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THESIS

Regional differences of molecular factors during demyelination and early remyelination in the CNS

by

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Moharregh-Khiabani D, Blank A, Skripuletz T, Miller E, Kotsiari A, **Gudi V**, and Stangel M. Effects of fumaric acids on cuprizone induced central nervous system de- and remyelination in the mouse PLoS ONE 2010; 5(7).

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1. Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS), a chronic inflammatory demyelinating disease of the central nervous system (CNS), affects approximately 2.5 million people worldwide. Because of its high prevalence, MS is the leading cause of non-traumatic neurologic disability in young adults in the United States and Europe. Women suffer from MS twice as often as men (Sospedra and Martin 2005; Pugliatti et al., 2006). Jean-Martin Charcot, who named the condition “Sclérose en plaques” recognized MS as a distinct disease (Charcot, 1868). Clinically, there are four main subtypes of multiple sclerosis: relapsing-remitting (RRMS), secondary-progressive (SPMS), primary-progressive (PPMS), and progressive-relapsing (PRMS) (Lublin and Reingold, 1996).

Common histophatological hallmarks of MS are inflammatory plaques with multifocal perivascular infiltration of mononuclear cells including T cells, B cells, and macrophages, glial scar formation, loss of myelinating cells (oligodendrocytes), subsequent breakdown of myelin, and axonal damage/loss, which is the major cause of irreversible disability in patients with MS (Lassmann et al., 2001). To date, MS is considered to be primarily an autoimmune disease with myelin specific T and B cell reactivity, multiple genetic susceptibility loci, and as yet not defined environmental risk factors (Weinshenker, 1996; Lipton et al., 2007; Haines et al., 1996; Lincoln et al., 2005; Sawcer et al., 2004, 2008). The mechanisms underlying chronic neurological deficits are still not completely understood and the main cause of multiple sclerosis remains unknown.

Demyelinating lesions are primarily found in the white matter of periventricular areas of the brain stem, cerebellum, optic nerve, and spinal cord (Noseworthy et al., 2000). Recent studies have shown that demyelination affects also the cerebral and cerebellar cortex (Gilmore et al., 2008; Kutzelnigg et al., 2007; Kutzelnigg et al., 2005; Albert et al., 2007). Cortical demyelination is particularly prominent in primary and secondary progressive MS, but is rare

in the acute or relapsing form (Kutzelnigg et al., 2007). The underlying pathophysiological mechanisms seem to differ between white and grey matter since cortical demyelinating lesions are associated with an intact blood brain barrier, alleviated infiltration of lymphocytes, and mild astrogliosis (Bo et al., 2003; van Horssen et al., 2007). Furthermore, the remyelinating capacity of grey matter lesions seems to be higher compared to white matter lesions (Albert et al., 2007). Demyelinating lesions can also arise within the hippocampus, the part of the limbic system functionally implicated in the processes of learning and memory (Papadopoulos et al., 2009; Moscovitch et al., 2006; Squire et al., 2004) and cerebellum (Kutzelnigg et al., 2005; 2007; Gilmore et al., 2008). However, demyelination is not always permanent in MS. Spontaneous remyelination occurs frequently after demyelinating events but is often not complete (Lassmann et al., 1983; Lucchinetti et al., 1999; Raine and Wu, 1993). Remyelination corresponds with the appearance of oligodendrocytes (Lassmann, 1983; Prineas et al., 1984; Prineas et al., 1993a; Bruck et al., 1994; Lucchinetti et al., 1999). Completely remyelinated plaques, so called shadow plaques, are extensive in a considerable proportion of multiple sclerosis patients. Remyelination is not restricted to early stages of the disease and occurs in all manifestations of the disease, including primary progressive MS (Patrikios et al., 2006; Patani et al., 2007; Albert et al., 2007). However, remyelinated shadow plaques may become affected by new bouts of demyelination (Prineas et al., 1993b) and remyelination is not an invariant response to a demyelinating event in MS, even though oligodendrocyte precursor cells (OPCs) can be present in the demyelinated lesions (Chang et al., 2001; Maeda et al., 2001). To understand the mechanisms of remyelination there is a large body of experimental data derived from *in vitro* cell culture models and from animal studies.

1.2 Animal models for experimental demyelination

To induce experimental demyelination there are four different experimental approaches: genetic myelin mutation, autoimmune inflammatory induced demyelination (experimental autoimmune encephalomyelitis, EAE), viral induced demyelination (e.g. Theiler-Virus), and toxic induced demyelination (e.g. cuprizone, ethidium bromide, or lysolecithin). All these models mimic only a part of MS pathology (Fig. 1).

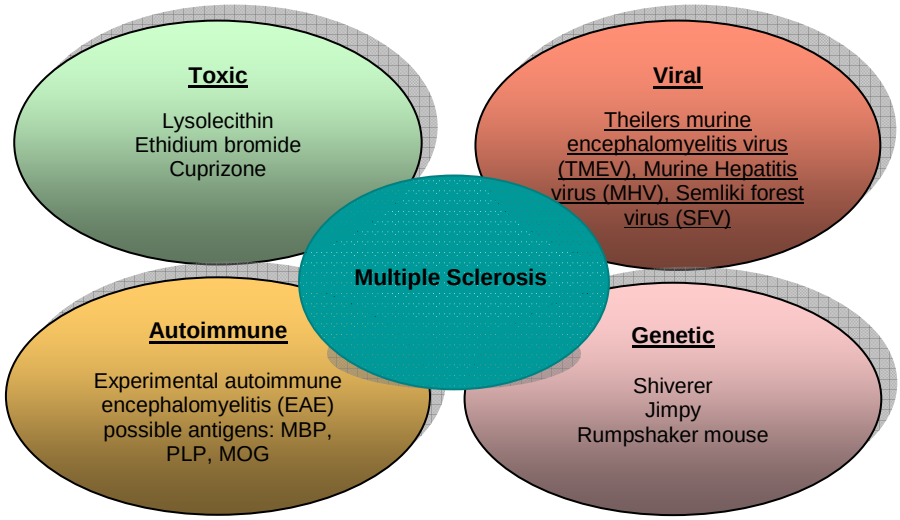

Figure 1. Experimental animal models mimic only a part of MS pathology

EAE, which reflects the inflammatory compound of MS is widely used model to study T cell mediated inflammatory demyelination in the CNS (Olitsky und Yager, 1949; Gold et al., 2000). EAE can be induced by injection of whole spinal cord lysate, purified myelin, or different myelin proteins such as myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP), or their encephalitogenic peptides. The severity of demyelination and inflammation in EAE lesions are variable and depend on the genetic background of animals and the injected antigen (Hemmer et al., 2002). Viral-induced demyelination models are also available to study inflammatory mediated demyelination. Inter alia, a mouse natural pathogen, the Theiler`s murine encephalomyelitis virus (TMEV), is used to induce CNS demyelination. Both models are characterized by scattered lesions; break down of the blood-brain barrier, and severe inflammation including T cells infiltration.

Toxic demyelination models include lysolecithin, ethidium bromide, and cuprizone. These models are characterized by good reproducibility and predefined areas of demyelination. In lysolecithin and ethidium bromide models, a focal lesion is induced by stereotactic injection of the compound into the rodent CNS (Yajima and Suzuki, 1979; Woodruff and Franklin, 1999). The toxic effect of lysolecithin is considered to be selective on myelin producing cells while ethidium bromide is toxic for all nucleolus containing cells (Woodruff und Franklin, 1999). Moreover, the stereotactic injection of toxins at least partially opens the blood-brain barrier and infiltration of peripheral inflammatory cells can not be excluded. The heterogeneity of myelinating cells, consisting of oligodendrocytes and Schwann cells, leads to increased complexity of this kind of demyelination and remyelination mechanisms (Woodruff und Franklin, 1999).

The cuprizone model is widely used to study toxin induced demyelination. In this model young adult mice are fed with the copper chelator cuprizone (bis-cyclohexanone oxaldihydrazone) for several weeks leading to a loss of oligodendrocytes and a subsequent demyelination accompanied by a strong microgliosis and astrogliosis. After cessation of the toxin, remyelination occurs within weeks. This model is reliable and has the advantage of good reproducibility regarding the amount and site of demyelination (Hiremath et al., 1998; Matsushima and Morell, 2001). Furthermore, the blood-brain barrier stays intact (Bakker and Ludwin, 1987) and remyelination can be analyzed without infiltration of T cells and peripheral macrophages, implying a reduction in the complexity of the system.

The mechanism of selective damage of oligodendrocytes is still not understood. The cuprizone induced disturbance of energy metabolism in oligodendrocytes was suggested as a main cause of oligodendrocyte death (Matsushima and Morell, 2001). The extent of de- and remyelination is strongly influenced by mouse age, gender, strain as well as the dose of cuprizone (Matsushima and Morell, 2001; Ludwin, 1980; Armstrong et al., 2002; Blakemore, 1972).

The cuprizone model has already been used for decades (Ludwin, 1978; Blakemore, 1981). Early studies were mainly focused on de- and remyelination in the superior cerebellar peduncles and in the corpus callosum (Arnett et al., 2004). Recently, severe de- and remyelination processes were also described in grey matter structures such as cerebral and cerebellar cortex, and hippocampus (Skripuletz et al., 2008, 2010; Koutsoudaki et al., 2009). Therefore, using the cuprizone model mechanisms of de- and remyelination in both grey and white matter can be analyzed.

1.3 Neurotrophic factors and their role in de- and remyelination

Differentiation of OPCs in demyelinating lesions seems to be the key determinant of efficient remyelination in MS. Inhibition of oligodendrocyte-axon interaction is also suggested to be a factor contributing to the failure of remyelination (Franklin, 2002; Lubetzki et al., 2005; Charles et al., 2000). Therefore, successful remyelination is the result of successful migration, proliferation, and differentiation of OPCs, contact with axons, and finally building of myelin sheaths (Fig. 2)

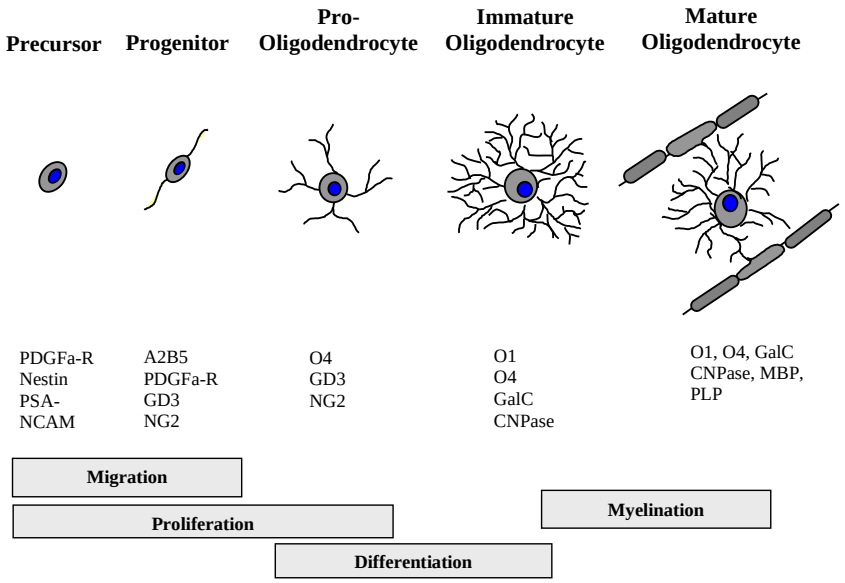

Figure 2. Stages and marker characteristic for development of the oligodendroglial lineage (modified from Stangel and Hartung, 2002, Progress in Neurobiology)

Neurotrophic factors are known to modulate migration, proliferation, and differentiation of OPCs, regulate oligodendrocyte-axon interaction and their myelination by a direct action on myelinating glial cells and, secondly, indirectly by influencing axonal signals. Neurotrophins, **nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF),** and **neurotrophin-3 (NT-3)** interact with two distinct types of transmembrane receptors, the Trks (tropomyosin related kinases) and p75NTR, which lack an intrinsic catalytic activity (Chao and Hempstead 1995; Teng and Hempstead, 2004). NT-3 has been reported to enhance proliferation and differentiation of OPCs, to support the survival of adult oligodendrocytes, and to promote myelination *in vitro* and *in vivo* (Cohen et al., 1996; Kumar et al., 1998; McTigue et al., 1998; Heinrich et al., 1999; Rubio et al., 2004). BDNF shows beneficial effects on proliferation and differentiation of OPCs and Schwann cells, and thus on myelination in the central and peripheral nervous system (McTigue et al., 1998; Tolwani et al., 2004). In EAE, BDNF delivery reduces demyelination and increases remyelination (Makar et al., 2009). Probably, BDNF also modulate developmental myelination of optic nerve (Cellerino et al., 1997). In MS lesions, BDNF is present in T cells, macrophages/microglia, and reactive astrocytes. Trk B, the full length receptor for BDNF, has been found on neurons and reactive astrocytes (Stadelmann et al., 2002). NGF, NT-3 and BDNF promote differentiation of basal forebrain oligodendrocytes. However, only NGF and NT-3 treatment increase the amount of MBP+ cells among the cortical OPCs population (Du et al., 2003). Maturation of dorsal root ganglion (DRG) OPCs is even inhibited by NGF (Chan et al., 2004). The survival of mature oligdendrocte derived from rodent brains is supported by NGF and NT-3 (Cohen et al., 1996). Additionally, NGF is involved in myelinating cell - axon interaction and promotes myelination of TrkA-expressing DRG neurons by Schwann cells, while it inhibits oligodendrocytes *in vitro* (Chan et al., 2004). Recent studies have been revealed that via TrkA NGF can induce the axonal expression of LINGO-1, one of known inhibitors of oligodendrocyte differentiation (Mi et al., 2004; Lee et al., 2007).

Epidermal growth factor (EGF) was reported to stimulate migration and proliferation of murine progenitor cells transplanted in adult rat striatum (Fricker-Gates et al., 2000) and expand the number of progenitors derived from the SVZ primary progenitors which migrate and differentiate into oligodendroglial cells (Gonzalez-Perez et al., 2009).

The influence of **basic fibroblast growth factor (FGF-2)** and **platelet-derived growth factor alpha (PDGF-A)**, as potent mitogens for OPC (McKinnon et al., 1990; Jiang et al., 2001; McMorris and McKinnon, 1996; Wolswijk and Noble, 1992) has been studied in vitro and in a number of animal models (Liu et al., 1998; Hinks and Franklin, 1999; Messersmith et al., 2000; Armstrong et al., 2002; 2006; Woodruff et al., 2004). In MS PDGFR-A expressing OPCs have been found within demyelinating lesions (Maeda et al., 2001).

Hepatocyte growth factor (HGF) is a pleiotrophic cytokine that can trigger proliferation, migration, and differentiation of various cell types. It has been reported that the functional HGF/c-Met system, which can influence proliferation, development, and cytoskeletal organization, is present in oligodendrocytes (Yan and Rivkees, 2002). HGF can induce chemotaxis of OPC *in vitro* (Lalive et al., 2005).

