Characterization of molecular alterations in normal appearing white matter of Multiple Sclerosis brain tissue and its animal model experimental autoimmune encephalomyelitis

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Content

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List of publications

This thesis is based on the following articles submitted to, or published in scientific journals:

Manuscripts

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Zeis, T., and N. Schaeren-Wiemers. 2008. Lame Ducks or Fierce Creatures? - The Role of Oligodendrocytes in Multiple Sclerosis. *J Mol Neurosci*. **Imp. Factor: 2.96**

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Kinter, J., **Zeis, T.**, and N. Schaeren-Wiemers. RNA profiling in MS brain tissue. *Int.MS.J. In press*

Book Chapters

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Abbreviations

APOE	-	Apolipoprotein E
CD	-	Cluster of Differentiation
CNP	-	Cyclic nucleotide phosphodiesterase
CNS	-	Central Nervous System
DA	-	Dark Agouti
EAE	-	Experimental Autoimmune Encephalomyelitis
EBV	-	Epstein-Barr Virus
HHV-6	-	Human Herpes Virus 6
HLA	-	Human Leukocyte Antigen
Ig	-	Immunoglobulin
IL	-	Interleukine
LT	-	Lymphotoxin
MAG	-	Myelin-Associated Glycoprotein
MHC	-	Major Histocompatibility Complex
MOG	-	Myelin Oligodendrocyte Glycoprotein
MRI	-	Magnetic Resonance Imaging
MS	-	Multiple Sclerosis
MSRV	-	Multiple Sclerosis-associated Retrovirus
NAGM	-	Normal Appearing Grey Matter
NAWM	-	Normal Appearing White Matter
nNOS	-	neuronal Nitric Oxide Synthase
OG	-	Oligodendrocyte
RT-PCR	-	Reverse-Transcription - Polymerase Chain Reaction
STAT	-	Signal Transducer and Activator of Transcription
Tc1	-	cytotoxic T cell type 1
Th1	-	T helper cell type 1
Th2	-	T helper cell type 2
Th17	-	T helper cell type 17
TNF	-	Tumor Necrosis Factor

Abstract

Multiple sclerosis is a chronic, inflammatory and demyelinating disease of the CNS. Although diffuse inflammatory damage as well as progressive axonal injury has been shown in the chronic phase of the disease, little is known about the molecular mechanisms underlying these pathological processes. In order to identify such mechanisms, the gene expression profile in MS normal appearing white matter (NAWM), was studied. Furthermore, the presence of such changes in a MS animal model was analyzed.

A differential gene expression analysis on NAWM revealed the upregulation of genes involved in anti-inflammatory mechanisms, such as STAT6, and genes involved in pro-inflammatory mechanisms, such as STAT4. By immunohistochemistry, a predominant expression of the components of the STAT6 signalling pathway in oligodendrocytes was demonstrated. These findings suggest an endogenous inflammatory activation throughout the whole MS NAWM, in which oligodendrocytes actively participate. Whether such changes represent also earliest pathological processes in MS or are due to a long, chronic disease course is unknown. Therefore, differential gene expression of a biopsy with NAWM taken from a 17 year-old woman during her first clinical incident was analyzed. This revealed a strong upregulation of neuronal nitric oxide synthase (nNOS) as well as STAT6, and genes involved in neuroprotection against oxidative stress. These findings suggest that intrinsic inflammatory- as well as neuroprotective mechanism activation are early events in MS NAWM, which sustain over time. To study these mechanisms in more detail, a gene expression study in an animal model for MS was performed. For this, normal appearing white and grey matter of DA rats with recombinant MOG-induced EAE was analyzed. However, an induction of immune-modulating or neuroprotective genes was not evident in EAE NAWM. Therefore, we conclude that MOG-induced EAE in DA rat may not be a suitable model to investigate the immune-modulating or neuroprotective mechanisms observed in MS NAWM. In contrast, a comparable downregulation of glutamate receptors and genes encoding mitochondrial proteins as in MS NAGM was detected in EAE NAGM.

In summary, gene expression changes characteristic of endogenous inflammatory as well as neuroprotective mechanisms were identified in the MS NAWM, whereas these mechanisms were not present in an animal model of this disease, leading to the conclusion that in MS intrinsic mechanisms may take place, independent of acute, autoimmune-mediated inflammation.

