Cuprizone-induced pathological changes in a rodent model of multiple sclerosis

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Ph.D. Thesis

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Pécs 2026

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (SM) is the most common chronic, inflammatory demyelinating disease that affects the central nervous system (CNS) and is widely considered to be autoimmune in nature. It affects approximately 3 million people worldwide, predominantly young people between the ages of 20 and 40 (global average 32 years) and this number is increasing. SM is twice as common in women as in men. Its prevalence varies significantly depending on ethnic background. In addition to genetic susceptibility, various environmental factors such as geographical location and standard of living may also influence its incidence. Low levels of sunlight, insufficient vitamin D intake, and low serum 25-hydroxyvitamin D levels, smoking, obesity, and a history of Epstein-Barr virus infection are all common environmental risk factors for SM. The etiology and pathogenesis of SM remain a major research challenge, as not allunderlying mechanisms have been fully elucidated.. Although conventional treatments for SM – anti-inflammatory and immunomodulatory agents – are essential in the management of the disease, they are unable to stop neurodegeneration and disease progression. Nevertheless, the development of new diagnostic methods and disease-modifying therapies provide opportunity to alleviate symptoms and increase patient survival. Further research is hindered by limited access to human neural tissue, and therefore various aspects of SM are often studied in mammals. The cuprizone animal model (bis(cyclohexanone)oxaldihydrazone), (CPZ), is a widely used and well-characterized experimental system for studying the mechanisms of demyelination and remyelination. After discontinuation of cuprizone administration, spontaneous remyelination occurs, making the model particularly valuable for studying regeneration in SM research. Recently, proteomic analysis of CPZ-treated mouse brain samples was suggested as a potentially useful approach for finding therapeutic targets enabling causal treatment of the disease. Mass spectrometry (MS)-based proteomics is widely used to uncover the pathogenesis of various complex and multifactorial diseases, as we can identify characteristic molecular changes already in the early stages of disease, thus preventing the disease from becoming more severe.

2 Aims and Objectives

SM is the most common neurological disease causing dysfunction in young adults. Currently, the symptoms and clinical course of the disease can be treated with traditional and novel drugs, but there is no definitive cure for the disease. In our work, we used the CPZ mouse model, which does not cover all aspects of SM, but de- and remyelination can be clearly outlined. Thus, we set the following goals:

- Comprehensive proteomic analysis of the corpus callosum of CPZ-treated mice using a bottom-up proteomic approach with MS-MS.
- Identification of potential molecular targets involved in the modulation of de- and remyelination.
- Defining the role of posttranslational modifications.
- Explore the molecular networks that are clearly associated with de- and remyelination processes, as well as predict and identify the stages in SM.

In the second part of our research, we aimed to investigate the effects of microRNAs on CPZ-induced demyelination and remyelination. To this end, we set the following goals:

- Identify the miRNAs involved in the pathology of de- and remyelination.
- Determine the expression of miRNA-146a in the organs affected in the CPZ model.
- Investigate the level of miRNA-146a during physiological myelination.
- Examine the effect of miRNA-146a deficiency on demyelination and axon loss.
- Assess the effect of miRNA-146a deficiency on the expression of cytokines, chemokines and cytokine receptors.
- Reveal the differences between the proteome of wild-type (WT) and miRNA-146a knockout (KO) mice

3 Materials and methods

3.1 Ethics statement

The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the experimental protocol was approved by the Animal Research Review Committee of the University of Pecs, Hungary and Danish Animal Health Care Committee.

3.2 Cuprizone treatment

The animals were randomly divided into 4 groups (n=5). Three groups were fed with powdered 0.2% CPZ-containing general rodent chow for 4 weeks *ad libitum* to induce demyelination. In two groups, 2 days of acute remyelination and 14 days of complete remyelination were performed after the end of cuprizone feeding. The control group received the same diet without CPZ. The animals were terminated by cervical dislocation under isoflurane anesthesia. The brains were removed from the skull, the corpus callosum was dissected out and frozen on dry ice. The samples were stored at −80°C until further processing.

3.3 Sample preparation, liquid chromatography, mass spectrometry (nano LC-MS/MS)

Corpus callosum samples from demyelinated, acutely and fully remyelinated, and control groups were homogenized in a buffer solution (pH 7.5) containing protease and phosphatase inhibitors, 10 mM dithioerythritol (DTT), 10 mM sodium orthovanadate, and benzonase (0.05%), and then sonicated with an ultrasonic cell disrupter. Proteins were precipitated by adding acetone and trichloroacetic acid, collected by centrifugation, and the protein precipitate was solubilized with 6 M urea, 2 M thiourea, and 10 mM DTT, then alkylated with iodoacetamide, digested with lysyl endopeptidase (Lys-c), and trypsin. The total protein content was then estimated by amino acid sequence analysis. and isobaric labels (iTRAQ 4-plex ^{TM}) for relative quantification. Phosphorylated and glycosylated peptides were enriched using the TiSH (TiO2-SIMAC-TiO2) protocol. Phosphorylated, deglycosylated and unmodified peptides were fractionated before nano LC-MS/MS identification. The analysis of each fraction was performed using a Thermo nano-Easy LC coupled to a Q-Exactive or Velos mass spectrometer. The peptide fraction was loaded onto a 2 cm, 100 µm inner

diameter precolumn using a nano-Easy LC and With multi-step gradient elution at a flow rate of 250 nl/min was eluted directly onto a reversed-phase C18 analytical column (3 μm, 75 μm x 150-200 mm.) Protein identification was performed by searching the NCBI and Swiss-Prot databases taxonomically restricted to house mouse (*mus musculus*) using the Mascot search server.