The impact of **Neuregulin 1 (NRG 1)** on the oligodendrocyte lineage and particularly on remyelination has been studied in several animal models. Systemic delivery of NRG 1 to mice exposed to EAE delayed signs of the disease, decreased the severity, and resulted in significant reduction in relapse rate (Cannella et al., 1998; Marchionni et al., 1999). Moreover, NRG 1 treated groups exhibited increased remyelination in CNS lesions than in controls. In contrast to EAE, in toxin-induced demyelination application of NRG 1 into demyelinated areas did not improve remyelination (Penderis et al., 2003).

The myelination promoting effects of **glial cell-derived neurotrophic factor (GDNF)** have been predominantly demonstrated in spinal cord injury animal models, in the peripheral nervous system *in vitro* by acting on Schwann cells and on neurons, and modulating Schwann cell – axon interactions (Zhang et al., 2009; Iwase et al., 2005; Hoke et al., 2003).

Transforming growth factor-beta (TGF-ß1) and **Insulin-like growth factor I (IGF-I)** are considered to be the key regulators of oligodendrocytes differentiation. TGF-ß1 is one of the essential TH2/TH3 cytokines and a potent immunosuppressor that can prevent EAE and suppress disease (Preller et al., 2007; Steinbrecher et al., 2001; Racke et al., 1991). TGF-ß1 inhibits proliferation of OPCs, promotes oligodendrocyte development (McKinnon et al., 1993), and enhances myelinogenesis (Diemel et al., 2003). It is over-expressed by reactive astrocytes within MS lesions (Peress et al., 1996).

Insulin-like growth factor I (IGF-I) seems to play the crucial role in the oligodendrocytes differentiation, survival of oligodendrocytes, and myelination (Mozell and McMorris, 1991; Barres et al., 1992; Ye and D'Ercole, 1999; Goddard et al., 1999). IGF-1 over-expressing mice show a significant increase of the number of myelinated axons and of the myelin thickness (Ye et al., 1995). IGF-I knock-out (KO) mice exhibit a decreased number of oligodendrocytes and myelinated axons in the corpus callosum and anterior commissure (Beck et al., 1995). Beneficial effects of IGF-1 on remyelination have been studied in various experimental demyelination models (Yao et al., 1995; Mason et al., 2003).

Despite of IGF-1 and TGF-ß1 also **ciliary neurotrophic factor (CNTF)** and **leukaemia inhibitory factor (LIF)** seem to be strongly involved in the regulation of the oligodendrocyte lineage. Both molecules act via gp130 receptors and could enhance the generation of oligodendrocytes in cultures of dividing O-2A progenitors and promote oligodendrocyte maturation, as determined by expression of myelin basic protein (Mayer et al., 1994). CNTF promotes genesis, differentiation, maturation, and survival of oligodendrocytes derived from developing and adult CNS (Mayer et al., 1994; Barres et al., 1996; Marmur et al., 1998; Talbott et al., 2007). Moreover, CNTF has been shown to enhance myelination in vitro (Stankoff et al., 2002). The promyelinating effect of CNTF is proposed to be mediated through the JAK/Stat pathway (Stankoff et al., 2002). CNTF, but also LIF, can prevent death of oligodendrocytes under pro-inflammatory conditions *in vitro* (Louis et al., 1993; Vartanian

et al., 1995). LIFRβ is expressed on oligodendrocytes and is activated in affected tissue (Butzkueven et al., 2002). There are also reports that LIF is involved in the differentiation of oligodendrocytes *in vivo* (Ishibashi et al., 2009). In cuprizone induced demyelination LIF-knock-out mice display more severe demyelination and impaired remyelination, although oligodendrocyte replenishment is not significantly compromised (Marriott et al., 2008). In the EAE, LIF receptor signalling limits the severity of inflammatory demyelination (Butzkueven et al., 2002). This study shows that LIF directly prevents oligodendrocyte death in EAE.

Understanding the role of individual growth factors and their complex interplay during de- and remyelination will open new opportunities to develop successful MS therapies (Fig. 3).

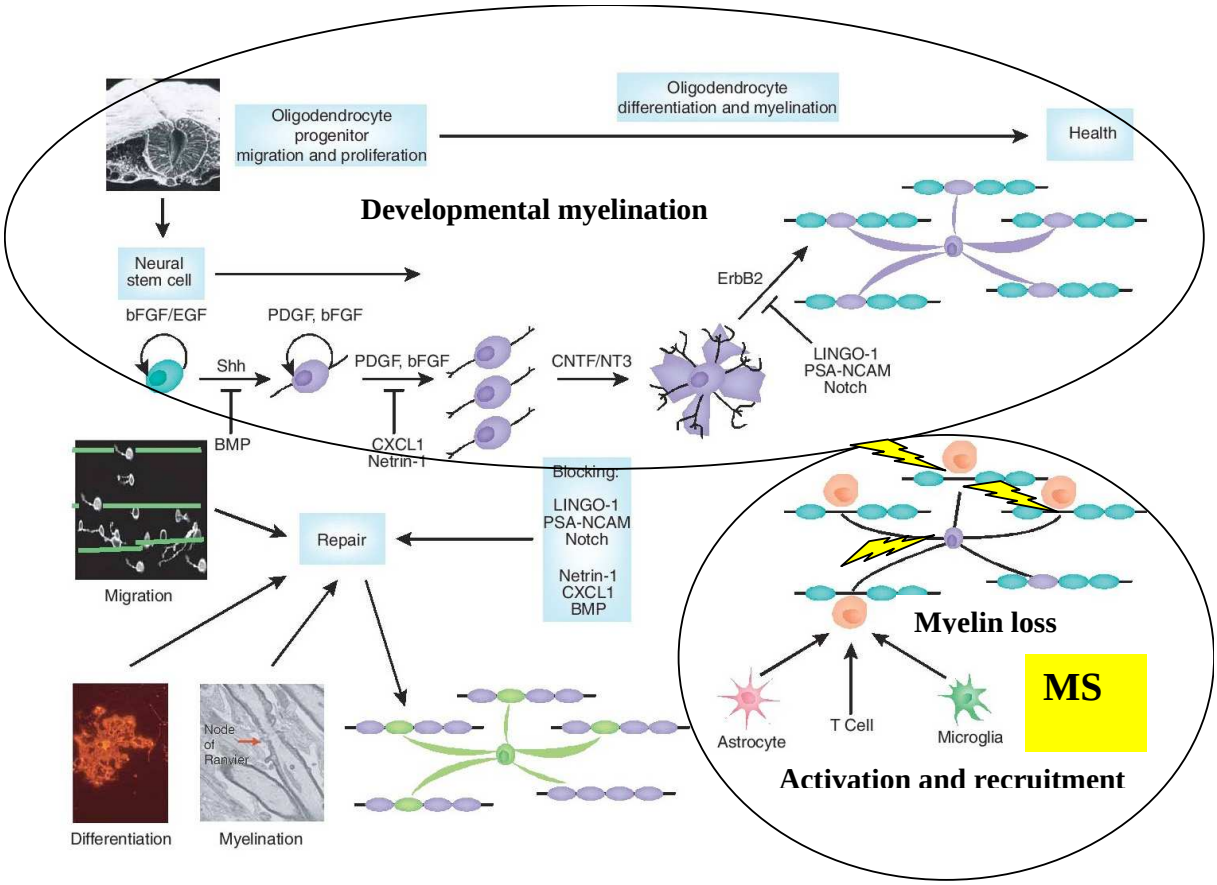

Figure 3. Myelination by oligodendrocytes in the CNS results from a sequential series of events, which are regulated by several factors. Successful myelin repair (green sheaths, bottom left) requires the recapitulation of the developmental stages with migration, differentiation and myelination by OPCs recruited from the adult CNS. Perturbation of critical steps at any stage will lead to a failure of myelin repair (modified from Miller and Mi, 2007, Nature Neuroscience).

1.4 Clinical trials and possible therapies

The primary cause of MS is still not known. Since dysfunctions of the immune system are supposed to be the main compound in the pathology of MS, most clinical trials focused on immunomodulatory therapies. The currently available MS therapies are also aligned to control the immunmediated mechanisms. These include immunosuppressive agents and immunomodulatory agents like several recombinant versions of cytokine Interferon beta (Johnson et al., 1990). Another approved drug is glatiramer acetate (GA, Copaxone©, Teva Pharmaceuticals, Petah Tikva, Israel), a synthetic copolymer of glutamic acid, lysine, alanine and tyrosine. New therapies, like treatment with Nataluzimab (Tysabri©, Biogen Idec, Massachusetts, USA), antibodies to alpha 4 integrin or Mitoxantrone (Novantrone©, OSI Pharmaceuticals, New York, USA), an antineoplastic agent that inhibits DNA and RNA synthesis of B and T cells are also directed toward the alleviation of autoimmune response. Currently, several clinical trials are approaching enhancement of remyelination and neuroprotection.

Fumaric acid esters (FAE) are a group of compounds which are currently investigated as an alternative oral drug for the treatment of relapsing remitting multiple sclerosis. In the placebo-controlled phase II study treatment with FAE resulted in a significant improvement of various MRI parameters in MS patients with relapsing-remitting MS (Kappos et al., 2008). The precise mechanism of FAE action is not yet clear, but both an immunomodulatory and a neuroprotective effects are suggested (Linker et al., 2008).

In *in vitro* studies, immunomodulatory effects of FAE were observed on T cells (Treumer et al., 2003), B cells (Mrowietz and Asadullah, 2005), and dendritic cells (Litjens et al., 2004; Zhu and Mrowietz, 2001). In the EAE animal model, treatment with FAE led to a significant therapeutic effect on the disease course. Furthermore, in the inflammatory lesions the numbers of microglia/macrophages but not of T cells were reduced.

Beside its immunomodulatory effects FAE showed the potential for a cell protective effects via activation of detoxifying pathways in different glial cells *in vitro* (Wierinckx et al., 2005). In human peripheral blood mononuclear cells (PBMC) FAE induced an increase of the anti stress protein heme oxygenase1 (HO-1), which led to a reduction of the intracellular glutathione content (Lehmann et al., 2007).

Minocycline, an antibiotic of the second generation tetracycline showed immunomodulatory and neuroprotective properties like inhibition of T cell proliferation (Kloppenburg et al., 1995), microglial activation and proliferation (Yrjanheikki et al., 1999; Dommergues et al., 2003; Fan et al., 2007). The decrease of microglial activation induced by minocycline has been postulated to be a neuroprotective mechanism in ischaemic models of stroke (Yrjanheikki et al., 1999). The effects of minocycline were also tested in EAE, where minocycline led to beneficial effects on inflammation, demyelination, and disease activity (Popovic et al., 2002).

2. Aims

In multiple sclerosis the pathomechanisms leading to demyelination seem to be different in grey and white matter since there are only mild lymphocytic infiltrates in the cortical MS lesions compared to the white matter lesions. Although new aspects of underlying pathomechanisms leading to demyelination are being discovered continuously, the complex biological interactions are far from being completely understood. Therefore, animal models like the cuprizone model may be helpful in exploring the different mechanisms.

The main aim of this study was to perform detailed analysis of cuprizone induced CNS demyelination in the grey and white matter. The dynamics of cuprizone induced demyelination and glial reactions were studied in different CNS regions including corpus callosum, cerebral cortex, hippocampus, cerebellar white matter, and cerebellar cortex.

Growth factors are known to play a crucial role in the development of the oligodendroglial lineage. Thus, in the next step it was aimed to analyze the mRNA expression pattern during de- and remyelination in grey and white matter regions of the cerebrum.

Currently, all available MS therapies are aligned to modulate the immune mechanisms. There is no reliable therapy is available that could support remyelination or promote repair. Thus, development of new regenerative therapies is an important issue in the MS research. In this study we investigated the impact of fumarc acid esthers (FAE) and minocycline on cuprizone induced de- and remyelination.

Chapter I

Regional differences between grey and white matter in cuprizone induced demyelination

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Brain Research, 2009

Preface – about this manuscript

Cuprizone feeding is a commonly used model to study experimental de- and remyelination.
In the first part of the dissertation, we analysed the dynamics of de- and remyelination in the cerebral cortex and the corpus callosum. Demyelination in the cortex was delayed as compared to the corpus callosum. Remyelination in the corpus callosum was observed even before the termination of cuprizone administration. The cellular response during the demyelination process was stronger in the corpus callosum as compared to the cortex. Overall, the cuprizone model is an excellent tool to investigate de-and remyelination in both white and grey matter and to uncover the regional molecular differences.

The text of the original publication can be found here: *Brain Research 2009,* Volume 1283, Pages 127-138.

Chapter II

Temporal analysis of growth factor mRNA expression in the white and grey matter during cuprizone induced demyelination and remyelination

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In preparation

Abstract

Growth factors are crucial for the development and maintenance of glial cells and are also strongly involved in the regulation of glial responses in various pathological conditions including de- and remyelination in the central nervous system. Its concert of signals and their temporal and spatial expression are not well characterized. Here we have analyzed the temporal mRNA expression profile of thirteen growth factors during cuprizone induced de- and remyelination using laser microdissection and real-time PCR techniques from the corpus callosum and cerebral cortex. When corpus callosum and cortex were compared, a similar pattern of growth factor mRNA expression was observed for demyelination We found a strong up-regulation of neuregulin 1 (NRG 1) and glial cell-derived neurotrophic factor (GDNF) and slightly increase of ciliary neurotrophic factor (CNTF) and epidermal growth factor (EGF) in the first week of cuprizone treatment in both the corpus callosum and the cortex. Hepatocyte growth factor (HGF), basic fibroblast growth factor (FGF-2), insulin-like growth factor I (IGF-I), and transforming growth factor-beta 1 (TGF-ß1) were up-regulated mainly during peak of demyelination at weeks 3-4.5. For remyelination different growth factor mRNA expression levels were detected in the regions analyzed. mRNA levels of GDNF, CNTF, HGF, FGF-2, and brain-derived neurotrophic factor (BDNF) were elevated in the corpus callosum but not in the cortex, suggesting differences in molecular regulation of remyelination in the white and grey matter. The knowledge of factors promoting successful remyelination may be a prerequisite for the design of therapeutic strategies for remyelination in MS. This study confirms a role of FGF-2, TGF-ß1, IGF-1, CNTF, and LIF also in cuprizone induced demyelination in both white and grey matter. Furthermore, new factors such as NRG 1, GDNF, and HGF could be identified as possible modulators of de- and remyelination as well glial responses in this animal model. mRNA expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) was on the control level during de- and remyelination in the cortex. Neurotrophins seem not to be involved in the cortical de- and remyelination. In contrast,

different mRNA expression pattern were seen for the neurotrophins during de- and remyelination in the corpus callosum, suggesting different molecular regulation of de- and remyelination in white and grey matter.

