Metabolic changes and differentiation-dependent motility responses of neural stem cells during in vitro neuron formation

Theses of the Ph.D. Dissertation

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Budapest, 2017

1. Introduction

Although neural stem/ progenitor cells are present in the brain during the entire life of mammals, neurons destroyed by injuries or by neurodegenerative diseases are not replaced by inherent new born neurons and can not be substituted by implanted neural stem/progenitor cells, which *in vitro* generate a wide variety of functional nerve cells. For understanding the conditions for successful clinical applications, both, the biological requirements of neural stem cells and the environment provided by the host brain tissue should be explored. During neuronal tissue formation, stem cells develop in an entirely different environment from that of the fully developed nervous system. It is now evident that neuron formation and the integration of novel neurons into functional neuronal circuits require specific extracellular matrix components, growth factor combinations, stimulus patterns, oxygen and metabolite supply that are different from those provided by the adult nervous tissue. For the time being, our knowledge on the specific metabolic needs of neural stem cells and the motility responses of neural progenitors to bioelectric stimuli are far from complete.

During my thesis work, the metabolic characteristics of NE-4C embryonic mouse neural stem cells were compared to those of NE-4C-derived neurons obtained by *in vitro* neurogenesis of stem cells. Studies on cell metabolism included the instrumental measurement of oxygen consumption and proton production (extracellular acidification), the monitoring of cell viability under different metabolite supplies, and the gene expression analyses of some key factors (enzymes, transporters and regulatory proteins) of the basic cell metabolism.

Using radial glia-like neural stem/progenitor cells carrying light gated cation (channelrhodopsin) channels we analysed the changes in cell motility in response to ionic stimulation.

Objectives

My aim was to analyse some environmental conditions that could significantly affect the survival and neuronal development of neural stem cells in the adult nervous tissue.

I. Metabolic characteristics of the early embryonic neuroectoderm derived NE-4C stem cells and their differentiating neuronal progenies were investigated in different stages of the *in vitro* neuron formation. The O_2 consumption and H^+ production of neuronal stem cells and developing precursors were measured and compared in the presence of different metabolites. The metabolic responses were measured on primary (directly isolated from the brain tissue) neuronal and astroglial cultures and were compared to data obtained from NE-4C cells.

The following questions were specifically addressed:

1. How does the metabolism of neuronal stem cells change during the neuronal differentiation?
2. What metabolites are used by the stem cells and the stem cell derived neurons?

3. What are the main routes of energy production in neural stem cells and in neurons?
4. Do NE-4C derived neurons display similar metabolic properties as primary neurons originated from the embryonic mouse brain?

II. Using image analysis and statistical evaluation, the affects of ionic stimulation on the migration of neural progenitor cells were analysed on time-lapse records of radial glia-like stem/progenitor cells.

The studies were focused on the following questions:

1. Are there statistically significant changes in the motility of the cells during the progression of *in vitro* neuron formation?
2. Are there statistically significant motility changes in response to ionic stimulation?

2. Methods

The developmental state of NE-4C (Schlett and Madarász, 1997; ATTC-CRL-2595) neural stem cells and NE-4C derived neurons were tested with immunocytochemical and qPCR methods.

The oxygen consumption (OCR) and proton production (ECAR) of NE-4C stem cells and NE-4C derived neurons were measured with Seahorse cell metabolism analyser device, in artificial cerebrospinal fluid (ACSF) solution. For comparison, the OCR and ECAR values were also measured in cultures of neurons and astrocytes cultured from embryonic and new born brain, respectively. After additions of single nutrients (glucose, lactate, pyruvate or β-hydroxi-butyrate) or nutrient free ACSF, the changes in cell metabolism were assayed in response to certain metabolites or to further starvation.

The viability of cells in normal medium and in solutions without nutrients was measured with MTT reduction assays. The mitochondrial function of cells in solutions without nutrients or in the presence of a single metabolite was checked by measuring the responses evoked by specific mitochondrial

blockers including oligomycin, FCCP, DNP, antimycin and rotenone.

The changes of expression of some metabolic key enzymes, transporters and regulatory proteins during the advancement of neuronal differentiation were investigated with qPCR method on the gene expression level.