3.4 Immunohistochemistry

Frozen native brains were formalin-fixed, paraffin-embedded, and sectioned. 8 μm sections were deparaffinized and subjected to microwave antigen retrieval. Endogenous peroxidase activity and nonspecific binding sites were blocked. Primary and horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies were exposed. After development, the nuclei were stained with Meyer's hematoxylin, then the sections were dehydrated and coverslipped with Canada balsam. Sections were digitized with a Panoramic midi scanner. Quantitative analysis was performed with Molecular Devices' MetaXpress® image analysis software.

3.5 Gene ontology, pathway and functional correlation analyses

Deregulated proteins were categorised into protein classes using Protein Analysis Through Evolutionary Relationships (PANTHER) classification system software (http://www.pantherdb.org) and the general annotation from UniProt (http://uniprot.org). For clustering, we used the Perseus software platform (http://www.perseus-framework.org) developed by the MaxPlanck Institute of Biochemistry (https://maxquant.net/perseus/). Gene ontology (GO) analysis of biological processes and cellular components was performed with PANTHER soft ware. Regulatory network prediction was performed by Ingenuity Pathway Analysis (IPA) software (Qiagen Inc. https://digitalinsights.qiagen.com/products-overview/discovery insights-portfolio/analysis-and-visualization/qiagen-ipa/) utilising Ingenuity Knowledge Base, a highly structured repository of biological interactions and functional annotations.

3.6 Statistical analysis

The intensity of proteins and phosphopeptides was normalized to the total peptide amount. The extent of changes occurring under given conditions was estimated based on the average of 5 replicate control samples. Log2 transformed protein ratios and differential expression were analyzed using Linear Models for Microarray Data (Limma) and Stats packages. The

amount of phosphopeptides was further normalized against the amount of unmodified proteins. Changes between conditions were considered significant if the q- value was less than 0.05. One-way ANOVA was performed to compare groups, followed by Tukey's post-hoc test. Groups were considered significantly different if the p-value was less than 0.05. Expression differences of regulatory genes predicted by IPA software between human SM lesion types and unaffected white matter sections were identified using the edgeR package (3.8) using the previously created database. To determine significant differences, p-value filtering corrected by the Benjamini and Hochberg method was used.

4 Results

4.1 Quantification of proteins involved in de- and remyelination

Except for the control group (C), demyelination was induced by feeding powdered rodent chow containing 0.2% cuprizone for 4 weeks (4wD), and then remyelination was allowed for 2 days (2dR) and 2 weeks (2wR) in one group after the end of the treatment. A total of 3183 unmodified proteins were identified from the 20 samples (5 per group). In addition, 6017 single- or multiply phosphorylated and/or glycosylated peptides were detected. Based on the previous peptide sequences, 1703 PTM proteins were identified. Expression changes in demyelination and remyelination were visualized by protein clustering. Therefore, we selected 7 clusters for both unmodified and PTM proteins. For further analysis, 1970 unmodified and 1255 PTM proteins were used, of which 93 significantly increased and 68 decreased during de- and remyelination, respectively. Of the 1255 single or multiple phosphorylated and/or glycosylated proteins, 40 were significantly affected by de- and remyelination. Only 9 proteins were identified as both PTM and unmodified. 30% of the upregulated PTM proteins were phosphorylated, the rest were N-glycosylated. In contrast, 92% of the downregulated PTM proteins were at least monophosphorylated**.** In total, we identified 39 demyelination-specific, 24 early and 48 late remyelination-specific unmodified, and 18 demyelination-specific, 3 early and 9 late remyelination PTM proteins. On the other hand, although to different extents, 30 unmodified and 6 PTM proteins were affected in both groups, while 20 unmodified and 3 PTM proteins underwent significant changes in all three groups.

4.2 Immunhistochemical examination of phosphorilation changes during de- and remyelination

Immunohistochemical analysis was performed using primary antibodies specific for p-Ser, p-Thr and p-Tyr on brain sections from all four experimental groups. The nuclei and cell bodies were stained in a low percentage, but no specific pattern or staining characteristic of a given cell type could be identified. In order to correlate phosphorylation with de- or remyelination, the staining intensity between brain sections was measured using image analysis software. We found a 9.17 and 26% difference in phosphorylation between the Ser, Thr and Tyr groups. Furthermore, the staining intensity was lower in early remyelination than in the control group and the demyelination group based on the phosphorylation of Thr and Tyr.