Introduction

Multiple sclerosis (MS) is a chronic, immune-mediated, demyelinating, neurodegenerative disease of the central nervous system (CNS) affecting predominantly young adults. The primary cause of MS is still unknown. The underlying pathophysiological mechanisms seem to differ between white and grey matter since cortical demyelinating lesions are associated with an intact blood brain barrier, alleviated infiltration of lymphocytes, and mild astrogliosis (Bo et al., 2003; van Horssen et al., 2007). In recent years a bunch of neurotrophic factors has been characterized to be involved in the pathology of MS (Mirowska-Guzel, 2009; Frota et al., 2009). Neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neuropoietic cytokines, ciliary neurotrophic factor (CNTF), transforming growth factor-b (TGF-ß1), leukaemia inhibitory factor (LIF), and other growth factors such as hepatocyte growth factor (HGF), platelet-derived growth factor alpha (PDGF-A), basic fibroblast growth factor (FGF-2), insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), glial cell-derived neurotrophic factor (GDNF), and neuregulin 1 (NRG 1) are suggested to support migration, proliferation, and differentiation of glial cells and to regulate myelin synthesis (reviewed by Althaus et al., 2008; Rosenberg et al,. 2006; Butt and Berry, 2002). Using these powerful agents to protect glial cells and neurons from damage or enhance remyelination may open new opportunities for MS therapy.

Animal models like the murine cuprizone model are commonly used to study experimental de- and remyelination. Cuprizone (bis-cyclohexanone oxaldihydrazone) feeding leads to oligodendrocyte death and a subsequent reversible demyelination in the corpus callosum and cortex (Skripuletz et al., 2008; Torkildsen et al., 2008). Recently, we have shown that the temporal course and dynamics of de- and remyelination differ in the corpus callosum and cortex (Gudi et al., 2009). To further investigate these differences and the implication of growth factors during de- and remyelination the temporal and spatial profile of growth factors was analyzed for mRNA expression levels in the CNS white and grey matter.

Experimental procedures

Animals, induction of demyelination, tissue preparation

C57BL/6 male mice were obtained from Charles River (Sulzfeld, Germany). Animals underwent routine cage maintenance once a week and were microbiologically monitored according to Federation of European Laboratory Animal Science Associations recommendations (Rehbinder et al., 1996). Food and water were available ad libitum. All research and animal care procedures were approved by the Review Board for the Care of Animal Subjects of the district government (Lower Saxony, Germany) and performed according to international guidelines on the use of laboratory animals.

Demyelination was induced in 8-week-old male C57BL/6 mice by feeding 0.2% cuprizone (bis-cyclohexanone oxaldihydrazone, Sigma-Aldrich Inc., St.Louis, MO, USA) mixed into a ground standard rodent chow for 4.5 weeks. For remyelination animals were returned to normal chow for an additional 1.5 weeks.

Tissue preparation

At different time points (weeks 1, 2, 3, 3.5, 4, 4.5 for demyelination, weeks 5, 5.5, and 6 for remyelination) mice were sacrificed and perfused via the left cardiac ventricle with RNAse free phosphate buffered saline (PBS) for gene expression analysis or with 4% paraformaldehyde (PFA) in phosphate buffer for immunohistochemistry studies. A group size of four or five animals was investigated at each time point. For gene expression analysis the brains were removed and immediately embedded in Tissue Tek® Compound (Sacura, USA), frozen in liquid nitrogen and stored at -80°C until use. Under RNase free conditions, serial coronal sections (bregma 0.98 to -2.46; Paxinos and Franklin, 2001) with a thickness of 30µm were cut at -20° C. The sections were mounted on polyethylene-naphthalate (PEN) membrane slides (Carl Zeiss MicroImaging GmbH, Germany), fixed for 2 min in 70% icecold ethanol, rinsed with DEPC-treated water, and stained for 30 sec in 1% cresyl violet acetate solution

(Sigma-Aldrich, Germany) in 50% ethanol. Afterwards, sections were dehydrated in a graded ethanol series (70% and 100% ethanol) and finally air dried for several minutes. All solutions were prepared with DEPC-treated water.

For immunohistochemistry brains were postfixed in 4% PFA in PBS at 4°C overnight, cryoprotected in 30% sucrose in PBS for 24 h followed by embedding in tissue-freezing medium and flash-freezing on dry ice. For light microscopy, 10 µm coronal serial frozen sections were cut. Sections between bregma -0.70mm and -1.46mm (according to mouse atlas by Paxinos and Franklin, 2001) were analysed.

Laser microdissection

The Palm® MicroBeam System (Carl Zeiss MicroImaging GmbH, Germany) was used to precisely excise the cerebral cortex and medial part of the corpus callosum from coronal brain sections of pre-treated and age matched control mice (see Fig. 1). Dissected brain regions of the corpus callosum and cortex were collected separately with a sterile 21-gauge needle and stored until RNA extraction at -80°C.

RNA isolation and real-time quantitative RT-PCR

According to the manufacturer's recommendations total RNA was extracted from microdissected cortex and corpus callosum using the RNeasy®Mini Kit (Qiagen, Germany) and RNeasy® Micro Kit (Qiagen, Germany) respectively. The RNA concentration was measured with NanoDrop 1000 devise (Thermo Fisher Scientific, USA). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). RNA samples from a selected set of cuprizone treated and age matched control mice (cortex n=4, corpus callosum n=5) were parallel processed under the same conditions. Real-time quantitative RT-PCR analysis was performed using the StepOne™ Real-Time PCR

System and appropriate TaqMan assays (Applied Biosystems, USA). All primers were intron-spanning (Table 1). A negative control containing PCR amplification mix without reverse transcribed cDNA template was included for each PCR plate. Gene expressions of NGF, BDNF, NT-3, CNTF, IGF-1, NRG 1, NGF, EGF, FGF-2, GDNF, PDGF-A, LIF, and TGF-β1 were analyzed in the corpus callosum and the cortex at 9 time points (demyelination phase: weeks 1, 2, 3, 3.5, 4, 4.5; remyelination phase: weeks 5, 5.5, and 6). The ΔΔCt method was used to determine differences in expression between cuprizone treated and age-matched control mice. Changes in mRNA expression level were calculated after normalization to Hypoxanthin Phosphoribosyltransferase *(HPRT)*.

Histology and immunohistochemistry

Frozen sections were air dried at room temperature for 20 min. For inhibition of endogenous peroxidase activity, sections were treated with 3% H_2O_2 then blocked for 1 h with PBS containing 3% normal goat serum, 0.1% Triton X-100, and incubated with primary antibody at 4°C overnight. Anti-proteolipid protein (PLP) (1:500, mouse IgG, Serotec, Germany) and anti-Nogo-A (1:750, rabbit, polyclonal, Chemicon) were used as markers for myelin protein PLP and oligodendrocytes respectively. After washing, sections were incubated with biotinylated secondary antibody (1:500, anti rabbit/mouse/rat IgG (H+L), Vector Laboratories, UK) for 1 h, followed by peroxidase-coupled avidin-biotin complex (ABC Kit, Vector Laboratories, UK). Reactivity was visualized with diamino-3,3' benzidine (DAB, Dako Cytomation, Germany).

Activated microglia were detected using lectin ricinus communis agglutinin 1 (RCA-1) (1:1000, fluorescein coupled, Vector Laboratories). Glial fibrillary acidic protein (GFAP) (1:200, mouse IgG, Millipore, USA or rabbit polyclonal, Dako Cytomation, Germany) was selected as a marker for astrocytes.

Determination of de- and remyelination in the cortex and corpus callosum

The extent of cortical demyelination was studied as described previously (Skripuletz et al., 2008). In particular, myelin protein-stained sections for PLP were scored using a light microscope (Olympus DP 72, Germany). Scoring of demyelination was performed by three blinded observers, using a scale from 0 (complete lack of myelin) to 4 (normal myelin) (Skripuletz et al., 2008). For determination of demyelination in the corpus callosum PLP stained sections were scored on a scale from 0 (complete demyelination) to 3 (normal myelin) by three blinded investigators (Lindner et al., 2008).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Fisher-PLSD-test or Dunnett test for post hoc comparison. LIF mRNA was not detected in the control animals in both the corpus callosum and the cortex. The normalization for LIF was done with the data from week 6. All data are given as arithmetic means ± standard error of the mean (SEM). *P* values of the different ANOVAs are given in the results, while group comparisons derived from post hoc analysis are provided in the figures. In the latter cases, significant effects are indicated by asterisks (compared to the preceding time point) or rhombs (compared to control, $*^{\#}p < 0.05$; $**^{\#\#}p < 0.01$; $***^{\#\#\#}p < 0.001$).

Results

De- and remyelination induced by cuprizone feeding

To determine the de- and remyelination pattern in response to 4.5 weeks of cuprizone treatment, immunohistochemical stainings and real time PCR analyses for the myelin protein PLP were performed. As demonstrated in figure 2, severe loss of PLP was observed in the corpus callosum after 4.5 weeks of cuprizone feeding ($p<0.0001$) (Fig. 2C-F, K). At week 6 (after 1.5 weeks of remyelination on normal chow), immunoreactivity for PLP was nearly completely recovered in the corpus callosum (Fig. 2F, K). mRNA expression for PLP was strongly down regulated from the first week and remained at low levels during the following weeks of cuprizone treatment (Fig. 2M). After cuprizone withdrawal from the diet, PLP mRNA expression returned to normal level.

In the cortex, severe loss of PLP was detected at weeks 5 and 6 ($p<0.0001$) (Fig. 2G-J, L). PLP mRNA expression was massively decreased from the first week and continued at low levels during whole cuprizone diet (Fig. 2N). The normalization of PLP mRNA expression was achieved at weeks 5.5 and 6, approximately 0.5-1 week later than in the corpus callosum.

Glial reactions during cuprizone treatment

To follow the oligodendroglial cellular response to cuprizone feeding we used the marker Nogo-A. After 4.5 weeks of cuprizone treatment no Nogo-A positive cells were visible neither in the medial corpus callosum nor in the cortex (Fig. 3B, F). Oligodendrocytes reappeared first in the corpus callosum at week 5 (Fig. 3C). In the cortex, Nogo-A positive cells were seen only sporadically at week 5. Increased amounts of Nogo-A positive cells were detected in the cortex at week 6 (Fig. 3G, H).

Accumulation of activated microglia was studied by RCA-1 staining. In the corpus callosum RCA-1 positive cells were detected already after 2 weeks of cuprizone feeding. During the following 2.5 weeks the amount of activated microglia increased and reached a peak at week

4.5 concomitant with the demyelination peak (Fig. 3J). Thereafter, a continuous decrease of activated microglia was observed (Fig. 3K, L). In the cortex, microgliosis occurred in an obviously reduced density compared to the corpus callosum. Within the cortex a small extended infiltration of activated microglia was found at weeks 3.5, 4.5, and 5 (Fig. 3N-P). At week 6 microglia were only sporadically found in the cortex (Fig. 3P).

Astrogliosis was studied by GFAP immunostaining. Consistent to our previous results (Gudi et al., 2009), few GFAP positive astrocytes were seen in the corpus callosum in untreated controls, while in the cortex GFAP positive astrocytes were found sporadically (Fig. 3Q, U). After 2 weeks of cuprizone treatment a strong astrogliosis was observed in the cortex. However, in the corpus callosum astrogliosis occurred delayed, particularly at the week 3, and was still detectable in both regions until week 6 during remyelination (Fig. 3R-T, V-X).

Nestin positive cells were detected in the cortex at the week 2 (Fig. 4C). These cells showed astrocytic shape and were double positive for the astrocytic marker, GFAP (Fig. 4G). Interestingly, nestin positive cells were situated mostly in the fourth cellular layer of the lateral cortex. At the following weeks nestin positive cells were sporadically seen in the cortex, mainly in the fifth and sixth cellular level (Gudi et al., 2009). In the corpus callosum nestin positive cells occurred only by some animals sporadically at the week 2. The main amount of these cells were observed at the weeks 3 (Fig. 4D, H), 4, and 4.5 followed by gradually decrease, as described previously (Gudi et al., 2009)

Analysis of growth factor mRNA expression profile during de- and remyelination in corpus callosum and cortex

To identify growth factors secreted in the white and grey matter during de- and remyelination, mRNA expression of thirteen growth factors was studied in the medial corpus callosum and the lateral cerebral cortex of cuprizone treated mice. As shown in figures 5, 6, and 7 different pattern of growth factor mRNA expression could be identified. NRG 1 and GDNF mRNA

expression was massively up-regulated after the first week of cuprizone feeding in both the corpus callosum and cortex (for both $p<0.0001$). During the following weeks NRG 1 and GDNF mRNA expression normalized (Fig. 5C, D, E, F). Parallel to the remyelination start the amount of GDNF mRNA increased again in the copus calosum (approx. 8 times) though failed to be significant.

mRNA production of CNTF and EGF showed significant changes mostly in the white matter (corpus callosum: for both $p<0.0001$; cortex: for CNTF $p=0.01$; EGF $p=0.05$). Both factors were slightly up-regulated after the first week of demyelination (CNTF approx. 4 times in the corpus callosum and 2.5 times in the cortex; EGF approx. 2.5 times in the corpus callosum and 3.6 times in the cortex). Towards the end of the remyelination phase CNTF and EGF mRNA were strongly elevated in the white matter only (Fig. 5G, H, I, J).

Upon cuprizone treatment TGF-ß1 and IGF-1 mRNA expression increased significantly and reached its peak after 4.5 weeks in both investigated areas (for both $p<0.0001$). With progressing remyelination, mRNA expression of these two factors decreased gradually (Fig. 6C, D, E, F).

The mRNA syntheses of HGF and FGF-2 were strong regulated in both white and grey matter during the whole experimental time window (corpus callosum: for both factors $p<0.0001$; cortex: FGF-2 $p<0.0001$; HGF $p=0.05$) (Fig. 6G, H, I, J). Whereas in the cortex FGF-2 mRNA was increased at a constant level during the whole demyelination phase there was a peak of mRNA elevation at weeks 4 and 4.5 in the corpus callosum. During remyelination mRNA of HGF and FGF-2 continued to be elevated in the corpus callosum in contrast to the cortex where mRNA expression of these two factors was on control level. Upon cuprizone treatment mRNA expression of NT-3 and NGF decreased significantly (for both $p<0.0001$) in the corpus callosum (Fig. 7 C, E). With the cessation of the cuprizone diet NT-3 and NGF mRNA expression returned to normal level.

BDNF mRNA synthesis was only slightly elevated in the corpus callosum during the first 3.5 weeks of cuprizone feeding, and normalized within the following weeks including maximal demyelination. During the remyelination phase mRNA expression of BDNF increased significantly ($p = 0.001$) in the corpus callosum (Fig. 7G). In the cortex, mRNA expression levels of NT-3, NGF, and BDNF did not change and persisted on levels comparable to controls during both de- and remyelination phases (Fig. 7D, F, H).

PDGF-A mRNA synthesis was not changed during de- and remyelination in both regions (Fig. 7I, J).

The expression of LIF mRNA in both the corpus callosum and the cortex was hardly detectable. Thus, these mRNA expressions were calculated in comparison to the mRNA expression at week 6. In the white matter, LIF mRNA was particularly increased at weeks 2 and 3 of cuprizone feeding ($p=0.05$) (Fig. 7K). In the grey matter, LIF mRNA synthesis was strongly increased during the first weeks ($p=0.002$) and at the peak of demyelination (Fig. 7L). Results for different mRNA expression pattern are summarized in the table 2.

