Metabolic profiles of cells present in the central nervous system

PhD thesis booklet

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"Discovery consists in seeing what everyone else has seen and thinking what no one else has thought."

Albert Szent-Györgyi

INTRODUCTION

Approximately 20% of the oxygen and nearly 50% of the glucose consumed by the human body are dedicated to brain functions, however, the brain represents only 2% of the total body weight. In comparison to other tissues, the central nervous system has got a considerably high energy need that is fulfilled with fine-tuned spatial and temporal regulatory mechanisms. These tasks involve the dynamics of the functional anatomical cytoarchitecture and the transport and utilization of the energy metabolites present in the cerebrospinal fluid.

For a long time the exclusive role of glucose in brain energy metabolism and the bioenergetic functions of neurons were in the main focus of research. Results of the last three decades, however, represent a more complex view than the previously imagined neurocentric metabolism. From the metabolic point of view glial cells (astrocytes, oligodendrocytes and the microglia) present in the central nervous system were considered for a long time as indifferent cells compared with neurons. This dogma has been changed with the discovery of unique glial functions, particularly of their role in the energy substrate metabolism, production, storage and transport. FDG-PET and fMRI studies reveal functional anatomical cytoarchitecture and metabolic cooperation between the different types of glial cells and neurons. Investigating the bioenergetic properties of glial cells and their contribution to neuronal energy metabolism therefore have a significant role in the discovery of brain functions.

In this thesis the energy metabolism of cells in the developing and mature brain were investigated throughout *in vitro* experiments. Among glial cells bioenergetic properties of the dynamically

active microglia was in the focus of this research. Acting as a primary immune defense of the central nervous system, microglial populations show heterogenous and dynamic distribution in the brain. They migrate into different brain regions, where they encounter various metabolic conditions. A question arise: what kind of energy donor metabolites can support microglial energy provision? The available energy substrates in these brain microcompartments determine the metabolic route of microglial energy production. Glucose is the primary, but not the obligate energy metabolite available in the brain. Multiple other substrates can serve as energy donors for the cells in the brain. As a member of the glutamate-glutamine cycle, the amino acid glutamine is abundantly present in the CNS. The astrocytic production and neuronal consumption of lactate are well documented results highlighting the essential role of lactate in brain energy metabolism. The concentration of ketone bodies (β-hidroxybutirate and acetoacetate) can also reach the millimolar level in the CSF, particularly under starvation.

The main purpose of this research therefore was to explore, what the potential substrates are for microglial cells that can support their energy metabolism under different microenvironmental conditions. Beside the investigation of microglial bioenergetics in the mature brain, changes in the metabolism of neuronal differentiation during the development was also in the focus of this study.

AIMS AND OBJECTIVES

In vitro experiments were performed with CNS cells (derived from the developing and from the mature brain) in order to investigate the metabolism and utilization of energy substrates that are present in the cerebrospinal fluid under physiologic conditions.

I. Energy substrate-preference of primary microglial cultures (isolated from mice) and of the immortalized BV-2 cell line were investigated after starvation in an artificial media lack of energy substrates. Cellular oxygen consumption, H^+ production, intracellular ATP and ADP levels, cell viability, autophagy activity and the phosphorylation levels of the m-TOR signalling cascade were detected in the presence of single metabolites.

Main questions were the following:

1) What kind of substrates can serve as energy donors for the microglia?

2) What are the main metabolic pathways that are used for energy provision in the microglia?

3) What are the metabolic similarities and differences between the primary microglial cultures and the BV-2 microglial cells?

II. The effect of glucose and glutamine were investigated on NE-4C neural stem cells and on differentiated NE-4C neurons. For a better comparison the oxygen consumption measurements were also performed with primary neuronal and with astroglial cultures.

Main questions were the following:

1) Are there any changes in the metabolism of neural stem cells during differentiation?

2) Are the metabolism similar in the differentiated NE-4C neurons and in primary neuronal cultures isolated from embrionic mice?

