The Cuprizone-Induced Experimental Demyelination – A Promising Animal Model for Multiple Sclerosis Type III-IV

Ph.D. Thesis

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Pécs, 2012

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1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS). It is the most common neurological disorder in young adults in the Western hemisphere, being approximately 2 fold more common in females. MS is characterized by a broad spectrum of sensory-motor, cognitive and neuropsychiatric symptoms and appears in several distinct disease courses. The majority of the patients are first diagnosed with relapsing–remitting MS (RRMS), where patients develop alternating episodes of neurological symptoms and recovery. This clinical course can last for many years, but within 25 years close to 90% of RRMS patients enter the secondary-progressive stage (SPMS) with a steady progressive neurological decline. Primary progressive MS (PPMS) affects about 10% of MS patients and characterized by slowly accumulating neurological symptoms without recovery. Progressive-relapsing MS (PRMS), affecting 4-10% of MS patients, is characterized by steadily declining neurological disability associated with acute relapses with or without recovery. Recent reports suggest that different clinical courses of MS might be associated with different pathomechanism.

The classical neuropathological alteration in MS is the plaque-like demyelinated lesion. A detailed histopathological study by Lucchinetti et al. has highlighted the profound heterogeneity in the pattern of demyelination among MS patients. According to these observations lesions can be divided into four distinct patterns with the common hallmark of myelin destruction and the similarities in the inflammatory reaction (composed mainly of T cells and macrophages). Pattern I and II lesions can be described as macrophage- and antibody mediated demyelinating events, respectively. Pattern I lesions are characterized by T cell- and macrophage dominated inflammation and can be considered as sites of active demyelination. Pattern II lesions are similar to pattern I, but additionally show deposition of immunoglobulin and activated complement at sites of active

myelin destruction. Pattern III lesions are not centered by veins but preservation of a rim of myelin is frequently observed around an inflamed vessel within the demyelinated plaque. The borders of pattern III lesions are ill-defined. Another major difference compared to pattern I and II is that pattern III lesions are characterized by a preferential loss of MAG. Within these areas, myelin-forming oligodendrocytes (OLs) show typical signs of apoptosis (nuclear condensation and fragmentation). A pronounced loss of OLs is characteristic at the active plaque border; while OLs are absent in the inactive center. Pattern IV lesions are described with significant cell death in the periplaque white matter resembling that of pattern III lesions. These lesions also contain microglia/macrophages and T cells (as pattern I lesions). In summary, pattern III and IV lesions can be characterized by signs of OL dystrophy/apoptosis. Besides histological heterogeneity recent studies revealed temporal heterogeneity of MS plaques and proposed a novel hypothesis for lesion formation. Studying new symptomatic lesions in patients who died shortly after the onset of a relapse, extensive OL apoptosis (without caspase 3 activation) was observed. A subsequent analysis confirmed that OL apoptosis is the earliest event in prephagocytic lesions with still intact myelin and without T and B cell infiltration in tissue bordering rapidly expanding MS lesions. These observations challenge the generally accepted view of a T lymphocyte-initiated demyelination in MS and point out that OL apoptosis may not only occur in type III lesions, but rather represents the earliest stage of lesions underlying MS exacerbation.

1.2 The cuprizone-induced experimental demyelination

Cuprizone-induced demyelination model has attracted increasing interest during the last decade since contrary to other models of MS this one provides a highly reproducible system of primary OL apoptosis and secondary demyelination. The administration of the copper chelating agent cuprizone (bis-cyclohexanone oxaldihydrazone) to mice induces spatially and temporally (e.g. corpus callosum, superior cerebellar peduncle) well defined histopathological alterations in the CNS. The earliest event is the appearance of megamitochondria, followed by OL

apoptosis. The peak of the apoptotic events is between the 3rd and 10th days of the cuprizone challenge, but apoptotic OLs can be detected during the entire administration, and even during the recovery period 12 weeks post treatment. The massive OL apoptosis is followed by extensive demyelination. Another prominent pathological feature associated with OL apoptosis is the invasion of the demyelinated areas by activated microglial cells. Beyond the phagocytosis of disrupted myelin sheaths, the role of activated macrophages and microglia in the cuprizone model is controversial. These cells may further amplify the cuprizone initiated OL cell death by the production and secretion of pro-inflammatory cytokines. Alternatively, microglia may have a beneficial role by stimulating OL precursor cells and promoting remyelination.

