The β-amyloid progression effect on mitochondrial proteome and oxidative metabolism - the specific role of synaptic mitochondria

Doctoral Thesis

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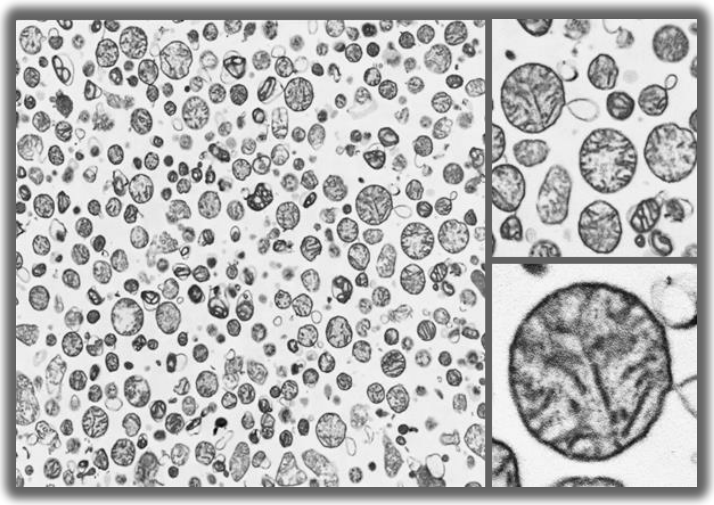

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Introduction

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder. The initial events that lead to the development of AD are still not known. When the first symptoms can be diagnosed, the disease is already in its late phase. Mild cognitive impairment (MCI) is a transitional state between normal aging and dementia. However, we have no well-established definition for MCI and this state is not always associated with the development of AD. The symptoms of AD might be described as an accelerated spontaneous dementia during aging and it is not always possible to easily distinguish between these phenomena. It would be highly necessary, therefore, to find new diagnostic markers that could be used for the early detection of AD, well before the first symptoms appear or to identify new molecular targets for an early therapy. The aims of the present work fit well into this trend.

The first symptom of AD that can be diagnosed by positron emission tomography using 2-deoxy-2-(^{18}F)fluoro-D-glucose (FDG-PET) is the decreased metabolic activity of brain tissues. Based on this fact, many scientific investigations aim to reveal changes in mitochondrial metabolism associated with neurodegenerative disorders. Results of experimental AD models and data of human AD patients clearly show that mitochondrial metabolism and reactive oxygen species (ROS) production in mitochondria change significantly in AD. This may also lead to different molecular changes in mitochondria. There are proteins in mitochondria that are known high-affinity binding partners of the misfolded amyloid beta (Aβ) peptide enriched in AD brains and are therefore directly influenced by this interaction. Consequently, the mitochondrial hypothesis of AD has recently become a widely accepted theory describing the molecular mechanisms of the early phase of AD.

Molecular architecture of mitochondria depends on their environment and position within the cell. The main reason of this is the different metabolic demand specific for the given subcellular region. The synapses are regions of remarkably high metabolic demand in the nervous system. Consequently, a large number of mitochondria are concentrated in a relatively small volume in the synaptic region of neurons. Involvement of synaptic mitochondria in neurodegenerative and psychiatric disorders makes it highly important to reveal their detailed molecular architecture and mechanisms. The number of synapses decrease in AD and the synaptic functions are also impaired. Learning disabilities are in part results of the decreased number of synapses and of the decreased synaptic plasticity.

The background of our working hypothesis was that in the earliest phase of AD there are molecular changes in synaptic mitochondria that might remain functionally not detectable due to other compensatory mechanisms for some time. These changes are caused by the high affinity binding of Aβ to several proteins in the synapses. This might cause altered expression of some synaptic proteins, therefore, investigation of the synaptic proteome must be highly informative and

indicative of AD. Presumably, the changes in proteome are associated with functional consequences, however, their appearance might be delayed in time because mitochondrial protein synthesis can compensate for these changes in the early period of the disease. We suppose that there are potential therapeutic targets among the proteins that are involved in the early phase of AD because the energetic demands of synaptic electrogenesis has a high priority in the metabolic regulation of the synaptic region. Our work could yield essential information for future studies aiming to target specific proteins that play essential roles in AD and therefore could reverse the development of the disease in an early state.

