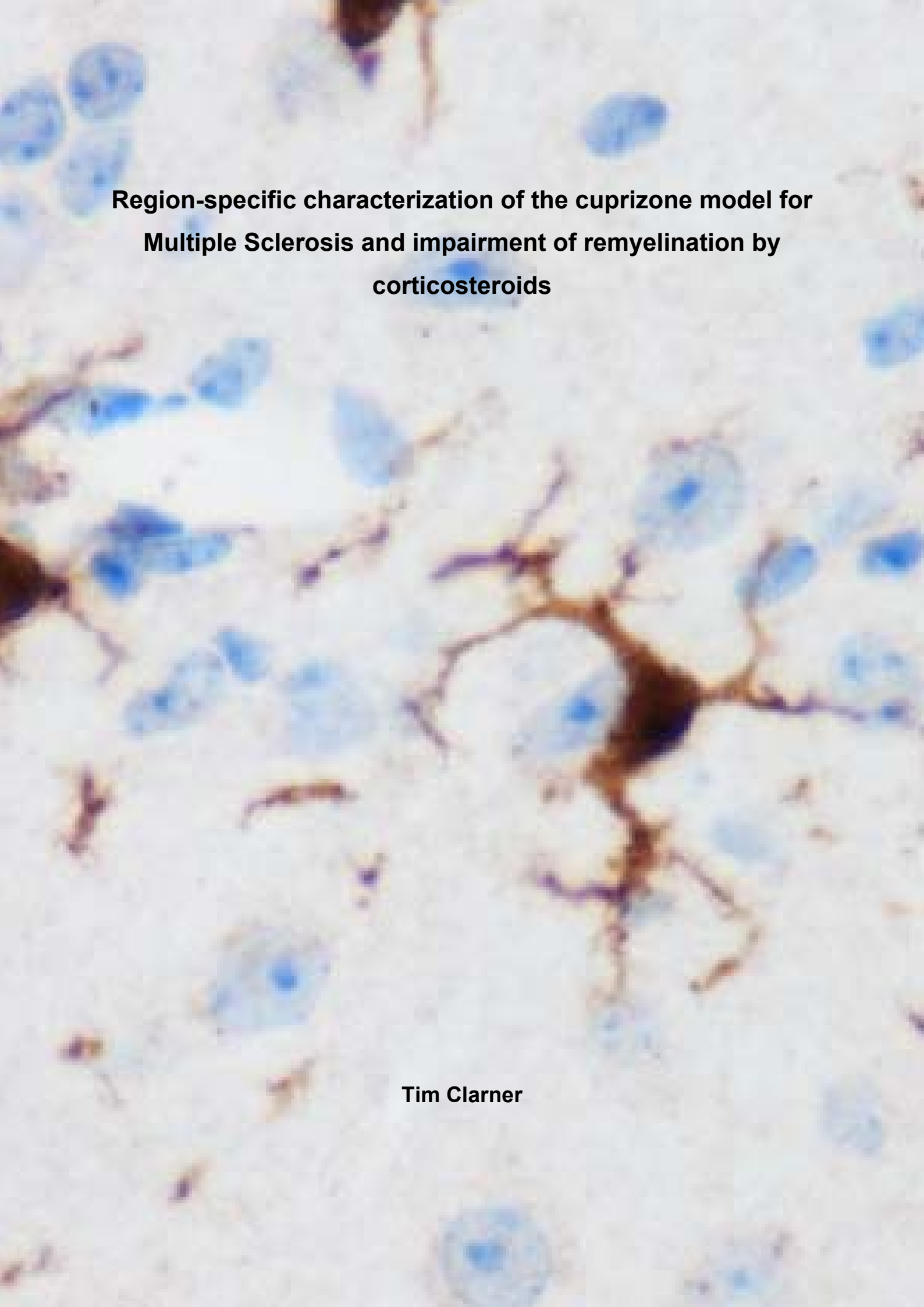

Region-specific characterization of the cuprizone model for Multiple Sclerosis and impairment of remyelination by corticosteroids

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„Region-specific characterization of the cuprizone model for Multiple Sclerosis and impairment of remyelination by corticosteroids”

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Index

Abbreviations

ABC	avidin biotin complex
AEC	3-amino-9-ethylcarbazole
ACTH	adrenocorticotropic hormone
AD	Alzheimers disease
ANOVA	analysis of variance
APC	adenomatosis polyposis coli
APP	amyloid precursor protein
BSA	bovine serum albumin
CA	cornu ammonis
CC	corpus callosum
CCx	cerebellar cortex
cDNA	complementary DNA
CM	cerebellar marrow
CNH	head of the caudate nucleus
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CNV	ventral part of the caudate nucleus
CP	caudate putamen
Dex	Dexamethasone
DHC	dorsal hippocampal commisure
DMEM	Dulbeccos modified eagle medium
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FGF2	fibroblast growth factor
GC	glucocorticoid
GCR	glucocorticoid receptor
GFAP	glial fibrillary acidic protein
GL	granular layer
GM	growth medium
GP	globus pallidus
HDG	hilus of the dentate gyrus
HLA	human leukocyte antigen
HPRT	hypoxanthine phosphoribosyltransferase

ICC	immunocytochemistry
IFN-β	interferone-β
IHC	immunohistochemistry
IN	inerpositus nucleus
lAlv	lateral Alveus
LCN	lateral part of the caudate nucleus
LFB	Luxol fast blue
lStr	lateral striatum
MAG	myelin associated glycoprotein
mAlv	medial alveus
MBP	myelin basic protein
MCN	medial caudate nucleus
ML	molecular layer
MMLV	moloney murine leukemia virus
MOG	myelin associated glycoprotein
MP	methylprednisolone
MS	multiple sclerosis
mStr	medial striatum
Olig2	oligodendrocyte transcription factor 2
OPC	oligodendrocyte progenitor cell
PBS	phosphate bufferd saline
PDGFRα	platelet-derived growth factor receptor alpha
PDGF-αα	platelet derived growth factor
PL	pyramidal layer
PLP	proteolipoprotein
rtRT-PCR	reverse-transcription real time polymerase chain reaction
sAPP	secreted amyloid precursor protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SVZ	subventricular zone
SLM	stratum lacunosum moleculare
T3	triiodothyronine
TCR	T-cell receptor
TNF	tumor necrosis factor
WM	white matter

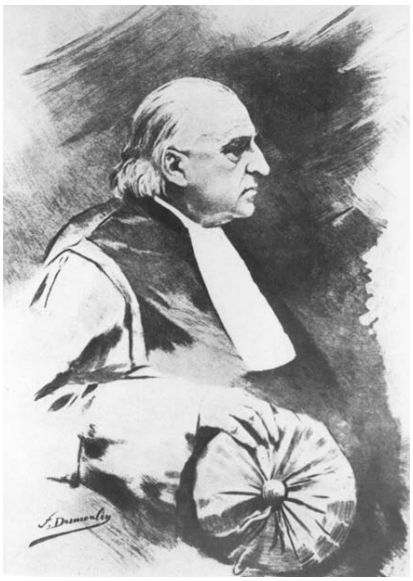
1. General introduction

1.1 Multiple sclerosis in history and present

Multiple sclerosis (MS) is a disorder of the central nervous system (CNS), characterized by myelin loss, varying degrees of axonal pathology, and progressive neurological dysfunction. Demyelination and a pronounced inflammatory infiltrate, composed of mainly lymphocytes, macrophages, and activated microglia are commonly observed in active MS lesions. Tissue changes, including neuronal damage, oligodendrocyte loss, as well as astrogliosis and signs of remyelination, accompany inflammation (Hemmer et al. 2002). The disease is preferably affecting young adults between 20 and 40 years with an average onset at 30 years. Patients younger than 15 or older than 55 are rarely diagnosed with MS. However, about 50 children, predominantly girls, are diagnosed with MS every year in Germany (female-to-male ratio, 2.8 vs. 1.8.). Children need longer from disease onset to reach the so called “secondary progressive stage”, but nevertheless do so at younger age (Renoux et al. 2007). Worldwide, 120,000 to 140,000 patients are diagnosed with MS (overall female-to-male ratio ~ 2:1) making it the most common chronic-inflammatory disease of the CNS (Friedrich 2008; Sellner et al. 2011).

Historically, MS-related symptoms can be traced back to the early middle ages. The Island Saga by St. Thorlakr reports of the Viking women Hala (1293-1323), suffering of speech disorders and blindness. Oblations and prayers relieved her symptoms as can be expected in a relapsing-remitting disease course (Friedrich 2008; Poser 1995).

The best known MS patient describing his disease course in a diary is Sir Augustus D’Este, grandson of King George III (Firth 1941; Pearce 2005). He describes in detail his afflictions, varying from “indistinctness of vision“ in 1822 to “spasmodic pains in (…) Feet and Legs” as well as strong paresthesia and serious “attacks of Bile” in 1846. Sir Augustus D’Este died in 1848 at the age of 54 years (Firth 1941). The first pathological elaboration on MS lesions can be annotated to Jean Cruveilhier (atlas completed in 1841) and Robert Carswell (atlas completed in 1838) (Compston 1988), whereas the first medical description was made by William McKenzie in 1840. He reported several cases of remitting visual loss in young people, compatible with optic neuritis (McDonald 1999; McKenzie 1840).



Picture 1 Jean Martin Charcot, Painting from J. Desmoulin, about 1885

In 1867, the French pathologist Jean Martin Charcot (1825-1893) gave the first full clinico-pathological description of MS. The illustration published in Ordenstein's 1867 Parisian thesis represents the earliest summary of MS pathology in the spinal cord and the brain (Pearce 2005). He was the first to use the expression "*sclerose en plaque*" and to define diagnostic criteria (Friedrich 2008). MS has no "typical" course that would make the disease course predictable for both, patients and physicians. It can manifest in various ways and temporal patterns which led to the often heard denotation of "the disease with 1000 faces." Already in those days, the heterogeneous nature of the disease was emphasized.

In 1996, Lublin and colleagues (Lublin and Reingold 1996) gave the following clinical criteria for MS-diagnosis:

1. Relapsing-remitting MS is defined as "…episodes of acute worsening of neurologic function followed by a variable degree of recovery, with a stable course between attacks (Lublin and Reingold 1996)." 80–90% of cases start with a relapsing–remitting course. Over time, the number of relapses decreases, but most patients develop a secondary progressive phase of the disease in later stages (Hemmer et al. 2002).

2. Secondary-progressive MS. Patients that initially present with a relapsing-remitting disease course may develop "…progression with or without occasional relapses, minor remissions, and plateaus (Lublin and Reingold 1996)."

3. Primary–progressive MS is defined as "a gradual nearly continuously worsening baseline with minor fluctuations but no distinct relapses (Lublin and Reingold 1996)." This disease course affects about 10-20% of the patients (Hemmer et al. 2002).

4. Progressive-relapsing MS. This is a rare clinical course, defined as …"progressive disease from onset, with clear acute relapses, with or without full recovery; periods between relapses characterized by continuing progression (Lublin and Reingold 1996)."

Additionally, they gave two clinical definitions based on the severity of symptoms: "**Benign MS**" which was defined as "…disease in which the patient remains fully functional in all neurologic systems 15 years after disease onset" and "**Malignant MS**" which was defined as "…disease with a rapid progressive course, leading to significant disability in multiple neurologic systems or death in a relatively short time after disease onset (Lublin and Reingold 1996)."

In 2000, Lucchinetti and colleagues described four distinct demyelinating lesions based on histological criteria. They postulated lesion types I and II as mainly T-cell–mediated or T-cell plus antibody–mediated, whereas lesions III and IV are resulting of primary oligodendrocyte dystrophy (Lucchinetti et al. 2000).

	Type I	Type II	Type III	Type IV
Pathology				
Inflammation				
T-cells	++	++	++	++
B-cells/plasma cells	+	+	+	+
Macrophages	+++	+++	+++	+++
Complement activation	–	++	–	–
Demyelination				
Perivenous pattern	+	+	–	±
Lesion edge	Sharp	Sharp	Ill-defined	Sharp
Concentric pattern	–	–	~30% of cases	–
Oligodendrocytes				
Density in plaque	+++	+++	+ (↓)	+ (↓)
DNA fragmentation	±	±	++ (apoptosis)	++ (periplaque white matter)
Myelin protein loss	Even	Even	MAG ≫ others	Even
Remyelination				
Shadow plaques	++	++	–	–
Clinical phenotypes	Acute, RR, SP, PP	Acute, RR, SP, PP	Acute, RR, SP	PP
Proposed immunopathology	T-cell-mediated autoimmunity	T-cell and antibody-mediated autoimmunity	Oligodendrogliopathy (? virus induced)	Oligodendrogliopathy (? Virus induced)

Table 1 Histopathological characteristics of different patterns of active MS lesions (modified from Lucchinetti et al., 2000 and Rejdak et al., 2010)

Given the complexity of MS, a clear diagnosis of MS is difficult. First neurological symptoms that are caused by demyelination or CNS inflammation are termed clinically isolated syndrome (CIS). A CIS does not fulfil the important hallmark of MS, dissemination in time. However, it may be indicative of MS (McDonald et al. 2001). In 2001, McDonald and colleagues presented guidelines for MS diagnosis that have

been modified since then, most recently by Polman et al. in 2010 (McDonald et al. 2001; Polman et al. 2011).

Although intense research has been done since the first descriptions of MS, the cause of the disease is still unclear but is thought to be a combination of environmental exposure and genetic susceptibility (Compston and Coles 2008).

1.2 Genetic risk factors

A monozygotic twin whose sibling suffers from MS has a 50% lifetime risk to develop MS. The risk in first degree relatives ranges from 2% (parents; children) to 5% (siblings) and decreases to 1% in second- and third-degree relatives (Compston and Coles 2008).

Most genes that have been identified to be involved in the susceptibility to develop MS are related to the immune system. In the 1970s, variations in the human leukocyte antigen (HLA) loci have been linked to a greater risk to develop MS. Since then, a growing number of genes have been identified, among genes that affect T-cell function, immune cell regulation in general, tumor necrosis factor-α (TNFα) signalling as well as interferon- and vitamin D metabolism. Although the non-HLA genes only moderately influence MS susceptibility, their identification is of importance since this might lead to a better understanding of pathways involved in the development of MS (Nischwitz et al. 2011).

1.3 Epidemiology

MS prevalence is higher in the northern and southern latitudes compared to latitudes closer to the equator. Migrants from high- to low-prevalence areas display a reduced risk to develop MS. Interestingly, migrants originating from countries with low risk do not develop a higher risk of getting MS when moving to high risk countries (Gale and Martyn 1995). Age during movement is discussed to be a critical factor in this context (Kurtzke 1993). Two different hypotheses have been developed to explain MS epidemiology and the results of migration research. The *prevalence hypothesis* by Kurtzke and colleagues (Kurtzke 1993) postulates that MS is caused by a not yet identified pathogen that is more common in areas of high MS prevalence. It mainly bases on observations of MS incidence on the Faroe Islands. Before 1940, no MS cases have been reported on the Faroe, however, a high incidence was observed after the occupation by British troops during World War II. It is speculated that British

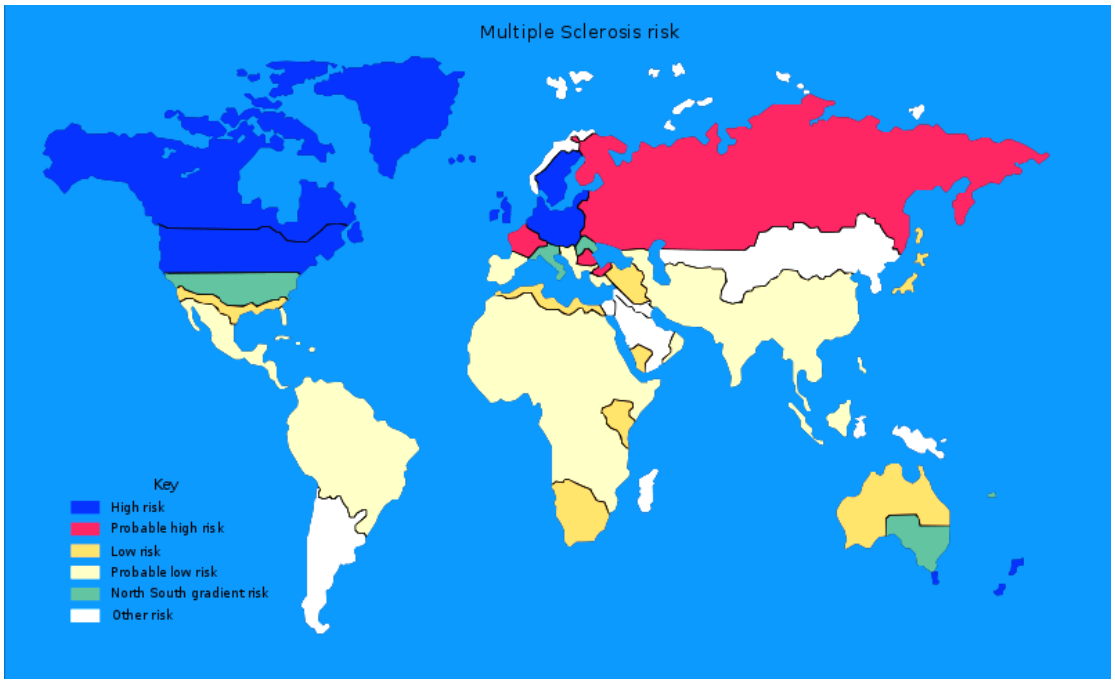
soldiers introduced a pathogen, formerly absent on the islands causing MS in a small proportion of those infected (Kurtzke 1993; Kurtzke and Heltberg 2001).

The second hypothesis is called the *hygiene hypothesis*. Accordingly, exposure to several infectious agents early in life is protective against MS, whereas the same infection might trigger autoimmune reactions in susceptible individuals when it occurs later in life (Ascherio and Munger 2007a). The Ebstein-Barr virus has been identified as a potential infectious agent and there is convincing epidemiological evidence that it is a strong risk factor for MS development (Ascherio and Munger 2011). Anyhow, an Ebstein-Barr virus infection alone cannot explain some aspects of MS epidemiology, including the reduction in risk among migrants from high- to low-risk areas (Ascherio and Munger 2007b).

Beyond that, several non-infectious factors have been identified to correlate with MS incidence (Ascherio and Munger 2007b). Sunlight exposure, vitamin D uptake and sex hormone levels are widely accepted to positively interfere with MS risk or disease progression. High sunlight exposure and vitamin D production and uptake have been found to reduce MS risk. For sex hormones, e.g. estrogen, it has been shown that they can decrease relapse rate and attenuate neuroinflammation (Ascherio and Munger 2007b; Confavreux et al. 1998; Kipp and Beyer 2009).

A negative factor that increases MS risk is smoking. Furthermore, it has been associated with an accelerated transition from relapsing-remitting to secondary progressive MS (Ascherio and Munger 2007b; Kipp and Beyer 2009).

Although many other factors (e.g. dietary factors or stress) have been discussed to be involved in MS risk or pathology, their role remains to be further investigated.



Picture 2 World distribution of MS risk.
Source: http://www.unitedspinal.org/msscene/2009/10/06/mapping-multiple-sclerosis

1.4 Therapeutic attempts for the treatment of MS

In Germany, the “Multiple Sklerose Therapie Konsensus Gruppe” and the “Deutsche Gesellschaft für Neurologie” defined guidelines for the treatment of MS patients. They distinguish between acute relapse-therapy, long term immuno-modulating- and symptomatic-therapy.

In accordance to their guidelines, acute relapses are usually treated with a high dose methylprednisolone (MP) pulse therapy (1000mg/day for 3 consecutive days) which is a synthetic glucocorticoid dampening the acute inflammatory responses and stabilizing the blood brain barrier. In rare cases, escalating relapses can be treated additionally with a plasmapheresis.

Currently, five immuno-modulating agents are approved for the long term treatment of MS patients in Europe: Beta-interferon (IFN-β), glatiramer acetate, a random polymer of 4 amino acids found in Myelin basic protein (MBP), Azathioprin and Mitoxantron, which are cytostatic drugs and Natalizumab, a monoclonal antibody. Due to their well toleration, first choice agents are IFN-β and glatiramer acetate that might be replaced by one of the other agents if indicated (for example in very aggressive MS courses). Medication is often completed by additional symptomatic treatments, depending on the individual patient’s needs. It should be pointed out that all available treatments are symptomatic and healing of MS is not possible so far. Amelioration of remyelination has come into the focus of research, since recent data

demonstrate that myelin sheaths play an important role in maintaining the axonal integrity and protecting neurons (Irvine and Blakemore 2008).

1.5 Animal models for MS

The vast majority of MS-related experimental studies rely on the autoimmune model of experimental autoimmune encephalomyelitis (EAE). EAE can be induced by the active sensitization to CNS tissue, myelin or different protein antigens of the CNS or by the passive transfer of auto reactive T-cells. Another option is to make use of T-cell receptor (TCR) transgenic mice, for instance using a myelin oligodendrocyte glycoprotein (MOG)-specific TCR. Such mice develop spontaneous inflammatory disease in the CNS (Bettelli et al. 2003; Lassmann 2008; Madsen et al. 1999). Chronic virus-induced inflammation and demyelination in the CNS is an alternative model for MS. Theiler's virus encephalomyelitis appears to be the most reliable and most frequently used virus-induced MS model. Despite major differences in the etiology and pathogenesis between autoimmune-mediated and virus-induced demyelinating diseases, similar patterns of demyelination and remyelination suggest common basic mechanisms for tissue injury and repair (Lassmann 2008; Lipton et al. 2005; Rodriguez 2007). These models, in their broad spectrum, are useful tools to study specific aspects of MS pathology and pathogenesis but none of them mimics the disease as a whole.