If mice return to normal diet after six weeks of cuprizone exposure, demyelination is followed by a spontaneous and complete remyelination driven by the repopulation and maturation of OL progenitor cells. If the cuprizone challenge is prolonged for 12 weeks, the degree of remyelination may be limited or remyelination may even fail to occur.

Histopathological features of the cuprizone-induced demyelination closely resemble those of the Lucchinetti et al - defined type III MS lesions. The most significant similarities include a prominent OL apoptosis and microglial activation in the actively demyelinating lesions, the lesions are not perivenous and their borders are ill defined, and there is an early and profound downregulation of the MAG mRNA level. Considering the above histopathological features, the cuprizone model is highly suitable for studying basic mechanisms of acute and chronic demyelination and remyelination, exploring the pathophysiology of OL apoptosis, and testing preclinically new interventions for promoting remyelination and repair in MS lesions.

2 Summary of the studies

The goal of our experiments was to investigate whether targeting the survival of OLs (by promoting remyelination or by inhibiting apoptosis) reduces the demyelination in the cuprizone model.

2.1 Effect of 17β-estradiol and progesterone treatment on cuprizone induced demyelination in C57BL/6 mice

2.1.1 Background and aim

The positive influence of female gonadal steroid hormones [17b-estradiol (E) and progesterone (P)] on neurodegenerative and neurotoxic processes is widely accepted. In patients with MS E and P plasma levels seem to be inversely related to the severity and progression of MS symptoms. The idea that female sex steroids might be a therapeutic option for MS derive from retrospective studies revealing remission of symptoms during pregnancy, disease exacerbation postpartum, and a decreased incidence rate of MS in multipara when compared with nullipara. Prospective studies support these observations and suggest that hormonal alterations during gestation have a protective effect on the course of MS, in particular during the third trimester when hormone levels are massively elevated.

The aim of this set of experiments was to determine whether the administration of 17β-estradiol and progesterone either alone or in combination affects the cuprizone induced demyelination. In our experiments, we used E and P, administered alone or in combination, to prevent demyelination independent of autoimmune reaction.

2.1.2 Results

Effect of hormone treatment on the cuprizone induced demyelination and mature OL loss.

In the cuprizone treated group in vivo T2 weighted MRI images revealed increased signal intensity within the CC indicating massive demyelination. The administration of E and P significantly reduced CC demyelination in T2-weighted MR images and diminished ventricle swelling. To confirm the results obtained by MRI, luxol fast blue (LFB) staining was performed and the myelin score of the CC was determined in the

experimental groups. Cuprizone feeding resulted in a profound and significant demyelination of the CC. The administration of E and P significantly reduced CC demyelination. Quantification of myelin scores (LFB) revealed that cuprizone significantly impaired the myelin score to 1 when compared with controls (score 3). Single applications of E or P only moderately abolished this negative effect. The administration of both hormones, however, returned myelin score to >2. Data always showed intermediate responses of single hormone application versus significantly better myelination score after coapplication of the hormones. For further quantification, gene expression of MBP and PLP, both markers for mature myelin synthesizing OLs, were analyzed by rtPCR. Cuprizone exposure led to a highly significant down-regulation of both myelin markers. The application of E and P caused a significant up-regulation of MBP and PLP, although it appears less obvious than myelin scores of the same animals would suggest. Levels were only restored to approximately 45% and 30% of controls for MBP and PLP, respectively. Single steroid hormones had almost no effect on both parameters. Immunostaining for APC to label mature OLs within the CC showed a massive decline (>60%) in the number of APC-positive cells after the cuprizone challenge which was significantly antagonized by both steroid hormones. Moreover, after E/P treatment, the number and distribution of OLs were comparable with the control group.

Because the modulation of astroglial or microglial function might be associated with the myelin restoration, we analyzed the effect of E and P on the presence/absence of these cell types in the demyelinated CC using immunohistochemistry. In addition, GFAP mRNA expression was quantified by qPCR (data not shown). We observed a pronounced astrocytosis in cuprizone-fed animals. Interestingly, this effect was multiplied after the application of E and P. Expression analysis confirmed these data and revealed an approximately 12-and 18-fold increase in GFAP mRNA levels in cup- and cup-plus E/P-treated mice, respectively, when compared with controls. Single administration of E or P together with the cup did not show a significant up-regulation of GFAP mRNA expression. Macrophage invasion demonstrated by the presence of Iba1-positive cells was seen in the lateral part of the CC in cuprizone-

fed mice. In E/P-treated animals, macrophage infiltration was not only observed in the lateral part but additionally in the midline of CC.