Objectives

As the molecular and cellular mechanism of AD is highly complex and there are more than 100.000 scientific papers associated with AD it is impossible include our hypotheses and results into a full picture. Therefore, we give bellow a short description of all the main facts and theories that were thought to be necessary for establishing our own working hypothesis.

A. AD is not a homogenous disease rather a complex group of diseases with many subtypes. The main common feature of these diseases is the dementia associated with amyloid deposits in the brain and the hyperphosphorylation of tau.

B. There are early molecular level changes in AD that are present long before the first symptoms influencing learning or spatial orientation abilities appear. The initial molecular mechanism has not been revealed yet and might be heterogeneous in the different subtypes of AD.

C. The most toxic forms of Aβ are not the plaques, but soluble oligomers in brain. These may have many different physiological effects according to the degree of aggregation. Our preliminary *in vivo* results also supported this hypothesis.

D. The neuronal functional deficit in AD due to intracellular amyloid oligomers appears long before the deposition of amyloid plaques would begin.

E. Mitochondrial metabolism is highly affected by intracellular Aβ, but the details of the molecular mechanism still have to be recovered and our current knowledge is often inconsistent and contradictory.

F. Synaptic mitochondria have a special role in impaired neuronal functions because they have a direct effect on the efficiency of neurotransmission.

G. Investigation of early mitochondrial changes and subsequent molecular rearrangements due to amyloid deposition is only possible by an “unbiased” strategy because the molecular architecture of the mitochondrion changes in really complex way in AD.

H. Since we do not have brain samples from early stage AD patients who have not yet shown behavioral symptoms, detection of early changes is only feasible using animal models.

I. As the available animal models can only partially reproduce the symptoms of AD it is highly important to choose the most appropriate one that can be used for the investigation of amyloid progression and for the detection of early molecular changes.

According to our working hypothesis we started a comprehensive mitochondrial AD mechanism research with the following questions:

I. The molecular analysis of synaptic mitochondria specific role background – the comparative analysis of synaptic and non-synaptic mitochondrial proteome

Accordingly our aims were the followings:

- *Metabolically active synaptic and non-synaptic mitochondrial isolation from Balb/c mouse brain tissue*
- *Mitochondrial sample validation with electron microscopy and FACS*
- *Comparative analysis of synaptic and non-synaptic mitochondrial proteome*
- *Functional clustering of significant mitochondrial protein changes*
- *The main protein changes validation*

II. The analysis of Aβ progression effect on mitochondrial proteome and oxidative metabolism – the comparative analysis of mitochondrial proteome and oxidative metabolism in 3, 6 and 9 months old APP/PS1 and B6 mice

Accordingly our aims were the followings:

- *Metabolically active synaptic and non-synaptic mitochondrial isolation from 3, 6 and 9 months old APP/PS1 and B6 mouse brain tissue*
- *Comparative analysis of synaptic and non-synaptic mitochondrial proteome of 3, 6 and 9 months old APP/PS1 and B6 mice*
- *Comparative analysis of synaptic and non-synaptic mitochondrial oxidative metabolism of 3, 6 and 9 months old APP/PS1 and B6 mice*
- *Functional clustering of significant mitochondrial protein changes*
- *The main protein changes validation*
- *Bioinformatics analysis of mitochondrial protein changes*

Methods

To synaptic and non-synaptic mitochondria comparative analysis we selected 3 months old Balb/c mice for proteomics (n=12), electron microscopy (n=5) and light microscopy experiments (n=4), while to Aβ accumulation effect proteomic (n=36), functional (n=30) and light microscopy (n=12) analysis we selected 3, 6 and 9 months old APP/PS1 and C57BL/6 (B6) control mice.