Using radial glia-like neuronal stem cells population with ($ChR2^+$) or without ($ChR2^-$) light gated channelrhodopsin cation channels and in different stages of *in vitro* differentiation, the motility changes were investigated in response to repeated illumination (λ=488 nm; 300 ms; 0.13 mW/mm^2 in every 5 minutes), for 12 hours. The move of cells was followed in consecutive frames of time-lapse records. The routes of cell displacements were determined by the x and y coordinates of tracked cell centers. The distances made by individual cells between consecutive frames (in 5 minutes) were determined by the two dimensional Euclidean distance calculation. The motility of the $ChR2^+$ and $ChR2^-$ cells in response to the illumination were analysed in the three stages of cell development: in the basic stem cells state, in the committed progenitor cells and in the neuron precursor phase. After summarizing the data of 60 cells from each stage, the

motility differences and responses to light stimulation were analysed by statistical methods.

3. Summary of new scientific results

Thesis 1.: NE-4C derived neurons and neurons isolated from the brain display similar metabolic properties.

NE-4C derived and primary neurons react similarly to starvation, to the addition of glucose, lactate, pyruvate and β-OH-butyrate. The oxygen consumption, proton production, and proton leak, as well as their *pdk4*, *atg12* and *tfam* expression of NE-4C derived and primary neurons changes in a similar way in response to single nutrients, and differs similarly from the responses of neuronal stem cells.

Thesis 2.: Nutrient deprivation damages neuronal stem cells to a greater extent than neurons.

Based on viability assays, the more developed cells can maintain the energy levels for several hours by consuming their own cellular material. Embryonic neural stem cells, on the other hand, are significantly damaged by a 3 hour starvation. (Figure 1)

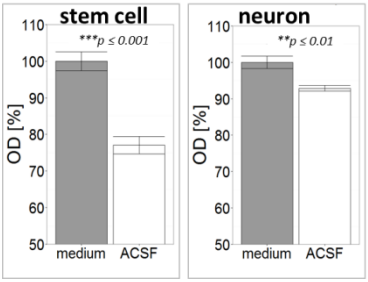

Figure 1.: The relative viability of NE-4C stem cells and NE-4C-derived neurons after 3 hour starvation in normal culture medium (100%) (grey) and in metabolite free ACSF (white). Averages and standard errors of mean ($n \geq 10$) are shown.

Thesis 3.: During the neuronal differentiation of NE-4C cells the maximum mitochondrial respiration increases and the proton leak decreases.

Based on the results of the metabolic measurements, the maximum mitochondrial respiration of NE-4C neurons is significantly higher and the quantity of protons used in non-mitochondrial ATP synthesis (proton leak) is significantly lower in comparison to the NE-4C stem cells. (Figure 2) The increase in the expression of the mitochondrial transcription factor A also confirms the mitochondrial changes during

neuronal development. During the differentiation the mitochondrial activity and the oxygen consumption increase.

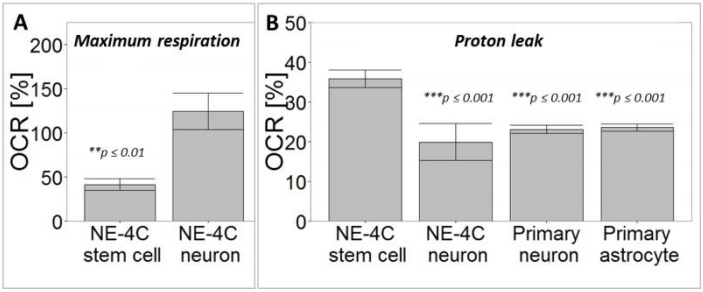

Figure 2.: The mitochondrial maturation during the differentiation (A) Maximum mitochondrial respiration of NE-4C stem cells and NE-4C-derived neurons in the percentage of basal OCR in starvation (100%). The columns represent averages and standard errors of means ($n \geq 8$). (B) The proton leak of NE-4C stem cells, NE-4C-derived neurons, primary neurons and astrocytes was determined as the percentage of OCR after blocking the mitochondrial activity in starvation (100%). The columns represent averages and standard errors of means ($n \geq 23$).

Thesis 4.: Glucose if added to starving cells, is utilised through aerobic glycolysis in both stem cells and neurons.