MATERIALS AND METHODS

Cell cultures

BV-2 immortalized murine microglial cells, a kind gift from Prof. Rosario Donato (Department of Experimental Medicine and Biochemical Sciences, Perugia, Italy), were grown on plastic tissue culture flasks in DMEM-based media. Cells were seeded onto microplates 24 h prior to the experiments in order to reach the appropriate attachment.

Primary microglial cells were isolated from the forebrains of 1- to 2-day old CD1 mice. The mice were euthanized via decapitation. Ten to thirteen animals were used per cell culture, resulting approximately 3×10^6 microglial cells at the end of the isolation. Primary microglia were co-cultured with astrocytes and were grown on plastic tissue culture petri dishes in MEM-based media. At 24–26 days after isolation, microglial cells were harvested and seeded onto microplates 48 h prior to the experiments in order to reach the appropriate attachment.

NE-4C p53-/- cells (derived from the forebrain of embrionic mice) were maintained in petri dishes in MEM-based media. Cells were let to grow to 85% of confluency. Semiconfluent cultures were split by trypsinization. After trypsinization, detaching cells were suspended in cell culture media, than counted in Bürker-chamber and seeded onto new petri dishes. Cell culture medium was changed three times a week. 10^{-6} M all-trans retinoic acid was added to the confluent NE-4C neural stem cell culture. After 48 h cellular medium was changed to a medium lack of retinoic acid and of serum. Metabolic studies on

differentiated NE-4C neurons were performed two weeks after the induction.

Primary neuronal cultures were isolated from the forebrains of embrionic mice (E15-E16). The mechanical dissociation of the cerebral tissue and the suspension of the cells were done in MEM-based cell culture media. Cell suspension was sieved over a nylon mesh with a pore diameter of 40– 42 μm, than cells were seeded onto 96 well microplates for oxygen consumption measurements. In the second week after plating, media were changed to serum-free neuronal media. The cultures were used for metabolic studies on the 11th–13th days after seeding.

Primary astroglial cultures were prepared from late fetal or perinatal CD1 mouse forebrains. MEM-based cell culture medium was used for the cultivation. 24 h prior to the measurements cells were seeded onto 96 well plates in order to reach the appropriate attachment.

Measurement of mitochondrial oxidation and glycolytic activity

The oxygen consumption rate (which reflects mitochondrial oxidation) and the extracellular acidification rate (which is considered a parameter of glycolytic activity) were measured using a microfluorimetric Seahorse XF96 Analyzer. Prior to the addition of any substrate, cells were starved in nutrient-free artificial cerebrospinal fluid (ACSF). The O_2 tension and pH values were detected, and the oxygen consumption rates and extracellular acidification rates were calculated via XF96 Analyzer software. The basal rate of respiration corresponded to the last 30 min of the two-hour-long starvation in ACSF. Substrates (glucose, glutamine, pyruvate, lactate, 3-hydroxybutyrate (BOHB) and acetoacetate or metabolic inhibitors/modulators (oligomycin, 2,4-dinitrophenol (DNP) or

carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) as uncouplers, antimycin A and sodium oxamate), prepared in assay media, were injected into each well during the measurement.

Measurement of intracellular ATP and ADP levels

The ATP and ADP levels were estimated using a bioluminescent ATP assay kit. Measurements were performed on a Galaxy Bio-Orbit 1258 Microplate Luminometer in white luminescent microplates. On the day of the experiment, the cells were washed and incubated for 2 h in ACSF medium (pre-warmed to 37 °C). The ACSF was subsequently supplemented with the appropriate substrate (with the exception of in the control wells), and the cells were further incubated for 2 h. This starvation protocol was followed in the other methods. Following 4 h of incubation, the medium was changed to an ATP assay medium, and the extraction of ATP and ADP from the cells was immediately achieved with the addition of an extractant. The ATP assay is based on the firefly luciferase reaction and was performed with a recombinant and thermostable luciferase. The ATP and ATP + ADP assays were performed in two separate reaction mixtures. The measurements were performed in an ATP assay medium, which contained phosphoenolpyruvate and pyruvate kinase enabling the detection of the initial ATP + ADP levels. Initial ADP levels can than be easily calculated using the known amount of initial ATP content. The assays were individually calibrated by the addition of a known amount of ATP standard.