We used light and electron microscopy to Aβ progression detection in 3, 6 and 9 months old APP/PS1 mice brain.

Metabolically active synaptic (sMito) and non-synaptic mitochondria (nsMito) were isolated with Percoll gradient centrifugation according to already published protocols with some minor modifications. The purity of mitochondrial sample was validated with electron microscopy and fluorescence-activated cell sorting.

We used two-dimensional differential gel electrophoresis (2D-DIGE) „Saturation Labeling" method to proteomic analysis. Differential protein analysis was performed using DeCyderTM 2D software 7.0 Differential In-gel Analysis (DIA) and Biological Variance Analysis (BVA) modules. For the identification of proteins in spots of interest, preparative 2D gel electrophoresis was performed. Resolved protein spots were visualized by Colloidal Coomassie Blue G-250. Significantly changed proteins identified by mass spectrometry (nanoUHPLC-MS/MS). Significantly altered proteins were clustered on the basis of the UniProt (http://www.uniprot.org/) and GeneOntology (http://geneontology.org/) databases. The proteins were clustered in groups according to their most relevant cellular functions and roles in human Alzheimer's disease pathology were also listed.

The highest fold-differences in the altered proteins were selected for further validation by Western blot, immunhistochemistry and immun-electronmicroscopy.

We analyzed the interactions between significantly changed mitochondrial proteins with Ariadne Genomics Pathway Studio® 9.0 software environment. Common regulator and common target analysis were made for all significant mitochondrial protein changes between APP/PS1 and B6 mice.

Mitochondrial oxygen uptake was measured with a Clark-type oxygen electrode in a high-resolution respirometry system Oxygraph-2K. The peroxide assay is based on H_2O_2 detection in the medium using the Amplex UltraRed fluorescent dye. For measurements, synaptic or non-synaptic mitochondrial preparations were used from 3, 6 and 9 months old B6 and APP/PS1 mice. Mitochondria were energized with glutamate plus malate or succinate substrates.

Results and conclusions

I. Main results and conclusions of the comparative analysis of the synaptic and non-synaptic mitochondrial proteome:

- We create a modified Percoll gradient centrifugation method to isolate ***metabolically active synaptic and non-synaptic mitochondria*** from the same tissue with ***more than 90% purity***, that was ***validated with electron microscopy and FACS***.
- We identified **56 different significant protein changes** (average ratio: ***-2.28 – +3.70***), ***22 significantly increased, 34 significantly decreased*** in sMito, compared to nsMito. According to these data we can conclude, that ***sMito has a specific proteome***, therefore, we raise the point that mitochondrial proteome dynamics is an important component of the synaptic function.
- The ***functional classification*** of the proteins showed that proteins involved in ***synaptic transmission, lactate, glutathion and nucleotide metabolism process*** enriched in ***sMito***. In contrast to sMito, proteins involved in ***glucose, lipid, and ketone metabolism, signal transduction, morphogenesis, protein synthesis, protein degradation and transcription*** are enriched in ***nsMito***. The other functional groups contain both synaptic and non-synaptic mitochondrial proteins.
- ***Sod1 and C1qbp*** oxidative stress related proteins and ***Idh3a and Sucla2*** citric acid cycle related proteins were also ***validated with Western blot and immunhistochemistry***. According to these results, synaptic energy provider citric acid cycle is significantly different in the two kind of mitochondria population, furthermore sMito has higher vulnerability to oxidative stress.
- Based on energy metabolism related protein changes in sMito, we proposed a model of increased efficiency of energy production by means of glial interactions implying the ***glutamine-glutamate cycle*** as a significant element of substrate supply for energy production in sMito.
- The special protein composition of ***sMito*** could be also important from ***therapeutical aspects*** since specific targeting of sMito is possible as mitochondrial proteins with elevated synaptic concentration (like ***C1qbp***) can be selected for future drug development.