Discussion

We have previously reported that the time course of cuprizone induced de- and remyelination differs in the corpus callosum and cortex (Gudi et al., 2009). We hypothesised, that different growth factors may influence de- and remyelination in the white and grey matter. Therefore, we analyzed the mRNA expression of thirteen neurotrophic factors during cuprizone induced demyelination and the subsequent remyelination in the corpus callosum and cerebral cortex in detail. Especially during remyelination differences between the corpus callosum and cortex were found in the profile of growth factor expression, suggesting regional differences in the regulation of remyelination.

As previously described (Hesse et al., 2009), diminished numbers of mature oligodendrocytes were detectable already after 1 week of cuprizone treatment. Subsequently, in both the corpus callosum and the cortex severe loss of myelin proteins was observed after 4-4.5 weeks of cuprizone feeding. Due to the termination of the cuprizone diet at week 4.5 newly generated oligodendrocytes, which were numerously present already at weeks 5 and 5.5 in the corpus callosum and at weeks 5.5-6 in the cortex promoted the expression of myelin proteins. The myelin protein changes could be confirmed by real-time PCR data for PLP that showed diminished mRNA levels during demyelination and up-regulated levels during remyelination. When cortex and corpus callosum were compared, the temporal myelination pattern was different in both regions, and maximal loss of myelin proteins occurred delayed in the cortex. Along with demyelination, microglial infiltration was observed in both regions analyzed and was remarkably increased in the white matter of the corpus callosum compared to the cortical areas, which is consistent with previous reports (Hiremath et al., 1998; Gudi et al., 2009). According to previous data, astrogliosis was as prominent in the cortex and as it was in the corpus callosum during de- and remyelination (Skripuletz et al., 2008; Gudi et al., 2009). The role of astrogliosis in cuprizone induced demyelination is not completely understood. Reactive astrocytes are known to be powerful providers of growth factors. The expression of

CNTF and its receptor, CNTFRa, can also be detected in astrocytes following brain injury (Ip et al., 1993; Rudge et al., 1994; Asada et al., 1995; Kirsch et al., 1998). Since application of exogenous CNTF induces reactive astrogliosis and the up-regulation of GFAP mRNA expression (Kahn, et al., 1997), CNTF is suggested to be a key player in astrogliosis. In our present work CNTF mRNA expression was up-regulated in the first two weeks of cuprizone feeding in both the corpus callosum and cortex and may account for the astrogliosis during demyelination.

For GDNF and NRG 1 mRNA expression we observed a strong up-regulation only after the first two weeks of callosal and cortical demyelination. In the human brain, the membrane associated form of NRG 1 was identified in cortical neurons, while released soluble NRG 1 activity was found on astrocytes in the white matter (Pankonin et al., 2009). It can be possible that neurons produce this factor, which stimulates astrocytes to subsequently secrete other growth factors. GDNF has a proliferative effect on C6 glioma cells (Suter-Crazzolara and Unsicker, 1996) and it has been demonstrated that GDNF is up-regulated in astrocytes during pathophysiological conditions such as spinal cord injury (Satake, 2000; Ikeda et al., 2002; Lee et al., 2006; Miyazaki et al., 2001). Thus, the up-regulation of GDNF may provide another activating signal for astrocytes. Such an early mRNA up-regulation of these three growth factors suggests that CNTF, GDNF, and NRG 1 may represent the key molecules to drive astrogliosis and microgliosis in the cuprizone model. This assumption can be supported with our observation of nestin expressing astroglia at week 2 in the cortex. Nestin expressing astroglia were seen in the corpus callosum sporadically at the week 2 with a clear increase at week 3 and 4.

CNTF, GDNF, and EGF showed a second peak in mRNA expression by the onset of remyelination in the corpus callosum, but not in the cortex. The involvement of GDNF in myelination has been predominantly described for spinal cord injury and in the peripheral nervous system, and *in vitro* by acting on Schwann cells and neurons (Zhang et al 2009; Iwase

et al., 2005; Hoke et al., 2003). CNTF was shown to promote OPCs differentiation in optic nerve, but it is not supportive for differentiation of cortical OPCs (Power et al., 2002). In addition, CNTF and CNTF receptor alpha expression pattern differ between white and grey matter astrocytes (Dallner et al., 2002). Generally, CNTF promotes genesis, differentiation, maturation, and survival of oligodendrocytes derived from developing and adult CNS (Mayer et al. 1994; Barres et al. 1996; Marmur et al. 1998; Talbott et al. 2007). Under *in vitro* conditions an enhancement of myelination was shown (Stankoff et al. 2002). CNTF has been detected in astrocytes in the remyelinating phase after viral-induced spinal cord demyelination (Albrecht et al., 2003). However, CNTF mRNA has not been detected in the lysolecithin rat animal model (Hinks and Franklin, 1999). Also exogenously applied recombinant CNTF has not shown any beneficial effects on OPC proliferation, differentiation, and survival in ethidium bromide induced demyelination (Talbott et al., 2007). The differences between CNTF effects on remyelination in different regions and demyelination models may be due to heterogeneity of OPC populations and involvement of peripheral inflammatory cells in the lysolecithin and ethidium bromide models.

BDNF mRNA expression was increased only in the corpus callosum in the first 3.5 weeks of cuprizone feeding and showed a second peak during remyelination. In the cortex no changes of BDNF mRNA expression were observed. BDNF has shown beneficial effects on differentiation and myelination of OPCs and can reduce demyelination and increase remyelination (Makar et al., 2009). Moreover, it has been suggested, that astrocytes and oligodendrocytes may exist as a heterogeneous population of cells, expressing different neurotrophin receptors in various combinations or separately. Since cortical OPCs do not express the full-length BDNF receptor, trkB, BDNF can not promote differentiation of cortical oligodendrocytes (Du et al., 2003). Probably, cortical OPCs do not require BDNF support for their differentiation, explaining observed BDNF mRNA expression differences in the white and grey matter.

NT-3 and NGF mRNA expression was significantly down-regulated in the corpus callosum after 2 weeks of cuprizone feeding. In the cortex, all analyzed neurotrophins seem not to play a role during either demyelination or remyelination. The mRNA expression of these three neurotrophic factors did not change during the whole experiment.

Since elevated TGF-ß1 expression was found in injured brain and in neurodegenerative diseases including MS, it was suggested that TGF-ß1 may play an important role in inflammatory processes. (Nichols et al., 1991; Klempt et al., 1992; Kiefer et al.,1993a,b; Logan et al., 1994; Laping et al., 1994). Astrocytic expression of IGF-I is markedly increased during and/or after a variety of CNS injuries (Gluckman et al., 1992; Lee et al., 1996; Liu et al., 1994; Garcia-Estrada et al., 1992; Komoly et al., 1992; Yao et al., 1995). In our study, the dynamics of IGF-1 and TGF-ß1 mRNA expression with gradual elevation of mRNA expression with a peak at week 4.5 (the time point of severe demyelination, especially in the corpus callousum) were similar in both regions studied. This is in line with data from other experimental demyelination models where IGF-1 and TGF-ß1 are strongly up-regulated during demyelination in different animal models (Hinks and Franklin, 1999; Fushimi and Shirabe, 2004). The time course of IGF-1 mRNA expression is consistent with the studies of Mason (Mason et al., 2000), where the whole brain samples were studied. Here we examined the IGF-1 mRNA expression separately in the corpus callosum and the cortex. During remyelination IGF-1 and TGF-ß1 mRNA expression continued to be elevated in both the corpus callosum and the cortex. This finding is consistent with studies in lysolecithin induced demyelination in rats (Hinks and Franklin, 1999). Based on data from different animal models and *in vitro* studies, IGF-1 is considered to be a key modulator of oligodendrocyte differentiation and myelination (McMorris et al., 1986; Saneto et al., 1988; Mozell and McMorris, 1991; Barres et al., 1992; Goddard et al., 1999; Ye et al., 1995; Beck et al., 1995).

mRNA expression of HGF and FGF-2 was slightly elevated during demyelination, primarily in the corpus callosum. HGF expression has been detected during acute demyelination in EAE

(Moransard et al., 2009). It has also been reported that a functional HGF/c-Met system, which can influence the proliferation, development, and cytoskeletal organization, is present in oligodendrocytes (Yan and Rivkees, 2002). HGF induces chemotaxis of OPCs *in vitro* (Lalive et al., 2005). Since FGF-2 expression is up-regulated in nearly every model of experimental CNS demyelination (Armstrong et al., 2002; Liu et al., 1998; Hinks and Franklin, 1999; Messersmith et al., 2000) it seems to be strongly involved in the regulation of distinct processes during demyelination. FGF-2 is considered to be a potent mitogen for OPCs (McKinnon et al., 1990; Jiang et al., 2001; McMorris and McKinnon, 1996; Wolswijk and Noble, 1992). In our previous study, we already reported that the strongest proliferating activity of OPCs was observed at weeks 4 and 4.5, during the peak of demyelination (Gudi et al., 2009). In contrast to the cortex, where mRNA levels of FGF-2 and HGF returned to that of controls, an increase of FGF-2 and HGF mRNA expression was observed in the corpus callosum also during remyelination. HGF producing OPCs were present in spinal cord EAE lesions during the recovery phase, but not in the acute stage of disease (Lalive et al., 2002).

For FGF-2 a significant inhibitory effect on progenitor differentiation and myelination has been shown (Bansal and Pfeiffer, 1997; Goddard et al., 2001; Armstrong et al., 2002, 2006). Thus, we assume that the down-regulation of FGF-2 in the cortex allows OPC differentiation and thus myelin formation. However, a different situation seems to be in the corpus callosum. Up-regulation of FGF-2 during remyelination has been reported in lysolecithin induced demyelination (Hinks and Franklin, 1999) and also *in vitro*, in myelinating aggregate cultures (Copelman et al., 2000). It can only be speculated that FGF-2 possesses multiple functions and acts not only directly on oligodendrocytes but also influences other cell types promoting myelination indirectly.

Further, based on the literature and our results IGF-1, FGF-2, and TGF-ß1 may also regulate migration, proliferation, and differentiation of OPCs. The peaks of IGF-1, FGF-2, and TGF-

ß1 mRNA expression corresponded, especially in the corpus callosum, with the phase of proliferation of OPCs and the start their differentiation.

In summary, the dynamics of growth factor expression differ in the corpus callosum compared to that in the cortex. During remyelination mRNA expression of CNTF, FGF-2, HGF, BDNF, and GDNF were up-regulated in the corpus callosum but not in the cortex, suggesting different regulation of remyelination in the white and grey matter. Taking our results and the published data in consideration, the following scenario of de- and remyelination orchestration in the corpus callosum and the cortex could be suggested: NRG 1, GDNF, and CNTF induce astrogliosis. In turn, reactive astrocytes produce TGF-ß or LIF that further support astrogliosis and are chemotactic for both astrocytes and microglia. Activated microglia and reactive astroglia may release IGF-1, FGF-2, and HGF, which support migration, proliferation, and finally initiate together with TGF-ß1 differentiation of OPCs. CNTF, GDNF, but also FGF-2, BDNF and HGF are suggested to be the key players in promotion of remyelination in the corpus callosum, either directly or indirectly. In the cortex, possibly due to differences in astroglia and OPC populations and the involvement of neurons, these latter factors may not be required or act in different time windows

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Table legends

Growth Faktor	Assay number
NGF	Mm00443039_m1
BDNF	Mm01334042_m1
NT-3	Mm01182924_m1
GDNF	Mm00599849_m1
NRG 1	Mm00626552_m1
CNTF	Mm00446373_m1
IGF-1	Mm00439560_m1
TGF-ß1	Mm00441724_m1
FGF-2	Mm00433287_m1
HGF	Mm01135184_m1
LIF	Mm00434761_m1
PDGF-A	Mm01205760_m1
EGF	Mm01316968_m1
HPRT	Mm00446968_m1

Table 1. Following TaqMan® Gene Expression Assays were used to investigate mRNA expression for different growth factors

Growth factors	Early demyelination (weeks 1-3)	Severe demyelination (weeks 3.5-4.5)	Early remyelination (weeks 5, 5.5)	Remyelination (week 6)
NGF Corpus callosum	↔	↓	↔	↔
NGF Cortex	↔	↔	↔	↔
BDNF Corpus callosum	↖	↔	↖	↖
BDNF Cortex	↔	↔	↔	↔
NT-3 Corpus callosum	↔	↓	↓	↔
NT-3 Cortex	↔	↔	↔	↔
GDNF Corpus callosum	↑	↔	↔	↖
GDNF Cortex	↑	↔	↔	↔
NRG1 Corpus callosum	↑	↔	↔	↔
NRG1 Cortex	↑	↔	↔	↔
CNTF Corpus callosum	↖	↔	↔	↑
CNTF Cortex	↖	↔	↔	↔
EGF Corpus callosum	↖	↔	↔	↖
EGF Cortex	↖	↔	↔	↖
IGF-1 Corpus callosum	↑	↑	↑	↑
IGF-1 Cortex	↔	↖	↖	↔

Growth factors	Early demyelination (weeks 1-3)	Severe demyelination (weeks 3.5-4.5)	Early remyelination (weeks 5, 5.5)	Remyelination (week 6)
FGF-2 Corpus callosum				
FGF-2 Cortex				
TGF-ß1 Corpus callosum				
TGF-ß1 Cortex				
PDGF-A Corpus callosum				
PDGF-A Cortex				
HGF Corpus callosum				
HGF Cortex				
LIF Corpus callosum				
LIF Cortex				

Strong up-regulation (≥10 times)

Mild up-regulation (<10 times)

No change

Down-regulation

Table 2. Schematic summary of mRNA expression patter

Figure legends

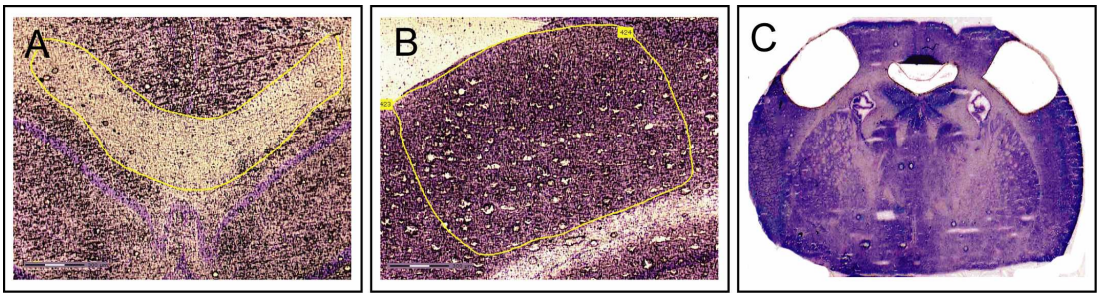

Figure 1

Laser microdissection. A) microdissected area of the corpus callosum; **B)** microdissected area of the cortex; **C)** Overview of coronal section with the dissected corpus callosum and the cortex, stained with cresyl violet.