4.3 GO analysis of genes affected by de- and remyelination

To identify specific biological processes, we performed an over-representation test. We combined the 192 PTM-regulated proteins with the unmodified proteins and then performed a biological process test. All but two proteins were classified into 76 categories. The categories predicted by the algorithm are: glial and neuronal function, metabolism, cell death, inflammatory response, protein and cation homeostasis, and cytoskeleton-related processes. In addition to the expected oligodendrocyte-compatible events, the over-representation test also indicated the involvement of additional astrocyte- and microglia-related processes, such as glutamate metabolic processes and inflammatory reactions. These data are consistent with the accepted view that CPZ-induced oligodendrocyte loss is accompanied by aggregation and activation of microglia and astrocytes. In biological processes Proteins present at less than 50% and two unclassified proteins were excluded. The remaining 157 proteins were classified as remyelination only (RO), and demyelination related (DR), groups and over-representation test were performed on them. The algorithm classified the DR proteins into 9 categories. In the case of RO proteins, the only significant category was nervous system development, which included myelination as the most specific subcategory. To further investigate the role of RO proteins in the mechanisms regulating remyelination, we used the Ingenuity Pathway Knowledge Base to select a set of connected nodes and assess their cellular changes during the 2dR and 2wR phases. Analysis of the two remyelination groups yielded the same eight-member network. In early remyelination, two of them were upregulated, while the other six members were not significantly affected, whereas the reverse pattern was observed in late remyelination. We searched for the occurrence of these eight genes among the genes differentially expressed between human MS lesion types and NAWM. As we found, four of them were differentially expressed in at least one lesion type,

and the perlecan (HSPG2) gene was the most upregulated in remyelination lesions. In remyelination MS lesions, the gene expression pattern of the identified experimental remyelination network orthologs was more consistent with early remyelination in the CPZ model, i.e., upregulation of HSPG2 and downregulation of signal transduction and transcription activator 1 (STAT1) and thrombospondin-4 (THBS4).

5 Discussion

The proteomic analysis of the corpus callosum yielded a total of 4886 proteins, however, initial clustering of these did not reveal any groups whose quantitative changes followed a clear pattern indicative of de- and remyelination regulators. In total, we were able to select 192 proteins whose concentrations were significantly different from the control in at least one experimental group. The functions of the chosen molecules are very diverse, few of which were specific to de- and remyelination processes. Among the 57 demyelination-specific proteins, 25 showed a more than 1.5 - fold increase, while14 showed a decrease in their concentration. Notably, myelin basic protein (MBP) decreased to 13.9%, while the myelin-associated oligodendrocyte basic protein decreased to 20.8% compared to the control, which is clearly consistent with the massive demyelination and oligodendrocyte loss occurring in the 4wD group. MBP was identified based on its three phosphopeptides; one of them contains two threonine phosphorylation sites**.** Phosphorylation of these sites by mitogen activated protein kinases (MAPK) dramatically reduce protein binding to negatively charged lipid bilayers. Considering that MAPKs are activated during treatment, these phosphorylation changes are consistent with CPZ-induced demyelination. In addition to the 17 proteins involved in lipid metabolism, we found two additional myelin-associated proteins; 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP), and ermin. Interestingly, ermin occurred in reduced concentrations in all three experimental conditions, which is difficult to reconcile with its role in cytoskeletal rearrangements and the maintenance of myelin sheath stability during myelinogenesis. As expected, the majority of the 27 early and 54 late remyelination-specific proteins were associated with the myelin sheath, neuronal processes, synapses, and cytoskeletal organization. However, among the major oligodendrocyte marker proteins, only the PTM form of myelin-associated oligodendrocyte basic protein and only MBP were found. On the other hand, we found reduced phosphorylated MBP levels, as previously described in other publications, in samples from MS patients and CPZ-treated mice. Furthermore, our finding that all but one of the reduced PTM proteins were phosphorylated is consistent with

the view that reduced phosphorylation may be part of the pathogenesis of demyelination. Immunohistochemical analysis of protein phosphorylation during de- and remyelination has not proven conclusive. Although we found that phosphorylation of Ser, Thr and Tyr did not change significantly between groups and no clear pattern or cell type-specific staining could be identified, in contrast to remyelination-only proteins, demyelination-related proteins did not correlate with the corresponding expected biological process categories such as oligodendrocyte apoptosis, demyelination, oxidative stress or mitochondrial damage. Instead, many DR proteins have been implicated in biological processes related to cytoskeletal and organelle reorganization, metal ion homeostasis, and migration, processes likely related to astrocytosis and microglial activation. Furthermore, the 60 demyelination only DR proteins could not be classified into any biological process category, indicating that a statistically acceptable classification of these proteins is only possible when combining them with proteins that have altered levels in two or all three experimental groups. Therefore, based on the above, proteomic analysis of the corpus callosum of CPZ-treated animals seems to be more informative for remyelination processes than for demyelination. IPA network analysis performed on early and late RO proteins resulted in a single network whose members were activated in a reversed pattern during early and late remyelination. Taken together, the role of this predicted network in the regulation of remyelination processes does not seem unquestionable, therefore further studies are required to elucidate the molecular mechanisms regulating de- and remyelination.