Expression of OL maturation-related genes in the cuprizone and hormone treated mice

Protective hormonal effects on myelination status in the CC in hormone treated groups may be the consequence of the recruitment of OL progenitors which account for new myelin sheets. To follow this hypothesis, we have analyzed expression levels of platelet-derived growth factor alpha receptor (PDGFα-R) in the CC, a stringent marker of premature OLs. PDGFα-R mRNA levels displayed a tendency to be higher in cup-fed animals when compared with controls (c 100 ± 3 vs. cup 122 ± 11) without reaching statistically significant values. In contrast, mRNA levels were significantly higher in the cup+E/P group when compared with controls (c 100 ± 3 vs. cup+E/P 145 ± 20, $P < 0.05$) implicating proliferation of premature OLs in response to hormonal treatment. This is strongly supported by immunohistochemistry for Ki-67, a marker for cell proliferation, in the CC. Only few Ki-67-positive cells were found in controls and after cup-treatment in the sub-ventricular zone and in the lateral part of the CC, whereas numerous Ki-67 positive cells were detected in all parts of the CC after E/P treatment. Insulin-like growth factor-1 (IGF-1) is a well-known mitogen for premature OLs, and astrocytes the major sources for brain IGF-1 synthesis. As we observed a massive astrogliosis within the CC, we studied gene expression of IGF-1 in the CC. IGF-1 mRNA levels were significantly increased by approximately 20-fold in cup-fed mice when compared with controls and by more than 40-fold in cup+E/P-treated animals. Similar to the regulation of GFAP expression, the application of single hormones together with cup did not significantly stimulate IGF-1 mRNA expression.

2.1.3 Conclusions

In our first study we aimed to investigate the role of gonadal steroids during experimentally-induced demyelination in the male CC using cuprizone as toxic agent. We provide here clear evidence that the application of both ovarian steroids, E and P, possess the capacity of inhibiting mature OL damage and/or of positively stimulating (re)myelination, whereas single steroid treatments did not exceed mild effects. Moreover, both hormones appeared to balance the interaction of astroglia, microglia, and/or OL and OL precursors resulting in a strong activation of resting premature OLs or prevention of mature oligodendrocyte damage. The activation and differentiation of premature OLs is assumed to be the major account for the observed counteraction of cuprizone induced demyelination by sex steroids.

2.2 Effect of poly(ADP-ribose) polymerase inhibition on cuprizone induced demyelination

2.2.1 Background

Apoptosis-like depletion of OLs has been described in the earliest MS lesions in pathological subtypes for patterns III and IV, suggesting degenerative processes. According to an alternative hypothesis of MS, OL apoptosis represents the first and earliest stage of all lesions. The nuclear enzyme PARP functions as a DNA damage sensor and signaling molecule, and forms long branches of ADP-ribose polymers on a number of nuclear target proteins, including PARP itself. Extensive DNA damage triggers overactivation of PARP, eventually resulting in cell dysfunction and death. Additionally, PARP activity is required for the translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus, supporting the hypothesis that nuclear mitochondrial crosstalk dependent on poly(ADP-ribosyl)ation is critical in determining the fate of injured cells. PARP-mediated cell death and inflammation have been implicated in the pathogenesis of several central nervous system diseases. Besides many beneficial role of PARP inhibition in animal models of central nervous system diseases, PARP inhibition attenuates inflammation in EAE, the autoimmune model of MS.

2.2.2 Hypothesis and the aim of the study

Oligodendrocyte apoptosis is a common feature of cuprizone-induced demyelination and certain hystopathological types of MS. In the cuprizone model OL apoptosis is – at least partly – induced by the disruption of mitochondrial respiratory chain and complex IV. This process induces DNA damage of the affected cells. We hypothesized that the mitochondrial impairment and consequent DNA damage is associated with poly(ADP-ribose) polymerase (PARP) overactivation contributing to OL cell loss in the cuprizone challenge.