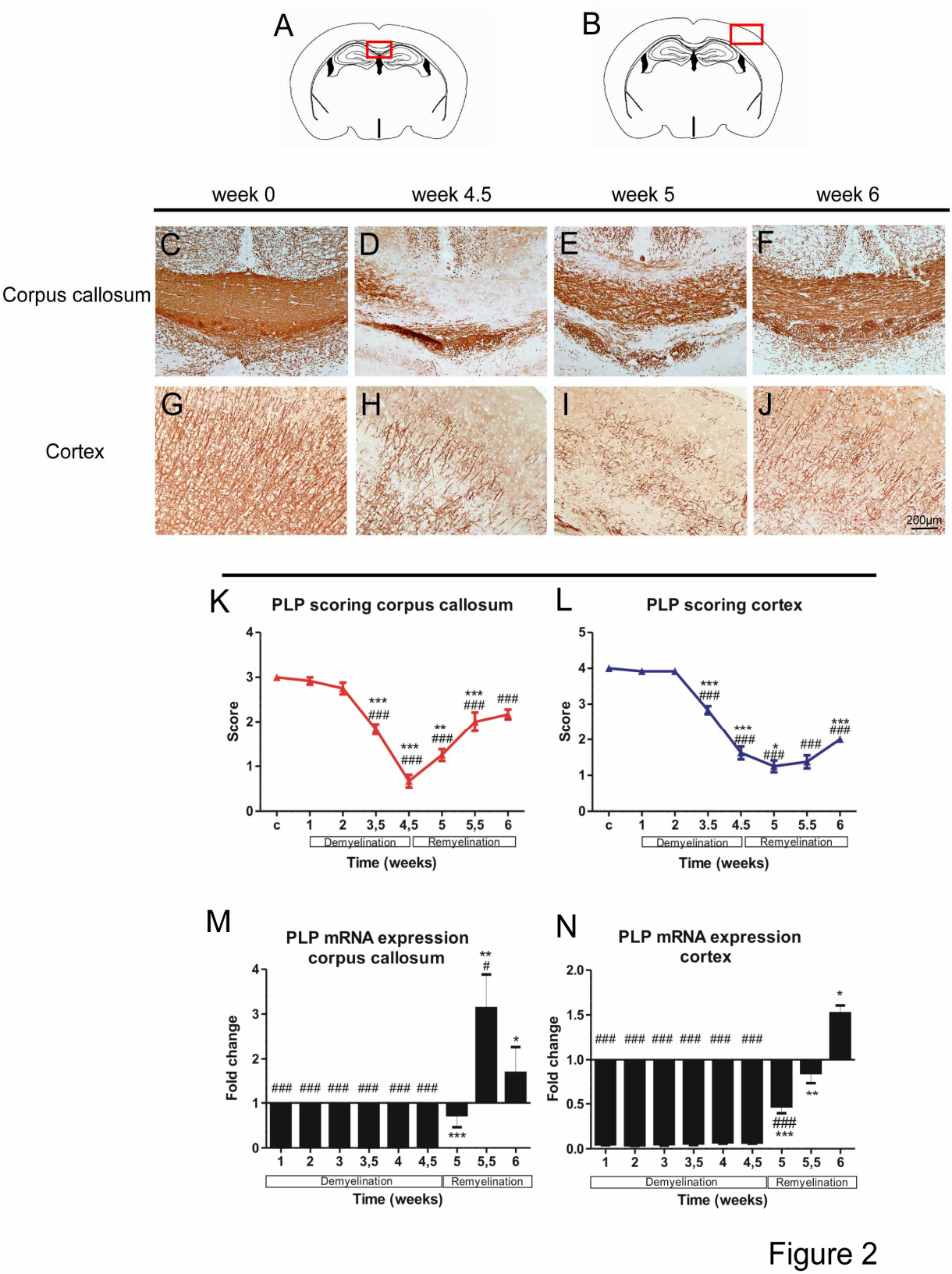
A
B
week 0
week 4.5
week 5
week 6
Corpus callosum
Cortex
C
D
E
F
G
H
I
J
200µm
K
PLP scoring corpus callosum
Score
Time (weeks)
Demyelination
Remyelination
L
PLP scoring cortex
M
PLP mRNA expression
corpus callosum
Fold change
N
PLP mRNA expression
cortex

Figure 2

Figure 2

Demyelination and remyelination in the corpus callosum and the cortex, studied by PLP mRNA and protein expression. A) Schematic diagram of the mouse brain in coronal sections. The red line shows the investigated middle part of corpus callosum. **B)** The red line marks the area of the investigated cortex. **C-F)** PLP-stained sections demonstrate severe demyelination in the corpus callosum at week 4.5. Remyelination starts already at the week 5 and is almost complete at week 6. **G-J)** PLP-stained sections show severe demyelination in the cortex at weeks 4.5, 5, and 6. At week 6 the PLP amount increases. **K)** PLP expression in the corpus callosum. Score of 0 represents complete myelin protein loss, score of 3 represents normal myelin protein amount in the corpus callosum. **L)** PLP expression in the cortex. Score of 0 represents complete loss of myelin protein; score of 4 shows a normal amount PLP. **M)** mRNA expression of PLP in the corpus callosum. **N)** mRNA expression of PLP in the cortex

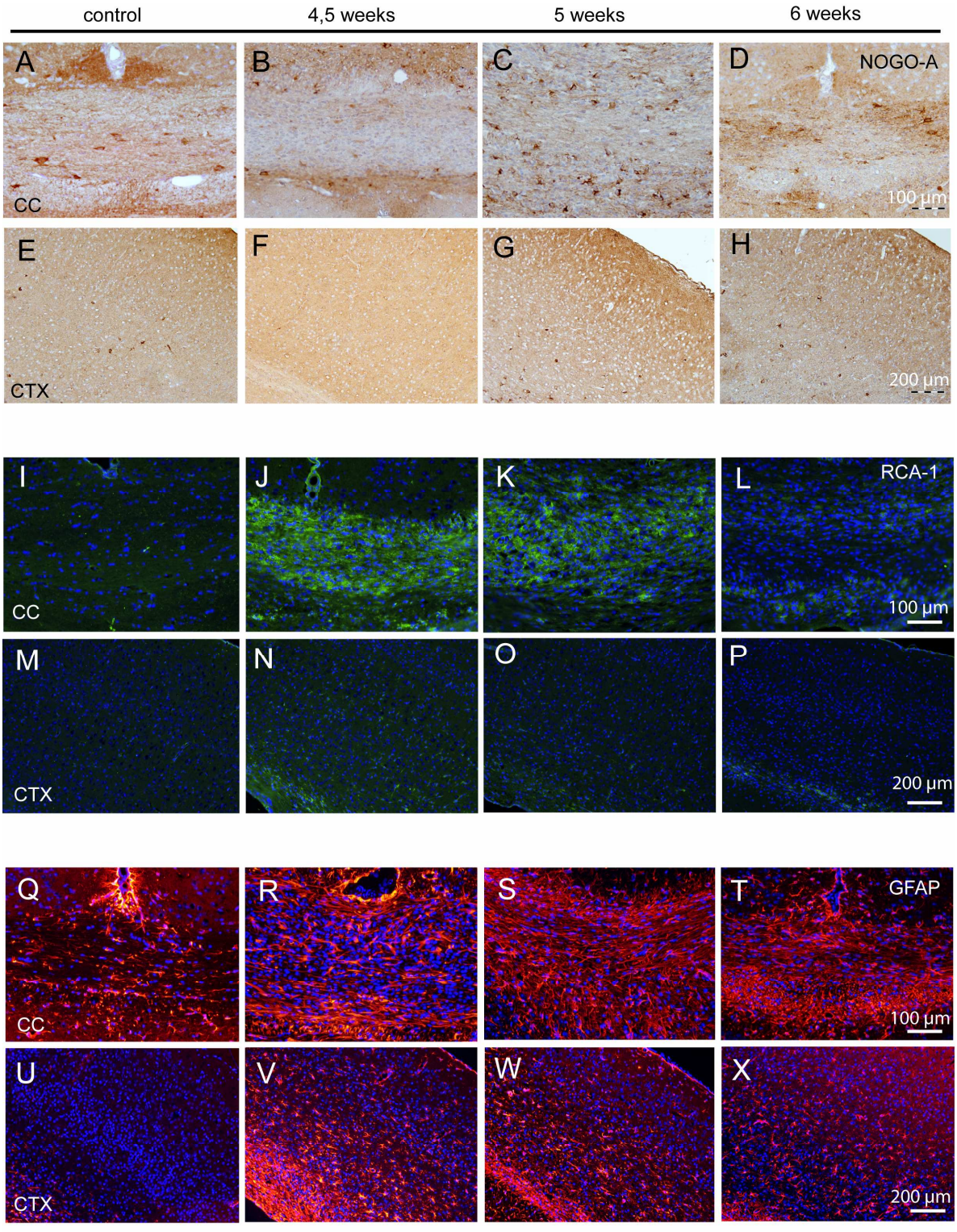
control
4,5 weeks
5 weeks
6 weeks
A
B
C
D
NOGO-A
CC
100 µm
E
F
G
H
CTX
200 µm
I
J
K
L
RCA-1
CC
100 µm
M
N
O
P
CTX
200 µm
Q
R
S
T
GFAP
CC
100 µm
U
V
W
X
CTX
200 µm

Figure 3

Figure 3

Oligodendrocyte, microglia, and astrocytes during peak demyelination (week 4.5) and remyelination (weeks 5-6) in the corpus callosum and the cortex. Representative sections show oligodendrocytes in the corpus callosum **(A-D**) and the cortex **(E-H)** visualized with anti-Nogo-A and DAB. No oligodendrocytes are seen in the corpus callosum and the cortex at week 4.5 **(B, F)**. Oligodendrocytes reappear at week 5 in both, the corpus callosum and the cortex **(C, G)**. Representative sections show microglia in the corpus callosum **(I-L**) and the cortex **(M-P)**, stained with fluorescein coupled anti-RCA-1 (in green). The peak of microgliosis was observed at week 4.5 in both the corpus callosum and the cortex. Astrogliosis is shown in the corpus callosum **(O-T**) and the cortex **(U-X)**, stained with anti-GFAP and Alexa 555 secondary antibodies (in red). In untreated animals numerous GFAP positive cells are found in the corpus callosum **(Q)** in contrast to only few GFAP positive cells in the cortex **(U)**. Upon cuprizone treatment reactive astroglia appear in the cortex and in the corpus callosum. At week 4.5 hypertrophic astrocytes are abundantly detected in both areas **(R, V)**. At weeks 5 and 6 astroglia are still presented in large numbers in the corpus callosum and the cortex, however, the shape of astrocytes alters and their processes become thinner **(S, T, W, X)**. For nucleus staining, slides were counterstained by DAPI.

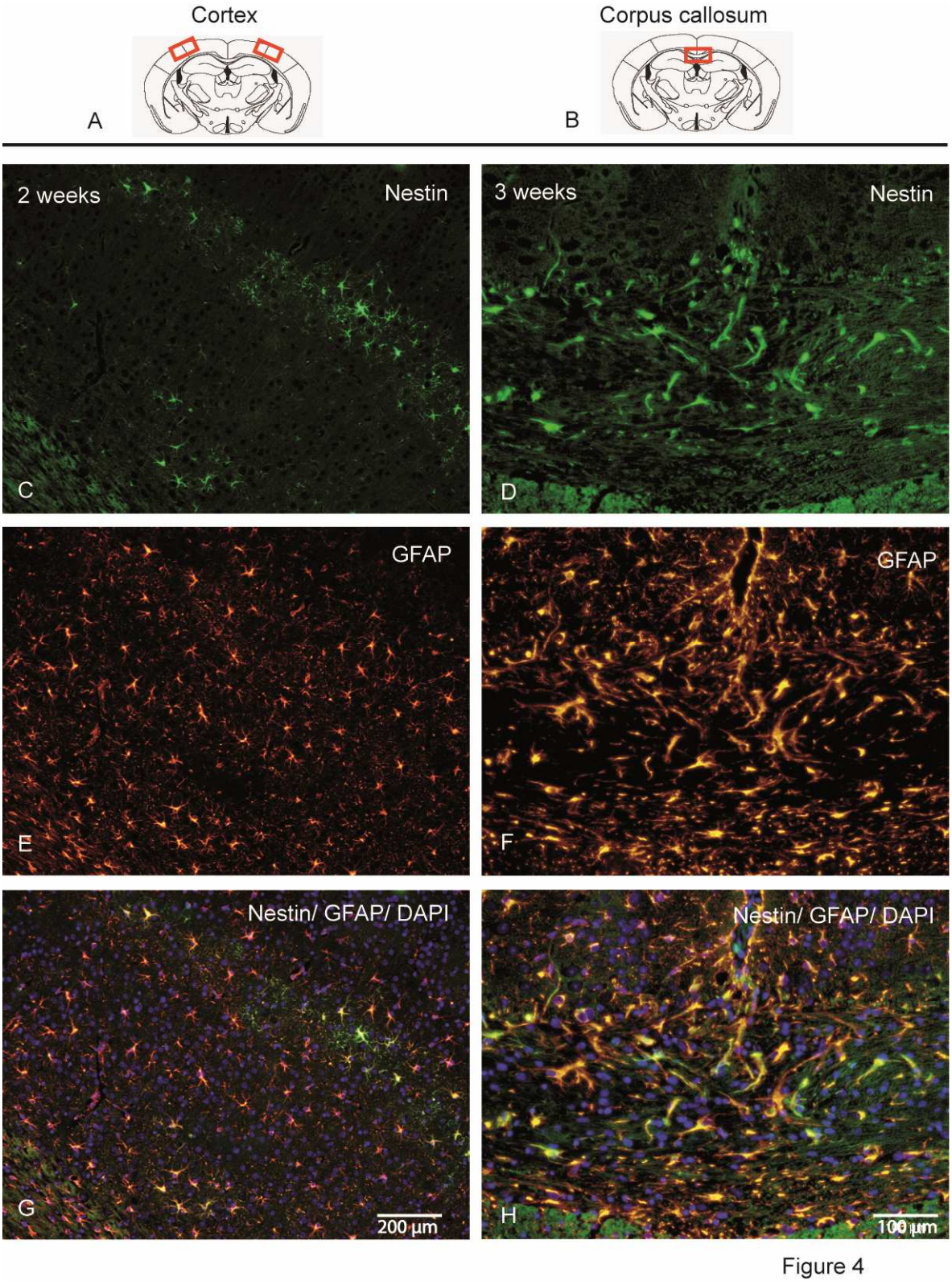
Cortex
A
Corpus callosum
B
2 weeks
Nestin
C
3 weeks
Nestin
D
GFAP
E
GFAP
F
Nestin/ GFAP/ DAPI
G
200 µm
Nestin/ GFAP/ DAPI
H
100 µm