6 Introduction

6.1 miRNA-146a

miRNAs are short, non-coding RNA molecules (19-25 nucleotides long) that act as post-transcriptional regulators of gene expression. It is estimated that more than 60% of all protein-coding mammalian genes can be regulated by them. In addition to individual silencing, they can also act synergistically, with pairs of miRNAs able to cooperatively repress translation of target mRNA, leading to increased efficiency and specificity of repression, and miRNAs can converge on the same pathway with alternative outputs. Another noteworthy aspect is that their characteristic expression patterns are early predictors of many diseases and therefore may be valuable diagnostic and prognostic markers, as they can be easily, reproducibly, reliably and non-invasively measured, even in formalin-fixed paraffin-embedded tissues. Many miRNAs show dynamic and specific temporal and localization expression in certain developmental and functional processes, therefore, in the case of MS, they can show the extent of neurodegenerative damage, the onset of remyelination, exacerbations, or indicate the production of immune and central nervous system cells and molecules. They have also become a popular topic in the design of new therapeutic interventions, where clinical trials are underway to restore miRNA function by administering miRNA mimetics or to inhibit their function with anti-miR oligonucleotides. One of the most abundant miRNAs expressed in the CNS, playing a role in both physiological and pathological processes, such as apoptosis, migration, growth, viral infection, and undoubtedly showing dysfunction in different types of neurological diseases, and even in different stages of the same neurological disease, is MiR-146a.

7 Materials and methods

7.1 Cuprizone treatment

Powdered standard chow mixed with 0.2–0.4% CPZ was administered *ad libitum* . To induce demyelination, 7–8-week-old mice were administered CPZ for 4 weeks (4 weeks demyelination: 4wd). Remyelination was examined at two time points: in the case of acute remyelination, 4 weeks of CPZ administration was followed by 2 days of normal diet (2 days remyelination: 2dr), while in the case of complete remyelination, 4 weeks of CPZ administration was followed by 2 weeks of normal diet (2 weeks remyelination: 2wr).

Control mice consumed normal rodent chow. During the experiments, the weights of the mice were measured every other day to determine whether significant body weight loss occurred. The experiments were terminated by euthanizing the mice with an overdose of pentobarbital and perfused with 4% paraformaldehyde.

7.2 Extraction of whole RNA and quantitative PCR (qPCR)

The brain was removed from the skull and the corpus callosum was isolated, immediately frozen, and then cut into coronal serial sections (200 μm thick sections). Using a stereomicroscope, the corpus callosum was excised from the sections with a fine Graefe knife along its rostro-caudal extent. RNA was extracted using the miRNeasy micro Kit . The quantity and quality of total RNA were assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent 2100 Bioanalyzer, respectively. To measure miRNA expression, primer sets for specific miRNA assays and sno135 endogenous control, as well as the MicroRNA reverse transcription kit, were used according to the manufacturer's protocol. qPCR measurements were performed using an Applied Biosystems 7000 Real-Time PCR system. The relative expression of each miRNA was calculated from the equation $2^{-\Delta Ct}$.

7.3 miRNA microarray

For miRNA profiling, the Agilent Mouse miRNA Microarray Kit (G4472A, 8 × 15k) was used according to the manufacturer's instructions (version 1.0) with 100–100 ng of quality-controlled total RNA. Labeled samples were hybridized for 20 h at 55°C. The arrays were scanned with an Agilent DNA Microarray Scanner BA, signal calculation was performed with Feature Extraction 10.7 Image Analysis Software, and the data were further analyzed with Genespring GX10.0. The microarray data have been deposited in the NCBI Gene Expression Omnibus database under accession number GSE100662.

7.4 Meso Scale Discovery Multiplex Electrochemiluminescent Assay

Cytokine levels in the corpus callosum were measured using the Meso Scale Discovery (MSD, USA) electrochemiluminescent proinflammatory mouse V-Plex Plus Kit (IL-1β , IL-4, IL-6, IL-10, TNF), a MULTI-SPOT 4-spot cytokine-specific plate (MIP1α , VEGF and MMP9) and a MULTI-SPOT 2-spot cytokine-specific plate (TNF-RI and TNF-RII). A SECTOR Imager 6000 (Meso Scale Discovery) plate reader was used, and the data were

analyzed using the MSD Discovery Workbench software according to the manufacturer's instructions.