In this study we aimed to investigate whether a.) PARP overactivation contributes to the OL cell loss in the cuprizone model, whether b.) inhibition of PARP has a beneficial role in the cuprizone induced demyelination. Additionally, our purpose was to learn if PARP overactivation is present in human MS plaques and plays a role in OL cell death.

2.2.3 Results

Cuprizone enhances PARP activation in the corpus callosum

To investigate the effect of PARP inhibition on experimental demyelination, we first examined the activation of PARP upon cuprizone treatment. Cuprizone induced auto-poly(ADP-ribosyl)-ation, i.e. activation of PARP in the CC of mice after 3 weeks of treatment. Expression of poly(ADP-ribose) immunoreactivity in the apoptotic nuclei of OLs was confirmed by confocal laser microscopy. In addition, 4HQ - a potent inhibitor of the enzyme - blocked both cuprizone induced and basal auto-poly(ADP-ribosyl)ation at a dose of 100 mg/kg used throughout this study. This dose of 4HQ was previously found to be effective and devoid of any apparent toxic effect.

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of astroglia, microglia, and/or OL and OL precursors resulting in a strong activation of resting premature OLs or prevention of mature oligodendrocyte damage. The activation and differentiation of premature OLs is assumed to be the major account for the observed counteraction of cuprizone induced demyelination by sex steroids.

PARP inhibition protects against cuprizone induced demyelination

Examination of the brain was performed by non-invasive in vivo MRI. In untreated mice, CC appeared hypointense on T2-weighted images. Upon cuprizone feeding, T2-weighted images of the CC showed hyperintensity corresponding to demyelination, that was most pronounced after 4 weeks. PARP inhibitor prevented cuprizone-induced hyperintensities in the CC. Inhibition of PARP prevented demyelination. When applied alone, 4HQ did not cause any changes in signal intensities. Pathological analysis with LFB-cresyl violet staining revealed a profound demyelination in the CC of cuprizone-fed mice. 4HQ reduced the cuprizone-induced demyelination ($P<0.001$). 4HQ alone did not affect myelination. Quantitative MBP immunoblotting revealed decreased MBP expression after 5 weeks of cuprizone feeding ($P<0.01$), which was reversed by the PARP inhibitor 4HQ ($P<0.05$). The administration of the PARP inhibitor alone did not affect the MBP level. Similar results were found by MBP immunohistochemistry.

Cuprizone induces caspase-independent AIF-mediated cell death, which is attenuated by PARP-inhibition

Parallel to demyelination, we observed elevated expression of AIF in the CC of mice treated with cuprizone for 3 weeks, an effect that was attenuated by 4HQ. Besides elevating its expression, cuprizone induced nuclear translocation of AIF. In cuprizone-treated mice, numerous cells showing typical shape and arrangement of OLs gave strong nuclear anti-AIF immunostaining in the midline and cingular part of the CC, which were prevented by the PARP inhibitor. In contrast, cuprizone did not induce caspase-dependent cell death, as revealed by the absence of procaspase-3 cleavage determined by immunoblotting and a fluorescent caspase-

3 assay. Taken together, these data indicate caspase-independent AIF-mediated cell death in the CC of cuprizone-fed mice.

PARP activation in multiple sclerosis lesions

To determine PARP activation in MS lesions, we demonstrated accumulation of the enzyme's product by using anti-poly(ADP-ribose) immunofluorescence or immunohistochemistry. We observed very strong poly(ADP-ribose) reactivity in the nucleus and cytoplasm of single cells. This was most pronounced in patients with acute MS, in active lesions showing the characteristic pathological hallmarks of pattern III demyelination and containing high numbers of apoptotic OLs. The expression was seen in cells that, by the anatomy of their processes, mainly resembled OLs. They contained a condensed, sometimes fragmented nucleus and their cytoplasm revealed, in part, fragmented cell processes or swelling and focal vacuoles. Quantitative analysis confirmed that poly(ADP-ribose) reactive glial cells were enriched in areas of initial and active myelin breakdown of pattern III lesions, as defined before. Similar poly(ADP-ribose) reactive OLs, although in lower numbers, were also seen at the active edge of slowly expanding lesions in progressive MS and in lowest numbers in patients with pattern II lesions. Double staining and confocal laser-scanning microscopy confirmed that the majority of cells with strong poly(ADP-ribose) immunoreactivity also expressed the OL marker carbonic anhydrase II.