II. Main results and conclusions of the analysis of Aβ progression effect on mitochondrial proteome and oxidative metabolism:

- We identified ***42 different significant synaptic mitochondrial protein changes (average ratio: -1.41 – +1.80) and 43 different significant non-synaptic mitochondrial protein changes (average ratio: -2.29 – +1.52)***, that reflect the progressive effect of APP overproduction and Aβ accumulation on mitochondrial processes. Our results demonstrate a ***different and age dependent effect on synaptic and non-synaptic mitochondrial proteome in APP/PS1 mouse model of APP overproduction and Aβ accumulation***.
- Before any behavioral changes and amyloid plaque accumulation, we observed ***the earliest, complex protein network change in sMito and nsMito, when there was no alteration in hydrogen-peroxid production***. This phenomenon could be the result of the functionaly compensated protein changes. However, ***in 6 months old APP/PS1 mice we found elevated hydrogen peroxide production, specifically in synaptic mitochondria, that was less abundant in 9 months of age.***
- The ***functional classification*** of the proteins showed that most of the significantly affected proteins play role in the ***mitochondrial electron transport chain, citric acid cycle, oxidative stress or apoptosis***.
- Expression levels of ***Htra2 and Ethe1*** apoptotic protein in sMito, showed parallel changes in different age groups (↓↑↓) of APP/PS1 mice, were confirmed also by ***Western blot***. ***ES1*** protein with unknown function showed the same protein level changes with age in ***nsMito***, thus they could be used as markers of ***Aβ progression effected mitochondrial protein network changes' different phase***.
- The result of ***common target and regulator bioinformatical analysis*** suggests a regulatory role of ***Tnf-α*** in Aβ mediated mitochondrial protein changes. Based on these findings in mice, we suggest conducting research for early non-invasive tests of Htra2, Ethe1 and Tnf level in human plasma which could lead to the establishment of early biomarkers for Alzheimer's disease risk population.
- The ***Tnf-α*** induced ***extrinsic*** and the Aβ-mediated ***Htra2, Ethe1, Pebp1 and Vdac1*** related ***mitochondrial apoptotic pathways connections*** suggest the importance of Aβ effect on ***NFκB signaling*** and ***caspase-cascade*** pathways.
- Our results are in accordance with the previous ***post mortem*** human brain proteomic studies in Alzheimer's disease in the case of many proteins.

- Our results could open a ***new path of research aiming early mitochondrial molecular mechanisms of Aβ accumulation*** as a prodromal stage of human AD. Our findings also suggest a ***very early analysis of mitochondrial proteom, before 3 months of age***.

Related publications

1.) **Völgyi K**, Gulyássy P, Háden K, Kis V, Badics K, Kékesi KA, Simor A, Györffy B, Tóth EA, Lubec G, Juhász G, Dobolyi A. „Synaptic mitochondria: A brain mitochondria cluster with a specific proteome." J Proteomics. 2015 Apr 29;120:142-57. doi: 10.1016/j.jprot.2015.03.005. Epub 2015 Mar 14. PMID: 25782751

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Other publiacations

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2.) **K. Völgyi**, B. Kellermayer, K. Háden, P. Gulyássy, V. Kis, A.E. Tóth, A. Simor, K. Kékesi, B. Penke, G. Juhász "The Early Effect of β-amyloid on Synaptic and Extrasynaptic Mitochondria Proteome and Membrane Potential in APP/PS1 Mice Brain" 11th International Conference on Alzheimer's and Parkinson's Diseases (AD/PD 2013, Florence, Italy)

3.) **K. Völgyi**, K. Háden, P. Gulyássy, A. Simor, B. Györffy, K.A. Kékesi, V. Kis, E.A. Tóth, Z. Szabó, T. Janáky, B. Penke, L. Drahos, L. Tretter, A. Dobolyi, G. Juhász "The β-amyloid progression effect on the proteome and function of the synaptic and non-synaptic mitochondria in APP/PS1 mice brain" 9th FENS Forum of European Neuroscience (Milan, July 5-9, 2014)