Assessment of cell viability

The cell reduction capacity was measured using the MTT assay and was considered a test for cell viability. Microglial cells were washed and incubated in nutrient-free ACSF for 2 h; they were further incubated for 2 h in ACSF assay medium, which was

supplemented with a single substrate (with the exception of the ACSF controls). After 4 h of incubation, MTT reagent was added, and the cells were further incubated at 37 °C for 1 h. The converted dye was subsequently solubilized by the addition of acidic isopropanol, and the absorbance intensity of $\lambda = 570$ nm light was measured.

Apoptosis/necrosis assay

Apoptotic cells were detected using Annexin V – Cy3.18. The cell permeability was tested by the acetoxymethyl-calcein ester method. Prior to the annexin and calcein stainings 2 h of starvation was followed by 2 h of incubation in substrate-supplemented media (with the exception of the ACSF controls). After staining cells were immediately analyzed by a Zeiss Axiovert 200 M microscope. The number of calcein and Annexin V positive cells was determined manually on images. Cells stained only with calcein were considered vital cells. The Annexin-V labelled cells were considered necrotic, and the double stained cells comprised apoptotic cells.

Western-blot

BV-2 microglial cells were seeded onto 6-well tissue culture plates. On the day of the experiment, the same starvation protocol was used as described in the other methods: 2 h of incubation of the cells in nutrient-free ACSF was followed by an additional two-hour long incubation with a single substrate (with the exception of the ACSF controls). The cells were homogenized in lysis buffer. A Bicinchonic Acid Assay kit was used to measure the protein concentration of the lysates. Approximately 12–16 μg of protein from each lysate were separated in a Tris–glycine SDS polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane. The primary antibodies were as follows: marker of autophagy: microtubule-associated

protein 1 light chain 3 II/I [LC3 II/I]; markers of mTOR pathway: phospho-mTOR; mTOR, phospho-AMP-activated protein kinase α, AMPKα. Immunoreactivity was detected with the corresponding horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The signals were detected with an enhanced chemiluminescence detection reagent by Chemidoc XRS+. For the removal of antibodies that detected phosphorylated epitopes, Pierce Stripping Buffer was used prior to incubation with antibodies that detected the total protein.

Flow cytometry

For the p-S6 expression analysis, 5×10^5 cells/condition were stained using the PerFix-nc Kit following the manufacturer's instructions. The p-S6 expression was detected with direct-labelled monoclonal p-S6 antibody. The samples were measured on a Navios flow cyt- ometer and analyzed using Kaluza software.

RESULTS

Energy metabolism in the mature brain
Focus on microglia

In vitro studies were performed with primary microglial cultures and on BV-2 microglial cells. In this study, the effects of different energy substrates, which may be present in the CSF, on the cellular respiration of primary and BV-2 microglia were investigated using a Seahorse XF96 Extracellular Flux Analyzer. The measurement of the oxygen consumption of intact, adherent cells is one of the most direct, dynamic methods to quantify in situ mitochondrial function. **In the absence of energy substrates**, both primary and BV-2 cells exhibited decreases in the basal oxygen consumption and a reduced maximal respiration with a decrease in the intracellular ATP levels, indicating a serious energy deficit. In primary microglia, in contrast to BV-2 cells starvation did not decrease the viability and did not significantly increase the apoptotic frequency. In BV-2 cells m-TOR and S6 phosphorylation activity was reduced in starvation, indicating a decrease in the rate of protein synthesis. The increased LC3 II/I ratio is consistent with the initiation of self-degradative processes. In term of viability primary microglial cells were less sensitive to nutrient deprivation than cells of BV-2 cell line.