1.6 The cuprizone model for MS

Since MS is a very complex and heterogeneous disease, reductionistic animal models are needed to investigate and uncover distinct pathological processes and repair mechanisms. In the cuprizone mouse model, the orally administered copper chelator cuprizone (bis(cyclo-hexanone)oxaldihydrazone) causes a highly reproducible demyelination of distinct brain regions, among them the corpus callosum (CC). The CC represents the most frequently investigated white matter tract in this model. Demyelination can be induced in an "acute" manner by supplementing the chow with 0.2% (w/w) cuprizone for 5-6 weeks or in a "chronic" manner by prolonged intoxication for 12 weeks or even longer. Acute demyelination is followed by spontaneous remyelination during subsequent weeks when mice are fed normal chow. In contrast, remyelination is highly restricted after chronic demyelination. Cuprizone susceptibility is dependent on strain, age and weight of the animals (Irvine and Blakemore 2008; Skripuletz et al. 2008).

The cuprizone model is thought to best mimic human pattern III lesions (Kipp et al. 2009; Lucchinetti et al. 2000). In fact, some similarities exist between human pattern III lesions and cuprizone-triggered demyelination: Myelin associated glycoprotein (MAG) mRNA levels seem to be more down regulated during early demyelination compared to other myelin protein markers, demyelination does not present in a perivenous distribution, borders are ill-defined and pronounced oligodendrocyte apoptosis can be found in active demyelinating lesions. However, other features of pattern III lesions, such as inflamed blood vessels and the presence of CD3+ T-cells, are not observed in the cuprizone model. The later might be due to a direct suppression of cuprizone on T-cell function (Mana et al. 2009). The biochemical events leading to oligodendrocyte damage in response to cuprizone treatment are cryptically. Although cuprizone is a copper chelator, there is a controversial discussion of changes in CNS copper levels during the toxic challenge. Furthermore, the mechanisms responsible for oligodendroglial death in MS lesions are not clear. It is questionable whether similar pathomechanisms are responsible for oligodendroglial loss in MS lesions and in the cuprizone model. Thus, one might ask whether cuprizone-induced demyelination models myelin loss observed in human MS patients. This assumption is often challenged by experts in the field. However, one should notice that MS is presently regarded as a disorder with many different features and facets. No current experimental animal model covers the entire complexity of MS. Instead, the various animal models and intoxications can be applied to selectively investigate and uncover distinct pathological processes and repair mechanisms of MS (Kipp et al. 2009).

1.7 Astrocytes in MS and during CNS inflammation

Besides lymphocytes, astroglia and microglia contribute to the cellular composition of inflammation in MS lesions (van der Valk and De Groot 2000). Astroglia are pivotal regulators of intra-cerebral inflammatory processes and are required to protect nerve cells from pathological threats. Activation, proliferation, and morphological alterations of astrocytes are histopathological hallmarks of MS and its related animals models, i.e. EAE and toxin- and virus-induced demyelination (Gold et al. 2006; Kipp et al. 2009; Lassmann 2008).

The sclerotic aspect in MS pathology is due to a tissue scar formed mainly by astrocytes but also other glia cells such as microglia and oligodendrocytes. In the

most common type of MS, plaques of demyelination are interspersed with and surrounded by reactive astrocytes. In addition, widespread focal reactive astrogliosis of varying intensity throughout the white and grey matter is characteristic for the disease. Astrocytes during MS may also demonstrate the phenomenon of ”emperipolesis”, i.e., an astrocyte apparently engulfing one or more other cells such as oligodendroglia (Ghatak 1992) and lymphocytes (Furer et al. 1993). The role of astrocytes during MS and related disorders is under intense investigation. Astrocytes play an active role during degeneration and demyelination by promoting inflammation, damage of oligodendrocytes and axons, and glial scarring but might also be beneficial by creating a permissive environment for remyelination and oligodendrocyte precursor migration, proliferation, and differentiation. Astrocytes secrete a multitude of harmful cytokines such as TNF-α or interleukin 1β (IL1β) as well as pro-inflammatory prostaglandins (Kipp et al. 2007; Kipp et al. 2008). This makes it likely that astrocytes are implicated in oligodendrocyte damage and, in consequence, in promoting demyelination. Astrocytes might contribute to regeneration and remyelination by secreting important growth factors, i.e., platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF1), and basic fibroblast growth factor (FGF-2). All of these factors are known to promote remyelination and prevent oligodendrocyte apoptosis. On the other hand, it has been shown that the glycosaminoglycan hyaluronan, synthesized by activated astrocytes, accumulates in chronically demyelinated MS lesions and inhibits oligodendrocyte progenitor (OPC) differentiation and consequently remyelination *in vivo* (Back et al. 2005). Of relevance is a recently published review article which focuses on astrocytes as producers of secreted factors that can either promote or impede myelination within the CNS and how these cells are currently thought to be involved in the myelin pathology of MS (Moore et al. 2011).

We stress at this point that the secretion of pro-inflammatory molecules such as TNF-α or IL-1β from astrocytes does not necessarily has a negative impact on MS disease progression. The importance of inflammation for reparative processes such as remyelination is well-documented (Patani et al. 2007). The cytokines TNF-α and IL-1β promote proliferation of OPC and, in consequence, remyelination (Arnett et al. 2001; Mason et al. 2001). Major histocompatibility complex-II (MHC-II)-null mice display delayed remyelination and regeneration of oligodendrocytes compared to their wild type littermates after cuprizone- or Theiler's virus-induced demyelination

(Arnett et al. 2003; Njenga et al. 1999). Furthermore, the activation of an inflammatory response by a combination of growth factors leads to myelin repair (Biancotti et al. 2008) clearly demonstrating a beneficial role for inflammation in the CNS. Other factors related to inflammation, such as interferon-γ (Lin et al. 2006; Mana et al. 2009), however, might be more detrimental for remyelination. Understanding of the dual role of inflammatory cytokines/factors to enable control and manipulation of these responses thus represents a major goal for the development of new therapies.

2. Scope of the study

Our research group aimed to characterize the cuprizone mouse model in detail with regard to regional susceptibility for myelin loss and innate immune resposes. One hallmark of MS is dissemination in space. We, therefore, investigated the mouse basal ganglia, hippocampus, and cerebellum, and performed histopathological and gene expression studies of these brain regions. The focus of my reserach was the intrinsic inflammatory response (e.g. astrocyte and microglia activation) and the kinetic and magnitude of demyelination in white and grey matter areas of the cerebellum. Results and discussion are given in **Chapter I**.

As already mentioned, glucocorticoids (GC) are the gold standard in treating acute MS relapses but not much is known whether and how they also interfere with regenerative processes. To be able to distinguish between the immunomodulating effects of GC and direct effects on repair capacity of the brain, we used the potential of non-immune driven cuprizone intoxication to serve as a model for spontaneous repair/remyelination. We investigated the effects of high dose GC treatment on the remyelination capacity of the mouse brain *in vivo* and addressed the underlying mechanisms by *in vitro* models. Results are summarized and discussed in **Chapter II**.

Recent and ongoing research in our lab focuses on the role of glia cells, in particular astrocytes, during reparative and inflammatory processes in the brain. Amyloid precursor protein (APP) positive glia cells can be found in MS lesions (Gehrmann et al. 1995). Although not much is known about the biology of glia-derived APP, it is thought to be involved in brain intrinsic inflammatory responses. In the third part, we addressed APP expression in the cuprizone model for MS. Results are given and discussed in **Chapter III**.

3. Materials and methods

3.1 Animal treatment and induction of demyelination and remyelination

C57BL/6 male mice were obtained from Harlan Winkelmann (Borchen, Germany). Animals underwent routine cage maintenance once a week and microbiological monitoring according to the Federation of European Laboratory Animal Science Associations recommendations. Food (Sniff Spezialdiäten, Germany) and water were available *ad libitum*. Research and animal care procedures were approved by the Review Board for the Care of Animal Subjects of the district government (North Rhine-Westphalia, Germany) and performed according to international guidelines on the use of laboratory animals. Demyelination was induced by feeding 20g +/- 1g (app. 8 weeks old) male C57BL/6 mice a diet containing 0.2% cuprizone (Sigma-Aldrich Inc., Germany) mixed into a powdered ground standard rodent chow (Sniff Spezialdiäten, Germany) for the indicated period (up to 7 weeks, depending on the experimental analysis) and animals were sacrificed at the indicated time point.

For remyelination studies, the animals were divided into experimental groups I – VI (compare figure 7). Group I received normal chow during the indicated time point, whereas animals from group II-VI were fed cuprizone for 5 weeks to induce acute demyelination of the CC. Group II was sacrificed at week 5 to demonstrate demyelination. Group III received 9 days, group IV 21 days vehicle (NaCl) injection, respectively to allow animals to recover (i.e. remyelination). Group V and VI were treated every day with intra peritoneal (i.p.) MP (Urbasol, Sanofi-Aventis, Germany; 15 mg/kg body weight) for the respective period. Animals that were used for triiodthyronin (T3) ELISA analysis were allowed to recover for 3 days, receiving MP (15mg/kg body weight) or NaCl, respectively, every day.

For histological/immunohistochemical analysis, mice were transcardially perfused with 2 % paraformaldehyde containing 1.5 % saturated picric acid (for perfusion solution and detailed procedure, see Appendix 1) after the indicated period as published (Groebe et al. 2009; Norkute et al.; Pott et al. 2009).

3.2 Tissue preparation

After perfusion (Appendix 1) and overnight post fixation in the same fixative, brains were dissected. Embedded brains (details of the embedding procedure are summarized in Appendix 2) were coronally sectioned each 5 µm in thickness. Investigated levels according to Sidman et al. (http://www.hms.harvard.edu/research/brain/atlas.html) were 455, 465, and 475 (cerebellum), levels 285, 305, and 325 (hippocampus) and levels 215 and 265 (APP, corpus callosum). For gene expression analysis, mice were killed by rapid decapitation. Brains were quickly removed and the brain areas of interest (entire cerebellum without the cerebellar peduncles, the entire hippocampus, and the basal ganglia at the level of the commisura anterior, the entire CC and the cerebral cortex) separated using a stereomicroscopic approach. All tissues were immediately shock-frozen in liquid nitrogen and kept at -80°C until used.

3.3 Luxol fast blue (LFB) staining and immunohistochemistry (IHC)

For LFB staining, 5 µm-thick sections were placed on superfrost glass slides (Thermo Fischer, Germany). Sections were deparaffinised in 2 x 10 min xylol, rinsed in 2 x 10 min 100% ethanol and 2 x 5 min 95% ethanol, then incubated in a LFB solution (0.01% in 95% ethanol) overnight at 60°C. Sections were processed in a lithium carbonate solution (0.05% in bidest. H_2O; Roth, Germany), differentiated in 70% ethanol and counterstained with cresyl violet (myelinated fibers appear blue, the neuropil pink, and nerve cells purple).

For IHC, 5 µm-thick sections were placed on superfrost glass slides (Thermo Fischer, Germany). Sections were rehydrated and heat-unmasked where advantageous (see table 2), blocked with PBS containing 2% normal horse serum for 1 h, and incubated overnight with the primary antibody diluted in blocking solution (for the detailed procedure, see Appendix 3). Anti proteolipoprotein (PLP) antibody, anti 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) antibody and anti-MAG antibody (rabbit IgG; Santa Cruz, Germany) were used as myelin markers, whereas glial fibrillary acidic protein (GFAP) was used to visualize astrocytes. Anti-oligodendrocyte transcription factor-2 (Olig2) antibody was used to visualize OPC, whereas anti-Adenomatous Polyposis Coli (APC) antibody was used to detect mature oligodendrocyte cell bodies. Ionized calcium binding adapter protein 1 (Iba-1) was used as a marker to detect microglia/macrophages. This protein has been proven as

a valid marker for microglia as well as macrophages (Cheung et al. 2008; Wells et al. 2005). An overview of all primary antibodies used in this work is given in table *2*.

Antibody (primary)	Manufacturer	Host	ICC	IHC and AGR	WB
Adenomatus Polyposis Coli (APC)	Calbiochem OP80	mouse		1:200 (citrate buffer)	
Amyloid Precurser Protein APP	Abcam ab15272	rabbit	1:200	1:100 (citrate buffer)	1:200
Beta-Actin	Sigma A 2066	rabbit			1:1000
CNPase (SMI91)	Abcam ab24566	mouse		1:200 (citrate buffer)	1:200
Fibroblast growth factor (FGF-2)	Santa Cruz sc-79	rabbit			1:200
Glial fibrillary acidic protein (GFAP)	Santa Cruz sc-65343	mouse		1:10	
Glial fibrillary acidic protein (GFAP)	Encore RPCA-GFAP	rabbit		1:1000	
Glucocorticoid receptor (GC)	abcam ab52190	rabbit	1:50	1:500	
Ionized calcium binding protein 1 (Iba1)	Wako 019-19741	rabbit		1:250 - 1:1000 (Tris-EDTA buffer)	
Myelin-associated glycoprotein (MAG)	Santa Cruz sc-15324	rabbit		1:50 (citrate buffer)	
Oligodendrocyte transcription factor 2 (Olig2)	Abcam ab81093	rabbit		1:500 (Tris-EDTA buffer)	
Proteo Lipoprotein (PLP)	Serotec MCA839G	mouse		1:500	

Table 2 Information about used primary antibodies and the respective concentrations are summarized. ICC = Immunocytochemistry; IHC = Immunohistochemistry; AGR = antigen retrival; WB = western blot

After washing for 2 x 5 min in PBS, sections were incubated with biotinylated secondary antibodies for 1 hour, followed by peroxidase-coupled avidin-biotin-complex (ABC kit, Vector Laboratories, UK). The immune-precipitated product was visualized with the ACE reaction (Invitrogen, Germany) following the suppliers protocol until satisfying staining intensity was achieved and reaction was stopped in H_2O. Slides were mounted with Kaiser's glycerine gelatine (Merck, Germany). Stained and processed sections were documented with a Nikon ECLIPSE 55i camera. For double-staining, the following fluorescently-labeled secondary antibodies were applied over night at 4°C: Alexa Flour 488 donkey anti-mouse IgG or Alexa Flour 568 goat anti-rabbit IgG. Sections were analyzed by laser-fluorescent

microscopy (Zeiss Axiophot) or confocal microscopy (LSM 710, Zeiss, Germany). An overview of all secondary antibodies is given in table 3.

Antibody (secondary)	Manufacturer	Host	ICC	IHC	WB
Alexa-Fluor 488	Invitrogen A11034	Donkey	1:500	1:500	
Alexa-Fluor 568	Invitrogen A11004	Goat	1:500	1:500	
Biotinilated secondary antibody anti mouse	Vector labs BA-2000	Horse		1:500	
Biotinilated secondary antibody anti rabbit	Vector labs BA-1000	Horse		1:500	
HRP-conjugated anti-mouse	Abcam ab6728	Rabbit			1:3000
HRP-conjugated anti-rabbit	BioRad Immune star #170-5046	Goat			1:3000

Table 3 Information about used secondary antibodies and respective concentrations are summarized. ICC = Immunocytochemistry; IHC = Immunohistochemistry; WB = western blot

3.4 Immunocytochemistry (ICC)

ICC to detect the expression of glucocorticoid receptor (GCR) and GFAP in primary astrocyte cultures was performed using appropriate secondary fluorescence antibodies (Alexa Fluor 488 and Alexa Fluor 568; table 3). Therefore, cells were grown on poly-L-ornithin (Sigma, Germany) coated glass slides in 24 well plates. Fixation was performed for 20 min in 3.7% formalin (Roth, Germany), washed 2 x 5min in PBS and kept at 4°C until used. Cells were permeabilized by incubating in 0.2% Tween 20 for 10 min. After washing for 2 x 5 min in PBS, the slides were incubated for 20 min with 1% bovine serum albumin (BSA) in PBS to block unspecific antibody binding. Primary antibody was diluted in 1% BSA in PBS and cells incubated over night at 4°C. After washing for 3 x 5 min in PBS, counterstaining with 1µg/ml Hoechst (Invitrogen, Germany) for 10 min, and additional washing for 3 x 5min in PBS, slides were mounted in Fluor Preserve^{TM} (EMD Biosciences, USA) and analyzed by laser-fluorescent microscopy (Axiophot, Zeiss, Germany).

3.5 Quantification of demyelination

Immunohistological investigations of myelination were performed at the levels 215, 265 (basal ganglia and CC), 285, 305, 325 (hippocampus), 465 and 475 (cerebellum) according to the mouse brain atlas by Sidman *et al*. Four to five animals/group were included. Each two to three slices (5µm) per animal, investigated brain region, and indicated time point were examined. All manual scorings were performed by at least 2 experienced and blinded observers.

See Figure 1A/B for cerebellar subregions. Additionally, densitometric measures of the CC were performed on binary converted pictures using the freely available ImageJ-software (National Institute of Health, USA) giving similar results. To this end, pictures were taken from the medial and lateral parts of the CC, resulting in a total of 6 pictures per animal, investigated brain region, and indicated time point.

3.6 Quantification of oligodendrocyte cell numbers

Quantification of oligodendrocytes was achieved by manual counting the number of APC-positive cells in the investigated regions. At least two slices per animal, investigated brain region, and indicated time point were analyzed by at least two independent observers. Cell numbers were calculated and expressed per mm^2. Cell counting was performed using an Axiophot microscope (Zeiss, Germany) and a 10x objective in the cerebellum, CC and hippocampus region (including cells in the DHC, the SLM and the hilus of the dentate gyrus (HDG) and the fimbria region) and a 40x objective at the basal ganglia level. Cell counting was performed with a Nikon ECLIPSE 55i microscope.

3.7 Quantification of astrocyte parameters

Since astrocytosis may result from hypertrophy or hyperplasia, quantification of astrocyte parameters was performed in the cerebellar cortex and cerebellar marrow, in the hippocampal SLM and the HDG, the basal ganglia and CC. To determine hyperplasia of astrocytes, GFAP-positive cells with a clearly visible nucleus were counted in three different sections per animal, investigated brain level, and time point. Cell numbers were calculated and expressed per mm^2. Cell counting was performed using the Axiophot microscope (Zeiss, Germany) and a 10x or 40x objective. To quantify hypertrophy of astrocytes, the area occupied by GFAP-positive astrocytes with a clearly visible nucleus was measured using a morphometric device and the

respective imaging software (NIS elements AR 2.30, Leica, Germany). This measurement was performed using a 40x objective. Data are finally expressed as μm^2 area/cell.

3.8 Real-time reverse transcriptase-polymerase chain reaction (rtRT-PCR)

Gene expression was measured using the rtRT-PCR technology (BioRad, Germany), QTM SYBR Green Supermix (Bioline, Germany), and a standardized protocol. Isolation of total tissue RNA was performed with NucleoSpin kit (Macherey-Nagel, Germany). For cell culture experiments, RNA was isolated with PeqGold (PeqLab, Germany). RNA concentration and purity were assessed using the OD260 and OD260/OD280 ratios, respectively, and reverse transcribed using an Invitrogen MMLV RT-kit and random hexanucleotide primers (both Invitrogen, Germany). PCR reactions were carried out in a reaction mixture consisting of 2 µl cDNA, 2 µl RNAse-free water (Invitrogen, Germany), 5 µl QTM SYBR Green Supermix (BioLine, Germany), and 0.5 µl of primer (100 pmol/µl) in standard tubes using the MyIQ rtPCR Detection System (BioRad, Germany) under following conditions: 10 min enzyme activation at 95°C, 45 cycles of 15 s denaturation at 95°C, 30 s annealing at individual temperatures, 30 s amplification at 72°C, and 5 s fluorescence measurement at 80°C. Primer sequences are shown in table 4. Relative quantification was performed using the ΔCt method which results in ratios between target genes and a housekeeping reference gene (HPRT) (Kipp et al. 2008). As the validity of this method critically depends on the constant expression of the housekeeping gene, constant expression of HPRT was tested against other housekeeping genes (not shown). In each run, external standard curves were generated by several fold dilutions of target genes. The concentration of the target genes was calculated by comparing Ct values in each sample with Ct values of the internal standard curve. Finally, data were expressed as the ratio of the amount of each transcript versus the concentration of HPRT. Melting curves and gel electrophoresis of the PCR products were routinely performed to determine the specificity of the PCR reaction (not shown).