In response to glucose addition NE-4C stem cells, NE-4C derived neurons as well as primary neurons and astrocytes fail to increase oxygen consumption and acidify the extracellular

environment indicating that the cells utilize glucose through aerobic glycolysis. (Figure 3)

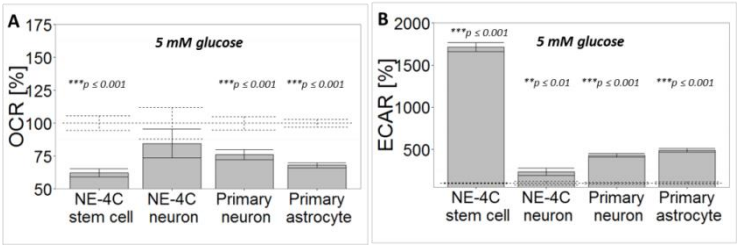

Figure 3.: The changes to addition of glucose in (A) oxygen consumption rate and (B) extracellular acidification rate in cultures in relation to basal starving (100%), mean ± SEM (n≥23)

Thesis 5.: The starving NE-4C stem cells utilise lactate, pyruvate and β-OH-butyrate for mitochondrial energy production, while NE-4C derived neurons and primary neurons do not increase their mitochondrial activity in response to these nutrients.

The starving NE-4C stem cells can utilise lactate, pyruvate and β-OH-butyrate molecules for mitochondrial energy production. (Figure 4)

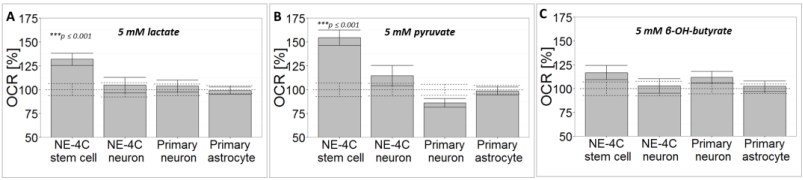

Figure 4.: The changes to addition of lactate (A), pyruvate (B) and β-OH-butyrate (C) in oxygen consumption rate in cultures in relation to basal starving (100%), mean ± SEM (n≥23)

The metabolism of NE-4C stem cells is characterised by fast adaptation to the available nutrients, while NE-4C neurons retain their high oxidative phosphorylation activity in starvation by catabolising their own materials. The latter fact is also confirmed by the higher expression level of the gene related to autophagy (*atg12*) in the neurons.

The insignificant change in oxygen consumption in the presence of lactate and pyruvate confirms that these molecules do not play an essential role in the metabolism of nutrient deprived cultured neurons which display low synaptic activity compared to the *in vivo* activity. Since we could not detect the mRNA of MCT2 transporter, it is assumed that these nutrient molecules do not get into the neuronal soma which provides the majority of the cultured cell material.

Thesis 6.: The inwardly directed cation current significantly enhances the migration activity of radial glia-like neuronal progenitor cells, while it reduces the motility of neuronal precursors derived from them.

4. Areas of application

Based on our data, the metabolic activities and accordingly the nutrient and oxygen requirements of neural stem cells and developing neurons are significantly different. There are also relevant differences between the motility reactions of neural progenitor cells and developing neurons in response to ionic stimulation. The results confirm that understanding of the optimal environmental conditions for the survival and differentiation of neural stem/progenitor and precursor cells is essential for the development of future cell therapies and recent tissue engineering methodology.

The publication of the author:

Journal Article

[1] **A. G. Jády**, Á. M. Nagy, T. Kőhidi, S. Ferenczi, L. Tretter, and E. Madarász, "Differentiation-Dependent Energy Production and Metabolite Utilization: A Comparative Study on Neural Stem Cells, Neurons, and Astrocytes," Stem Cells and Development, vol. 25, no. 13, pp. 995–1005, Jul. 2016.

[2] T. Köhidi, **A. G. Jády**, K. Markó, N. Papp, T. Andrási, Zs. Környei, E. Madarász, "Differentiation-Dependent Motility-Responses of Developing Neural Progenitors to Optogenetic Stimulation," Frontiers in Cellular Neuroscience, vol. 11, Dec. 2017.

Conference paper in journal

[3] Z. Bérces, Á. Horváth, **A. Jády**, A. Pongrácz, E. Madarász, and Z. Fekete, "Neural Cell Response to Nanostructured Biosensor Surfaces," Procedia Engineering, vol. 87, pp. 971–974, 2014.