Introduction

Multiple Sclerosis

Multiple sclerosis is a chronic inflammatory, demyelinating disease of the CNS. It is one of the most common diseases of the CNS in young adulthood. The hallmark of this disease is the inflammatory plaque. Despite extensive research, the clinical cause of MS is still unknown.

A major goal of this thesis was to unravel certain molecular aspects of this disease with the focus on the normal appearing white matter (NAWM), which is one of the most promising tissue to study earliest pathogenic mechanisms possibly leading to or protecting from the formation of lesions.

History of Multiple Sclerosis

There exist several historical reports of people probably suffering from MS. One of the first documented cases probably suffering from MS is thought to be the case of Saint Lidwina of Schiedam (1380-1433). The Dutch nun developed from the age of sixteen until her death at age 53 intermittent pain, weakness of the legs and vision loss, which are typical symptoms for MS. There exists also an Icelandic saga about a possible early case of multiple sclerosis in which a woman lost sight of both eyes and her speech which on the following days recovered again.

However, the first illustrations and descriptions of clinical details of multiple sclerosis were made by Robert Hooper (1773-1835), Robert Carswell (1793-1857) and Jean Curveilhier (1791-1873) during the 1830's (Figure 1). But it was Jean-Martin Charcot (1825-1893) who putted previous work and his own clinical and pathological observations together and developed the disease concept of MS (Charcot, 1868). Later, several subforms of MS were introduced, such as Devic's type of neuromyelitis optica, Marburg's acute MS and Balo's concentric sclerosis (Balo, 1928; Devic, 1894; Marburg, 1906).

In 1863, Eduard Rindfleisch (1836-1908) recognized that focal lesions are centred by blood vessels which are in a state characteristic of chronic inflammation (Rindfleisch, 1863). In contrast to Charcot, for which the disease was primarily a glia disorder and only secondary due to blood vessel changes, Rindfleisch and later Marburg (1906) considered Multiple Sclerosis to be an inflammatory demyelinating disease (Marburg, 1906; Rindfleisch, 1863). Following these first studies on MS, many others described in great detail structural changes in MS. In 1864, Carl Fromann described the occurrence of demyelination and astrocytosis (Fromann, 1864). Joseph Babinski (1857-1932), showed in 1885 the interaction of inflammatory cells, in particular macrophages, with demyelinated nerve fibres (Babinski, 1885). In his study, Babinski also showed axons which are surrounded by thin myelin sheaths with short internodes (Babinski, 1885). Later, this aspect was discussed by Otto Marburg (1874-1948), which also recognized the abundance of axons with thin myelin sheaths (Marburg, 1906). It was him who first suggested, that these axons might represent attempts of remyelination (Marburg, 1906). In 1916, James Dawson (1870-1927) published a summary of the significant knowledge on MS and also of the different ideas about the cause of the disease (Dawson, 1916). 5 years later, del Río Hortega discovered the myelin producing cell, which he named oligodendrocyte (Hortega, 1921). Then, in 1961, remyelination was first demonstrated by Richard and Mary Bunge (Bunge et al., 1961). 18 years later, it has been demonstrated that remyelination restores conduction in previously demyelinated lesions (Smith et al., 1979; Smith et al., 1981). Until now, huge effort has been made to elucidate the cause and disease mechanisms of MS. This is reflected by the over 37000 publications found today on PubMed (A service of the U.S. National Library of