Primer	sense	antisense	bp Length
Amyloid precursor protein (APP)	ACCCCAGATCGCCATGTTC	CCCACTTTCCATTCTGCACATTC	64bp
Fibroblast growth factor (FGF-2)	CCAACCGGTACCTTGCTATG	TATGGCCTTCTGTCCAGGTC	203bp
Glycerinaldehyd-3-phosphat-Dehydrogenase (Gapdh)	AACCCATCACCATCTTCCAG	GTGGTTCACACCCATCACAA	196bp
Glial fibrillary acidic protein (GFAP)	GAGATGATGGAGCTCAATGACC	CTGGATCTCCTCCTCCAGCGA	380bp
Hypoxanthin-Guanin-Phosphoribosyltransferase (HPRT)	GCTGGTGAAAAGGACCTCT	CACAGGACTAGAACACCTGC	248bp
Insulin like growth factor (IGF1)	CTGCACCAGACACCCTTTGC	GGACGGGGACTTCTGAGTCTT	264bp
Myelin Basic Protein (MBP)	CCATCCAAGAAGATCCCACA	CCTGTCACCGCTAAAGAAGC	193bp rat/ 187bp mouse
Platelet derived growth factor α receptor (PDGFRα)	AGGCTTGGGGCTCACTTTTT	TCAGCGGCACAATCTTCTCA	199bp
Platelet derived growth factor αα (PDGF-αα)	GAGGAAGCCGAGATACCCC	TGCTGTGGATCTGACTTCGAG	60bp
Proteo Lipoprotein (PLP)	TGGCGACTACAAGACCACCA	GACACACCCGCTCCAAAGAA	115bp both species

Table 4 Primer sequences and lenght of the resulting product in bp.

3.9 Adrenocorticotrop hormone (ACTH) and T3 ELISA

About 1 ml blood of mice was collected by cardiac puncture and incubated at room temperature for 30 min. After centrifugation at 2000 x g for 10 min, serum-supernatant was collected and stored at -20°C until used. ELISA was performed in 96-well plates according to the manufactures instructions (mdbiosciences, Switzerland (ACTH) and GenWay, USA (T3)). Absorbance was measured with a TECAN ELISA-reader at 450 nm (ACTH-concentrations to 150 pg/ml and T3 ELISA) and 405 nm to be able to interpolate to concentrations higher than 150 pg/ml (ACTH only), as recommended by the supplier.

3.10 Cell culture experiments

3.10.1 Primary oligodendrocyte progenitor cells (OPC) and neonatal astrocyte cultures

OPC cultures were established following a modified protocol as described previously (Richter-Landsberg and Vollgraf 1998). One to three day old rat pups were killed by rapid decapitation, cerebral cortices were dissected, meninges and blood vessels removed and dispersed in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Germany) containing 10 % heat inactivated foetal calf serum (FCS) (PAA, Austria) and the antibiotics penicillin/streptomycin (0.5 %, Gibco, Germany). Tissues were pooled, homogenized with a pipette, and cells were seeded in GM in 50 ml flasks (Greiner, Germany). They were allowed to grow for 4 days, followed by a GM change every third day. After approximately 14 days in culture, an astrocyte monolayer had formed on the bottom of the flasks with microglia and OPC growing on top. For OPC isolation, GM was replaced, cells incubated for 1 h in the incubator and agitated at 100 rpm for 2 h at 37°C to remove debris, dead cells, and microglia. Subsequently, flasks were washed with pre-warmed PBS, incubated with 7 ml of GM for 1 h at 37°C 5 % CO_2, and placed on the shaker at 240 rpm and 37°C for 18 h to de-attach OPC from the astrocyte monolayer. GM was removed, flasks were washed gently with pre-warmed PBS and both, collected GM and PBS, were combined. Collected OPC were centrifuged for 10 min at 800 x g, pellet containing OPC re-suspended in 10 ml GM, and seeded at a density of 2 x 10^6 cells/well on poly-L-ornithin (Sigma, Germany) coated 6-well plates. After 1 h, cells were gently washed with 37 °C PBS and incubated in 37 °C DMEM plus supplement (insulin- (5 µg/ml) transferrin- (5 µg/ml) sodium selenite- (25 ng/ml; Boehringer, Germany). Dexamethasone (Dex) treatment was started directly after washing (10^{-6} M; Sigma-Aldrich, Germany).

Primary astrocytes were prepared from 2-to-4-day-old Balb/c mice (Harlan Winkelmann, Germany) as described in detail previously (Braun et al. 2009). Briefly, cerebral cortices were dissected, meninges removed and dispersed in Dulbecco's phosphate-buffered saline containing 1% trypsin and 0.02% ethylene-diaminetetraacetic acid (EDTA), filtered through a 50-mm nylon mesh, and centrifuged at 400 x g for 5 min. Cells were re-suspended in DMEM (Invitrogen, Germany) containing 20% heat-inactivated fetal calf serum (PAA, Austria), streptomycin (0.5%) and fungizone (0.1%, both from Invitrogen, Germany). Finally,

cells were seeded in 100 mm plates coated with poly-L-ornithin (Sigma, Germany) at a density of 1–2 x 10^6 cells per cm^2. Astrocytes were cultured at 37 °C in a humidified incubator with 5% CO_2. Medium was replaced every third day. Upon reaching confluency, cells were trypsinized and replated at a lower density. This procedure was repeated twice. The resulting final astroglia culture is characterized by >95% homogeneity of glial fibrillary acidic protein positive cells (Braun et al. 2009; Pawlak et al. 2005).

3.10.2 Cell culture treatment

For MP or Dex treatment, steroids (both from Sigma-Aldrich, Germany) were dissolved in ethanol (100 % p.a. quality) at concentrations of 10^{-3} M, and further diluted in 1 x PBS to yield final dilutions. Appropriate concentrations of ethanol were added in control groups. CS treatment of OPC was performed at day 0 and, after changing the medium, at day 3. CS treatment of astrocytes was performed on day 0 for 24h.

Fibroblast growth factor (FGF2) and platelet derived growth factor (PDGF-αα); (both Peprotech, Germany) were dissolved in 1 x PBS and applied in a final concentration of 5 ng/ml each.

LPS (Sigma-Aldrich, Germany; 100 ng/ml), TNFα (Invitrogen, Germany; 100 ng/ml), glutamate (Sigma-Aldrich, Germany; 4 µM) or H_2O_2 (Roth, Germany; 200 µM) treatment of astrocytes was performed for 24 h. Previous studies revealed that the applied concentrations are sufficient to activate astrocytes, however, are not toxic in the applied concentrations (Braun et al. 2009).

3.11 Western blot

Western blot analysis was performed following a modified protocol as published previously (Kramer et al. 2010; Norkute et al. 2010). For *in vivo* studies, the appropriate brain areas were dissected and snap frozen as described above, proteins isolated and finally dissolved in PLB-TCEP provided with the NucleoSpin® RNA/Protein kit (Machery-Nagel, Germany). Protein concentrations were determined using the trichloracetic acid method (Kramer et al. 2010). Subsequently, same amounts of protein (10-14 µg) were loaded on each lane, separated by 8% SDS poly acrylamide gel electrophoresis (SDS-PAGE; for details see Appendix 4) and blotted

onto a nitrocellulose membrane (Hybond, amersham pharmacia biotech, Great Britain) for 1 h at 10V.

For *in vitro* studies, cell culture-derived protein was isolated using RIPA buffer (50 mM Tris HCl pH = 8; 150 mM NaCl; 1 % NP-40; 0.5 % sodium deoxycholat; 0.1 % SDS) containing complete Mini Protease inhibitor cocktail tablets (Roche, Germany) according to the manufacturer's recommendation. Cells were harvested by scraping, washed and centrifuged. Supernatant was collected and protein concentration determined by Pierce BCA™ Protein Assay Kit (Thermo Fischer, USA). Same amounts of protein (10 µg) were electrophoretically separated on 8% to 10 % polyacrylamide gels (depending on the size of the target protein) and then transferred onto nitrocellulose membranes (BioRad, Germany) for 1 h at 10 V. The nitrocellulose membranes were blocked after washing in PBS (3 x 5 min) with 5 % fat-free milk solution in PBS for 30 min. The membranes were then incubated with primary antibodies diluted in 5 % milk in PBS-Tween over night at 4 °C. After washing with PBS, appropriate secondary antibodies conjugated to horse-radish peroxidase were added to the membranes and incubated for 2 h at room temperature (see tables 2 and 3 for antibodies and dilutions). The membranes were washed 3 x 5 min with PBS and then incubated with commercial enhanced chemoluminescence reagent kit (ABC Kit, Amersham Bioscience, UK) and subsequently exposed to X-ray film (Kodak, Germany).

3.12 Statistical analysis

Chapter I: 4-5 mice each were used for immunocytochemistry and gene expression analysis per experimental group in all experiments. For cell counting, staging of myelination, and analyzing cell volumina, an independent sample t-test was performed. For gene expression analysis and quantification of cells, differences between groups were statistically tested by an analysis of variance (ANOVA) followed by a Tukey's post-hoc multiple-range test using SPSS software (SPSS Inc., Chicago, IL, USA). All data are given as arithmetic means ± SEM. P values of the different analyses with $p<0.05$ were considered to be statistically different.

Chapter II: Number of animals used: Group I: 9 animals; II: 7 animals; III: 8 animals; IV: 3 animals; V: 9 animals; VI: 3 animals. Results of cell counting and analysis of myelination index were compared by independent sample t-tests. For gene expression analysis, statistics were made using data from all experiments. GraphPad

Prism version 5.00 for Windows, GraphPad Software, San Diego California USA was used for statistical analysis and preparation of graphs. For gene expression analysis, differences between groups were statistically tested by ANOVA followed by a Tukey's post-hoc multiple-range test using GraphPad software. All data are given as arithmetic means ± SEM. P values <0.05 were considered to be statistically different. Cell culture experiments regarding OPC differentiation consisted of $n = 4$ independent experiments. Experiments regarding growth factor supplementations of OPC were performed twice. All other cell culture experiments were conducted at least three times.

All *in vivo* experimental groups included in **Chapter III** consisted of at least 4 animals per group. Cell culture experiments have been conducted 3 times independently and differences between groups were statistically tested by ANOVA followed by a Tukey's post-hoc multiple-range test using GraphPad software.

4. Chapter I

Characterization of demyelination and innate immune responses in the cuprizone mouse model

Parts of this chapter have been published in:

Angela Groebe*, Tim Clarner*, Werner Baumgartner, Cordian Beyer and Markus Kipp

"Cuprizone treatment induces distinct demyelination, astrocytosis and microglia cell invasion/proliferation in the mouse cerebellum."

Cerebellum, (2009) 8:163–174

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4.1 Introduction

Our group intended to uncover histopathological and molecular changes within three different brain regions in cuprizone-treated animals. The CC and the cerebral cortex are well-investigated brain regions in this animal model and served as white and grey matter controls.

One area of interest was the mouse cerebellum, a well-known part of the brain involved in demyelinating events in MS (Gilmore et al. 2008; Kutzelnigg et al. 2007; Pokryszko-Dragan et al. 2008) and EAE (Craner et al. 2003; Tonra 2002). In MS, the brain stem and cerebellum are frequently sites of damage and appear to play a decisive role for clinically-isolated syndromes associated with MS. It is very likely that lesions located in these brain structures have an important impact on the development of disability in the definite forms of the disease (Yousry et al. 2000). Cerebellar dysfunction in MS is a significant contributor to disability, is relatively refractory to symptomatic therapy, and often progresses despite treatment with disease-modifying agents. Thus, there is a need for better understanding of its pathophysiology.

Determination of behavioural deficits is important to reveal beneficial therapeutic effects reflecting functional stabilization and/or recovery in various animal models affecting the CNS. Some studies reported behavioural deficits in the cuprizone model. It was reported that after 5 weeks of cuprizone treatment, sensorimotor reactivity was affected as determined by the rota-rod test (Franco-Pons et al. 2007). Motor skill deficits were also reported by another group after cuprizone exposure using a more advanced testing battery, namely the motor skill sequence test (Liebetanz and Merkler 2006). Affected motor skill performance in cuprizone exposed animals is thought to be due to commissural demyelination such as the CC (Matsushima and Morell 2001). However, poor outcome on motor skill performance testing might also be due to an involvement of other brain areas, above all the cerebellum.

We have recently published a study of the basal ganglia system (Pott et al. 2009) which is frequently involved in MS pathology as well (Hasan et al. 2009; Henry et al. 2008; Nociti et al. 2008; Pirko et al. 2007). In this report, we came to the conclusion that "Smaller values in the absolute volume of the caudate nucleus (CN) and in relation to total intracranial volume were observed in relapsing-remitting patients compared to healthy controls (Hasan et al. 2009). Demyelination and

neurodegeneration within the basal ganglia has been linked to several MS symptoms such as fatigue (DeLuca et al. 2008; Leocani et al. 2008), impairment of memory (Benedict et al. 2009; Brassington and Marsh 1998), and dysfunction of motor skills (Wei et al. 2010). At present, no adequate animal model for investigating mechanisms related to basal ganglia demyelination has been described. The basal ganglia represent sub-cortical nuclei interconnected with the cerebral cortex, thalamus, and brainstem which are implicated in a variety of functions such as motor control, cognition, emotion, and learning. The five individual nuclei that make up the primate basal ganglia along with their major subdivisions are the striatum (i.e. putamen and caudate nucleus), the external and internal segment of the globus pallidus, the subthalamic nucleus, and the substantia nigra (Bakker and Ludwin 1987). The striatum is a heterogeneous mosaic of two neurochemically, developmentally, and functionally distinct compartments, i.e. the mu-opioid receptor-enriched striosomes and the matrix (Miura et al. 2007; Pott et al. 2009; Sato et al. 2008)."

As a third brain structure we included the hippocampus formation (Norkute et al. 2009) which is believed to be crucial for long-term episodic memory,, yet, its precise role still remains elusive (Bird and Burgess 2008; Burns and Young 2000; Kumaran and Maguire 2005): To the end, we found and discussed that "Cognitive impairment has been documented in MS (Brex et al. 2002; Potter 2002). A large number (40–65%) of patients with established MS experience cognitive deterioration of variable severity (Summers et al. 2008; Tintore et al. 2006). Deficits in verbal and visual recall (DeLuca et al. 1998; Lazeron et al. 2005), speed of information processing (DeLuca et al. 2004), and aspects of executive function, particularly abstract reasoning (Foong et al. 1999), are commonly reported. Memory impairment is a striking feature within the spectrum of cognitive deficits in MS patients (Bobholz and Rao 2003; Rao et al. 1991a; Rao et al. 1991b). The hippocampus comprises distinct regions, including the dentate gyrus and the literal hippocampus, which is subdivided into three fields, cornu ammonis (CA) regions 1-3 (Frotscher 1992; Frotscher et al. 1991; Leranth and Frotscher 1986; Lubbers and Frotscher 1987). Geurts et al. (2007) recently reported extensive hippocampus demyelination in MS patients in parallel with microglia and/or macrophage invasion and activation that strongly correlates with cognitive decline. This coincidence suggests that hippocampal demyelination is directly related to cognitive impairment. Despite the frequency and high functional impact, the

knowledge about effective strategies for managing cognitive impairment in MS is low. Research on therapeutic options for cognitive impairment in MS is still in the early stages. Available disease-modifying therapy strategies in MS only provide modest benefit for cognitive performance and improvement of cognitive dysfunction. As for nonpharmacological interventions that are based on cognitive rehabilitation, few studies have used an experimental approach, and in general, results have been disappointing. Therefore, further research strategies and animal models are needed in this area (Amato et al. 2006). Although novel insights into pathological processes related to MS gain in importance (Keegan et al. 2005; Lassmann 2008), the complexity of pathophysiological interactions are poorly understood (Norkute et al. 2009)."

In the cerebellum, the status of myelination and oligodendrocyte depletion was determined by histological staining and IHC for PLP and APC. Gene expression analysis of mature oligodendrocyte markers such as PLP and MBP was additionally conducted. Astrocyte and microglia responses were investigated by IHC, rt-RTPCR and quantification of both cell types.

4.2 Results

4.2.1 Demyelination of distinct cerebellar areas

Figures 1A/C/E and 1B/D/F show an anti-PLP stained cerebellum of a male control and cuprizone treated mouse, respectively. The effect of cuprizone (0.2%, 5 weeks) on demyelination was studied in different white and grey matter regions, namely the molecular layer (ML), the granular layer (GL), the pyramidal layer (PL), and the white matter layer (WM) of the cerebellar cortex (CCx), as well as the cerbellar marrow (CM) containing the rodent lateral cerebellar nucleus (LCN), medial cerebellar nucleus (MCN), and interpositos nucleus (IN). The regions are outlined in figure 1.

Effect of cuprizone on myelination of these regions is shown in figures 1A (control animal) and 1B (cuprizone-treated) in an overview. Intact myelin in anti-PLP stained sections appears as dark fiber tracts. Cuprizone exposure resulted in a massive demyelination of the cerebellar marrow as detected by a loss of anti-PLP stained fibers in this region. Demyelination of the cerebellar marrow was mainly observed in all three cerebellar nuclei (figs. 1A-D). The surrounding area of the cerebellar nuclei, i.e. the white matter of the cerebellar marrow, was not affected by cuprizone treatment (fig. 1A/B). Interestingly, myelin sheaths of the cerebellar cortex including the molecular layer, the granular layer, the pyramidal layer and the white matter layer were also not affected (fig. 1E/F). As shown in figure 1G, scoring of anti PLP-stained sections from zero to three revealed that the myelin status of cerebellar nuclei is significantly reduced by approx. 80% after the 5 weeks cuprizone challenge (control: 3, cuprizone: 0.42±0.14, $p<0.01$).

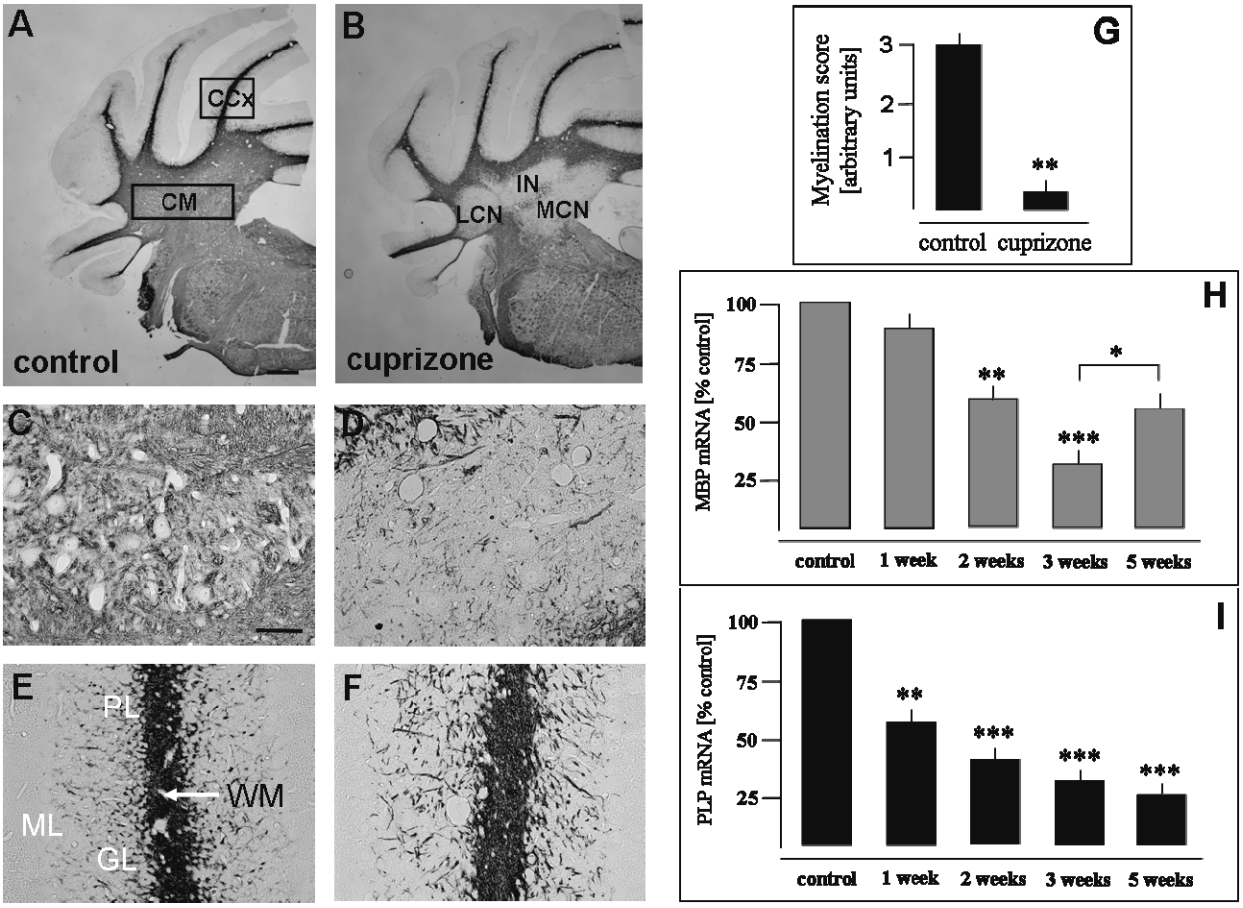

Figure 1 Effect of cuprizone (5 weeks, 0.2%) on myelination of different cerebellar white and grey matter areas in male C57BL/6 mice. (A) shows anti PLP-staining of a control animal and gives and overview of the two principal regions included in this study. (B) shows anti-PLP staining of a mouse treated 5 weeks with cuprizone. The upper box in (A) indicates the cerebellar cortex region (CCx) including the molecular layer (ML), the pyramidal layer (PL), the garnular layer (GL) and the cerebellar white matter (WM) as detailed indicated in (E). The lower box in (A) indicates the cerebellar marrow containing the rodent cerebellar nuclei, namely the lateral cerebellar nucleus (LCN), medial cerebellar nucleus (MCN), and interpositos nucleus (IN), as detailed indicated in (B). (C) and (D) show the interpositus nucleus of a control and cuprizone treated animal, respectively, whereas (E) and (F) show the cerebellar cortex of a control and a cuprizone treated animal, respectively, in a higher magnification. Note profound demyelination in the rodent cerebellar nuclei, whereas, the cortical region is not affected. As shown in (G), quantitative assessment of cerebellar nuclei myelination revealed a significant reduction in myelin score by >95% compared to control (**p<0.01). (H) and (I) show quantitative analysis of MBP and PLP gene expression during 5 weeks of cuprizone treatment in the entire cerebellum.. Values were normalized against a housekeeping gene (HPRT) and expressed as % of controls (**p<0.01, and ***p<0.001 compared to controls; * p<0.05 3 weeks compared to 5 weeks). Scale bars: 200µm (A/B); 20µm (C-F).