Nuclear translocation of AIF in pattern III multiple sclerosis lesions

Since AIF is essential in mediating PARP-dependent cell death, we examined its expression in MS lesions. AIF reactivity in the normal brain, and with some exceptions in the normal appearing white matter of MS patients was confined to the mitochondria of neurons and glia cells. In MS lesions, AIF reactivity in mitochondria was enhanced and seen not only in neurons and glia but also in macrophages. Within initial and active areas of MS pattern III lesions and much less in other active MS lesions, we found a variable number of glia cells with nuclear AIF reactivity co-localized with increased anti-poly(ADP-ribose) staining in

condensed nuclei, showing features of apoptosis. These data suggested that activation of PARP may result in AIF-mediated OL death in pattern III MS lesions.

2.2.4 Conclusions

Our data indicate that OL death occurs via very similar mitochondrial pathomechanisms in the cuprizone model and pattern III MS lesions. Inhibition of PARP effectively attenuated OL depletion and protected against experimental demyelination mediated through a caspase-independent pathway involving nuclear translocation of AIF. Considering that PARP inhibition was also highly effective in EAE, the autoimmune inflammatory model of MS, it may provide a therapeutic approach protecting against two basic processes in MS, inflammation and demyelination. Moreover, it may target all subtypes of MS either by preventing OL death, a key event in the formation of all new lesions or additionally, by targeting inflammation.

3 Summary of the thesis

In this study - by using a mouse model that mimics pattern III MS subtype - we aimed to explore new therapeutical approaches to MS forms mainly characterized by OL apoptosis either by promoting the remyelination process or by inhibiting the apoptosis of mature OLs. Additionally, our goal was to investigate pathomechanisms that may have a role in OL loss in MS.

3.1 Effect of 17β-estradiol and progesterone treatment on cuprizone induced demyelination in C57BL/6 mice

- In vivo MRI investigation, routine histopathology and immunohystochemistry revealed that mice administered the combined hormone therapy have a better myelin status than the cuprizone treated mice.

- Combined hormone therapy induced massive astrocyte reaction and macrophage accumulation during the cuprizone challenge. Astrocytosis and macrophage activation are well known features of the cuprizone induced demyelination, but the hormone treatment strongly enhanced these reactions.

- The administration of 17β-estradiol and progesterone induced the expression of remyelination-related genes (IGF-1 and PDGF-alpha R) in the corpus callosum of cuprizone treated mice. Additionally, hormone treated mice revealed more dividing cells in the subventricular zone.
- Our data implicate that the combined hormone therapy promote the maturation of OL precursors and thus the remyelination in the cuprizone induced demyelination model.
- Our results suggest that a combined17β-estradiol and progesterone hormone therapy might have a beneficial role in the non-immune mediated forms of MS.

3.2 Effect of poly(ADP-ribose) polymerase inhibition on cuprizone induced demyelination

- Cuprizone administration induces PARP activation, and this effect is attenuated by the administration of the PARP inhibitor 4HQ.
- Through PARP overactivation, cuprizone induces AIF mediated, caspase-independent apoptosis of OLs.
- Inhibition of the nuclear enzyme PARP by 4HQ treatment diminishes the demyelination induced by cuprizone, as demonstrated by in vivo MRI, histology, immunohystochemistry and immunoblot analysis.
- PARP inhibition provides a protective effect on OL apoptosis and thus attenuates demyelination in the cuprizone model.
- PAR overactivation and consequent AIF mediated, caspase independent OL apoptosis is present in type III MS lesions.
- Our data indicate that PARP inhibition might provide a therapeutic approach against OL death in MS.

4 Publications directly related to the thesis:

Articles

P. Acs*, M. Kipp*, C. Beyer, S. Komoly; Estrogen and progesterone treatment prevents cuprizone induced demyelination in C57Bl/6 male mice Glia. 2009 Jun; 57(8):807-14 IF: 4.932
*contributed equally

Sara Veto*, Peter Acs* , Jan Bauer, Hans Lassmann, Zoltan Berente, Gyorgy Setalo, Jr, Gabor Borgulya, Balazs Sumegi, Samuel Komoly, Ferenc Gallyas, Jr and Zsolt Illes: Inhibiting poly(ADP-ribose) polymerase: a potential therapy against oligodendrocyte death Brain, 2010, Volume 133, Number 3, Pp. 822-834 IF: 9.23
*contributed equally