Figure 4

Figure 4

Astrocytic expression of nestin during early demyelination in the corpus callosum and the cortex. A) Schematic diagram of the mouse brain in coronal section. The red line marks the area of the investigated cortex. **B)** The red line shows the investigated middle part of the corpus callosum. Nestin positive cells (in green) are present at week 2 of the cuprizone diet in the cortex **(C)** and at the week 3 in the corpus callosum **(D)**. GFAP stained activated astrocytes (in red) are numerously present at week 2 of the cuprizone diet in the cortex **(E)** and at the week 3 in the corpus callosum **(F)**. Several nestin positive cells are double positive for GFAP in both the cortex **(G)** and the corpus callosum **(H)** (Nestin is shown in green, GFAP in red, nuclear staining with DAPI in blue)

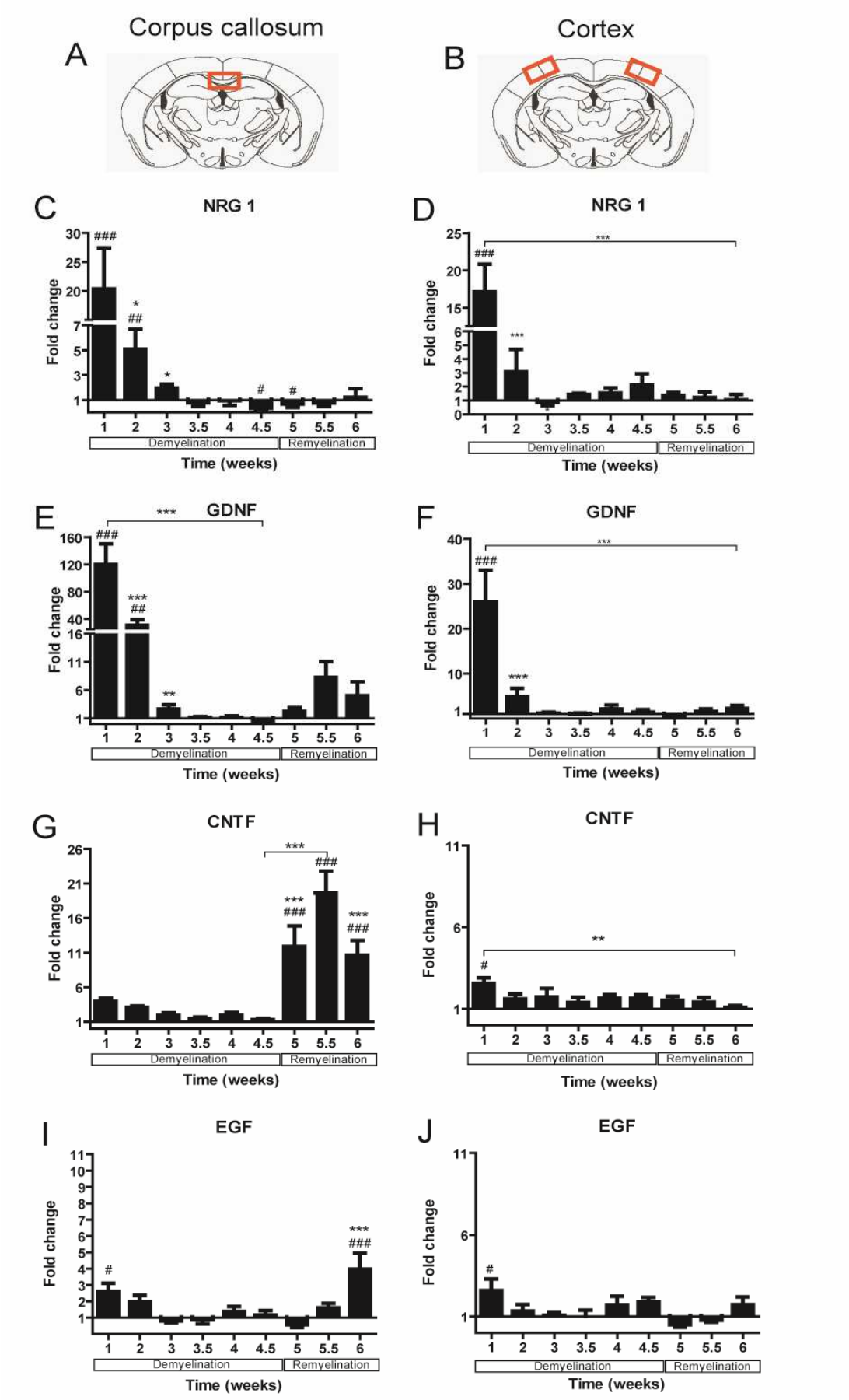
Corpus callosum
Cortex
A
B
C
NRG 1
D
NRG 1
E
GDNF
F
GDNF
G
CNTF
H
CNTF
I
EGF
J
EGF
Fold change
Time (weeks)
Demyelination
Remyelination
1 2 3 3.5 4 4.5 5 5.5 6

Figure 5

Figure 5

Expression of growth factor mRNA in the corpus callosum and the cortex of mice undergoing demyelination / remyelination. A) Schematic diagram of the mouse brain in coronal section. The red line shows the investigated middle part of the corpus callosum. **B)** The red line marks the area of the investigated cortex. **C-J)** Graphs show mRNA expression fold changes of NRG1, GDNF, CNTF, and EGF in the corpus callosum (C-I) and in the cortex (D-J) compared to the age-matched controls and normalized with HPRT using the ΔΔCt method. Significant changes are indicated by rhombs (compared to control) or asterisks (compared to the preceding time point, $*^{\#}p < 0.05$; $**^{\#\#}p < 0.01$; $***^{\#\#\#}p < 0.001$, ANOVA).

Figure 6

Expression of growth factor mRNA in the corpus callosum and the cortex of mice undergoing demyelination / remyelination. A) Schematic diagram of the mouse brain in coronal section. The red line shows the investigated middle part of the corpus callosum. **B)** The red line marks the area of the investigated cortex. **C-J)** Graphs show mRNA expression fold changes of TGF-ß1, IGF-1, HGF, and FGF-2 in the corpus callosum (C-I) and in the cortex (D-J) compared to the age-matched controls and normalized with HPRT using the ΔΔCt method. Significant effects are indicated by rhombs (compared to control) or asterisks (compared to the preceding time point, $*^{\#}p < 0.05$; $**^{\#\#}p < 0.01$; $***^{\#\#\#}p < 0.001$, ANOVA).

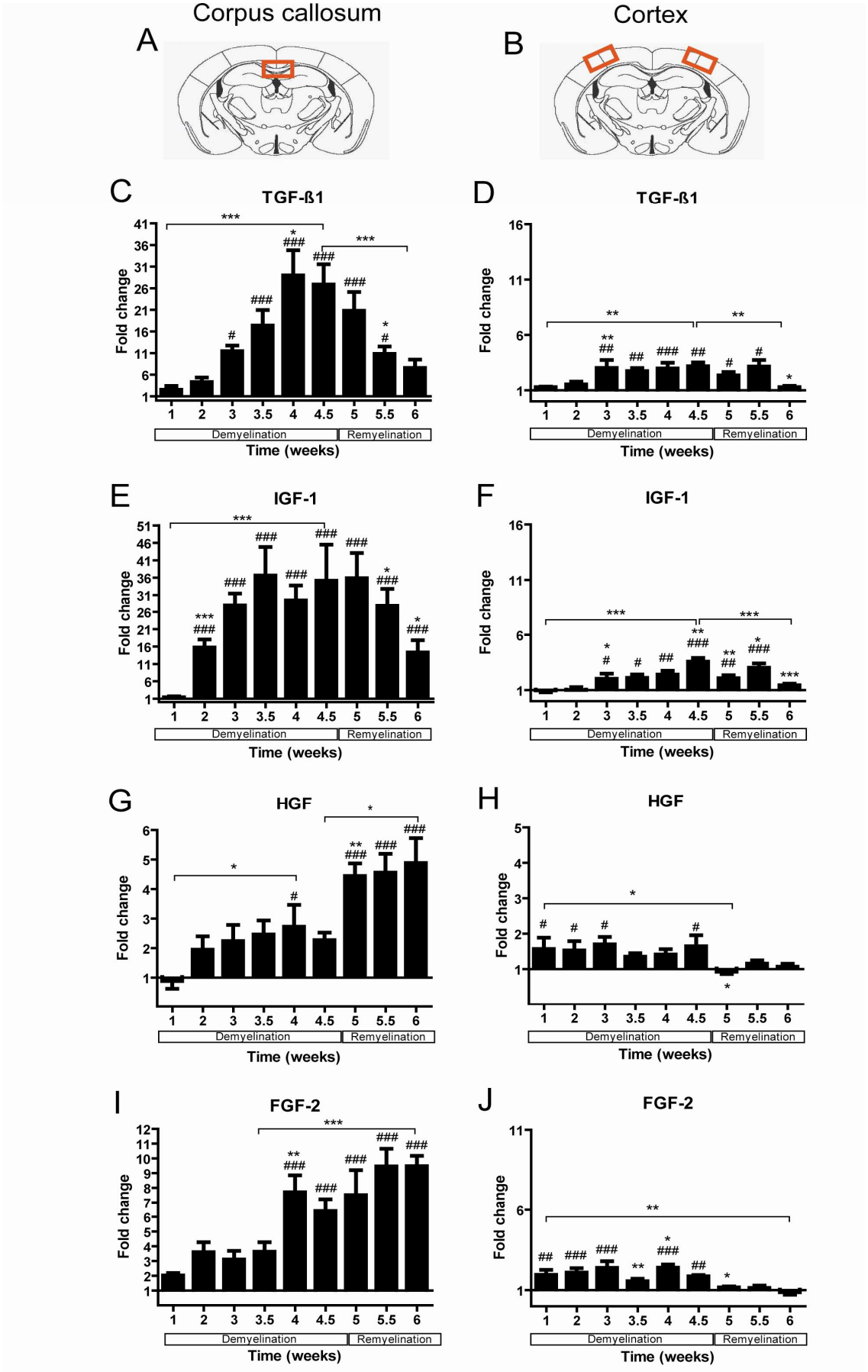
Corpus callosum
Cortex
A
B
C
TGF-ß1
D
TGF-ß1
E
IGF-1
F
IGF-1
G
HGF
H
HGF
I
FGF-2
J
FGF-2
Fold change
Time (weeks)
Demyelination
Remyelination

Figure 6

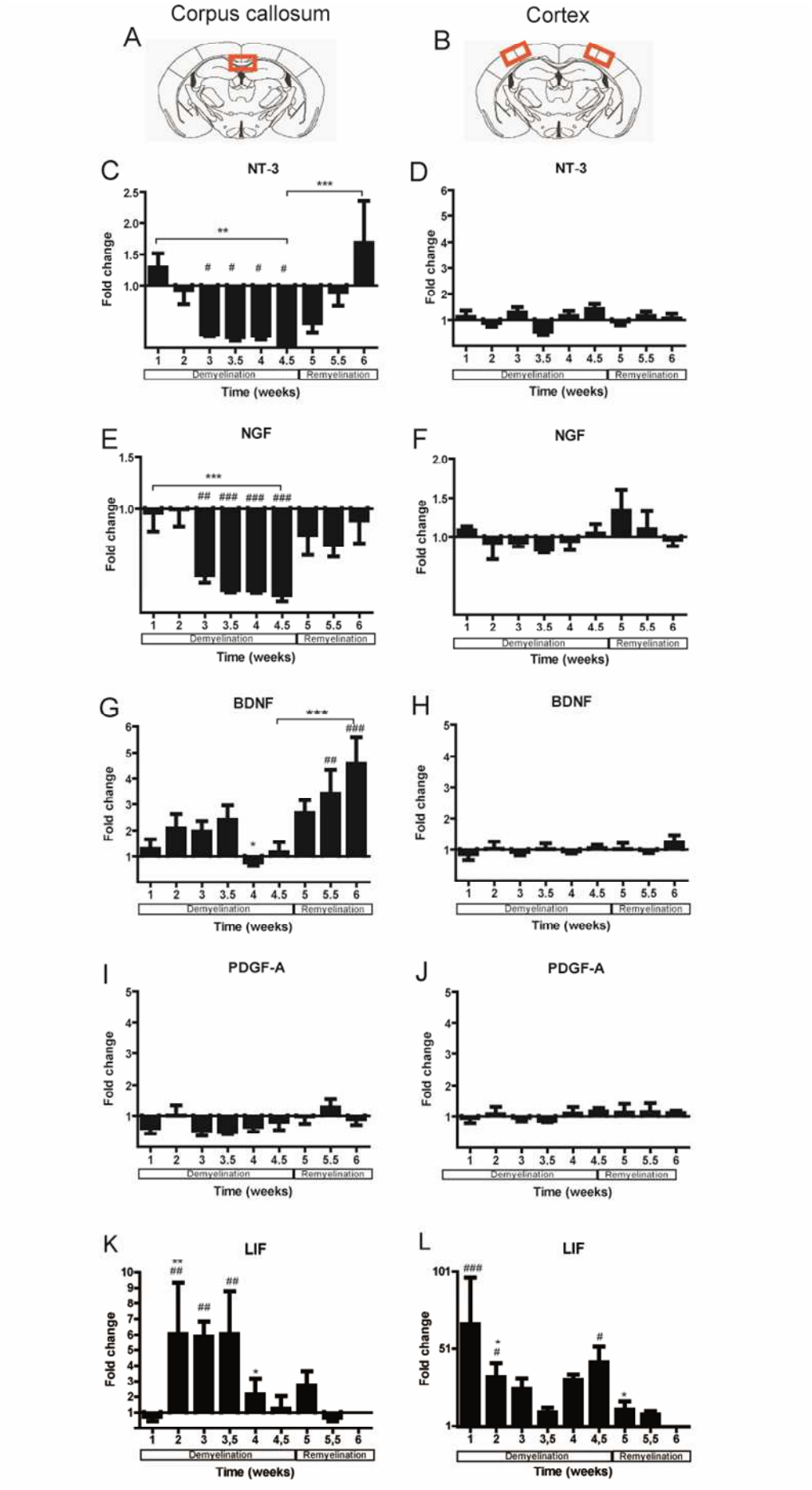
Corpus callosum
Cortex
A
B
C
NT-3
D
NT-3
E
NGF
F
NGF
G
BDNF
H
BDNF
I
PDGF-A
J
PDGF-A
K
LIF
L
LIF
Fold change
Time (weeks)
Demyelination
Remyelination

Figure 7

Figure 7

Expression of growth factor mRNA in the corpus callosum and the cortex of mice undergoing demyelination / remyelination. A) Schematic diagram of the mouse brain in coronal section. The red line shows the investigated middle part of the corpus callosum. **B)** The red line marks the area of the investigated cortex. **C-L)** Graphs show mRNA expression fold changes of Nt-3, NGF, BDNF, PDGF, and LIF in the corpus callosum (C-K) and in the cortex (D-L) compared to the age-matched controls and normalized with HPRT using the ΔΔCt method. Significant effects versus controls are indicated by rhombs or asterisks if compared to the preceding time point, $*^{\#}p < 0.05$; $**^{\#\#}p < 0.01$; $***^{\#\#\#}p < 0.001$, ANOVA).