For further quantification, real-time gene expression analysis of two marker proteins of mature oligodendrocytes (MBP and PLP) for the total cerebellum not containing the cerebellar peduncles was performed to obtain quantitative information about the time-dependent decline in oligodendrocyte myelin formation capacity after cuprizone exposure (0.2%, different time intervals). Quantification of MBP and PLP mRNA expression levels in the total cerebellum supports the above observation. As shown in figures 1H and I, both myelin marker genes decreased stepwise until week 3. First significant reductions ($p<0.01$) were already observed after 2 weeks cuprizone for MBP (30% compared to controls) and after 1 week for PLP (45% compared to controls). Levels of PLP further declined by approx. 70% compared to controls till week 5 ($p<0.001$). Levels of MBP declined till week 3 by approx. 70% compared to controls ($p<0.001$) whereas a partial recovery was detectable till week 5 ($p<0.05$).
Concordantly with results obtained by anti-PLP immunohistochemistry, the number of mature oligodendrocyte cell bodies gradually decreased in areas of the cerebellar nuclei during the 5-weeks cuprizone exposure period (figs. 2A-C).

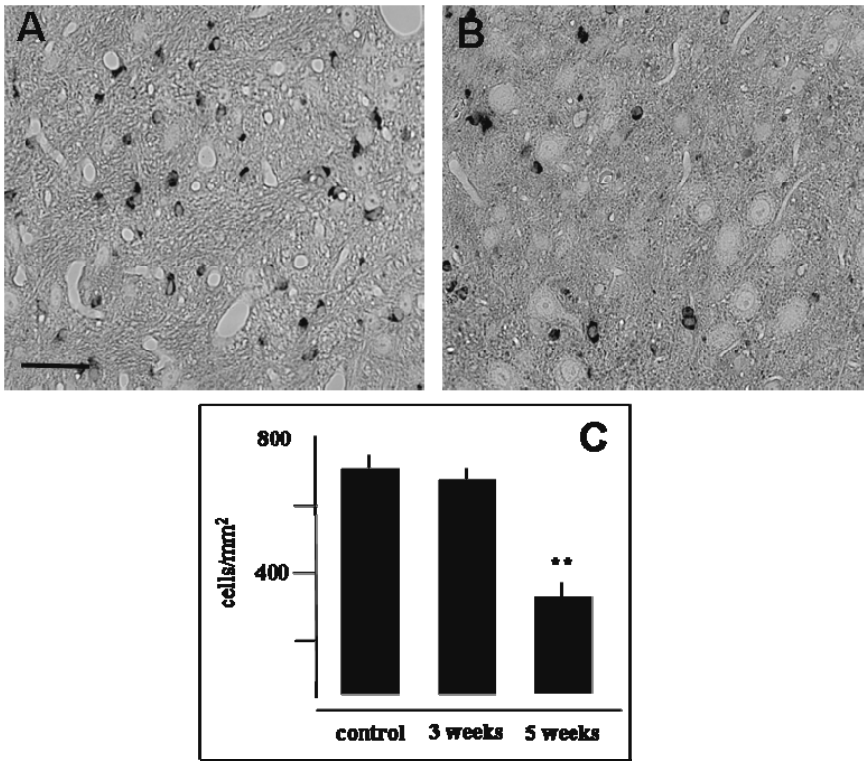

Figure 2 Effect of cuprizone exposure (0.2%, 5 weeks) on the distribution of APC-positive oligodendrocytes in the cerebellar nuclei. (A) and (B) show anti APC-staining of a control and cuprizone treated animal in the rodent interpositus cerebellar nucleus, respectively. Arrows in (A) indicate APC-positive cells. Note the decline of mature oligodendrocyte cell bodies after 5 weeks of cuprizone exposure. (C) shows quantification of the number of APC-positive cells in the rodent interpositus cerebellar nucleus. Numbers of APC-positive cells are expressed as cells/mm^2. **$p<0.01$ compared to controls. Scale bar: 20µm

Exemplarily, the quantitative evaluation of mature oligodendrocyte numbers in the interpositus nucleus is presented (fig. 2C). It reveals a significant ($p<0.01$) reduction of APC-positive cells (control: 709±12, cuprizone 5-weeks: 333±19). The medial and the lateral cerebellar nucleus exhibited a similar quantitative loss in APC-positive cells (quantification and picture not shown).

4.2.2 Astrocyte and microglia responses during cerebellar demyelination

In a next step, we analyzed astrocyte and microglia reactions in the affected cerebellar areas. All images and bars show results from the interpositus nucleus. However, the medial and the lateral cerebellar nuclei exhibited similar changes (data not shown). During cuprizone-induced demyelination, higher numbers of GFAP-positive astrocytes are seen within the cerebellar nuclei and the surrounding cerebellar marrow (figs. 3B and D) compared to controls (figs. 3A and C). In addition, the morphology of astrocytes revealed thin cells only possessing a few short processes under control conditions (fig. 3C). In contrast, during cuprizone intoxication, astrocytes became hypertrophic and multipolar (fig. 3D). Furthermore, we observed a pronounced increase in astrocyte cell numbers in the CC and adjacent cortex that served as control regions (data not shown). Quantification of GFAP mRNA expression in the total cerebellum supports the above observation. GFAP induction was seen after 5 weeks (fig. 3 E, $p<0.001$). In order to further characterize astrocyte reaction and to precisely relate astrogliosis or to exclude hyperplasia, we have performed morphometric measurement of GFAP-positive cells in the areas of the cerebellar nuclei. First, the number of astrocytes per unit (mm^2) increased significantly ($p<0.001$) until week 5 compared to controls (fig. 3F). Absolute numbers were 218±14 cells/mm^2 (control) and 461±23 cells/mm^2 (cuprizone). Furthermore, the average area occupied by an astrocyte was 13.5±12 μm^2/cell (control) compared to 89±13 μm^2/cell at week 5 (fig 3G). This hyperplasia reaction was highly significant ($p<0.001$). As shown in figs. 3A and B, such increase in GFAP positive cells was not visible in any investigated cerebellar cortex region.

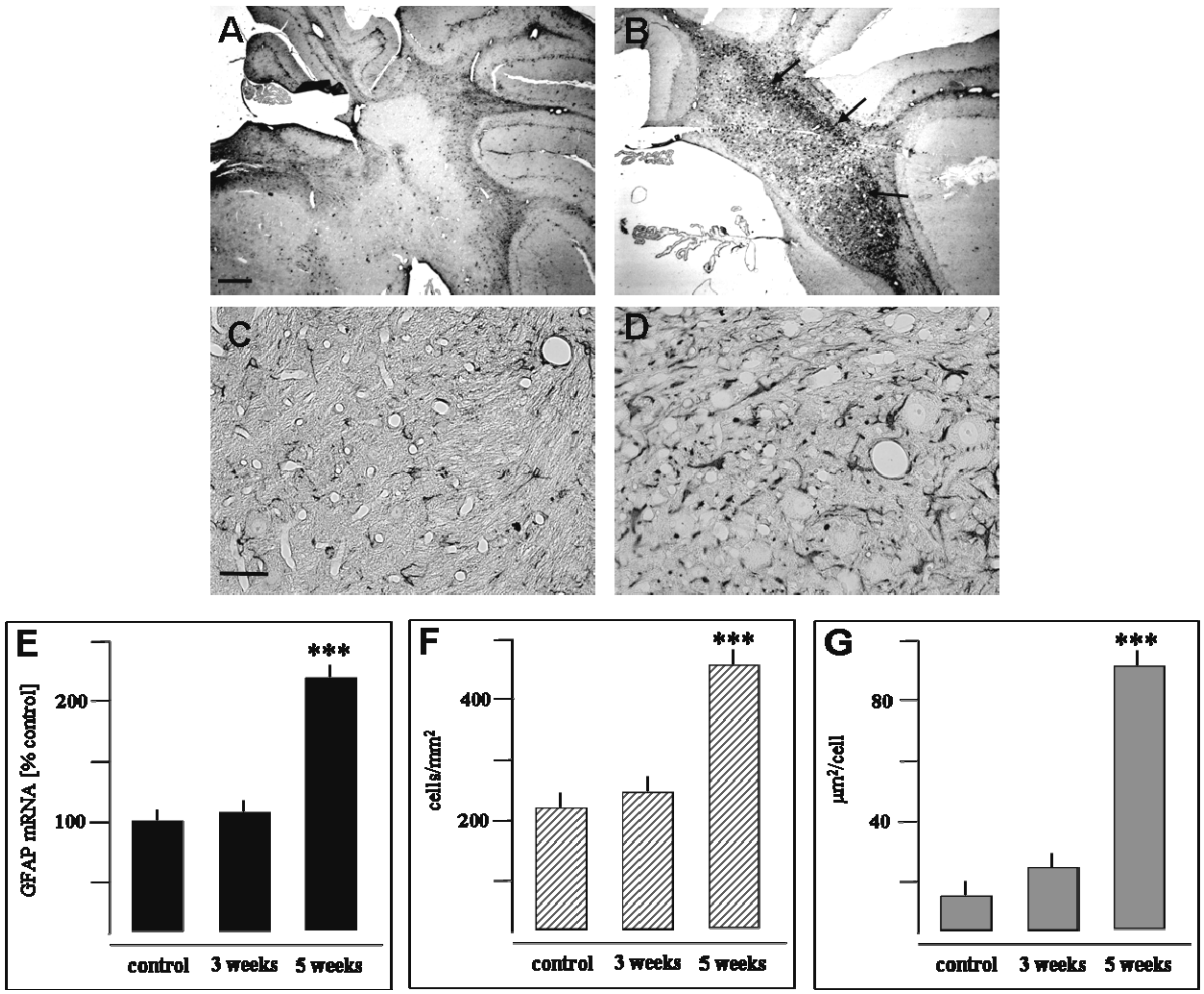

Figure 3 Effect of cuprizone exposure (0.2%, 5 weeks) on the distribution of GFAP-positive astrocytes (A-D), the expression of GFAP mRNA (E), the number of GFAP-positive cells (F), and the astrocyte hypertrophy (G). (C and D) show the interpositus cerebellar nucleus in a higher magnification. After 5 weeks of cuprizone treatment, a massive astrocytosis can be seen in and around the cerebellar nuclei. GFAP expression was maximally induced after 5 weeks (***$p<0.001$) compared to controls and 3-week treatment. At 5 weeks, astrocyte numbers increased from approx. 220 (control) to 460 cells/mm^2 (cuprizone, ***$p<0.001$) compared to controls. In addition, the volume occupied by a GFAP-positive cell increased from approx. 20 to 85 µm^2/cell after cuprizone (***$p<0.001$). 50 cells were analyzed per experimental group. Scale bars: 200µm (A/B); 20µm (C/D).

IHC for Iba-1 to detect microglia/macrophages demonstrated that (i) microglia is uniformly distributed in the cerebellar marrow (fig. 4A and C) and (ii) intense increase of Iba-1-positive cells can be observed after 5 weeks of cuprizone exposure (figs. 4B and D) in and around the cerebellar nuclei. As shown in figure 4, an increase in Iba-1 positive cell numbers is not evident in any investigated cerebellar cortex region. Microgliosis is evident between week 3 and 5 in the investigated regions. Absolute numbers were 82±21 cells/mm^2 (control) and 2623±43 cells/mm^2 (cuprizone) (fig. 4E). This effect was highly significant ($p<0.001$).

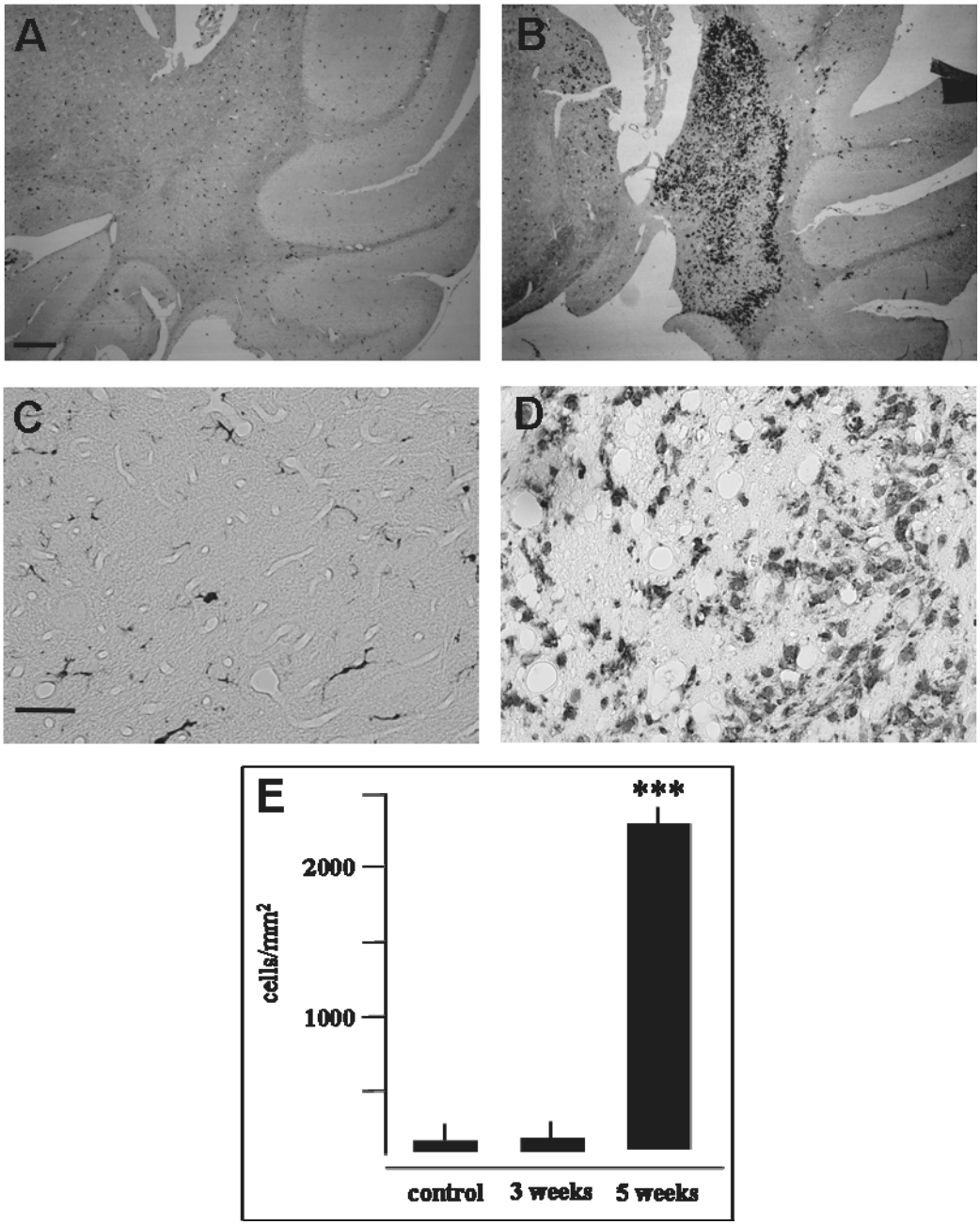

Figure 4 Effect of cuprizone exposure (0.2%, 3 and 5 weeks) on the distribution of Iba1-positive microglia/macrophages in the cerebellum (A-D) and the number of Iba-1-positive cells (E). (C and D) show the interpositus cerebellar nucleus in a higher magnification. After 5 weeks of cuprizone treatment, a massive microglia/macrophage increase can be seen in and around the cerebellar nuclei. At 5 weeks, microglia/macrophage numbers increased from approx. 100 (control) to 2600 cells/mm^2 (cuprizone, ***$p<0.001$) compared to controls. Scale bars: 200µm (A/B); 20µm (C/D).

4.3 Discussion

One of the important criteria for the diagnosis of MS is the demonstration of white matter lesions in the brain (Polman et al. 2011). This type of white matter lesions is the major pathological hallmark of MS and disseminate in space and time. However, factors that contribute to clinical deterioration include grey matter lesions and axonal damage as well. Cuprizone was thought to mainly affect the CC white matter tract and the cerebellar peduncles (Matsushima and Morell 2001; Silvestroff et al. 2010). Grey matter lesions in the cerebral cortex of cuprizone treated mice have been described only recently (Skripuletz et al. 2008). In this Chapter, I present our data addressing the question whether the cuprizone model mimics the spatial dissemination of MS lesions. Regions included in this discussion are (i) the cerebellum (Groebe et al. 2009), (ii) the basal ganglia system (Pott et al. 2009), and (iii) the hippocampus (Norkute et al. 2009). The cerebellum is a well-known part of the brain affected by demyelinating events in both, MS (Gilmore et al. 2008; Kutzelnigg et al. 2007; Pokryszko-Dragan et al. 2008) and EAE. Concordantly, patients with MS frequently develop symptoms owing to cerebellar lesions, such as dysmetria leading to hand dexterity impairment. The cerebellum takes part in several motor functions through its influence on the motor cortex and corticospinal output. Furthermore, the cerebral cortex and the cerebellum are tightly interconnected by a large descending fiber system, the cerebro-ponto-cerebellar projections. Besides the superior cerebellar peduncles, other regions of the cerebellum are affected by cuprizone administration. The most striking feature of cerebellar demyelination is the intense affection of the cerebellar marrow containing the rodent cerebellar nuclei, namely the lateral cerebellar nucleus, medial cerebellar nucleus, and interpositos nucleus. This demyelination event can be demonstrated by staining myelin fibers and mature oligodendrocyte cell bodies. The cerebellar nuclei are the primary output targets of the cerebellum (Kleim et al. 1998). A vast amount of cortical and sensory information that converges onto the cerebellum is integrated by cerebellar Purkinje cells and subsequently conveyed to the neurons of the deep cerebellar nuclei. Those neurons further process this information and thus generate the major output of the cerebellum (Alvina et al. 2008). They are involved in a plethora of functions such as complex motor skill learning (Kleim et al. 1998; Sanchez-Campusano et al. 2007), modulating food intake behaviour, as well as other non-somatic functions by modulating the hypothalamus (Pu et al. 1995). In this model, demyelination of distinct

cerebellar cortical regions can not be detected, at least if conventional immunohistochemical techniques are used. This is in contrast to human MS pathology. In MS, the cerebellar cortex is a major predilection site for demyelination, in particular in patients with primary and secondary progressive MS. Cerebellar cortical demyelination occurs mainly in a band-like manner, affecting multiple folia (so called subpial lesions). The lesions are characterized by primary demyelination with relative axonal and neuronal preservation, although some axonal spheroids and a moderate reduction of Purkinje cells are present (Kutzelnigg et al. 2007). In our study, we were not able to show involvement of the cortical grey matter including the superficial molecular layer, the middle pyramidal cell layer, as well as the deep granular cell layer by conventional light microscopy or electron microscopy studies (Groebe et al. 2009). The white matter of the cerebellar cortex, however, displayed distinct minor myelin involvement, namely swelling of myelin sheath on the electron microscopy level (Groebe et al. 2009). We, therefore, have to conclude that cuprizone induced demyelination mimics well cerebellar deep grey matter involvement but not cerebellar cortex pathology of MS.

In the basal ganglia which are also frequently affected in MS pathology (Brownell and Hughes 1962; Hasan et al. 2009; Henry et al. 2008; Nociti et al. 2008; Pirko et al. 2007), a medial-to-lateral gradient of demyelination with more severe effects in the lateral striatal complex was observed (Pott et al. 2009). This region-specific effect appears to be the result from diminished local microglia activation but also from higher numbers of oligodendrocyte precursors which might contribute to simultaneous remyelination during myelin destruction. These data support the view that demyelination in the basal ganglia follows a medio-lateral distribution without stopping at anatomical borders. Demyelination was highly reproducible and predictable with respect to the temporal and spatial occurrence. This makes the cuprizone mouse model attractive for investigating the underlying mechanisms of demyelination and remyelination (Matsushima and Morell 2001) in this brain region (Pott et al. 2009). Interestingly, the observed preferential myelin loss of laterally-located white matter tracts (within striosomes) does not apply for axons located in the grey matter (within the matrix). Microglia accumulation was also more pronounced in the lateral part and might therefore contribute for preferential demyelination of the lateral orientated striosomes (Pott et al. 2009). Mechanisms related to white and grey matter demyelination within this brain complex appear to differ.