To investigate the substrate utilization, cells were maintained in ACSF medium supplemented with different glycolytic or oxidative substrate. Energy metabolites selected for this study (glucose, glutamine, lactate, pyruvate and ketone bodies) are present in the CSF under physiological conditions. The respiration quotient is close to 1 for the brain (cortex), which

indicates aerobic oxidation of glucose. In our experiments, the administration of **glucose** decreased oxygen consumption in both cell types. This acute effect of glucose (Crabtree-effect) is similar to that identified in cancer cells that are mainly dependent on glycolysis. However, the oligomycin induced decrease in oxygen consumption indicated the function of oxidative phosphorylation. Glucose highly stimulated the rate of acidification of the extracellular medium, indicating the increased rate of glycolysis. Glucose partially preserved the ATP level in primary microglial cells but not in BV-2 cells. Moreover, glucose did not stimulate survival and did not decrease the apoptotic frequency in BV-2 cells. Glucose was also not efficient to protect against the increased rate of autophagy and was unable to stimulate the phosphorylation of mTOR; thus, it could not reverse the starvation induced catabolic pathways in BV-2 microglial cells. The possible reasons for the inhibition of the oxidative metabolism of glucose were also investigated. The low level of pyruvate dehydrogenase complex activity (PDHc) could not be responsible for the low rate of oxidative metabolism; i) the addition of uncoupler augmented the rate of respiration in primary microglia, ii) inhibition of lactate dehydrogenase by oxamate stimulated the entry of glycolytic pyruvate into mitochondria, increased cellular respiration and decreased the rate of acidification in both primary and BV-2 microglial cells.

When glucose was replaced by **pyruvate**, ATP could not be produced in glycolysis; therefore, to produce ATP, pyruvate should enter into the TCA cycle. This need for ATP may activate PDHc enzymes in primary microglial cells. Pyruvate-supported oxidation significantly enhanced the cellular ATP levels in primary cells. However, this effect was not identified in BV-2 microglial cells. In accordance with this finding, pyruvate did not

increase cell viability, as measured by MTT and did not decrease the percentage of apoptotic cells.

Lactate is a proven physiologic substrate in the CNS. Primary and BV-2 microglial cells utilized lactate and exhibited an increased rate of basal and maximal respiration. ATP levels of the primary microglial cells were also significantly increased in the presence of lactate compared with the ACSF controls. Although lactate is an effective oxidative substrate for BV-2 cells, the ATP and MTT results demonstrate that these cells were not able to preserve their ATP levels and viability in the presence of lactate. It is interesting to compare the effects of lactate and pyruvate on oxygen consumption. In primary microglial cells, both the basal and the maximal OCR were more stimulated by pyruvate than by lactate. One potential explanation may be that pyruvate may enter into mitochondria and directly serve as a substrate for PDHc, whereas lactate should first be oxidized by lactate dehydrogenase (LDH). NADH, formed in the reaction catalysed by LDH, ought to enter into mitochondria using shuttle mechanisms (malate-aspartete shuttle and glycerophosphate shuttle) because the cytosolic NADH utilizing capacity is low. Under starvation the concentration of the substrates are low, resulting functional inactivity of the shuttle mechanisms.

In the present study we demonstrate that under starving conditions, the basal respiration is increased by the addition of **glutamine** as a single metabolic fuel, and the glutamine-induced oxygen-consumption is associated with ATP synthesis in primary and BV-2 microglial cells. Uncoupled respiration, which demonstrates a maximal utilization of the substrate, was clearly the highest in BV-2 cells when glutamine was present in the ACSF. Primary microglial cells and BV-2 cells exhibited a high ATP/ADP ratio and maintained the MTT viability in the

presence of glutamine. Significantly increased mTOR activity and p-S6 levels were observed in the presence of glutamine that suggest an increased rate of protein synthesis compared with the ACSF control cells. Although there was not a significant difference between glutamine-treated and starving (ACSF only) BV-2 cells concerning the p-AMPK/t-AMPK, and the LC3 II/I ratios, respectively, but the tendencies were obvious, the well-established reciprocal regulation between mTOR and autophagy. Glutamine reversed the general catabolic pattern of microglial cells.