7.5 Enzyme-linked immunosorbent assay

mothers against decapentaplegic homolog 4 **)** and SNAP25 (Synaptosomal-associated protein 25) protein levels in the corpus callosum were examined and compared between miR-146a KO mice and WT mice using pre-prepared Sandwich ELISA kits according to the manufacturer's instructions.

7.6 Histopathology

Brains were postfixed overnight in 4% PFA before embedding in paraffin. Coronal sections of 8 µm were then prepared at levels 161, 181, 209 and 221. Demyelination was assessed using Luxol fast blue and cresyl violet. Axonal pathology was examined using Bielschowsky stain. Sections in the corpus callosum were covered with a 100-point grid, which was used to first determine the size of the entire lesion. The size of the area showing remyelination was then measured. Immunocytochemistry was performed on paraffin sections using antibodies against Iba1, Mac3, NG2 and CNP. Stained cells were counted in sections covered with a morphometric grid.

7.7 Experimental design and statistical analysis

Statistical tests were performed using Prism 7 software (GraphPath, USA, CA, USA), and quantitative data are presented as mean ± SEM. Exact p-values are given for each ANOVA test , with $p > 0.0001$ and $p < 0.05$ considered significant. Each ANOVA test was followed by an appropriate post hoc test. Raw miRNA expression data were obtained from 3–4 mice per group. The microarray data are presented in Table 75. were normalized to percentile signal intensity, and those that were present in all samples of a given condition were filtered out. Differentially expressed genes were selected if they passed the signal intensity filter and showed at least a twofold statistically significant change between groups. For validation of miRNA expression by qPCR, 5–8 mice per group were used, and the data were analyzed by one-way ANOVA followed by LSD post hoc tests. For analysis of body weight, thymus and spleen weight, and lesion size, 4–16, 4–8, and 4–7 mice were used at each time point, and the data were analyzed by two-way ANOVA followed by Bonferroni post hoc tests. For proteome analysis, 5 mice were included. The ratio (r) of each protein in any of the three

comparisons was compared to the standard error (SE), such that if $r \leq 1 / (1 + 2SE)$ or if $r \geq (1 + 2SE)$, the protein was changed. If the protein was measurable in only one or two of the 5 mice, they were not examined in this analysis. ELISA analyses of SMAD4 and SNAP25 and Meso Scale Discovery multiplex analyses of cytokines, chemokines and TNF receptors were performed on 4–8 mice in each group, and data were analyzed by two-way ANOVA followed by Bonferroni post hoc tests.

8 Results

8.1 Differential expression of microRNAs in the corpus callosum during CPZ-induced demyelination and remyelination

To identify miRNAs involved in the pathology of demyelination and remyelination, we isolated the corpus callosum of mice exposed to CPZ and performed Agilent microscopic analysis of 627 miRNAs. We identified three miRNAs, miR-146a, miR-181b, and miR-193a, that showed differential expression compared to controls confirmed by qPCR. The expression of miR-146a increased in response to CPZ exposure and continued to increase during the remyelination phase. In contrast, the expression levels of miR-193a and miR-181b decreased in response to CPZ-induced demyelination and returned to baseline during the complete remyelination phase.

8.2 Expression of miR-146a in response to CPZ in different organs

In addition to the corpus callosum, we also analyzed miRNA-146a expression levels in the thymus, liver, spleen, and muscle tissue. In contrast to the corpus callosum, no CPZ-induced miR-146a increase was observed in these organs. Among the organs examined, the highest miR-146a expression level was in the spleen.

8.3 Expression of miR-146a in the brain during physiological myelination in postnatal mice

To investigate whether miR-146a levels are also increased during physiological myelination, we examined its expression in the corpus callosum isolated from postnatal mice at 1–14 days (P1–P14), a period that is most critical for physiological myelination in mice. We found no

change in miR-146a expression, suggesting that the increase observed in response to CPZ exposure is related to the demyelination pathology.

8.4 Systemic effects of CPZ exposure in miR-146a-deficient mice

Weight loss is a characteristic systemic effect of CPZ exposure in mice. As expected, both WT and miR-146a KO mice showed weight loss upon CPZ exposure, but miR-146a KO mice lost significantly less weight than WT mice during the period of demyelination. CPZ is known to cause thymic atrophy. Therefore, we examined the weight of the thymus and spleen upon CPZ exposure. Atrophy of the thymus and spleen was observed in WT mice, whereas atrophy of both organs was less severe or absent in miR-146a KO mice.

8.5 Effect of miR-146a deficiency on CPZ-induced demyelination and axonal loss

Demyelination and axonal damage were significantly reduced in miR-146a KO mice. The number of 2',3'-cyclic nucleotide 3'-phosphodiesterase+ (CNP+) myelinating oligodendrocytes was higher in miR-146a KO mice than in wild type during demyelination, while the number of neuronal glial antigen 2+ (NG2+) oligodendrocyte precursors showed no difference. In addition, we observed a decrease in the number of lysosomal membrane protein 2+ (Mac3+) cells and a trend towards a decrease in the number of ionized calcium-binding adaptor molecule 1+ (Iba1+) cells in the corpus callosum of KO mice during demyelination. Two weeks after CPZ withdrawal, the demyelinated lesions were largely remyelinated, containing Iba1+ cells, but only a minority of these cells were Mac3+. During remyelination, the number of CNP+ cells increased moderately and the number of NG2+ cells decreased; no difference was observed between KO and WT mice in terms of CNP+ oligodendrocytes, but the number of NG2+ oligodendrocyte precursor cells decreased.