The hippocampus, located in the medial temporal lobe, is part of the forebrain and integrated into the limbic circuitry. This subcortical structure appears to be crucial for cognitive performance and plays a major role for short term memory and spatial navigation (Bird and Burgess 2008). Cognitive impairment is a well-known phenomenon described for MS (Brex et al. 2002; Potter 2002). In MS patients, demyelination lesions have been seen in pathological specimens, and areas of signal abnormality have been detected within the hippocampus by double inversion recovery MRI (Geurts et al. 2007; Norkute et al. 2009; Vercellino et al. 2005).

We recently presented findings demonstrating that feeding with cuprizone causes a highly reproducible and severe but distinct demyelination within the hippocampus grey and white matter. Demyelination displayed a gradual time course between 3 and 7 weeks and was complete after 5 to 7 weeks (Norkute et al. 2009). Both white matter tracts of the hippocampus and grey matter areas (such as the stratum lacunosum moleculare containing the perforant path, the hilus of the dentate gyrus and the stratum pyramidale in CA-2/3), are strongly affected by cuprizone treatment. Demyelination in grey matter structures was more prominent and severe compared with white matter (Norkute et al. 2009). Hippocampal demyelination in white matter tracts was only seen in the medial alveus and the dorsal hippocampal commisure, whereas the fimbria and the lateral alveus were not affected. Although no clear-cut answer to this phenomenon can be given at present, a possible underlying reason could be differences in regional hippocampal blood supply (Pereira et al. 2007). Diversity among hippocampus subfield vascularisation has been documented (Grivas et al. 2003; Lovick et al. 1999). A recent study further suggests that local microglia response is implicated in this scenario (Kipp et al. 2011).

Besides regional differences in demyelination pattern, a second important outcome of our study was that cuprizone application results in astroglial proliferation and/or migration toward the injured area. We described astrogliosis which is due to increased numbers of astrocytes on one hand and hypertrophic astroglial responses on the other hand. Both effects, finally, result in an increased area occupied by astrocytes in demyelinated regions (Groebe et al. 2009; Norkute et al. 2009; Pott et al. 2009). Astrocytes may play an active role during degeneration and demyelination by promoting inflammation, damage of oligodendrocytes and axons, and glial scarring, but might also be beneficial by creating a permissive environment for remyelination and oligodendrocyte precursor migration, proliferation, and

differentiation (Markus et al. 2000). The same is true for microglia cells. Despite intense astrocytosis, demyelinated areas usually display intense accumulation of microglia, either by local proliferation and/or invasion. The role of both microglia and astrocytes during acute demyelination in the cuprizone model is currently under intense investigation by our group and is briefly discussed in the main discussion of this work. In summary, the first part of this thesis clearly shows that certain aspects of MS, such as grey matter pathology (Kidd et al. 1999), dissemination in space (Shaw et al. 2009) and brain intrinsic inflammation (Hemmer et al. 2002) are closely mirrored in the cuprizone model.

5. Chapter II
Corticosteroids during de- and remyelination in the cuprizone animal model

Parts of this chapter have been published in:

Tim Clarner, Ana Parabucki, Cordian Beyer and Markus Kipp
"Corticosteroids impair remyelination in the corpus callosum of cuprizone-treated mice."
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5.1 Introduction

Corticosteroids (CS) are regularly used for the tretament of MS, since they can decrease the inflammatory response by dampening the inflammatory cytokine cascade, inhibiting the activation of T-cells, decreasing the extravasation of immune cells into the CNS, facilitating the apoptosis of activated immune cells, and, indirectly, by decreasing the cytotoxic effects of nitric oxide and TNF-α (Sloka and Stefanelli 2005).

Demyelination is still a characteristic entity for the pathology of MS but it has become increasingly apparent in recent years that substantial axonal and neuronal loss are equal important features (Baracskay et al. 2002; Geurts et al. 2007). The loss of nerve's myelin sheath is not a definitive condition in demyelinating disorders. Progenitor cells of the oligodendrocyte lineage, a cell population which is responsible for myelination during development, can be (re)-activated and give rise to new myelinating mature oligodendrocytes. This endogenous repair process is termed "remyelination". Remyelination is the best-documented and most robust example of tissue repair in the human CNS. Approximately 40% of post-mortem MS tissues demonstrate signs of remyelination which can occur early and late during the course of MS (Franklin and Ffrench-Constant 2008; Irvine and Blakemore 2008; Patrikios et al. 2006). This process is important for axonal protection in demyelinating disorders. Remyelination is believed to restore the structural integrity of the axon-myelin unit and axonal conduction properties that are lost following demyelination (Beck et al. 1993; Halliday 1981). It is unresolved why in some patients remyelination is widespread while in others, it is sparse. The increasing importance of axonal damage and loss as the pathological basis for progressive loss of function demands a better understanding of the remyelination processes in MS and possible reasons for its failure. In this context a better understanding of steroidal interference with endogenous remyelination processes is essential. Beyond its anti-inflammatory function, previous reports have linked CS-mediated signalling with impaired (Chari et al. 2006; Triarhou and Herndon 1986) as well as accelerated (Pavelko et al. 1998) repair process after experimentally-induced demyelination (i.e. remyelination).

OPC express glucocorticoid (GC) receptors (Bohn et al. 1991). Direct interactions of CS and cells of the oligodendrocyte lineage have been reported (Barres et al. 1994). Methylprednisolone (MP) reduced TNF-α-induced nitric oxide release in OLN93 cells (Holzknecht and Rohl 2010) and protected oligodendrocytes against AMPA-induced

excitotoxicity *in vitro* (Sun et al. 2010; Xu et al. 2009). There are additional studies suggesting that CS influence OPC maturation and/or differentiation (Barres et al. 1994; Joubert et al. 2010; Kumar et al. 1989). During glial differentiation, the expression of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase) was induced by dexamethasone (Dex) treatment, a potent synthetic glucocorticoid-receptor agonist that stimulates oligodendroglial differentiation and myelination during the early phase of myelination in reagregating forebrain cultures (Almazan et al. 1986). Dex had a stimulatory or inhibitory effect on differentiation depending on the developmental stage of the cells (Almazan et al. 1986). In co-cultures of Schwann- and neuronal cells, Dex accelerated the time of initiation and enhanced the rate of myelin synthesis (Chang et al. 2002).

The most common MS animal model, EAE, is a mainly lymphocyte-driven model, which makes it difficult to distinguish between direct CS effects on remyelination and indirect effects due to CS immuno-modulation. Therefore, we used the toxic, non auto-immune driven cuprizone mouse model which induces a highly reproducible demyelination of distinct brain regions, among the CC (Acs et al. 2009; Clarner et al. 2010; Groebe et al. 2009; Pott et al. 2009). Spontaneous repair occurs after removal of the toxin from the diet, allowing investigation of spontaneous repair mechanisms such as OPC differentiation and restoration of myelin, as well as effects of pharmacological interventions on these processes (Kipp et al. 2009). Since no invasion of peripheral T-cells takes places, this model additionally allows studying direct steroidal effects on brain intrinsic inflammatory responses, such as the accumulation of microglia and astrocytosis.

In the present study, we aimed to analyze whether CS interfere with spontaneous endogenous remyelination during progressive disease stages where lymphocyte infiltration is less distinct. Furthermore, using primary astroglia and OPC cultures, we attempted to analyze cell-specific effects of CS that might be related to the remyelination capacity.

5.2 Results

5.2.1 Glucocorticoids accelerate spontaneous differentiation of cultured OPC

For studying the influence of GC on oligodendroglial differentiation *in vitro,* we used an OPC culture system which displays spontaneous differentiation within several days (5-7 days) under defined medium conditions (Richter-Landsberg and Vollgraf 1998). In a first set of experiments, we analyzed the kinetics of OPC differentiation. As shown in figure 5, OPC spontaneously differentiate within several days in serum-free medium supplemented with insulin, transferrin, and sodium selenite. Differentiation is reflected by a gradual increase in the expression of the myelin marker genes MBP, PLP and CNPase (figures 5A/D). Induction of myelin markers is paralleled by morphological maturation (figure 5B). After one day in culture, cells display a typical bipolar OPC morphology with long extending processes. After 5 days, cells are multipolar and show highly-branched processes indicating differentiation (Barres et al. 1994; Zakzanis 2000). Additional experiments revealed that maturation progresses till day 7, both on the morphological and gene-expression level (data not shown). In order to assess the influence of GC on OPC differentiation, cells were exposed for 5 days (subtotal differentiation) to Dex or MP. Figures 6A/C depict that both CS accelerated OPC differentiation *in vitro*. Expression analysis of MBP and PLP revealed significantly higher mRNA transcript levels in Dex- and MP-treated cultures. For Dex, ~2-fold and 3-fold induction was found for MBP and PLP, respectively (compared to controls). For MP, ~1.5-fold and 2-fold induction was observed for MBP and PLP, respectively (compared to controls). In line with these findings, Western-blot analysis revealed higher CNPase protein levels in Dex-treated groups as shown in figure 5D. These results convincingly show that both CS positively interfere with the endogenous differentiation program of OPC.

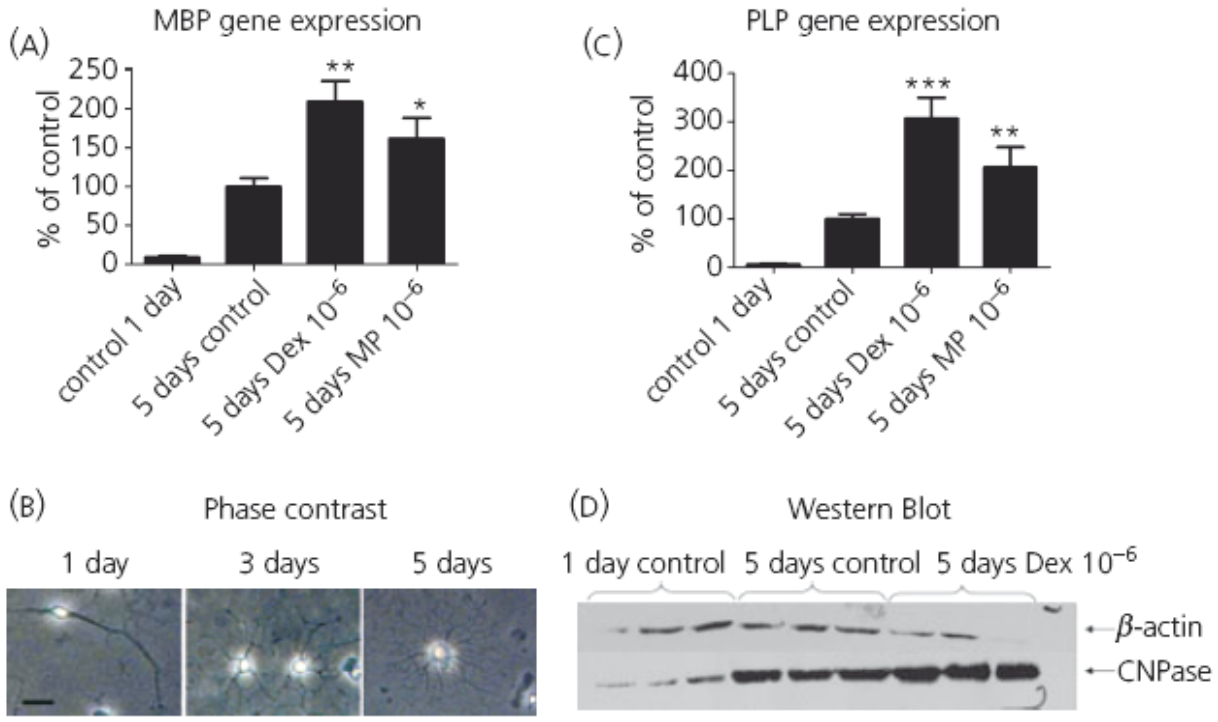


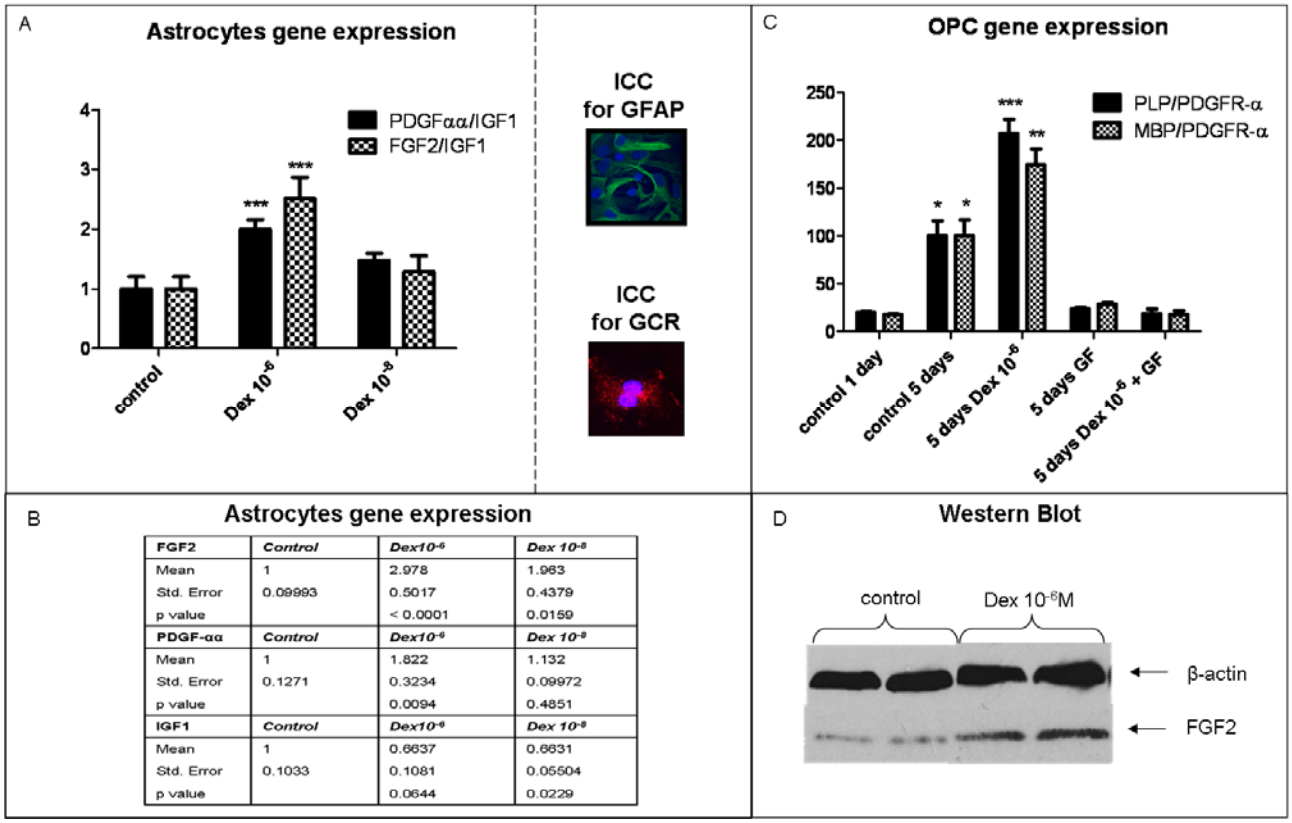
Figure 5 MBP and PLP mRNA expression levels in primary oligodendrocyte cultures determined by rtRT-PCR are shown in (A) and (B), respectively. Results of gene expression analysis are normalized against a housekeeping enzyme (GAPDH) and expressed as percentile of controls. Representative phase contrast views of cultured oligodendrocytes are presented in (C). Results of anti-CNPase Western blotting experiments are presented in (D). ß-actin was used as loading control. Note the increase of oligodendrocyte differentiation-related genes (i.e. MBP and PLP) during a 5 days culture period which is potentiated by GC treatment. Scale bars in (B): 10 µm, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to controls.

5.2.2 CS treatment regulates growth factor expression in astrocytes

There is evidence that the effect of CS on OPC differentiation depends on the presence or absence of mitogens such as PDGF-αα and FGF2 (Barres et al. 1994). Since astrocytes are a well-known source of certain growth factors that are able to modulate remyelination processes, we investigated CS effects on growth factor mRNA expression. PDGF-αα and FGF2 were included in the analysis due to their well known promotion of OPC proliferation. Expression levels of IGF1 were analyzed because this growth factor is well-known to promote OPC maturation.

Labelling for GFAP and CS receptor showed that virtually all cells in the applied primary astrocyte culture system expressed CS receptors (figure 6A). Expression levels are expressed as a ratio of those factors which promote OPC proliferation (PDGF-αα and FGF2) to IGF1 which promotes maturation. A high ratio indicates a proliferating, anti-maturating effect, while a low ratio indicates a maturating influence on OPC. Treatment with Dex for 24 h significantly increased the ratio of PDGF-αα

and FGF2 to IGF1 expression in primary astrocytes cultures (promoting a premature/proliferative stage of OPC). Expression levels of investigated genes were additionally normalized against a housekeeping gene (HPRT), and control values were set as 1. These data are given in figure 6B. Treatment with CS caused a significant induction of FGF2 and PDGF-αα, whereas IGF1 mRNA levels were decreased by CS treatment. Figure 6D shows a representative western blot for FGF2 protein, confirming that mRNA changes are paralleled by respective protein level changes after CS administration.



FGF2	Control	Dex10-6	Dex 10-8
Mean	1	2.978	1.963
Std. Error	0.09993	0.5017	0.4379
p value		< 0.0001	0.0159
PDGF-αα	**Control**	**Dex10-6**	**Dex 10-8**
Mean	1	1.822	1.132
Std. Error	0.1271	0.3234	0.09972
p value		0.0094	0.4851
IGF1	**Control**	**Dex10-6**	**Dex 10-8**
Mean	1	0.6637	0.6631
Std. Error	0.1033	0.1081	0.05504
p value		0.0644	0.0229

Figure 6 Ratio of expression levels of fibroblast growth factor 2 (FGF2) or platelet-derived growth factor-αα (PDGF-αα) to insulin-like growth factor-1 (IGF1) in Dex-exposed astrocytes and expression of glucocorticoid receptors in cultured murine GFAP-positive astrocytes (A). Gene expression levels of FGF2, PDGF-αα, and IGF-1, normalised against the housekeeping gene hypoxanthin-guanin-phosphoribosyltransferase (HPRT), is presented in (B). In (C), ratio of expression levels of proteolipid protein (PLP) and myelin basic protein (MBP) to platelet-derived growth factor receptor-α (PDGFR-α) in cultured oligodendrocytes after treatment with Dex or Dex plus growth factors (GF) PDGF-αα and FGF2 is shown. Results are expressed as percentage of controls at day 5. A representative anti-FGF-2 western-blots in Dex-treated astrocytes is shown in (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to controls.

5.2.3 Acceleration of Dex-mediated OPC differentiation is abolished by FGF2 and PDGF-αα

Our data obtained with astroglia cultures clearly show that those factors which promote OPC proliferation are induced by CS, whereas pro-maturating factors are reduced, respectively. We assumed that direct effects of GC on OPC maturation might be modulated by GC-induced release of FGF2 and PDGF-αα. Therefore, we analyzed the influence of Dex alone or in combination with both growth factors on MBP and PLP mRNA expression in isolated OPC cultures. As expected, the ratio of MBP or PLP (preferentially expressed in mature oligodendrocytes) to PDGFRα (preferentially expressed in OPC) is low in OPC cultured for one day. This ratio increases during spontaneous differentiation until day 5 (fig. 6C), reflecting OPC differentiation. Dex treatment potentiated this ratio increase. In contrast, the application of growth factors completely prevented OPC differentiation, and Dex was no longer able to induce OPC differentiation. These data convincingly show that the modulation of astrocytic growth factor release can interact with direct steroidal effects on OPC differentiation.

5.2.4 MP treatment interferes with endogenous myelination capacity after cuprizone-induced demyelination

Our *in vitro* data imply that corticosteroids directly interfere with OPC maturation which might be modulated indirectly by growth factors released by astrocytes. Therefore, interference of MP treatment with spontaneous repair mechanisms that are crucial for remyelination was tested after acute cuprizone-induced demyelination. Figure 7A illustrates the experimental design of our *in vivo* experiments. To assure sufficient systemic application of MP and to rule out that cuprizone feeding induces activation of the *hypothalamo-hypophyseal-adrenal axis*, serum levels of ACTH were determined by ELISA prior to scarification. The application of MP caused a significant reduction of ACTH serum levels compared to placebo-injected controls (fig. 7B). No reduction was found in cuprizone + vehicle-treated animals compared to controls.

Myelin index staging (both, manually and via densitometry) and quantification of APC-positive oligodendrocytes were performed in experimental groups. Figure 7C shows the results of PLP reactivity staging. Representative anti-PLP stained sections of the medial CC are presented in figure 7D. As expected, 5 weeks cuprizone feeding decreased the myelination index of the CC by ~50%. This was paralleled by a

significant reduction of APC-immunoreactive oligodendrocyte numbers (fig. 7E). After 9 days recovery, PLP staining intensity was unchanged in placebo-treated animals whereas demyelination progressed in MP-treated mice. These results indicate the suppression of early remyelination events after cuprizone-induced acute demyelination by systemic MP application. Spontaneous myelin recovery was evident after 21 days in PLP-stained sections (group IV). The intensity of PLP immunoreactivity was significantly lower in MP-treated (group VI) compared to placebo-treated animals (group IV). The re-appearance of APC-positive oligodendrocytes during early (9 days) and intermediate (21 days) endogenous remyelination was not affected by MP treatment. Additionally, we determined the density of PLP-stained CC on binary-converted pictures and found comparable results.

