNA isolation and qRT-PCR

Total RNA was isolated at day 7 of culture using Isol-RNA lysis reagent (Fisher Scientific, Whitby, ON) followed by DNase treatment according to manufacturer's instructions (Qiagen Sciences, Germantown, MD). RNA samples were then transcribed into cDNAs and individual transcript expression was assessed using gene specific Taqman® probe (Applied Biosystems, Foster City, CA) in an ABI 7000 thermocycler. Fold change calculations for neuronal and

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