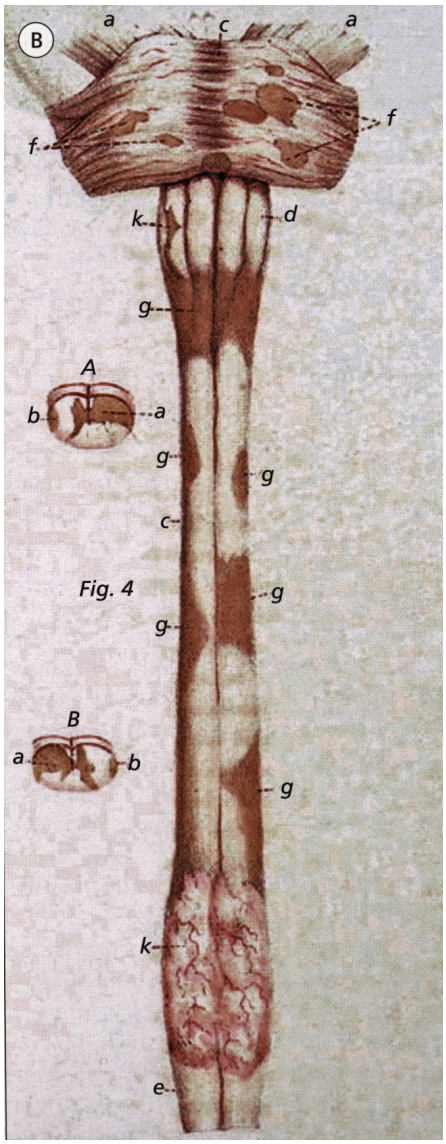

Figure 1 Drawing of a spinal cord from a MS patient by Robert Carswell (1793-1857
g: patches of the same kind on the spinal cord. k: Softening of a portion of the cord. A and B represent transverse sections of the cord to show that the discoloration commences on the surface of the white and extends inwards to the grey substande.
Copied from Carswell, 1938.

Medicine that includes over 17 million citations from MEDLINE and other life science journals for biomedical articles back to the 1950s) dealing with MS. Nevertheless, despite of extensive and detailed analysis of MS tissue pathology, the cause and underlying pathogenetic mechanisms are still unknown.

The Epidemiology of Multiple Sclerosis

The distribution of Multiple Sclerosis

Distribution by gender

In all representative prevalence studies, prevalence rates are higher for women than men. Rates range from 11 to 282 per 100'000 in women compared to 10 to 123 per 100'000 in men, which corresponds to a female:male ratio between 1.1 and 3.4 (Pugliatti et al., 2006). The highest ratio was found in North America, whereas the lowest reported ratio was found in Israel (Compston, 2006a). The average female:male ratio is about two females to one male (2F:1M) (Confavreux, 2006). This could be also demonstrated in Switzerland, in which a female:male ratio of 1.8 was reported in the Canton of Berne (Beer and Kesselring, 1994) and a ratio of 2.2 in the north-western region (Groebke-Lorenz et al., 1992). As additionally, pregnancy has an inhibitory effect on the relapse-rate in MS, it is speculated that the hormonal state has a great influence on disease mechanisms (Confavreux, 2006).

Distribution by age

Despite the problems to determine the age of onset in MS, there is a general consensus about the peak onset around the age of 30 (Figure 2) (Confavreux, 2006). There is a high variation of the total prevalence by age group in different countries (Pugliatti et al., 2006). In Switzerland, the prevalence for different age groups ranges from 5 per 100'000 (0-17 years) until 230 per 100'000 (35-49 years). The mean age at clinical onset was reported to be at 31.6 years for the north-western region (Groebke-Lorenz et al., 1992).

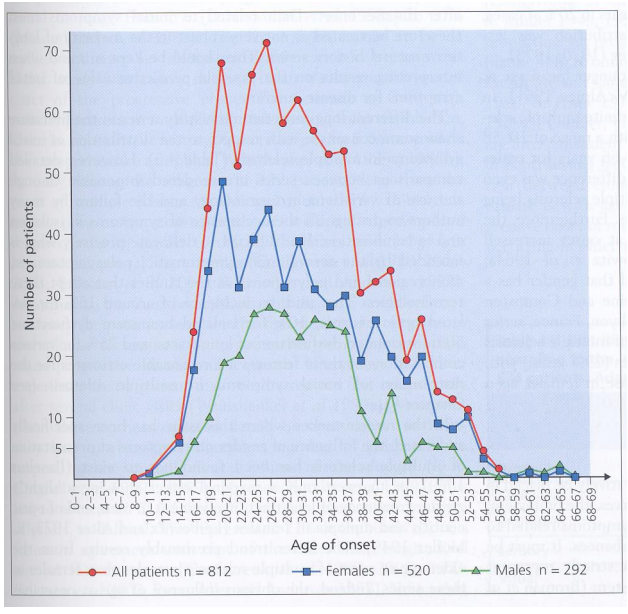

Figure 2 Distribution of patients by age af onset of the disease, among 812 patients with multiple sclerosis.
Copied from Compston, 2006