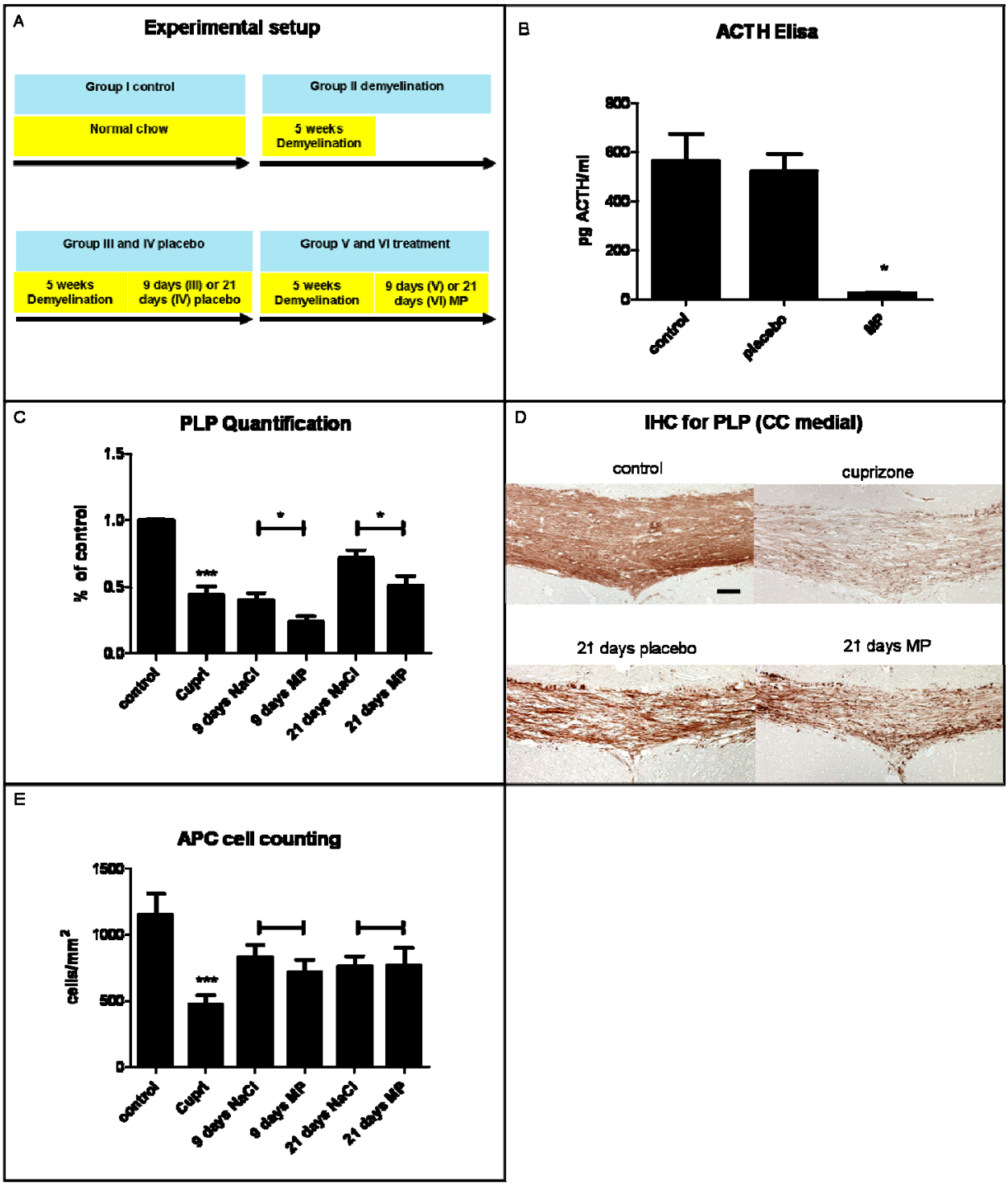


Figure 7 Experimental set-up of animal experiments is shown in (A). ACTH levels determined by ELISA in the serum of treated and untreated animals are shown in (B). Myelination index determined by staging PLP-stained sections is shown in (C). In (D), representative pictures of IHC for PLP of the medial CC are shown for groups I, II, IV and VI. Quantification of APC-positive oligodendrocytes within the CC is shown in (E). Placebo-treated mice showed no reduction in ACTH-serum levels compared to controls, whereas MP-treated animals displayed significant reduction. Note that recovery of anti-PLP staining intensity of the CC is significantly impaired in GC-treated animals after 21 days recovery. Reappearance of APC-positive cells is not affected by GC treatment. * $P < 0.05$, *** $P < 0.001$. Scale bar: 50 µm.

5.2.5 MP treatment does not affect microglia and astrocyte cell numbers during endogenous remyelination

We have further analyzed the effect of MP on the presence/absence of microglia and astrocytes in the CC by IHC during the remyelination period using the pan-macrophage/microglia marker Iba1 and the astrocyte marker GFAP. As shown in figure 8A, the number of microglia was significantly lower 9 days after cuprizone-induced demyelination (groups III) compared to the acutely demyelinated CC (group II). No further reduction was observed until day 21. In contrast, the number of GFAP-positive astrocytes remained at high levels during early and intermediate remyelination (fig. 8B). As demonstrated, kinetic changes of microglia and astrocyte counts were not affected by MP treatment.

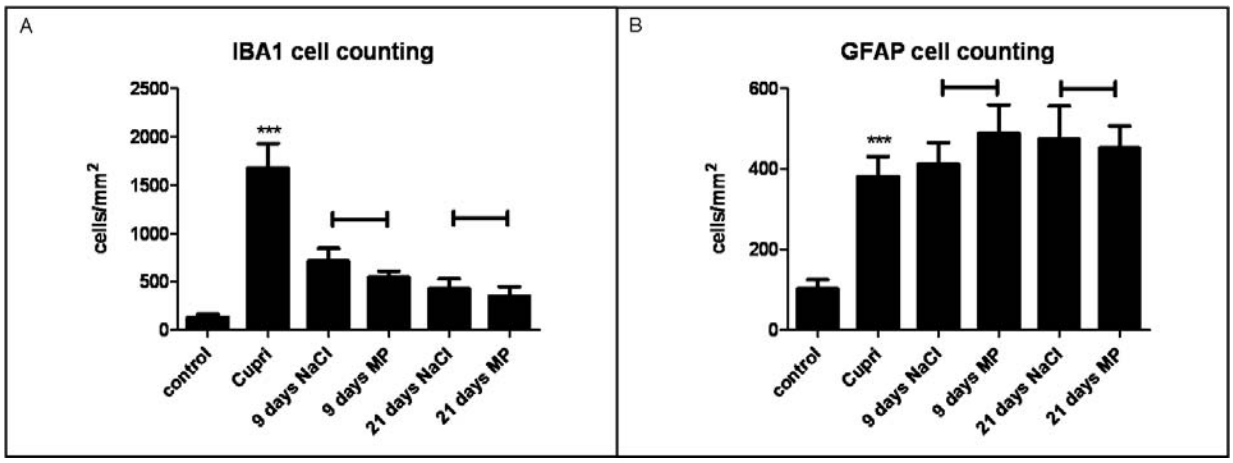


Figure 8 Results of Iba1-positive microglia (A) and GFAP-positive astrocytes (B) quantification in the CC is shown. Results are expressed as cell numbers/mm^2. Note that no significant difference in cell numbers could be observed in MP-treated animals compared to placebo controls for both cell types. *** $P < 0.001$

5.2.6 Effect of CS on thyroid hormone plasma levels

Thyroid hormones are unequivocally playing important roles during remyelination (Calza et al. 2005; Fernandez et al. 2004; Franco et al. 2008). Since CS are known to interfere with thyroid hormone levels (Haugen 2009; Nicoloff et al. 1970), we measured total T3 serum levels of experimental animals by solid phase competitive ELISA-analysis. Cuprizone treatment did not influence basal T3 serum levels after 5 weeks (fig. 9). Remyelinating mice displayed slightly increased T3 serum levels ($P = 0.075$ compared to controls) after three days of recovery. MP treatment during remyelination had no significant influence on T3 serum levels ($P = 0.095$, 3 days recovery MP-treated vs. 3 days recovery NaCl).

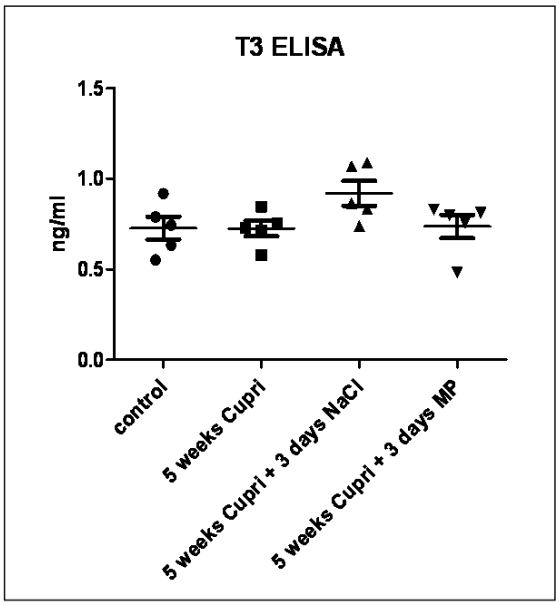


Figure 9 Quantification of T3 serum levels by solid phase competitive ELISA analysis is shown as scatter blot. Data are expressed as ng/ml. No significant decrease in the total amount of T3 could be found in MP-treated animals when compared to placebo-treated controls (n = 5).

5.3 Discussion

Clinical treatment of MS is to date based on the assumption arising from preclinical and animal studies that the suppression of autoimmune reactions can positively modify disease pathogenesis and neuronal damage. A further important goal is to reduce the number of relapses or to decrease their impact on the accumlation of neurologic symptoms. Beyond that, the promotion of tissue repair to reverse neurologic symptoms is another important aim (Compston and Coles 2002). Starting in 1950s, GC are routinely used as therapeutic instrument for MS treatment due to their potent anti-inflammatory effects. Today, a widely used treatment option is the administration of high doses of GC. GC are very helpful tools to effectively manage acute relapses in MS (Keegan and Noseworthy 2002). Typically, the drug is supplied intravenously for 3–5 days. This treatment can additionally be tapered by oral CS by several days up to weeks. CS act through the classical CS receptor, leading to an inhibition of the synthesis of pro-inflammatory cytokines and cell surface molecules required for immune function and modulation (Sloka and Stefanelli 2005). Despite their widespread use, not much is known about the effects of CS treatment on regenerative processes in the adult CNS. The possible mechanisms by which CS might influence the most important repair process in MS, the remyelination process, are poorly understood.

It has been postulated that GC are able to interfere with the course of MS. This class of steroid hormones might prevent new exacerbations and, therefore, modify the natural history of MS by reducing the accumulation of neurologic symptoms and disability.

Patients suffering of first episode optic neuritis that were treated with MP showed lower conversion rates towards definite MS (Beck et al. 1993). Zivadinov and colleagues presented data in 2001 that the treatment with pulses of i.v. MP in relapsing-remitting MS patients slowed the development of T1 black areas. They also showed that whole-brain atrophy and disability progression can be prevented or delayed by CS (Zivadinov et al. 2001). MS patients that were treated with high dose intravenous CS during six months *post partum* might have a reduced risk of an acute relapse after childbirth (de Seze et al. 2004).

Studies concerning the effects of long-term GC treatment in MS show a substantial heterogeneity between the different studies (recently reviewed by Ciconne et al. (2008)),

For example, the risk of disability progression was reduced by i.v. application of high dose MP in relapsing remitting MS after 5 years (Zivadinov et al. 2001), whereas low dose and orally-applied prednisolone did not lead to reduced risk after 18 months treatment (Miller et al. 1961). Because of this heterogeneity and the lack of further studies, the routine use of long-term GC treatment cannot be justified at the moment.

When it comes to CS effects on repair mechanisms in the CNS, studies on toxic demyelination animal models (lysolecithin-induced spinal cord demyelination) yielded conflicting results. On one hand, MP treatment accelerated spontaneously occurring repair/remyelination that was paralleled by reduced invasion of macrophages (Pavelko et al. 1998). On the other hand, an earlier study using the same lesion model demonstrated a delay in remyelination of rats treated with Dex (Triarhou and Herndon 1986). In a different toxic demyelination model (CNS injection of ethidium bromide into the rat spinal cord), Dex treatment led to delayed remyelination that occurred independently from the colonization of the lesion with PDGFRα positive OPCs (Chari et al. 2006).

In the above mentioned animal models, a disruption of the blood-brain-barrier can be observed. We, therefore, have chosen the cuprizone animal model for our study, since it allows to investigate the impact of CS on the remyelination process that follows acute toxic demyelination without interference of invading peripheral blood cells (due to an intact BBB) (Bakker and Ludwin 1987; Kondo et al. 1987; Liu et al. 2010) In our *in vitro* approaches, we aimed to study direct CS effects on OPC and astrocyte cultures.

We could demonstrate an acceleration of OPC maturation by CS *in vitro* (Clarner et al. 2011b). Similar effects have been reported by other groups (Barres et al. 1994; Joubert et al. 2010; Kumar et al. 1989). It is well-known, that OPC express GC receptors (Bohn et al. 1991). Direct interactions of CS and cells of the oligodendrocyte lineage have also been reported (Barres et al. 1994). Pathways that can be directly influenced by CS include the TNF-α-induced nitric oxide release (as shown for OLN93 cells) (Holzknecht and Rohl 2010). CS are also protective for oligodendrocytes which were exposed to AMPA-induced excitotoxicity *in vitro* (Sun et al. 2010; Xu et al. 2009). However, these direct CS effects upon OPC could be modulated by indirect stimuli, e.g. modulated growth factor release by astrocytes *in vivo*. Several growth factors have been shown to interfere with OPC proliferation and maturation and, in consequence, might modulate endogeneous remyelination. OPC

differentiation and proliferation is under the control of PDGFRα and FGF2 receptor signalling *in vitro* and in animals (Bogler et al. 1990; McKinnon et al. 1991; Pringle et al. 1989; Raff et al. 1983). IGF1 represents another important member of the TGFß family of growth factors and has been shown to induce myelination *in vitro* and *in vivo.* This factor also exhibits protective effects on mature oligodendrocytes under neuropathological challenges (D'Ercole et al. 1996; Mason et al. 2000). Since astrocytes are well-know to be an important source of various growth factors (Acs et al. 2009; Braun et al. 2009), we decided to investigate CS-induced changes in the growth factor expression pattern of these cell type.

Our results clearly argue for the possibility that direct steroidal effects on OPC can be modulated by astrocyte-derived growth factors. As shown, those growth factors which have been described to participate in OPC proliferation (i.e. PDGF-αα and FGF2) are induced, whereas pro-maturating growth factors (such as IGF1) are slightly repressed by GC treatment, respectively (Clarner et al. 2011b). Comparable results have been recently reported by Chesik and De Keyser who found a decrease in the expression of IGF1 as a response to Dex treatment in both astrocytes and OPC (Chesik and De Keyser 2010).

We performed a preliminary gene expression study using cuprizone-treated animals that were allowed to recover for three days and were treated either with MP or a placebo. Both investigated pro-proliferating growth factors (PDGF-αα and FGF2) were found to be induced by MP treatment, whereas IGF1 transcrips were slightly repressed. This is in line with our previous *in vitro* findings. We might speculate from these observations that the GC-mediated release of PDGF-αα and FGF2 from astrocytes in parallel with an inhibition of astrocytic IGF1 secretion impairs direct effects on OPC (i.e. acceleration of the endogenous maturation program) (Clarner et al. 2011b).

In vitro, we could show that the presence of PDGF-αα and FGF2 in the OPC culture medium completely antagonizes both spontaneous as well as GC-induced OPC maturation. Astrocytes might not be the only cell type that shows CS-induced changes in the growth factor release. A feedback mechanism of the OPC differentiation signalling cascade might exist, since Dex could interfere with OPC growth factor release as well (Chesik and De Keyser 2010). OPC-derived IGF1 could promote OPC differentiation, unless suppressed by CS.

Results from our *in vitro* studies further suggest that the net effect of complex cell-cell interactions and steroidal interference with these pathological processes results in impaired remyelination (Clarner et al. 2011b).

For several reasons, we decided to use the cuprizone model to study the impact of GC treatment on spontaneous endogenous remyelination properties. As already mentioned above, no disruption of the BBB takes place in the cuprizone model (Bakker and Ludwin 1987; Kondo et al. 1987). Therefore, no invasion of peripheral immune cells occures (Liu et al. 2010). Because of these characteristics, the cuprizone model allows us to investigate direct interference of CS with remyelination. Second, removal of the toxin from the chow leads to spontaneous rapid remyelination within a matter of weeks (Clarner et al. 2011b; Irvine and Blakemore 2006; Kipp et al. 2009). Third, changes of growth factor expression levels in response to cuprizone intoxication have been investigated (unpublished data from our group) and showed a significant induction of IGF1 that remained high for several days after removal of the toxin from the diet. With respect to FGF2 and PDGF-αα mRNA levels, we found only a slight increase of both transcripts after 5 weeks of cuprizone feeding. Transcript levels rapidly decreased to the level of control animals when cuprizone was removed from the diet. Taken together, the cuprizone model reflects several aspects of the situation *in vitro* and thus simplifies comparison of *in vivo* and *in vitro* results (Clarner et al. 2011b).

Endogeneous CS play an important role during myelination in CNS development (Gumbinas et al. 1973; Redzic et al. 2005) but repeated exogenous CS administration has been shown to result in an impaired CNS development (Dunlop et al. 1997; Raschke et al. 2008). However, in the adult CNS and especially during pathological and inflammatory events, CS actions might substantially differ from those observed during development. Our *in vitro* results could be interpreted as a positive interference of CS with the progression of the intrinsic differentiation program which leads to an acceleration of myelin marker gene expression in this cell culture system. These direct effects on OPC differentiation suggest pro-myelinating effects of CS in a local environment that facilitates myelination (Clarner et al. 2011b). Contrariwise, accelerated differentiation might have a negatively impact on repair processes, since there is a need to synergize with other processes (e.g. those associated with inflammation) (Franklin and Ffrench-Constant 2008).

The different mechanisms underlying steroidal interference with OPC maturation need further investigation. CS have recently been shown to modify the histone acetylation status in natural killer cells, thereby reducing promoter accessibility (Krukowski et al. 2010). Effective remyelination requires deacetylation of nucleosomal histones, thus executing this complex transcriptional program during OPC differentiation (Shen et al. 2008) might be representing one of the CS-associated mechanisms.

One might speculate, that CS cause a reduction and/or disappearence of astrocytes and microglia during remyelination. However, numbers of both cell types were not reduced in MP-treated animals. This does not necessarily argue that pro-inflammatory properties are not affected by MP because absolute cell numbers do not reflect cellular activity (Clarner et al. 2011b). This assumption is supported by data coming from another ongoing project. In this study, we were able to show that Dex treatment does not interfere with cuprizone-induced demyelination and concomitant glia cell activation (unpublished data). It appears that GC are powerful in dampening the peripheral immune response but lack efficacy in modulating brain intrinsic inflammatory pathways when the BBB is not affected.

As a further mechanism that could explain the observed impaiment of remyelination by CS, interference with thyroid hormone plasma levels should be considered. The thyroid hormones T3 and T4 are thought to play important roles in the activation of OPC, remyelination, and neuronal protection (Fernandez et al. 2004; Franco et al. 2008; Harsan et al. 2008). In cuprizone-treated remyelinating mice and rats, T3 supplementation led to increased numbers of oligodendrocytes ($Olig2^+$ cells), enhanced remyelination, and improved clinical symptoms (Franco et al. 2008; Harsan et al. 2008). In an autoimmune rat MS model (EAE), T4 supplementation led to improved remyelination, most likely via stimulating endogenous OPCs (Fernandez et al. 2004). Since CS are known to be able to suppress thyroid stimulating hormone secretion (Nicoloff et al. 1970; Wilber and Utiger 1969), we assumed that reduced levels of plasma thyroid hormones might contribute to the negative net effect of CS on remyelination in the cuprizone animal model. Although our experimental design cannot completely rule out this possibility, we consider this mechanism to be unlikely, since we only observed a marginal increase of plasma T3 in the spontaneous remyelination group. Additionally, CS treatment did not lead to hypothyroidism but the animals showed T3 serum levels comparable to controls (Clarner et al. 2011b).

In conclusion, we provide further evidence that exogeneous CS might impair reparative processes in the CNS. Our results additionally indicate that beneficial steroidal effects during CNS inflammation have to be balanced against possible remyelination-inhibiting effects in MS.