Chapter III

Cerebellar cortical demyelination in the murine cuprizone model

Thomas Skripuletz[1], Jens-Heiko Bussmann[1], Viktoria Gudi[1,2], Paraskevi N. Koutsoudaki[1,2], Refik Pul[1], Darius Moharregh-Khiabani[1], Maren Lindner, and Martin Stangel[1,2]

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[2] Center for Systems Neuroscience, Hannover, Germany

Brain Pathology, 2010

Preface – about this manuscript

The current study provided a detailed analysis of the dynamics of de- and remyelination in the cerebellar cortex and white matter. To induce demyelination, C57BL/6 mice were fed with a 0.2% cuprizone diet for 12 weeks followed by a recovery phase of 8 weeks. Significant loss of myelin could be detected after 12 weeks of cuprizone feeding in cortical and white matter structures of the cerebellum. Remyelination occurred after withdrawal of cuprizone but was less prominent in the more caudal region of the cerebellum. Infiltration of activated microglia was abundant in all analyzed cerebellar areas. Astrogliosis could also be observed but did not reach the extent observed in the cerebrum. Demyelination, microglia, and astrocyte changes were different in the cerebellum as compared to the cerebrum suggesting region-dependent different pathomechanisms.

Brain Pathology 2010, Volume 20, Issue 2, Pages 301-312.

Chapter IV

Demyelination of the hippocampus is prominent in the cuprizone model

Paraskevi N. Koutsoudaki[1,2], Thomas Skripuletz[1], Viktoria Gudi[1,2], Darius Moharregh-Khiabani[1], Herbert Hildebrandt[2,3], Corinna Trebst[1], and Martin Stangel[1,2]

[1] Department of Neurology, Medical School Hannover, Hannover, Germany
[2] Center for Systems Neuroscience, Hannover, Germany
[3] Department of Cellular Chemistry, Hannover Medical School, Hannover, Germany

Neuroscience Letters, 2009

Preface – about this manuscript

The current study provides a detailed characterization of hippocampal demyelination in the cuprizone model. Demyelination affected all hippocampal structures analyzed and was accompanied by astrogliosis and microgliosis. However, between the distinct hippocampal structures the temporal pattern of demyelination as well of cellular response intensity varied considerably. Cuprizone feeding provides a useful model for studying demyelination processes in the murine hippocampus.

The text of the original publication can be found here: *Neuroscience Letters 2009,* Volume 451, Issue 1, Pages 83-88

Chapter V

Beneficial effects of minocycline on cuprizone induced cortical demyelination

Thomas Skripuletz[1], Elvira Miller[1], Darius Moharregh-Khiabani[1], Alexander Blank[1], Refik Pul[1], Viktoria Gudi[1,2], Corinna Trebst[1], and Martin Stangel[1,2]

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[2] Center for Systems Neuroscience, Hannover, Germany

Neurochemical Research, 2010

Preface – about this manuscript

In this study, the potential of minocycline to influence cuprizone induced de- and remyelination in the grey and white matter was investigated. The administration of minocycline was associated with reduced demyelination in both the corpus callosum and the cortex. In addition to the beneficial effects on demyelination, treatment with minocycline improved the motor coordination behaviour of the cuprizone treated mice. For remyelination, astrogliosis, and the numbers of OPC and oligodendrocytes no treatment effects were found. In this study presented findings also demonstrated regional differences in tissue reactivity and microglia activation induced by minocycline in the white and the grey matter.

The text of the original publication can be found here: *Neurochemical Research 2010,* Volume 35, Number 9, Pages 1422-1433.

Chapter VI

Effects of fumaric acids on cuprizone induced central nervous system de- and remyelination in the mouse

Darius Moharregh-Khiabani[1], Alexander Blank[1], Thomas Skripuletz[1], Elvira Miller[1], Alexandra Kotsiari[1], Viktoria Gudi[1,2], and Martin Stangel[1,2]

[1] Department of Neurology, Hannover Medical School, Hannover, Germany
[2] Center for Systems Neuroscience, Hannover, Germany

PLoS ONE, 2010

Preface – about this manuscript

In this study the impact of fumaric acid esters (FAE) on de- and remyelination was investigated using the toxic cuprizone model. After the termination of the cuprizone diet, treatment with FAE could only marginal accelerate remyelination in the corpus callosum but not in the cortex as compared to controls. No FAE treatment effects were found for demyelination and for glial reactions. Since no breakdown of the blood-brain-barrier occurs in the cuprizone induced demyelination, the minor effects of fumarates in the cuprizone model might be related to the lack of influence of the peripheral immune system.

The text of the original publication can be found here: *PLoS ONE 2010,* Volume 5, Issue 7

4. Discussion

4.1 Regional differences of de- and remyelination in white and grey matter

The cuprizone model is a powerful tool to study de- and remyelination in the CNS. Previously, it has been shown that cuprizone induced demyelination also affects the cerebral cortex (Skripuletz et al., 2008). In diseases like MS, de- and remyelination seem to follow different mechanisms in grey and white mater (Albert et al., 2007; Bo et al., 2003; van Horssen et al., 2007). In the current study, we performed a detailed analysis of the demyelination dynamics in the cerebral cortex in comparison to the corpus callosum in C57BL/6 mice treated with 0.2% cuprizone. We also studied the demyelination in the hippocampus and the cerebellar white and grey matter (Koutsoudaki et al., 2009; Skripuletz et al., 2010). Interestingly, the time course of demyelination differed between the regions analyzed. In the cerebral cortex a complete demyelination was observed after 6 weeks of cuprizone feeding while in the medial corpus callosum a complete myelin loss occurred already after 4.5-5 weeks of cuprizone feeding. Differences between white and grey matter demyelination were also shown for the cerebellum, where only moderate demyelination was detected for all time points investigated. Cerebellar cortical demyelination could first be observed from week 6 onward and reached its maximum by week 12. Cerebellar white matter demyelination was already detected after 4 weeks of cuprizone feeding with the maximum at week 12 (Skripuletz et al., 2010). In the hippocampus demyelination was complete after 6 weeks of cuprizone feeding (Koutsoudaki et al., 2009). However, there were temporal differences of demyelination between different hyppocampal structures. Interestingly, hyppocampal fimbria did not demyelinate at all. A zonal distribution of cuprizone induced demyelination has been also described for the caudal and rostral corpus callosum (Stidworthy et al., 2003). Probably, due to the regional differences in the myelin content or due to different tissue reactivity de- and remyelination follow different temporal or possibly also

pathomechanistic pattern. In order to understand these regional differences we performed immunohistochemical studies of glial responses in all brain areas mentioned.

Cuprizone feeding led to a complete depletion of oligodendrocytes in all regions investigated after 4 weeks. Despite of continuing cuprizone feeding oligodendrocytes re-appeared in the cerebral cortex and the corpus callosum after 5-5.5 weeks of cuprizone feeding. In the cerebellum oligodendrocytes re-appeared between 6-8 weeks of cuprizone feeding followed again by a repeated depletion at week 12, when cuprizone was administrated for 12 weeks (Skripuletz et al., 2010). Obviously, repair mechanisms are common in demyelinating lesions in the cuprizone model and partial remyelination can occur. This phenomenon is in line with the literature and particularly affirmed by observation in the corpus callosum (Matsushima and Morell, 2001; Lindner et al., 2008). Proliferating OPCs could be observed one week before adult oligodendrocytes re-appeared suggesting an attempt to active remyelination.

Along with demyelination accumulation of microglial cells occurred in all demyelinated areas being clearly more prominent in the white matter compared to the grey matter of both cerebrum and cerebellum, probably due to different myelin amounts (Skripuletz et al., 2010). The peak of microglial infiltration was observed at week 4.5 of cuprizone feeding in both the corpus callosum and the cerebral cortex. In addition, microglia was the cell population with the strongest proliferation capacity in both areas. In cerebellar cortex the strongest microglial activation/ infiltration was seen at week 6, two weeks earlier than in the cerebellar white matter (Skripuletz et al., 2010). In most hippocampal structures the peak of microgliosis was detected already at week 3 of the cuprizone treatment (Koutsoudaki et al., 2009).

As previously described, astrogliosis was extensive in both the corpus callosum and the cortex and was constantly present from week 3 until week 6 of the cuprizone diet (Skripuletz et al., 2008). In the cerebellum, astrogliosis was less prominent compared to that in the cerebral cortex or corpus callosum (Skripuletz et al., 2010). Strong astrogliosis was seen in all hippocampal structures (Koutsoudaki et al., 2009). 99% of nestin positive cells in the corpus

callosum and 70 % of nestin positive cells in the hippocampus (Koutsoudaki et al., 2009) were also positive for GFAP and exhibited an astrocytic shape as well. Thus it seems that nestin is re-expressed in reactive astrocytes or alternatively these astrocytes may derive from neuronal precursors. The role of astrogliosis is still not completely understood.

Generally, zonal variations in demyelination may be explained by different region-specific functions of astrocytes, microglia or OPCs. In this regard, different astrocyte populations are known to be present in white and grey matter. Region dependent differences in morphology, expression of immunoregulatory proteins, migratory response to adenosine triphosphate (ATP) and phagocytic capacity are known for microglia (Haas et al., 2007, 2008).

4.2 Identification of factors involved in the de- and remyelination of white and grey matter

In order to clarify the regional differences, particularly between cerebral grey and white matter de- and remyelination and to identify factors involved in these processes, the mRNA expression of thirteen growth factors was analyzed. Using laser microdissection we were able to precisely separate both regions of interest. During demyelination we found no distinct differences in the growth factor mRNA expression pattern between the corpus callosum and cortex. In both regions GDNF and NRG 1 were significantly and CNTF and EGF were slightly up-regulated at the first two weeks of cuprizone feeding, when no demyelination was microscopic detectable. It has been demonstrated that GDNF is up-regulated in astrocytes during pathophysiological conditions such as spinal cord injury (Satake, 2000; Ikeda et al., 2002; Lee et al., 2006; Miyazaki et al., 2001). Since the application of exogenous CNTF induces reactive astrogliosis and the up-regulation of GFAP mRNA expression (Kahn, et al., 1997), CNTF is suggested to be a key player in the induction of astrogliosis. CNTF has also been reported to promote myelination *in vitro* (Mayer et al., 1994; Stankoff et al., 2002). In myelinating aggregate cell cultures CNTF mRNA expression increased continuously over the

period of MBP accumulation (Copelman et al., 2000). However, in lysolecithin induced demyelination CNTF was not elevated at any time points analyzed (Hinks and Franklin, 1999). The delivery of bioactive CNTF into ethidium bromide induced demyelinating lesions neither enhanced survival nor differentiation of endogenous OPCs *in vivo* (Talbott et al., 2007). In our study CNTF mRNA up-regulation corresponded with elevation of MBP and PLP mRNA expression during remyelination. We think that CNTF plays a dual role in the cuprizone induced demyelination. First, it may be involved in the initiation of astrogliosis in both the cortex and the corpus callosum. Second, it may promote remyelination of the corpus callosum but not of the cortex.

HGF and FGF-2 mRNA expressions were up-regulated during demyelination in both areas. HGF expression has been shown to be elevated in EAE (Moransard et al., 2009). Up-regulation of FGF-2 mRNA expression in demyelination has been reported in various animal models such as EAE, murine hepatitis virus induced demyelination, lysolecithin, and cuprizone induced demyelination (Amstrong et al., 2002; Liu et al., 1998; Hinks and Franklin, 1999; Messersmith et al., 2000). It has been suggested that the main impact of FGF-2 is the support of OPC proliferation and inhibition of OPC differentiation and myelination (McKinnon et al., 1990; Bansal and Pfeiffer, 1997; Goddard et al., 2001). Indeed, in cuprizone induced demyelination the absence of FGF-2 promotes regeneration of oligodendrocytes after demyelination but does not diminish OPCs proliferation activity (Armstrong et al., 2002). Moreover, using in situ-hybridization methods, FGF-2 has been shown to be up-regulated in the corpus callosum after 5-6 weeks of 0.3 % cuprizone feeding. The 0.3 % cuprizone dosage treatment seems to produce a different demyelination pattern as compared to 0.2 % cuprizone dosage (Lindner et al., 2008). In our study we induced demyelination with 0.2 % cuprizone for 4.5 weeks. In the corpus callosum FGF-2 was strongly up-regulated at the time of the demyelination peak, corresponding to the intensive proliferation of OPCs. In the cortex FGF-2 mRNA expression was elevated during the whole

demyelination. We conclude that FGF-2 may be involved in the regulation of OPC proliferation in both white and grey matter, however, based on the literature seems not to be an essential factor. During remyelination mRNA levels of HGF and FGF-2 were significantly up-regulated in the corpus callosum but not in the cortex, suggesting different molecular regulation of remyelination in white and grey matter. FGF-2 has been shown to be up-regulated during remyelination after lysolecithin induced demyelination (Hinks and Franklin, 1999) and in myelinating aggregate cultures *in vitro* (Copelman et al., 2000). Thus, it remains speculative whether FGF-2 may play an immediate role in the callosal remyelination or interplays with other factors.

IGF-1 and TGF-ß1 mRNA were strongly up-regulated in both the corpus callosum and the cortex, beginning after 3 weeks of the cuprizone diet and persisting during the period of severe demyelination. At the same time there was a strong astrogliosis, microgliosis, and OPC proliferation. With progressing remyelination mRNA synthesis of IGF-1 and TGF-ß1 gradually decreased. The up-regulation of IGF-1 in the cuprizone model has been previously described (Mason et al., 2000a). However, in that study investigators used the whole forebrain tissue and did not distinguish between grey and white matter. The elevation of IGF-1 mRNA expression has also been reported following ethidium bromide induced demyelination (Fushimi and Shirabe, 2004). In lysolecithin induced demyelination IGF-I and TGF-ß1 mRNAs were up-regulated in the spinal cord by 5 days post-lesion induction (Hinks and Franklin, 1999). IGF-1 has been considered to have strong beneficial effects on differentiation of OPCs and myelination (McMorris et al., 1986; McMorris and Dubois-Dalcq, 1988; Saneto et al., 1988; Mozell and McMorris, 1991; Barres et al., 1992; Ye and D'Ercole, 1999; Goddard et al., 1999). IGF-1 transgenic mice, which continuously express IGF-1, remyelinate more readily compared to wild type mice after cuprizone induced demyelination of the corpus callosum (Mason et al., 2000b). Additionally, in type 1 IGF receptor null mice remyelination does not occur adequately and progenitors do not proliferate or survive as well without IGF-

1R (Mason et al., 2003). TGF-ß1 induces oligodendroglial differentiation (McKinnon et al., 1993) and enhances myelinogenesis *in vitro* (Diemel et al., 2003). We believed that IGF-1 and TGF-ß1 are important factors for differentiation of oligodendrocytes in both the corpus callosum and the cortex. However, since IGF-1 and TGF-ß1 mRNA was already elevated after 2 or 3 weeks of cuprizone feeding it is also possible that these two factors interact in multiple processes.

mRNA expression of the neurotrophins NGF, BDNF, and NT-3 did not change in the cortex neither during demyelination nor during remyelination. In contrast, in the corpus callosum, however, especially BDNF seems to play an important role during de- and remyelination. The existence of two different types of astrocytes populating grey and white matter could contribute to the different regulation of remyelination in the corpus callosum and cortex. Furthermore, it has been suggested that different populations of oligodendrocytes and OPCs expressing different neurotrophin receptors in various combinations are present in different CNS regions (Du et al., 2003).