4.) **K. Völgyi**; K. Háden; V. Kis; P. Gulyássy; K. Badics; B. A. Györffy; A. Simor; Z. Szabó; T. Janáky; L. Drahos; L. Tretter; Á. Dobolyi; B. Penke; Kékesi A, K., G. Juhász "Synaptic and non-synaptic mitochondrial proteome and function reflects tge progress of beta-amyloid expression in APP/PS1 model of Alzheimer's disease" 12th International Conference on Alzheimer's & Parkinson's Diseases (AD/PD 2015 March 18-22, Nice, France)

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10.) B. Gyorffy, Z. Kovacs, P. Gulyássy, A. Simor, K. **Völgyi, G**. Orbán, P. Baracskay, Z. Szabó, T. Janáky, A. Dobolyi, G. Juhász, A. Czurkó, K.A. Kékesi "Brain protein expression changes in WAG/RIJ rats, a genetic rat model of absence epilepsy after peripherial lipopolysaccharide treatment" 9th FENS Forum of European Neuroscience (Milan, July 5-9, 2014)

11.) B. Gellén, P. Gulyássy, **K. Völgyi**, B. Györffy, A. Simor, A. Czurkó, I. Hernádi, A. Dobolyi, G. Juhász, A.K. Kékesi "Proteomic investigation of the amygdala and prefrontal cortex in the clomipramine model of depression" 9th FENS Forum of European Neuroscience (Milan, July 5-9, 2014)

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1.) **K. Völgyi**, G. Orbán, J. Kardos, B. Penke, G. Juhász "Inhibitory or excitatory effects of Abeta oligomers are dependent on degree of oligomerization" A Magyar Idegtudományi Társaság XII. Konferenciája 2009. Január 22-24.

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3.) B. Kellermayer, P. Gulyássy, A. Simor, V. Kis, A. E. Tóth, E., K. A. Kékesi, B. Penke, G. Juhász, **K. Völgyi** "Brain Protein differences between synaptic and extrasynaptic mitochondria: possible functional consequences" A Magyar Idegtudományi Társaság XIV. Konferenciája (2013. január 17-19., Budapest)

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5.) **K. Völgyi**, E. Udvari, É. Hunyadi-Gulyás, K. Medzihradszky, G. Juhász, K. A. Kékesi, Á. Dobolyi „Proteomic analysis of the medial prefrontal cortex and the hypothalamic preoptic area in mother rats" Molekuláris Élettudományi Konferencia (2015, Eger)

6.) B. Györffy, D. Madarasi, P. Gulyássy, V. Kis, **K. Völgyi**, K. Badics, É. Forgács, L. Gert, Á. Dobolyi, G. Juhász, A. K. Kékesi „Prenatal immune activation induced alterations in the synaptic proteome of adolescent rats" A Magyar Idegtudományi Társaság XV. Konferenciája (2015. január 22-23., Budapest)

7.) B. Gellén, **K. Völgyi**, B. Györffy, T. Janáky, Z. Szabó, A. Czurkó, I. Hernádi, G. Juhász, Á. Dobolyi, K. A. Kékesi „Proteomic investigation of the prefrontal cortex in the clomipramine model of depression" A Magyar Idegtudományi Társaság XV. Konferenciája (2015. január 22-23., Budapest)

8.) E. Udvari, **K. Völgyi**, B. Györffy, P. Gulyássy, V. Kis, K. A. Kékesi, G. Lubec, G. Juhász, Á. Dobolyi „Synaptic protein changes in the maternal hypothalamus" A Magyar Idegtudományi Társaság XV. Konferenciája (2015. január 22-23., Budapest)

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1.) OMICS napok 2.0 (Budapest, 2014. április 24-25.) "A szinaptikus és nem szinaptikus mitokondriumok proteomikai elemzése amiloid overexpresszáló egerekben."