8.6 Expression of experimentally validated miR-146a target genes and protein products in response to CPZ exposure

We also identified two downregulated protein products of miR-146a target genes in our proteomic dataset, one of which is SMAD4, and SNAP25. We used ELISA assays to investigate whether SMAD4 and SNAP25 are differentially expressed between miR-146a KO and WT mice in the corpus callosum in response to CPZ exposure. As expected, we observed an increase in SNAP25 protein levels in the corpus callosum of KO mice compared to control

mice. In KO mice, SNAP25 protein levels decreased during de- and remyelination, whereas there was no change in WT mice. SMAD4 protein levels did not change significantly in either mouse strain, and SMAD4 protein was not differentially regulated at any time point examined.

8.7 Expression of cytokines, chemokines and TNF receptors in response to CPZ exposure

Both TNF-RI and TNF-RII expression were significantly increased in WT mice upon CPZ exposure, but were not changed in miRNA-146a KO mice. In WT mice, TNF expression was significantly lower during remyelination compared to demyelination. In miR-146a KO mice, the highest TNF expression was found in the control group, which was significantly higher than the expression measured during acute remyelination. However, no significant differences in TNF expression levels were detected between the two groups of mice at any time point. A significant increase in CCL2 levels was also observed in response to CPZ exposure in WT mice, but there was no change in miR-146a KO mice. In addition, CCL2 levels were significantly lower in miR-146a KO mice compared to WT during demyelination ($p < 0.01$). For additional cytokines and chemokines, we found that IL-1β was upregulated, while IL-2, IL-5, IL-6, and IL-12p70 were downregulated in miR-146a KO mice, while IL-10 and VEGF were downregulated in both miR-146a KO and WT mice upon CPZ exposure. However, there was no significant difference in the expression levels of these cytokines and chemokines between miR-146a KO and WT mice at the time points examined.

9 Discussion

CPZ treatment resulted in differential changes in miRNA-146a, miRNA-181b and miRNA-193a levels compared to controls. In the later stages of the study, the role of miR-146a was further investigated. miR-146a is highly expressed in brain microglia. However, the observed increase in the corpus callosum after CPZ exposure cannot be explained solely by an increase in the number of infiltrating microglia and macrophages, as the number of infiltrating microglia cells decreases already 1 week after CPZ treatment and the highest level of miRNA-146a was found in the phase of complete remyelination, i.e. 2 weeks after CPZ treatment was discontinued. Despite the elevated level of miR-146a expression in the central nervous system, CPZ did not induce increased expression in other organs, including the liver and thymus, which are also affected by CPZ. This may suggest that the increased expression of miR-146a is unique to the CNS in response to CPZ-induced de- and remyelination. Therefore, we also examined the levels of miR-146a during physiological myelination. We found no change in miR-146a expression, indicating that the observed increase in response to CPZ exposure is associated with demyelination pathology.

In the next phase we compared the systemic and central nervous system effects of CPZ exposure between KO and WT mice. Mice are known to lose weight following CPZ exposure, and we recently recognized that thymic atrophy is associated with the loss of double-positive thymocytes as an additional systemic effect of CPZ exposure. Here we found that CPZ also induced atrophy of another immune organ, the spleen. It is conceivable that the additive effect of CPZ on primary and secondary immune organs may contribute to the lack of immune responses. In contrast, we found that miR-146a KO mice were protected against these systemic effects of CPZ, as thymus and spleen atrophy and body weight loss were both reduced in KO mice. These data suggest that miRNA-146a may participate in the regulation of toxic responses and mitochondrial dysfunction, considering the mitochondrial effect of CPZ. Moreover, miR-146a is one of the mitochondrial miRNAs with potential targets on mitochondrial mRNAs and is most upregulated in senescent cells with mitochondrial dysfunction, altered fission and fusion.

The reduction in lesion size observed in miR-146a KO mice was accompanied by lower numbers of Mac3+ and Iba+ macrophages/microglia and increased number of CNP+ myelinating oligodendrocytes in the corpus callosum during demyelination. In our study, miR-146a deficiency had no effect on remyelination and did not affect the number of OPCs

during remyelination. However, it reduced demyelination and axonal loss, as well as the increase in the number of oligodendrocytes during demyelination. Since we did not observe an increase in NG2+ OPCs during demyelination in KO mice, the higher number of myelinating oligodendrocytes in the myelinating corpus callosum may indicate an enhanced oligodendrocyte survival. Interestingly, a decrease in the number of oligodendrocyte precursors was observed in KO mice during remyelination, suggesting that miR-146a may have a beneficial role during remyelination. These findings may suggest a complex role of miR-146a in de- and remyelination when examined at the tissue level.