Beta-hydroxybutyrate and acetoacetate (ketone bodies) may also serve as effective energy substrates for microglia in starvation. In the present study, we demonstrated that the basal and maximal levels of respiration were increased in the presence of BOHB. However, this increase was the lowest among the substrates. To further characterize the role of ketone bodies in microglial metabolism, another ketone body, acetoacetate, was added to microglia. In the absence of glutamine, acetoacetate did not significantly influence the basal level of oxidation; however, it transiently stimulated the maximal rate of respiration.

When glutamine was present in the media, acetoacetate maintained a long-lasting stimulation of oxygen consumption. It is concluded that in starvation, the oxidation of ketone bodies is seriously limited by the low availability of succinyl-CoA. This finding is reflected in the fast decline of acetoacetate oxidation. In glutamine-supported microglia, the flux of oxidized intermediates of glutamine metabolism likely generates sufficient succinyl-CoA to support ketone body metabolism. These findings indicate that starvation of microglial cells resulted in a depletion of TCA-cycle intermediates, which hampered the

oxidation of ketone bodies; however, with a proper combination of substrates, the cycle function may be improved.

Another substrate combination proved to be efficient to stimulate respiration of BV-2 cells. Aspartate pre-treatment stimulated the oxidation of glucose, pyruvate and lactate.

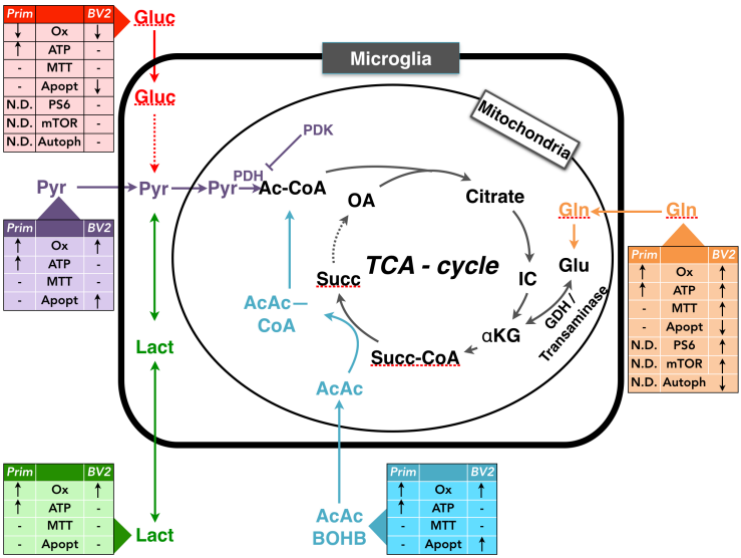

Figure 1.
Summary of results obtained with primary and BV-2 microglial cells

One direction arrows indicate irreversible reactions or uniports, while the bidirectional arrows show reversible reactions or bidirectional transports. Dashed arrow shows the summary of multiple consecutive reactions. Abbreviations: Prim (primary microglia), Apopt (apoptosis), ↑ (increase), ↓ (decrease), N.D. (not detected), – (no significant change versus ACSF control), ox (oxygen consumption), Ac-Ac (Acetoacetate), Glu (glutamate), IC (isocitrate), α-KG (α-ketoglutarate), Succ (succinate), OA (oxaloacetate), Gluc (glucose), Gln (glutamine), Glu (glutamate), Pyr (pyruvate), Lac (lactate), KB (ketone bodies),

Changes in the energy metabolism during neuronal development

In order to investigate the metabolic changes in the developing brain, *in vitro* experiments were performed with NE-4C neural stem cells and NE-4C differentiated neurons. For a better comparison primary neuronal cultures and primary astrocytes were also involved in the study. Starvation was induced in the cells, cellular media were supplemented with the two main energy substrates glucose and glutamine that are present in the brain in high concentrations. Mitochondrial oxygen consumption and glycolitic activity were measured and compared among the four cell cultures. In non-differentiated NE-4C cells glycolitic flux is active, while in differentiated cells the oxidation of glucose is the more effective pathway for energy production. A difference was also observed between stem cells and differentiated cells in the utilization of glutamine. Stem cells displayed high oxidation of glutamine, however, differentiated cells did not show any change in oxygen consumption upon the addition of glutamine.