Peter Acs, Samuel Komoly: Selective ultrastructural vulnerability in the cuprizone induced experimental demyelination; Ideggyógyászati Szemle, accepted for publication

Book chapter

P. Acs, B. Kalman: Pathogenesis of multiple sclerosis: what can we learn from the cuprizone model. In: A. Perl (Ed) Autoimmunity. Humana 2012

Abstracts

Veto S, Acs P, Bauer J, Lassmann H, Berente Z, Sumegi B, Komoly S, Gallyas F Jr, Illes Z, 2010. Inhibiting poly(ADP-ribose) polymerase: a potential therapy against oligodendrocyte death in multiple sclerosis. Clinical Immunology 135: S30-S30

Veto S, Acs P, Bauer J, Lassmann H, Berente Z, Sumegi B, Komoly S, Gallyas F Jr, Illes Z, 2010. Poly(ADP-ribose) polymerase (PARP) is activated in multiple sclerosis pattern III lesions and its inhibition prevents experimental demyelination and oligodendrocyte death. Journal of Neuroimmunology 222:16-16

Veto S, Acs P, Bauer J, Lassmann H, Berente Z, Sumegi B, Komoly S, Gallyas F Jr, Illes Z, 2009. Poly(ADP-ribose) polymerase (PARP) is activated in multiple sclerosis pattern III lesions and its inhibition prevents experimental demyelination and oligodendrocyte death. Journal of the Neurological Sciences 285: S105-S106 Suppl.

Veto S, Acs P, Bauer J, Lassmann H, Berente Z, Sumegi B, Komoly S, Gallyas F Jr, Illes Z, 2009. Poly(ADP-ribose) polymerase is activated in multiple sclerosis pattern III lesions and its inhibition prevents experimental demyelination and oligodendrocyte death. Multiple Sclerosis 15: S88-S88

Veto S, Acs P, Tapodi A, Lendvai Z, Komoly S, Illes Z, Gallyas F, 2008. Poly(ADP)ribose polymerase inhibitor modifies the activities of various kinase cascades in the cuprizone-induced demyelination model. Multiple Sclerosis 14: S81-S81

Veto S, Acs P, Dolowschiak T, Doppler H, Kanizsai A, Berente Z, ifj Gallyas F, 2007. Cuprizone hatása a mitogén aktiválta protein kináz (MAPK) rendszerekre a corpus callosumban. Folia Hepatologica 11: S40-S40

Veto S, Acs P, Dolowschiak T, Lendvai Z, Gallyas F, Komoly S, Berente Z, 2007. In vivo MRI follow-up of cuprizone-induced CNS changes. Multiple Sclerosis 13: S255-S255

Veto S, Acs P, Berente Z, Szabo A, ifj. Gallyas F, Komoly S, 2006. 4 hydroxiquinazoline hatása a cuprizone indukálta központi idegrendszeri elváltozásokra. Biokémia 30:80-81 S.

5 Acknowledgements

I generously and gratefully thank to my mentor, teacher and project leader, Prof. Samuel Komoly M.D., Ph.D., D.Sc. for his leadership, support, advices and for providing me the opportunity to perform this project.

I am grateful to Prof. Zsolt Illes M.D., Ph.D., D.Sc for his excellent contribution and support in the studies.

I wish to thank to Prof. Ferenc Gallyas Jr M.D., Ph.D, D.Sc. and to Sara Veto M.D., Ph.D. for the fruitful collaboration throughout the whole study.

I owe special thanks to Hans Lassmann and the Centre for Brain Research, Medical University of Vienna for the flourishing collaboration.

I appreciate the collaboration and great support of Univ.-Prof. Dr. hum. biol. Cordian Beyer and Markus Kipp M.D., Ph.D at the University Hospital Aachen, Institute of Neuroanatomy.

I am grateful to Prof. Gallyas Ferenc Sen for his technical support and advices.

I thank to Maria Mazlo M.D., Prof. Laszlo Seress M.D. Ph.D., D.Sc. and Hajnalka Abraham M.D., Ph.D. for their help.

I cordially acknowledge the help of Krisztina Fulop, Adam Feldmann and Erno Bognar.

I am grateful to my family for their support and unconditional love.