In the next step, we searched for proteins of validated target genes in our proteome database obtained during CPZ-induced de- and remyelination. We found that two proteins were downregulated during demyelination: SMAD4 and SNAP25. SMAD4 is involved in OPC migration and differentiation, while SNAP25 plays an important role in neuronal signaling and neurotransmitter release. Therefore, we examined the levels of these two proteins during de- and remyelination in corpus callosum lysates excised from WT and miR-146a KO mice. As expected SNAP25 concentrations were increased in miR-146a KO mice. However, SNAP25 was downregulated in miR-146a KO mice during demyelination, but did not differ significantly from levels in WT mice. Thus, ELISA results did not suggest differential regulation of SMAD4 and SNAP25 in KO mice during demyelination.

miR-146a is a well-known negative regulator of the immune system. We found that CCL2 was upregulated in WT mice upon CPZ treatment. However, in miR-146a KO mice, it was expressed at significantly lower levels during demyelination, in parallel with a decrease in the number of Mac3 + cells. In addition, we found that TNF-RI and TNF-RII levels were increased in WT mice, which was not seen in miR-146a KO mice. Previous data showed that TNF promoted the toxic effect of CPZ on oligodendrocytes *in vitro* and induced the depletion of microglia *in vivo*, which is the main source of cytokine and chemokine expression in the brain. Therefore, it is likely that the reduction of TNF-RI, TNF-RII and CCL2 in miR-146a KO mice contributed to the protection against CPZ-induced demyelination.

10 Summary

This study provides a comprehensive overview of the molecular mechanisms underlying demyelination and remyelination in the CPZ mouse model, highlighting the roles of miRNAs and proteins in the corpus callosum. miR-146a knockout (KO) mice showed remarkable protection against immune organ damage, axonal injury, and demyelination. This suggests that miR-146a contributes to immune regulation and oligodendrocyte survival. Furthermore, proteomic analysis revealed that remyelination is associated with clearer and more distinct molecular changes compared to demyelination, marked by increased levels of myelin-related, synaptic, and cytoskeleton-associated proteins. Although the absence of miR-146a did not significantly affect remyelination itself, it did influence key inflammatory mediators, including CCL2 and TNF receptors. Additionally, a slight increase in NG2+ OPCs was observed during remyelination in the KO mice, supporting previous data suggesting a beneficial role of miR-146a in the remyelination process. Moreover, several demyelination-related proteins were not part of canonical demyelination pathways, pointing to the involvement of tissue remodeling, astrocyte cytokine secretion, and microglial activation. Overall, these findings emphasise the importance of combining RNA, proteomic, and functional studies to better understand how demyelination and remyelination differ at the molecular level. Moreover, focusing on pathways linked to miR-146a may provide novel therapeutic or diagnostic approaches for diseases like multiple sclerosis.

Acknowledgement

I would like to express my sincere gratitude to all those who contributed to the realization of my doctoral work, supported me professionally and personally throughout this journey, and believed in me.

First and foremost, I would like to thank my supervisors, Ferenc Gallyas and Zsolt Illés. Their insightful guidance, continuous support, and extensive experience played a decisive role in the successful completion of my doctoral research. I am truly grateful for the time, patience, and dedication they have shown toward me. I would also like to express my gratitude to Jolán Hársfalvi, whose support and mentorship guided my first steps in the field of scientific research.

I am thankful to Dr. László Márk, Dr. Katalin Fekete, János Schmidt, Dr. Krisztián Erős †, Arkadiusz Nawrocki, Dr. József Pál, Dr. Veronika Nagy, Dr. Gábor Veres, Martin Røssel Larsen, and my co-authors for our collaboration, shared thinking, and for the professional and personal support provided in everyday work.

I would also like to express my appreciation to the University of Pécs Interdisciplinary Doctoral School of Medicine. Furthermore, I wish to acknowledge with deep respect Balázs Sümegi † for his support and generosity. I am incredibly grateful to Hatem Okba, my dear friend and office mate, for his unwavering friendship and daily encouragement. I sincerely thank Dr. Éva Szabó, Dr. Ágnes Bóna, Ömer Furkan Kaçar, Dr. Alexandra Hencz, Miklós Máté Réti, and Anita Horváth for contributing to a friendly and inspiring working environment.

Finally, I am deeply grateful to my loving family. Your constant support and endless encouragement have been the guiding thread throughout this journey. I am especially thankful to my parents for their devoted sacrifices and unwavering faith in me, and to my children and my partner, Dr. Viktória Pusztai, for always standing by my side.

I dedicate this dissertation to you with all my gratitude and love. None of this would have been possible without you.