CONCLUSION

Our results demonstrate the remarkable metabolic flexibility of microglial cells, using a wide range of substrates potentially present in the cerebrospinal fluid (glucose, pyruvate, lactate, amino acids and ketone bodies). This plasticity may help these cells to adapt their energy homeostasis to different metabolic compartments in the brain. Of the various substrates suitable to provide energy for microglial cells, glutamine plays a prominent role. There is a relatively high transamination capacity in these cells to create acetyl-CoA acceptor oxaloacetate from aspartate (and from malate). Thus, microglia may function as a potential regulator of the glutamate/glutamine pool. We also propose the possibility of metabolic cooperation among microglia, oligodendrocytes, astrocytes and neurons that contribute to the balanced energy supply of various CNS cell types. These findings form a basis for functional studies in vivo, with the aim of identifying the contribution of microglia to brain energy metabolism.

From the results obtained with differentiated and non-differentiated neural stem cells it is concluded that neural stem cells undergo metabolic changes during differentiation that provide them the appropriate amount of energy and intermediates for neural development. During differentiation a switch was observed from the glycolitic to the oxidative metabolism of glucose.

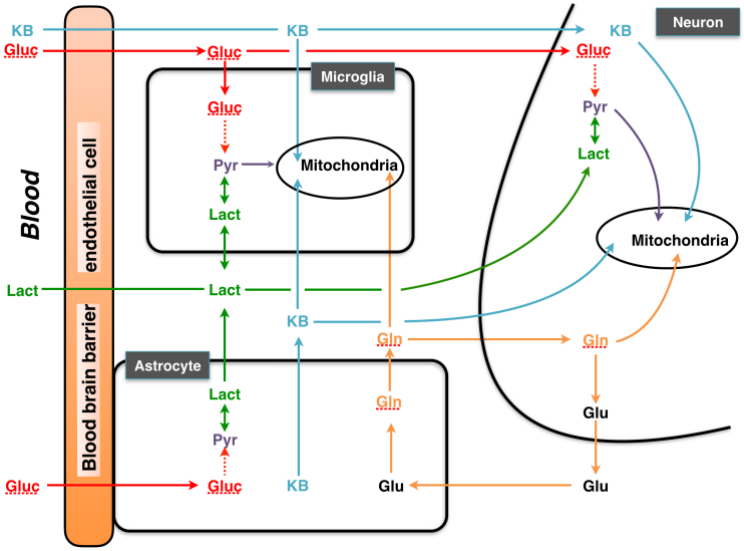

Figure 2.
Proposed metabolic cooperation between microglia and other cell types of the brain.

One direction arrows indicate irreversible reactions or uniports, while the bidirectional arrows show reversible reactions or bidirectional transports. Dashed arrow shows the summary of multiple consecutive reactions. Abbreviations: Gluc (glucose), Gln (glutamine), Glu (glutamate), Pyr (pyruvate), Lac (lactate), KB (ketone bodies)

LIST OF PUBLICATIONS

Publications related to the thesis:

Nagy AM, Fekete R, Horvath G, Koncsos G, Kriston C, Sebestyen A, Giricz Z, Kornyei Z, Madarasz E, Tretter L. 2018. Versatility of microglial bioenergetic machinery under starving conditions. Biochim Biophys Acta. 1859:201-214. *IF: 4.280*

Jady AG, **Nagy AM**, Kohidi T, Ferenczi S, Tretter L, Madarasz E. 2016. Differentiation-Dependent Energy Production and Metabolite Utilization: A Comparative Study on Neural Stem Cells, Neurons, and Astrocytes. Stem Cells Dev 25:995-1005. *IF: 3.562*

Publications not related to the thesis:

Harami-Papp H, Pongor LS, Munkacsy G, Horvath G, **Nagy AM**, Ambrus A, Hauser P, Szabo A, Tretter L, Gyorffy B. 2016. TP53 mutation hits energy metabolism and increases glycolysis in breast cancer. Oncotarget. 7:67183-67195.

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