6. Chapter III

The cuprizone model as a tool to study glia-derived amyloid precursor protein function

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6.1 Introduction

The role of amyloid precursor protein (APP) has been intensely studied under pathological and non-pathological conditions in the brain. The metalloprotein APP is abundantly and ubiquitously expressed in many cell types and spans membranes in the endoplasmic reticulum, trans-Golgi, and the cell surface (Cahill et al. 2009). Its abnormal proteolytic processing is associated with Alzheimer's disease (Senechal et al. 2006). Depending on the secretase cleavage, different fragments of APP are produced. APP cleavage by β- and γ-secretase generates the pathogenic Aβ peptide that aggregates to form the main component of amyloid plaques during Alzheimer's disease. Alternatively, processing of APP by the α-secretase prevents the formation of Aβ peptides and leads to secreted APP-α (sAPP) release in the so called non-amyloidogenic processing pathway (Senechal et al. 2006). sAPPs are known to have neurotrophic or neuroprotective properties (Lee and Wurtman 2000; Thinakaran and Koo 2008).

Besides neurons, astroglia and microglia cells are potential sources of APP within the healthy and diseased brain. The glial isoforms of APP consist of 770 or 751 amino acids compared to the 695 amino acid isoform predominantly found in neurons (Senechal et al. 2006). APP 770 was described to be more amyloidogenic than APP 695 in cultured mouse astrocytes and an increased immunoreactivity for glial-derived APP was related to higher plaque density in AD brains (Lee and Wurtman 2000; Zhan et al. 1995). Neuronal derived APP was in the focus of most studies conducted so far. Far less is known about the role of astroglia derived APP. The presence of APP-positive astrocytes has been described in different experimental cerebral ischemia models (Banati et al. 1995b; Kalaria et al. 1993; Stephenson et al. 1992). Furthermore, it has been demonstrated that activated astrocytes express APP at high levels in MS lesions (Gehrmann et al. 1995). Induced APP expression was also reported within EAE foci. These results suggest a role of APP expression by brain resident inflammatory cells as a response to tissue damage or injury.

In the present study, we investigated the induction and accumulation of APP within the acutely demyelinated CC. Cellular co-localization of APP with glial markers was achieved by double-immunofluorescence labelling. The general expression pattern was analyzed by RT-PCR and Western blotting. Neonatal mouse astroglia cultures were employed to study the APP responsiveness of astrocytes to different toxic factors.

6.2 Results

6.2.1 Astrocytes express APP in the CC of cuprizone-treated mice

After 5 weeks cuprizone treatment, demyelination can be detected within all parts of the CC after 5 weeks of cuprizone treatment indicated by a loss of PLP-reactive fibres. To study expression of APP in the demyelinated CC, IHC was performed using anti-APP antibodies. As shown in fig. 10B, high numbers of APP-positive cells with an astrocyte-like morphology are detectable in the CC after 5 weeks cuprizone. In contrast, no APP immunoreactivity was observed in control animals (fig. 10B). Due to the characteristic morphology, we assumed that APP expression can be allocated to (activated) astrocytes. Immunofluorescence double-labelling for GFAP and APP and confocal laser microscope analysis revealed that APP is exclusively present in astroglia (fig. 10C). APP shows an overlapping cellular distribution with GFAP. Both proteins are mainly located in the cytoplasm and cellular processes sparing the nuclear compartment. Notably, no other cell types, such as Iba-1 positive microglia cells, expressed APP within the CC, although microgliosis is apparent within the demyelination region (fig. 10A).

6.2.2 APP gene and protein expression in the CC of cuprizone-treated mice

As shown in fig. 10A, APP mRNA was low in control animals. In contrast, a time-dependent induction of APP expression was observed as early as one week cuprizone treatment. PCR analysis for GFAP mRNA revealed a similar pattern of expression. Expression of HPRT remained stable during the investigated period. mRNA expression data for APP were confirmed by Western blot analysis using an antiserum (anti-APP, see table 2) that detects the APP770 isoform which is predominantly expressed in glial cells (Lee and Wurtman, 2000). Anti-Actin antibodies were used to visualize actin as loading control (anti-actin, see table 2; (data not shown).

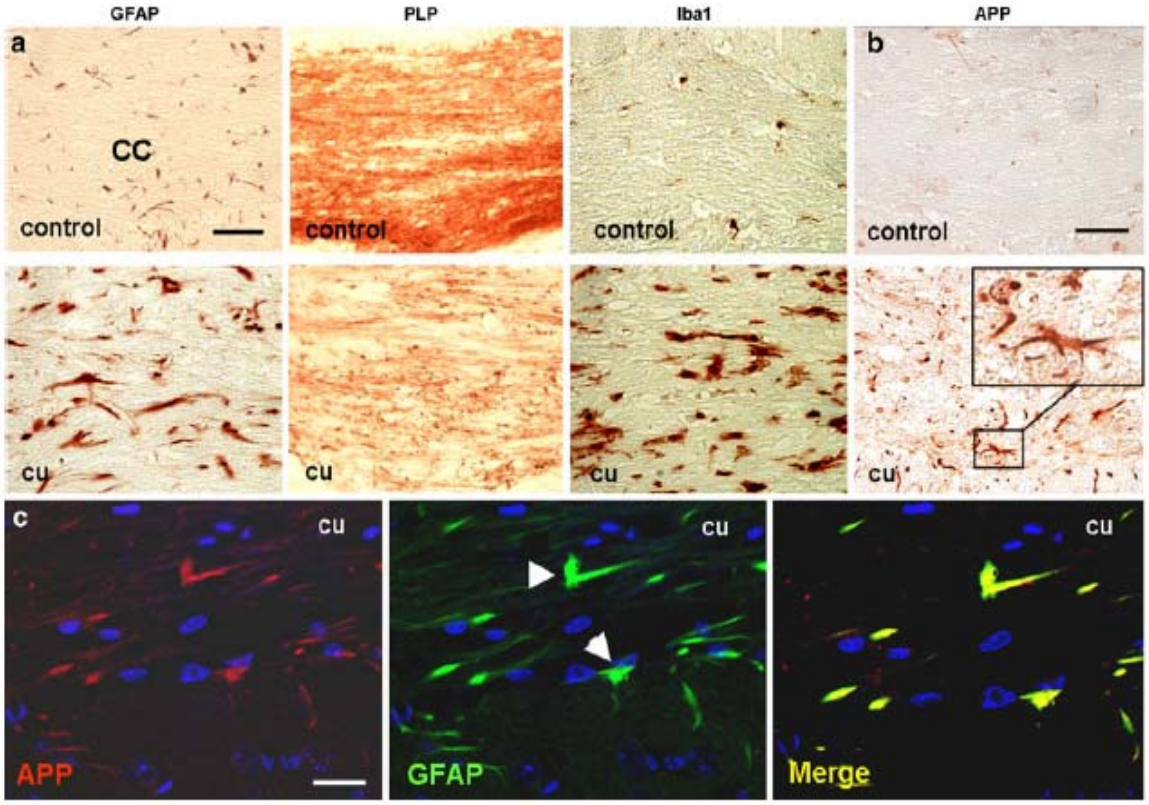

Figure 10 (A) Myelination and inflammatory cell composition in the midline of corpus callosum (CC) of control (upper row) and cuprizone-induced demyelinated (cu) brains (lower row). Anti-PLP antibodies were used to visualize myelin, anti-GFAP antibodies to visualize astrocytes, and anti-Iba1 antibodies to visualize microglia/macrophages. Anti-APP immunohistochemistry of control and cuprizone brains are presented in (B). Immunofluorescence double-labelling for APP and GFAP of cup animals are given in (C). Note that massive myelination (brown staining) is apparent in the CC of control animals, whereas after acute demyelination (5 weeks cuprizone treatment) the midline of CC is almost completely demyelinated. Further note the robust increase in GFAP and Iba1-positive cells, respectively. APP-positive cells are rarely detectable in control animals. In contrast, numerous APP-positive cells are visible after cuprizone exposure. The insert in (B) shows an APP-positive cell at higher magnification. APP positive cells also express GFAP (arrowhead in C). Scale bars: 25 µm in A; 50 µm in B; 20 µm in C.

The applied APP antibody detects the 110 kDa APP holoprotein as well as various smaller cleavage products and immature isoforms (information kindly provided by the manufacturer). A representative blot is presented in figure 11B. Increased APP reactivity could be observed after one week cuprizone treatment. When demyelination processes, APP protein levels remained higher compared to controls. Thus, the time course of APP protein induction parallels APP and GFAP mRNA levels. As shown in figure 11B, predominantly proteins with a molecular weight at 50 kDa appear to accumulate within the demyelinated CC compared to controls. These products most likely represent cleaved APPs. In contrast, accumulation of the APP

holoprotein with a molecular weight at 110 kDa was not observed in response to demyelination.

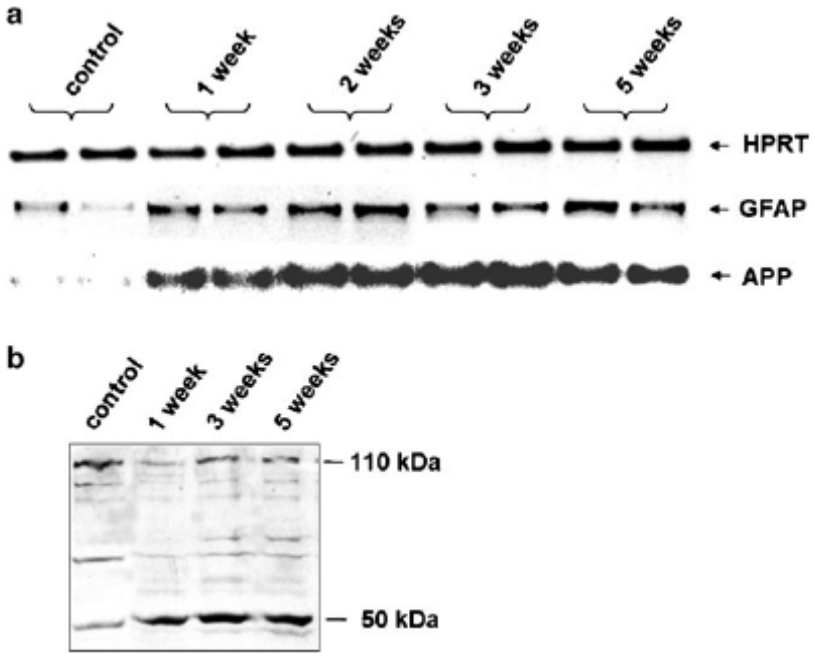

Figure 11 Time-dependent expression of APP mRNA (A) and protein (B) is shown. Induction of APP could be observed already after one week of cuprizone exposure. PCR showed an early up-regulation of both APP and GFAP mRNA expression, indicating a similar temporal induction pattern. HPRT served as loading control in PCR experiments. Note the selective increase in cleavage products (50 kDa) of the APP-holoprotein (110 kDa) reactivity.

Astrocytes fulfil many important functions within the CNS ranging from directing neuronal differentiation to regeneration, regulating inflammatory processes, and protecting nerve cells under pathological conditions. In a previous study, we have analyzed growth factor expression of differentially stimulated cultured cortical astrocytes. These data show that astroglia selectively respond to pathological compounds by the expression of growth factors (Braun et al. 2009). In contrast to the cuprizone model where only astrocytes seem to express APP, microglia cells in the EAE model are also APP-positive (Banati et al. 1995a). In human MS lesion, both cell types are potential sources of APP depending on the stage of the disease (Banati et al. 1995a; Gehrmann et al. 1995). It appears that complex cell-cell interactions are required to induce APP expression in astrocytes. We therefore reason that the induction of APP in astroglia is highly selective and not the result of an unspecific activation. This assumption was reassessed in primary astrocyte cultures prepared from one to three-day-old Balb/c mice cortices. Cultures were treated with LPS,

TNFα, glutamate or H_2O_2 for 24 h. Previous studies revealed that the applied concentrations are sufficient to activate astrocytes, however, are not toxic in the applied concentrations (Braun et al. 2009). APP mRNA expression was analyzed by real-time RT-PCR. As shown in fig. 12A agarose gel analysis of PCR products (30 cycles) revealed only moderate levels of APP mRNA in untreated cultures. As expected, the exposure to the different toxic compounds did not further increase endogenous APP levels in astrocytes (fig. 12B) significantly.

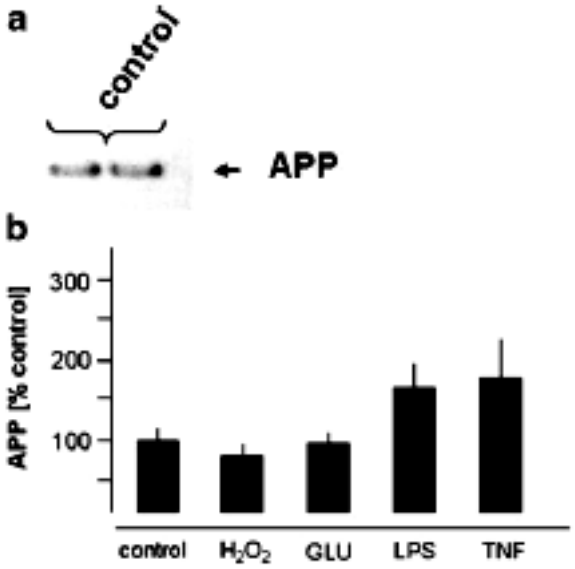

Figure 12 (A) Basal expression levels of APP in cortical astrocytes analyzed by semi-quantitative PCR (30 cycles) and agarose gel analysis. Results of real-time RT-PCR gene expression analysis are shown in (B). Toxin data are given as percentiles with control values set to 100%. All data were normalized against HPRT expression as a housekeeping gene. Data represent means ± SEM. APP is already expressed in control cultures. None of the stimuli applied induces significantly APP expression.

6.3 Discussion

The amyloid precursor protein (APP) has been under intensive study in recent years mainly due to its critical role in the pathogenesis of Alzheimer's disease (Guo et al.). APP is a type I trans-membrane glycoprotein that is constitutively expressed in different mammalian cell types. It is post-translational processed by protelytic secretase cleavage, leading to different APP-fragments (Tanzi and Bertram 2005). Despite intensive research and the fact that APP is ubiquitously expressed in many mammalian cells,, not much is known about the physiological roles of this protein under non-pathological conditions (Clarner et al. 2011a). It has been shown that the soluble and secreted form of APP (sAPP) shows similarities with growth factors and increases the *in vitro* proliferation of embryonic neural stem cells. In the subventricular zone (SVZ), i.e. the brain region where postnatal neurogenesis takes place, sAPP induces proliferation of EGF-positive neuronal progenitors (Caille et al. 2004). A clear expansion of the SVZ, based on the increase in cell density (2-3 fold) and proliferation has been shown in MS patients compared with controls. These findings correlated with elevated numbers of PSA-NCAM and GFAP-positive cells (Nait-Oumesmar et al. 2007). Our observations and reports from other groups show that the SVZ is activated in cuprizone-treated animals as well. However, we did not find increased APP immunoreactivity within the SVZ of cuprizone-treated animals. Therefore, APP might be exclusively involved in neurogenesis but not gliogenesis (Clarner et al. 2011a).

Beta-amyloid (Aβ), a peptide generated from APP, is widely believed to underlie the pathophysiology of Alzheimer’s disease (Minano-Molina et al. 2011). In the so called “amyloidogenic pathway”, APP cleavage by β- and γ-secretases generates Aβ-peptides which are the main constituents of senile plaques in this disease. Familial early-onset Alzheimer’s disease can be connected to mutations in the APP gene (Tanzi and Bertram 2005). Not only Alzheimer’s disease,but also the pathogenesis of other neurodegenerative disorders such as Niemann-Pick type C disease (Jin et al. 2004) and Down Syndrome can be associated to APP (Isacson et al. 2002). Beyond APP involvement in disease pathogenesis, the protein has been shown to be multi-facetted in function and can be induced as an acute phase protein by several cell types in the brain as response to cellular injury (Banati et al. 1993) or ischemia (Stephenson et al. 1992). If sufficently stimulated, virtually all brain resident cell types seem to be able to express APP (Gehrmann et al. 1995).

In demyelinating disorders like MS, not much is known about the role and expression of APP. Gehrmann et al. investigated six histopathologically normal control and six MS cases for presence of APP-positive cells (Gehrmann et al. 1995). They found low APP immunoreactivity in healthy subjects, whereas high levels of APP immunoreactivity can be seen in actively demyelinating lesions The amount of positively-stained cells depended on the lesion stage and was generally lower in more chronic lesions compared to acute forms. While in active lesions astrocytes, microglia, foamy macrophages, and T-cells stained positive for APP, the immunoreactivity was restricted to reactive astrocytes, their processes, and a few macrophages/microglia in more chronic lesions. The results presented by the Gehrmann group clearly demonstrate that APP is induced in reactive glial cells but equally in T-cells during demyelination (Gehrmann et al. 1995). The same group also demonstrated, that APP is expressed by microglia/macrophages in T-cell-mediated EAE (Banati et al. 1995a).

To obtain further information about expression pattern and possible role of APP during CNS demyelination, we have extended our study to toxic demyelination lesions using the cuprizone model (Clarner et al. 2011a). APP staining can be used as a marker for acute neuronal damage in this model (Lindner et al. 2009). However, induction of APP immunoreactivity in glia cells as a response to cuprizone intoxication has not been reported elsewhere. Three major isoforms of APP, consisting of 695 (small amounts), 751 or 770 amino acids (APP 770, APP 751 and APP 695) have been shown to be produced by astrocytes in culture (Rohan de Silva et al. 1997; Shepherd et al. 2000). The ratios of these isoformes appears to be connected to changes in the protein function (Gray and Patel 1993; Shepherd et al. 2000). The antibody we used in cuprizone-intoxicated mice is thought to react with the APP 770 isoform,but is predicted to react with APP 751 and 695 as well (Clarner et al. 2011a). We, therefore, cannot state which of the isoforms is present in astrocytes of cuprizone-treated animals (Clarner et al. 2011a). Importantly, we did not detect APP-positive oligodendrocytes or microglia cells in this animal model. As already mentioned and in contrast to the EAE model where APP-positive oligodendrocytes and microglia can be found (Banati et al. 1995a), no invasion of autoimmune T-cells takes place during cuprizone-induced demyelination (Kipp et al. 2009; Matsushima and Morell 2001). Our results indicate that different mechanisms of demyelination in the mentioned models lead to different responses of astrocytes,

microglia, and oligodendrocytes (Clarner et al. 2011a). A possible explanation for these differences could be the need of T-cell-derived stimuli to induce APP-expression in microglia and oligodendrocytes but not astrocytes. However, results of our cell culture experiments strongly suggest that, in astrocytes, the simultaneous application of different stimulatory factors appears to be critical to induce APP expression (Clarner et al. 2011a). We cannot completely exclude the possibility that the observed immunoreactivity in our model is due to internalized APP of a non-astrocytic source, since it is well-known that astrocytes can bind and internalize APP products *in vitro* and *in vivo* (Alarcon et al. 2005; Nagele et al. 2003). However, if this is the case, one would expect that also microglia would stain positive for APP, since it has also been shown that microglia are able to internalize APP products (Mandrekar et al. 2009). Furthermore, the applied antibody reacts most strongly with the glial 770 amino acid isoform of APP (Clarner et al. 2011a).

The precise role of astroglia-derived APP during toxic demyelination and reparative processes in the CNS needs to be further investigated. What we have learned from our model is that it perfectly mimics several aspects of MS disease progression and pathology and, therefore, is a suitable *in vivo* tool to further follow up the open questions.

7. General discussion

Research concerning neurodegenerative diseases, such as MS, largely depends on valuable animal models. Due to the extreme complexity of MS, various substantially distinct mouse models are frequently used to investigate certain aspects of the disease. One of these models is the cuprizone mouse model for de- and remyelination. In the following part, I will discuss in detail the advantages and limitations of cuprizone intoxication as a model for MS in the cerebellum, the basal ganglia system, and the hippocampus region. One outstanding hallmark of MS pathology which is dissemination in space was addressed in this animal model.

In all investigated brain areas, regions which are vulnerable to the cuprizone challenge have been found (Groebe et al. 2009; Norkute et al. 2009; Pott et al. 2009). In contrast, some regions seem to be partially protected. The cerebellar white matter, the medial basal ganglia compartment, and the hippocampal fimbria are almost completely resistant to cuprizone intoxication. In contrast, the CC, the cerebellar nuclei located in the cereballar marrow, and the lateral parts of the basal ganglia are highly vulnerable. One reason for this “partial protection” might be the spatial relation of the affected regions to the liquor system. The brain areas which were found to be protected are often located close to the ventricle system and might therefore be more effectively supplied with trophic and angiogenic factors via the cerebrospinal fluid (CSF). The CSF additionally plays an important role in the clearance of toxins and drugs (Redzic et al. 2005) and might therefore reduce the accumulation of toxins in the tissue. This might, at least in part, be the reason for the observed basal ganglia demyelination pattern. What argues against this hypothesis are our observations made in the cerebellum. There, the cerebellar peduncels as well as the nuclei located within the deep cerebellar marrow are not resistant to the cuprizone-challenge (Groebe et al. 2009).

A quite obvious reason for the observed region-specific vulnerability could be differences in the repair capacity of distinct brain regions. This seems to be especially true for the medial basal ganglia, a compartment in very close contact to the sub-ventricular zone (SVZ) where OPC are believed to originate (Gonzalez-Perez et al. 2009). Our observations indicate that the SVZ is activated during early cuprizone administration. Compared to controls, the SVZ showed a 2-3 fold increase in cell density during cuprizone treatment. Concordantly, proliferating cells can be found more frequently within the SVZ of the acute demyelinated brain. Further studies have

to show whether brain region specific vulnerability to the cuprizone challenge are attributable to repair processes.