In contrast to lysolecithin induced demyelination (Hinks and Franklin, 1999) in our study PDGF-A mRNA expression was not changed during de- and remyelination in both the corpus callosum and the cortex. However, using the cuprizone model Mason et al., similarly did not observe any changes of PDGF-A mRNA expression during de- and remyelination (Mason et al., 2000a).

Certainly, numerous factors may be arbitrative for a coordinated interaction to orchestrate the complex process of remyelination. Some growth factors have been shown to act indirectly which, in turn, influence the expression of other growth factors or their receptors (Albrecht et all., 2003; Jiang et al, 1999). With respect to the induction of demyelination, influence of peripheral inflammatory activity and heterogeneity of myelinating cells there might be differences between animal models.

In summary, different temporal patterns were found for demyelination and glial reactions in the corpus callosum and cerebral cortex, suggesting different tissue pathophysiology. In this study we performed detailed analysis of mRNA expression pattern of thirteen growth factors, to uncover differences between de- and remyelination in white and grey matter and to identify factors playing an important role during remyelination. For several growth factors different mRNA expression pattern were found during remyelination in the corpus callosum and the cerebral cortex that may underlie region specific mechanisms. CNTF, GDNF, and NRG 1 may play an important role in the induction of astrogliosis in both white and grey matter. In turn, activated astroglia may produce factors like LIF that stimulate migration, proliferation and activation of microglia. Astrocytes and microglia may produce factors like FGF-2, IGF-1 and TGF-ß1 that then regulate proliferation and differentiation of OPCs. CNTF, BDNF, FGF-2, HGF, and GDNF seem to be involved in remyelination in the corpus callosum but were not expressed at such high levels in the cortex. This lack of factors may be responsible for the delayed remyelination in the cortex as compared to the corpus callosum. IGF-1 and TGF-ß1 are suggested to regulate remyelination in both the corpus callosum and the cortex. The knowledge about growth factors regulated during cuprizone induced demyelination may provide a new insight in the modulation of de-and remyelination of white and grey matter and may be useful for development of new strategies in MS therapies.

4.3 Therapeutic interventions

The most current MS therapeutics are directed toward immunsupression or immunomodulation. Therefore, development of new regenerative therapies that support repair mechanisms is an important issue in MS research.

In this study we tested minocycline, an antibiotic of the second tetracycline generation with several immunomodulatory and neuroprotective properties, on de- and remyelination in the murine toxic demyelination model. The beneficial effects of minocycline were also reflected

in an improved neurological function, decreased lesion volume and reduced IL-1b production as shown in cortical traumatic injury studies (Sanchez Mejia et al., 2001). In EAE, the beneficial effect of minocycline on inflammation, demyelination, and disease activity was reported (Brundula et al., 2002; Popovic et al., 2002). The effects of minocycline were also tested in the toxic ethidium bromide model of demyelination, where minocycline inhibited microglia activation, suppressed OPC response, and decreased the extent of oligodendrocyte remyelination (Li et al., 2005). Still the mode of minocycline action is not known. In our study minocycline treatment diminished cuprizone induced demyelination in both white and grey matter and improved motor function compared to sham treated animals (Skripuletz et al., 2010).

Again, there were regional differences in microglial activation in the white and grey matter. However, oligodendroglial and astroglial responses were not affected by minocycline treatment, suggesting no direct protective effects on the oligodendrocyte lineage.

Recently, beneficial effects of fumaric acid esters (FAE) were shown in MOG-EAE studies in mice as well as in a phase II study in MS patients (Schilling et al., 2006; Kappos et al., 2008). In the current study the effect of FAE on de- and remyelination was investigated in the cuprizone toxic demyelination model (Moharregh-Khiabani et al., 2010, accepted). FAE are believed to have cell protective and immunomodulating properties (Lehmann et al., 2007). In contrast to EAE, where FAE was shown to reduce the amount of activated macrophages/microglia (Schilling et al., 2006), we could not observe any significant effects of FAE neither on microglial nor on astroglial activation in cuprizone model. Probably, FAE acts in EAE via peripheral immune cells that are not present in cuprizone induced lesions, where the blood brain barrier remains intact. Furthermore, the oligodendrocyte loss, as well as proliferation and differentiation of OPCs were not influenced by FAE. However, remyelination was slightly accelerated in the corpus callosum but not in the cortex again suggesting different pathomechanisms in the white and grey matter.

In summary, both agents FAE and minocycline could show therapeutic potential reflecting in the reduction of the demyelination (minocycline) or improvement of the remyelination (FAE). Our findings corroborated the hypothesis about beneficial effects of FAE and minocycline on de- or remyelination and uncover regional differences in the function of these two agents in the white and grey matter.

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5. Summary

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) affecting more than 2.5 mil people worldwide. Demyelinating lesions in MS occur in different brain areas and are not limited to the white matter tracts. Demyelination in the white and grey matter follows different temporal and qualitative pattern. There are strong evidences that these differences are based on different pathomechanisms of de- and remyelination in the white and grey matter. Animal models like cuprizone toxic induced demyelination are powerful tools to investigate this hypothesis. In the cuprizone model, demyelination is induced by chronic oral administration of the copper chelator cuprizone. Cuprizone is toxic for oligodendrocytes, however the precise mode of action is not known. After termination of the cuprizone diet spontaneous remyelination occurs. The model is well characterized, reliable, predictable, and well reproducible.

In the current work we analyzed the de- and remyelination in the cerebral and cerebellar white and grey matter, as well as in the hippocampus in the murine cuprizone model. Thereby we focused on the expression of myelin proteins and glial cell responses during de- and remyelination. In all studied brain regions de- and remyelination followed a different temporal and qualitative pattern suggesting different pathomechanisms. Demyelination was first observed in the corpus callosum, followed by hippocampus and the cerebral cortex. Demyelination in the cerebellum was delayed and reached its maximum first after 12 week of cuprizone feeding. There were regional differences in the demyelination pattern and glial responses between white and grey matter of the cerebrum and cerebellum, suggesting different underlying pathomechanisms. Growth factors are known to play important role in the orchestration of the oligodendrocyte lineage development. Therefore, mRNA expression profiles of thirteen growth factors were performed for both the corpus callosum and the cerebral cortex. The following growth factors were up-regulated during demyelination in both investigated areas: GDNF, NRG 1, CNTF, TGF-ß1, HGF, FGF-2, and IGF-1. However,

single growth factors showed differences in the expression pattern. GDNF, NRG 1, CNTF, and EGF were maximal up-regulated at the first week of cuprizone feeding, where myelin loss was not microscopic detectable. mRNA expression of IGF-1, TGF-ß1, HGF, and FGF-2 reached their peaks during the period of strongest demyelination parallel to the intensive OPCs proliferation and the start of their differentiation. During remyelination, IGF-1 and TGF-ß1 mRNA expressions were up-regulated in both areas, whereas CNTF, GDNF, BDNF, HGF, FGF-2 mRNA levels were elevated only in the corpus callosum, suggesting to promote remyelination in this white matter tract. PDGF-A was not enhanced in both regions during de- and remyelination. NGF and NT-3 mRNA expressions were down-regulated during demyelination and restored during remyelination in the corpus callosum. In the cortex NGF, NT-3 and BDNF expression was not altered compared to age-matched controls. These findings further corroborate the hypothesis of different regional pathomechanisms during de- and remyelination in the white and grey matter. We also investigated the effects of minocycline and fumaric acid esters (FAE) on de- and remyelination. Both agents are believed to have cell protective and immunomodulating properties. In our study we could show accelerated remyelination in the white but not in the grey matter in response to the FAE treatment, again suggesting different pathomechanisms of remyelination in white and grey matter. However, no FAE effects were found for demyelination. Minocycline treatment diminished myelin loss in both the corpus callosum and the cortex but showed no effect on remyelination. These new data corroborate the hypothesis that minocycline has beneficial effects on demyelination *in vivo*. Our findings also demonstrate regional differences in tissue reactivity and microglia activation in response to minocycline exposure.

The cuprizone animal model of toxic demyelination is an excellent model to study de- and remyelination mechanisms in grey and white matter of the cerebrum and cerebellum and is a potential platform to study effects of exogenously administered therapeutic agents to promote oligodendrocyte survival and remyelination.

6. Zusammenfassung

Multiple Sklerose ist eine chronisch-entzündliche Erkrankung des zentralen Nervensystems (ZNS), die zu einer Demyelinisierung von Axonen führt. Weltweit sind mehr als 2,5 Millionen Menschen von dieser Erkrankung betroffen.

Neuere Untersuchungen zeigen, dass die für MS typischen Demyelinisierungsherde nicht nur in der weißen Substanz auftreten, sondern auch in der grauen Substanz zu finden sind. Der Verlauf der Demyelinisierung in der weißen und grauen Substanz unterscheidet sich und scheint auf regional grundlegend verschiedenen molekularen Mechanismen zu beruhen.

Tiermodelle, wie zum Beispiel das Toxin induzierte Cuprizone Demyelinisierungsmodell, bieten eine solide Plattform um diese Mechanismen zu untersuchen. Bei diesem gut charakterisierten Tiermodell wird eine Demyelinisierung des Balkens (Corpus callosum) und anderen Gehirnarealen durch die chronische orale Einnahme des Kupferchelators Cuprizone induziert. Der genaue Wirkungsmechanismus von Cuprizone ist nicht geklärt Cuprizone bewirkt ein Absterben der Myelin bildenden Zellen, den Oligodendrozyten. Nach dem Absetzen des Toxins kommt es zu einer spontanen Remyelinisierung.

In der vorliegenden Studie wurde die De- und Remyelinisierung in der weißen und grauen Substanz des Zerebrums and Zerebellums sowie im Hippocampus untersucht. Dabei standen die Expression verschiedener Myelinproteine, die Reaktion verschiedener Gliazellen sowie die Expression von Wachstumsfaktoren im Hauptfokus. Die Demyelinisierung wurde erst im medialen Corpus callosum (nach 4.5 Wochen Cuprizone Fütterung), dann nach und nach in den einzelnen Strukturen des Hippocampus (4-6 Wochen) und schließlich in allen kortikalen Zellschichten beobachtet. Die zerebelläre Demyelinisierung erreichte ihr maximales Ausmaß erst nach 12 Wochen der Cuprizone Diät. Sowohl im Großhirn als auch im Kleinhirn verlief die Demyelinisierung in der weißen Substanz schneller als in der grauen Substanz. Auch die Rekrutierung bzw. Aktivierung der Mikroglia wiesen zeitliche und regionale Unterschiede auf. Diese Beobachtungen führten zu der Annahme, dass regional spezifische molekulare

Regulierungsmechanismen existieren. Daraufhin wurde die mRNA Expression von 13 Wachstumsfaktoren im medialen Teil des Balkens und lateralen Kortex analysiert. Folgende Wachstumsfaktoren wurden während der Demyelinisierung in der weißen sowie in der grauen Substanz vermehrt exprimiert: GDNF, NRG1, CNTF, EGF, TGF-ß1, HGF, FGF-2, LIF und IGF-1. Die zeitlichen Expressionsmuster der einzelnen Faktoren unterschieden sich deutlich voneinander. So wurden NRG1, GDNF, CNTF und EGF in der frühren Phase der Demyelinisierung hoch-reguliert, wo der Myelinverlust noch nicht mikroskopisch in Erscheinung getreten war. Die Faktoren IGF-1, TGF-ß1, FGF-2 und HGF erreichten ihre maximale Expression dagegen in der Phase der maximalen Demyelinisierung. Während der Remyelinisierung wurden die Wachstumsfaktoren in der weißen und grauen Substanz unterschiedlich reguliert. Während im Balken die Expression von CNTF, GDNF, HGF, FGF-2 und BDNF deutlich erhöht war, wurden im Kortex keine Veränderungen der mRNA Expression im Vergleich zu altersgleichen Kontrolltieren festgestellt. Die mRNA Synthese der Neurotrophine NT-3 und NGF war im Kortex konstant, während sie im Corpus callosum während der Demyelinisierung deutlich vermindert war und sich später wieder normalisierte. Die PDGF-A mRNA Expression blieb unverändert in beiden untersuchten Arealen. Die festgestellten Unterschiede in der mRNA Expression der verschiedenen Wachstumsfaktoren im Kortex und Corpus callosum bekräftigen die Hypothese der regionalen Unterschiede in der De- und Remyelinisierung der weißen und grauen Substanz. Die Kenntnisse zur Regulation der Faktoren, die bei der Remyelinisierung eine günstige Rolle spielen könnten, eröffnen die Möglichkeiten zur Entwicklung neuer Therapien.

In unseren Studien wurden ebenfalls potentielle protektive und remyelinisierende Therapeutika untersucht. Die Wirkung der Substanzen Fumarsäure (FAE) und Minozyklin wurden im Rahmen der Cuprizone induzierten De- und Remyelinisierung untersucht. Die neuroprotektiven und immunsuppressiven Eigenschaften wurden für beide Substanzen in den mehreren wissenschaftlichen Studien beschrieben. In unserem Tiermodell beobachteten wir

für FAE eine Beschleunigung in der collosalen aber nicht in der kortikalen Remyelinisierung, was wiederum auf die regional unterschiedlichen molekularen Mechanismen deutete. Die Minozyklin Behandlung milderte die Demyelinisierung in den beiden Arealen, der weißen und der grauen Substanzen. Diese Daten unterstützen die Vermutung, dass Minozyklin einer Demyelinisierung entgegen wirken kann. Die Hemmung der Mikroglia Aktivierung wurde jedoch nur im Kortex beobachtet, was mit der generellen Beobachtung von regionalen Unterschieden in der weißen und grauen Substanzen im Konsens ist und mit der regional spezifischen Gewebereaktivität zu erklären ist. Minozyklin zeigte allerdings keine positiven Effekte auf die Remyelinisierung.

In der vorliegenden Studie wurden regionalen Unterschiede zwischen De- und Remyelinisierung in der weißen und der grauen Substanzen auf den molekularen und zellulären Ebenen gezeigt. Dabei erwies sich das Cuprizone Tiermodell als eine zuverlässige Plattform für die Untersuchungen der De- und Remyelinisierung sowohl im Großhirn als auch im Kleinhirn und Hippocampus. Weiterhin zeigte sich die Cuprizone induzierte Demyelinisierung als ein geeignetes Tiermodel für die Identifizierung und Erforschung neuer therapeutischer Präparate.

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Declaration

I herewith declare that I autonomously carried out the PhD-thesis entitled **"Regional differences of molecular factors during demyelination and early remyelination"**.

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institutions: Department of Neurology, Hannover Medical School

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

Viktoria Gudi _____

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