11 Publication list

11.1 Publications serving as the basis for the dissertation

Gábor T Szilágyi, Arkadiusz M Nawrocki, Erős Krisztian, Schmidt János, Fekete Katalin, Maria L Elkjaer, Kirsten H Hyrlov, Martin R Larsen, Illés Zsolt, Gallyas Ferenc Jr. Proteomic changes during experimental de- and remyelination in the corpus callosum. PLOS ONE 15 : 4 Paper: e0230249, 21 p. (2020), **IF: 3.24;** Q1/D1

Nellie A Martin, Viktor Molnár, **Gábor T Szilágyi**, Maria L Elkjaer, Arkadiusz M Nawrocki, Justyna Okarmus, Agnieszka Wlodarczyk, Eva K Thygesen, Miklós Palkovits, Ferenc Gallyas Jr, Martin R Larsen, Hans Lassmann, Eirikur Benedikz, Trevor Owens, Asa F Svenningsen, Zsolt Illés. Experimental demyelination and axonal loss are reduced in MicroRNA-146a deficient mice. FRONTIERS IN IMMUNOLOGY 9 Paper: 490, 14 p. (2018), **IF: 4.716;** Q1

11.2 Other announcements

Éva Szabó, Tamás Marosvölgyi, **Gábor T Szilágyi**, László Kőrösi, János Schmidt, Kristóf Csepregi, László Márk, Ágnes Bóna. Correlations between Total Antioxidant Capacity, Polyphenol and Fatty Acid Content of Native Grape Seed and Pomace of Four Different Grape Varieties in Hungary. ANTIOXIDANTS 10: 7 Paper: 1101, 12 p. (2021), **IF: 7.675;** Q1

Hencz Alexandra Júlia, Magony Andor, Thomas Chloe, Kovács Krisztina, **Gábor T Szilágyi**, Pál József, Sik Attila. Mild hypoxia-induced structural and functional changes of the hippocampal network. FRONTIERS IN CELLULAR NEUROSCIENCE 17 Paper: 1277375, 14 p. (2023) **IF: 4.2;** Q2

Hencz Alexandra Júlia, Magony Andor, Thomas Chloe, Kovács Krisztina, **Gábor T Szilágyi**, Pál József, Sik Attila. Short-term hyperoxia-induced functional and morphological changes in rat hippocampus. FRONTIERS IN CELLULAR NEUROSCIENCE 18 Paper: 1376577, 13 p. (2024) **IF: 4.0;** Q1

11.3 Other publications

Hencz Alexandra Júlia, **Gábor T Szilágyi**, Győrfi Nina, Tenzlinger Kristóf, Széchenyi Alexander, Odry Ákos, Odry Péter, Karádi Zoltán, Vizvári Zoltán, Tóth Attila, Pál József .. Comparison of graphene and indium tin oxide electrodes with low-frequency electrical impedance spectroscopy measurements. In: Tóth Attila; Vizvári Zoltán (eds.) Biomedical questions - multidisciplinary, bioimpedance-based answers: Collection of publications of the PTE Metabolic Regulation and Bioimpedance Research Group, conference volume pp. 115-123. University of Pécs, Pécs. (2022)

11.4 Conference appearances

Hencz Alexandra Júlia, Magony Andor**, Gábor T Szilágyi**, Pál József, Sik Attila The impact of hypoxia and hyperoxia on the number of compacted neurons and brain activity. International Neuroscience Conference (INC), Pécs. (2024).

Hencz Alexandra Júlia, **Gábor T Szilágyi**, Győrfi Nina, Tenzlinger Kristóf, Széchenyi Alexander, Odry Ákos, Odry Péter, Karádi Zoltán, Vizvári Zoltán, Tóth Attila, Pál József. Comparative electrical impedance spectroscopy study of graphene and indium tin oxide electrodes during low frequency measurements. IEEE 15 th International Symposium on Applied Computational Intelligence and Informatics (SACI) (2021).

Gábor T Szilágyi, Arkadiusz M Nawrocki, János Schmidt, László Márk, Zsolt Illés, Katalin Fekete, Martin R Larsen, Ferenc Gallyas Jr. Proteomic study of remyelination processes in the cuprizone model. V. Interdisciplinary Doctoral Conference (IDK) Pécs (2016).

Gabor T Szilágyi, Arkadiusz M Nawrocki, Janos Schmidt, Ferenc Gallyas Jr, Zsolt Illés. Regulation of de- and remyelination in the central nervous system. Annual Meeting of the Hungarian Biochemical Society, Szeged (2016)

Gábor T Szilágyi, Identification of new molecular targets in a cuprizone-induced demyelination model. Spring Wind Conference. Budapest (2016)

Gabor T Szilágyi, Janos Schmidt, Arkadiusz M Nawrocki, Zsolt Illés, Ferenc Gallyas Jr. Proteomic analysis of gene products that regulate de- and remyelination. IX Annual Congress of European Proteomics Association (EUPA) Milano. (2015).