Region-specific brain-intrinsic inflammatory processes might be another underlying mechanism. Our group was recently able to show that primary astrocyte cultures, isolated from two different brain regions (midbrain vs. cortex), differ with respect to the expression and release of inflammatory mediators under control conditions and after stimulation (Kipp et al. 2008). Thus, astrocytes and microglia likely display region specific responses *in vivo*, as well. We and others were able to show that apoptotic cells and loss of oligodendrocytes can be found in both, affected (for example within the CC) as well as "protected" areas, i.e. the fimbria region or the spinal cord (Herder et al. 2011; Kipp et al. 2011). Furthermore, early microglia activation is evident in protected and vulnerable brain regions. One might speculate that the progression of those early lesions to demyelinated ones depends on brain region-specific astrocyte and microglia function. In this context, findings from Hesse and colleagues are noteworthy. Their results suggest that the underlying mechanism of oligodendrocytes cell death is not the same during early vs. late cuprizone treatment (Hesse et al. 2010). They observed that in early but not in later stages dying oligodendrocytes express activated caspase 3. It might be that region specific microglia activity modulates lesion progression during caspase 3-independent oligodendrocytes death stages.

A common feature of all affected brain regions is the activation and proliferation of astroglia inside or nearby the demyelinated areas. An ambivalent role has been discussed for this cell type during acute demyelination, since both, astrocyte-derived harmful chemokines and prostaglandins as well as important growth factors are synthesized during acute demyelination (Barres 2008; Brambilla et al. 2009). It is apparent that astrocytes possess a dual role in disease progression which appears to be destructive at the onset and during acute demyelination but might become supportive in later stages of disease. As shown in chapter 2, astrogliosis persists during effective endogenous remyelination, whereas microglia numbers are diminished at both 9 and 21 days of remyelination (Clarner et al. 2011b), arguing for a supportive role of astrocytes during ongoing remyelination.

At a very early time point of cuprizone intoxication (2 days), microglia activation and proliferation can be found preceding accumulation and activation of astrocytes and demyelination by weeks (unpublished data from our group). Most of the highly

affected brain areas showed strong microglia activation/proliferation and higher microglia numbers could be related to more severe demyelination (Pott et al. 2009). Interestingly, demyelinated grey matter parts of the hippocampus showed no increased microglia numbers (Norkute et al. 2009). The role of microglia in the progression of an early (microglia activation and oligodendrocyte apoptosis but no demyelination or astrogliosis) towards an actively demyelinating lesion, remains speculation so far and is currently under investigation by our group.

In summary, the first part of this thesis clearly shows that certain aspects of MS, such as grey matter pathology, dissemination in space and brain intrinsic inflammation are closely mirrored in the cuprizone model. Therefore, this model can be used to elucidate the principle mechanisms of both acute demyelination and reparative processes, i.e. remyelination. One should notice that the importance of lymphocyte-driven inflammation declines with disease duration. While widespread lymphocyte infiltration is characteristic for the relapsing-remitting disease course (Lucchinetti et al. 2000), microglia/macrophages become more dominant during the progressive disease stages (van der Valk and De Groot 2000). Hence, the cuprizone model can be used to study progressive MS pathology but is certainly less valuable for studying early MS disease course.

Preventing the onset of demyelination would be the most effective treatment for MS patients. However, understanding the cause of the disease is a prerequisite to achieve this goal. So far, high doses of CS are the standard treatment during acute inflammatory relapses. Undoubtedly, CS are beneficial during acute inflammatory MS exacerbations. They effectively manage autoimmune relapses by dampening the deleterious inflammatory events in the brain. Besides relapse management, facilitating remyelination became a important research goal, since remyelination can protect axons from degeneration (Irvine and Blakemore 2008) and thereby prevents the progression of irreversible functional deficits.

Substances that are known to promote the differentiation of OPC *in vitro* are retinoic acid, thyroid hormone T3, and CS (Barres et al. 1994; Clarner et al. 2011b). These substances are thought to act via a quite similar mechanism, timing oligodendrocyte development (Barres et al. 1994). Both, retinoic acid and T3 hormones have been investigated for their potential to facilitate remyelination *in vivo*. A recent study utilizing a toxic demyelination/remyelination model convincingly showed that retinoid receptor signalling accelerates CNS remyelination (Huang et al. 2011). Furthermore

T3 hormone injections led to differentiation of cells originating in the SVZ towards progenitors with migrating properties. These OPC can actively invade demyelinated tissue and facilitate remyelination (Franco et al. 2008; Harsan et al. 2008). In contrast, and despite the already mentioned effects on OPC *in vitro,* our own study clearly shows that CS are not promoting remyelination, but impair myelin restoration in the CC. The underlying mechanisms of these effects seem to be independent from thyroid hormone signalling (Clarner et al. 2011b). We hypothesize that the negative net effect on remyelination is mediated by indirect effects on OPC. Most likely, CS-induced changes in the growth factor release of astrocytes are involved in influencing OPC myelination capacity. We could show that not only OPC but also astrocytes respond to CS treatment. We observed changes in the growth factor expression levels, e.g. FGF2 and PDGF-αα in relation to IGF-1. The role of these growth factors has been investigated with respect to remyelination in the cuprizone animal model. On one hand, the combination of FGF-2, PDGF-αα, IGF-1 and Neurotropin 3 (NT3) has been shown to activate proliferation of neural cells and drive their differentiation into mature oligodendrocytes that can remyelinate axons in demyelinating lesions (Kumar et al. 2007). On the other hand, studies on cuprizone-treated FGF2-null mice showed that FGF2 signalling is an effective component limiting remyelination of chronically demyelinated lesions (Tobin et al. 2011). For PDGF-αα, it has also been reported that elevated expression of human (h)PDGF by astrocytes prevented oligodendrocyte apoptosis following cuprizone treatment (Vana et al. 2007). In addition, a single intracerebral injection of PDGF in a chemically induced demyelination rodent model (lysolecithin) increased numbers of mature oligodendrocytes and enhanced remyelination (Allamargot et al. 2001; Moore et al. 2011). In this context, the results of chapter II highlight the need of appropriate animal models to validate *in vitro* data dealing with OPC differentiation and the importance to further understand the complex interplay of different brain cell types.

Recently, Wang and colleagues showed that GC treatment leads to increased deposition of astrocyte derived amyloid-β protein (APP-β) and decreased APP clearance in both, cultured astrocytes and *in vivo* (Wang et al. 2011). APP is a protein that has been found to have neurotrophic or neuroprotective properties in its secreted form (Lee and Wurtman 2000; Thinakaran and Koo 2008). Beyond that, it also is involved in the pathogenesis of Alzheimer's disease when spliced in the so called amyloidogenic pathway that generates APP-β. Astrocytes are thought to be an

important source of APP-β (Blasko et al. 2000; Wang et al. 2011) and APP might therefore play a role in both the neuroprotective as well as the deleterious properties of astrocytes. Since ongoing research of our group focuses on astrocyte function during acute demyelinating CNS damage and since astrocytes were found to express high levels of APP in MS lesions (Gehrmann et al. 1995), we aimed to investigate, whether the cuprizone model might be a suitable tool to study glia derived APP functions. We found activation of APP expression exclusively in astrocytes as a response to cuprizone challenge. Our results (Chapter III) indicate that APP expression by astrocytes depends on the simultaneous stimulation of different factors and is not a general reaction to activation. Interestingly, microglia does not express APP in the cuprizone model. Therefore, cuprizone intoxication might be a unique tool to study astrocyte-derived APP functions without interference of other glia cells.

8. Summary

(English)

Multiple sclerosis is a widespread demyelinating disease where primary oligodendrocyte dysfunction represents a possible underlying mechanism of myelin loss. Cuprizone intoxication which causes primary oligodendrocyte apoptosis, mimics some aspects of this disease. In the first part of my thesis, the extent and pattern of demyelination in this MS animal model was analyzed. Additionally, the innate immune responses to the cuprizone challenge were investigated. Our results regarding the cerebellum were included in this thesis, and the different pattern of demyelination in the basal ganglia, hippocampus, and cerebellum was highlighted. Striking differences were found in the magnitude of demyelination between different white and grey matter areas. Distinct cerebellar white matter regions, the cerebellar cortical grey matter, the medial basal ganglia, and the hippocampal fimbria were resistant to the cuprizone challenge. In contrast, other regions displayed severe myelin loss and activation of microglia and astroglia. We discuss the advantages and limitations of this model with regard to regional differences and “protection”.

In the second part of this work, we used the cuprizone model to investigate the impact of corticosteroid (CS) treatment on spontaneously remyelinating lesions and addressed the underlying mechanisms by several *in vitro* and *in vivo* approaches. We are able to demonstrate that while CS-treatment accelerates the differentiation of oligodendrocyte progenitors *in vitro,* it impairs endogenous remyelination *in vivo.* We additionally identified CS-induced changes in the growth factor expression profile of cultured astrocytes that might explain the impairment of repair processes in the brain. The importance of astrocytes for early and late repair processes was further highlighted by our finding that astrocytosis but not microgliosis persists after an acute demyelinating event. Our data clearly show that promotion of the intrinsic oligodendroyte differentiation program might negatively affect myelin repair *in vivo*. We speculate that CS treatment induces a preterm oligodendrocytes differentiation and, thus, might impair endogenous remyelination cascades. Furthermore, beneficial steroidal effects during inflammation have to be balaced against possible remyelination-inhibiting effects.

In the third part of the presented thesis, we focused on astrocyte’s response during an acute demyelinating event. The amyloid precursor protein (APP) which is well known to be expressed in activated astroglia, microglia and stressed neurons in

several disease models, was in the focus of the study. We found that the expression of APP is strongly induced during acute cuprizone-induced demyelination. Exclusively astrocytes express APP during cuprizone-induced demyelination. Our *in vitro* experiments using primary astrocyte cultures indicate that this is a highly specific response to toxic demyelination. The cuprizone model, therefore, is a suitable tool to study the role of astrocyte derived APP in a MS animal model.
The results of my thesis significantly broaden the knowledge regarding the potential of the cuprizone model as a tool for studying underlying mechanisms of de- and remyelination.

(German)

Die Multiple Sklerose ist eine weitverbreitete Entmarkungserkrankung, bei der die primäre Apoptose von Oligodendrozyten einen grundelegenden Mechanismus des Myelinverlustes darstellt. Das Cuprizone Tiermodell verursacht primäre Oligodendrozytenapoptose und spiegelt daher einige Aspekte der Erkrankung wider.
Im ersten Teil meiner Dissertation wurden das Ausmaß der Demyelinisierung und ihr Verbreitungsmuster in diesem Modell analysiert. Zusätzlich wurden die Reaktionen des angeborenen Immunsystems untersucht. Unsere das Cerebellum betreffenden Befunde wurden in diese Arbeit mit aufgenommen und ein Vergleich der Demyelinisierungsmuster zwischen den Basalganglien, dem Hippocampus und dem Cerebellum gezogen.
Wir konnten auffällige Unterschiede in der Ausprägung der Demyelinisierung zwischen den untersuchten Bereichen der grauen und weißen Substanz feststellen.
Bestimmte Bereiche der cerebellaren weißen Substanz und der grauen Substanz des cerebellaren Cortex, die medialen Basalganglien und die Fimbrienregion des Hippocampus waren resistent gegen Cuprizone induzierte Demyelinisierung.
Im Gegensatz hierzu zeigten andere Bereiche ausgeprägten Myelinverlust und Aktivierung von Mikro- und Astroglia. Wir diskutieren die Vorteile und Limitationen des Cuprizone Modells mit Bezug auf regionale Unterschiede und „Protektion."
Im zweiten Teil dieser Arbeit verwendeten wir das Cuprizone Modell um den Einfluss von Korticosteroiden (KS) auf spontan remyelinisierende Läsionen zu untersuchen. Sowohl *in vitro* als auch *in vivo* Versuchsansätze wurden verwendet um die zugrundeliegenden Mechanismen dieses Einflusses zu untersuchen. Wir konnten zeigen, dass KS zwar die Ausreifung von Oligodendrozyten Progenitorzellen *in vitro* beschleunigen, die endogene Remyelinisierung *in vivo* jedoch verschlechtern.

Zudem fanden wir Änderungen im Expressionsprofil astrozytengenerierter Wachstumsfaktoren in Zellkultur. Diese Änderungen könnten die Verschlechterung der Reparaturprozesse im Gehirn erklären. Unsere Ergebnisse zeigen das die Astrogliose, im Gegensatz zur Mikrogliose, in der Phase nach einem akuten demyelinisierenden Ereignis persistiert. Diese Beobachtungen untermauern die Bedeutung von Astrozyten für frühe und späte Reparaturvorgänge. Unsere Ergebnisse zeigen zudem, dass das Vorantreiben des intrinsischen Differenzierungsprogramms von Oligodendrozyten die Myelinreparatur *in vivo* negativ beeinflussen kann. Wir spekulieren, dass die Behandlung mit KS ein verfrühtes Ausreifen von Oligodendrozyten verursacht und dadurch die Remyelinisierungsvorgänge negativ beeinflusst. Daher sollten die vorteilhaften Steroideffekte während akuter Entzündungsreaktionen gegen mögliche remyelinisierungshemmende Effekte abgewogen werden.

Im dritten Teil dieser Dissertation fokussierten wir uns auf die Astrozytenantwort während akuter Demyelinisierung. Das Amyloid Vorläuferprotein (APP), von dem man weiß das es sowohl von aktivierten Astro- und Mikroglia, als auch von gestressten Neuronen exprimiert wird, stand dabei im Mittelpunkt. Wir fanden heraus, dass die APP-Expression nach Cuprizone induzierter Demyelinisierung verstärkt wird. Die APP Expression ist dabei auf Astroyzten beschränkt. Unsere *in vitro* Experimente weisen darauf hin das dies eine hochspezifische Reaktion auf toxische Demyelinisierung ist. Daher stellt das Cuprizone Modell ein geeignetes Werkzeug zur Untersuchung der Rolle von astrozytärem APP dar.

Die Ergebnisse meiner Dissertation erweitern das Wissen darüber, inwiefern das Cuprizone Modell als Instrument für das Studium der zugrundeliegenden Mechanismen von De- und Remyelinisierung dienen kann.

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11. Curriculum vitae

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12. List of publications

Articles in scientific journals:

1. Acs P *, Kipp M *, Norkute A, Johann S, **Clarner T**, Braun A, Berente Z, Komoly S, Beyer C: ***17beta-estradiol and progesterone prevent cuprizone provoked demyelination of corpus callosum in male mice*.**
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2. Baertling F, Kokozidou M, Pufe T, **Clarner T**, Windoffer R, Wruck CJ, Brandenburg LO, Beyer C, Kipp M: ***ADAM12 is expressed by astrocytes during experimental demyelination.***
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9. **Clarner T**, Parabucki A, Beyer C, Kipp M: ***Corticosteroids impair remyelination in the corpus callosum of cuprizone-treated mice.***
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13. Appendix

13.1 Appendix 1: Transcardial perfusion of mice

Perfusion Solution

- Heat 800ml of distilled water to app. 60°C
- Dissolve 3.3g $NaH_2PO_4xH_2O$ (Natriumdihydrogenphosphat-Monohydrat, Carl-Roth, Germany) and 45g $Na_2HPO_4x12H_2O$ (di-Natriumhydrogenphosphat-Dodecahydrat, Carl-Roth, Germany)
- Dissolve 20g paraformaldehyde (Sigma-Aldrich; Germany)
- Add 100ml saturated picric acid (Sigma-Aldrich; Germany)
- Let cool down to room temperature, set the pH to 7.4 and add bi-distilled water to 1000ml
- Filter, and cool down to 4°C.

Procedure

- 30min prior to start, apply 10µl Clexane-40mg (Sanofi-aventis, Germany) by intraperitoneal injection.
- Deeply anaesthetise animals with Isolfuran (5%)
- Fix the animal, open the thorax by using forceps and scissors and cut a small opening into the right heart atrium.
- Insert a small needle into the left heart ventricle and perfuse 50ml of ice-cold perfusion solution.
- Cut the head and/or the trunk
- Incubate the tissue samples overnight at 4°C in the same fixative.
- Dissect the brain/spinal cord and store in 1:5 diluted (in PBS) perfusion solution at
  4°C until used.

13.2 Appendix 2: Paraffin-embedding of mice tissue samples

Since paraffin is immiscible with water, tissue needs to be dehydrated by progressively more concentrated ethanol baths. This is followed by a clearing agent, usually xylene or Histo-clear, to get rid of the ethanol. Finally, tissue is put into molten paraffin wax (60°C). The wax is changed 3 times to get red of xylene residues.

Tissue samples were embedded in paraffin by immersing them under gentle agitation for the desired time in

- 70% Ethanol: 3 x 30min
- 96% Ethanol 3 x 30min
- 100% Ethanol 3 x 30min
- Histo-Clear (Biozym, Germany) 3 x 60min
- Paraffin (xx, Germany) 3 x 120min and then overnight at 60°C

Thereafter, tissue samples were embedded in paraffin.

13.3 Appendix 3: Immunohistochemistry

Reagents:

Tris/EDTA buffer:

- Dissolve 1.21 g Tris-(hydroxymethyl)-aminomethan (Carl-Roth, Germany) and 0.37 g ethylenediaminetetraacetic acid (Carl-Roth, Germany) in 900 ml bi-distilled water.
- Set pH to 9.0 and add bi-distilled water to 1000ml
- Add 0.5ml Tween® 20 (Sigma-Aldrich, Germany)

Sodium citrate buffer:

- Dissolve 2.94 g Sodium citrate dehydrate (Sigma-Aldrich Germany) in 900 ml bi-distilled water
- Set pH to 6.0 and add bi-distilled water to 1000ml
- Add 0.5ml Tween® 20 (Sigma-Aldrich, Germany)

Procedure:

- Deparaffinise and rehydrate cutted sections in xylol (4 x 5min), 100% Ethanol (3 x 2min), 96% Ethanol (2 x 2min), 70% Ethanol (2 x 2min) and PBS (1 x 5min).
- Perform heat-mediated antigen retrieval where necessary (compare to table 2) by boiling slides in the desired buffer. Either use Tris/EDTA (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0) or Sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0). Set a microwave to full power and wait until the solution comes to the boil. Heat for 20 minutes from this point without boiling. Let slides cool down to room temperature
- Wash in PBS (3 x 5min)
- Block endogenous peroxidase activity by incubating slides for 30 min in 0.3% hydrogen peroxide (Carl-Roth, Germany) diluted in PBS.
- Wash in PBS (3 x 5min)
- Block unspecific bindings of secondary anti-mouse antibodies by incubating the slides in blocking solution for 1 h at room temperature.
- Decant the blocking solution and apply the primary antibody over night at 4 °C diluted in blocking solution. Use a wet chamber to avoid drying-out of the slides.
- The next day, decant the primary antibody and wash in PBS (3 x 5min)
- VECTASTAIN® ABC (peroxidase) system (vectorlabs, USA) are used to label primary antibody-antigen conjugates following the manufacturer`s recommendation.
- Wash in PBS (3 x 5min)
- Incubate the slides in aminoethyl carbazole (Invitrogen, Germany) for up to 30 min in room temperature to visualize antibody-antigen conjugates.
- Counterstain with haematoxylin to visualize cell nuclei if desired
- Mount slides in Kaiser’s glycerine gelatine.

12.4 Appendix 4: SDS Polyacrylamid gel electrophoresis

Protein expression can be semi-quantitatively determined by separating denaturated proteins by SDS gel electrophoresis on polyacryamid gels followed by western blot analysis. Proteins with a high molecular mass cross the gel more slowly compared to smaller proteins. Separated proteins are then blotted on nitrocellulose membranes and can be labeled by appropriate primary antibodies. Visualization is performed on x-ray films utilizing horse radish peroxidase coupled secondary antibodies and a chemoluminescence reagent.

Reagents:

4x Resolving buffer
Tris-OH (1,5M = 36,94g)
SDS (0,4% = 0,8g)
pH 8,8
fill up to 200ml with H_2O

4x Stacker buffer
Tris-Cl (0,6 M = 18,91g)
SDS (0.4% = 0.8g)
pH 6,8
fill up to 200ml with H_2O

Preparation of gels
Polyacrylamid gels consist of two phases, a high concentrated resolving gel and a lower concentrated stacker gel.

For 1 resolving gel (8%) mix:

Water	**2.2ml**
40% Acrylamide (Rotiphorese gel 40, Roth, Germany)	**0.8ml**
Resolving buffer	**1.0ml**
10% APS (Sigma, Germany)	**40µl**
Temed (Roth, Germany)	**4µl**

Let the resolving gel polymerize (app. 30min at room temperature), then a lower concentrated stacker gel is made consisting of:

Water	**0.781ml**
40% Acrylamide (Rotiphorese gel 40, Roth, Germany)	**0.162ml**
Stacker buffer	**0.312ml**
10% APS (Sigma, Germany)	**12.5µl**
Temed (Roth, Germany)	**1.2µl**

After complete polymerization (additional 30min), the gel is ready for use in SDS-PAGE. Proteins are separated by 120V for 10min followed by 140V for 1h.