Geographic distribution

The prevalence of MS varies in different regions around the world. The total estimated prevalence rate for MS is about 83 persons per 100'000. It is highest in northern Europe, southern Australia and in the middle part of North America (Noseworthy et al., 2000), whereas other regions like most of Australia, southern US and parts of South America are areas of medium prevalence (Figure 3). In earlier studies, prevalence rates for Europe correlated with geographic latitude. However, this latitude gradient seems to disappear as high frequency zones are now also found in southern Europe, such as Portugal and Greece (Ascherio and Munger, 2007; Kurtzke, 2000). Fitting in the gradient of MS prevalence in Europe, prevalence rates for Switzerland are between 110 and 164 per 100'000 (Beer and Kesselring, 1994; Groebke-Lorenz et al., 1992). As the global distribution of prevalence rates follow the distribution of Caucasian people, this points to genetic triggers for the disease. Nevertheless, regional diversities in prevalence rates further imply the involvement of environmental factors in the pathogenesis of MS.

Figure 3 Worldwide distribution of MS as of 1998 with high (prevalence 30+; solid), medium (prevalence 5 ± 29; dotted), and low (prevalence 0 ± 4; dashed) regions defined. Blank areas are regions without data. Copied from Kurtzke and Wallin, 2000.

Environmental factors

In a study of European immigrants to South Africa, it was reported that by migration from a high risk area into a location of low risk area before the age of 15, the prevalence for these immigrants equals the prevalence of the low risk area. In contrast, prevalence rate of migrants older than 15 was the same as expected for their high-risk homelands (Dean and Kurtzke, 1970; Elian et al., 1990; Kurtzke, 2000; Kurtzke et al., 1970). Although in other reports, migrants retain their prevalence rate independent of their age (Ebers, 2008), these results further suggested that in MS an environmental cause or precipitant may be active.

Infections

There is evidence that a viral exposure, e.g. measles, mumps and rubella, at a later age increases the risk of getting MS (Compston et al., 1986). This effect is even higher in individuals infected with EBV (Martyn et al., 1993). In particular, MS risk is about 10 times greater among individuals with an EBV infection in childhood and about 20 times greater among individuals who developed mononucleosis (Ascherio and Munger, 2007). Other examples of causative agents implicated in MS pathogenesis are HHV-6 (Challoner et al., 1995), MSRV (Dolei et al., 2002) and Chlamydia pneumoniae (Gilden, 1999; Sriram et al., 1999). To date, numerous reports claimed the involvement of viruses as MS triggers, but none of these observations withstood scrutiny so far (Soldan and Jacobson, 2001; Sospedra and Martin, 2005).

Non-infectious environmental events

There are several reported non-infectious environmental risk factors which may contribute to MS pathogenesis. One of the most likely candidate is sunlight exposure and linked to this, circulating Vitamin D. But also other factors such as diet, sex hormones, cigarette smoking or trauma have been and still are discussed (for review see Ascherio and Munger, 2007).

Genetic factors

Susceptibility to MS is not only modified by the environment but also by genetic factors. This is supported by studies showing a racial susceptibility to MS (Compston et al., 1986). For example, in Aborigines or Maoris, MS is rarely diagnosed, whereas it occurs commonly in the Caucasian population (Hammond et al., 1988; Miller et al., 1990; Skegg et al., 1987). There is a familial recurrence rate of about 15% for MS (Compston and Coles, 2002). For monozygotic twins, the recurrence rate is about 30-35% (Ebers et al., 1986; Ebers et al., 1995; Mumford et al., 1994; Sadovnick et al., 1993). Age-adjusted risk for siblings is around 3%, followed by parents (2%) and children (2%), which is much higher than general population risk (Figure 4) (Compston, 2006b; Compston and Coles, 2002; Sadovnick and Baird, 1988; Sadovnick et al., 1988). Despite extensive searches, no major susceptibility gene has been identified through full genome screens. Nevertheless, some regions of interests have been identified (Compston, 2000). Population studies have suggested a candidate gene within the HLA class II, encoded on chromosome 6q21.1 - 21.3, exerting an influence on susceptibility of MS (Burton et al., 2007; Compston, 2006b). Association of gene polymorphisms with disease course was shown among others for APOE (Chapman et al., 2001; Evangelou et al., 1999) or IL-1β receptor and IL-1β receptor antagonist genes (Kantarci et al., 2000; Schrijver et al., 1999). More recently, an allelic and functional association of the IL-7 receptor alpha chain was reported (Gregory et al., 2007).

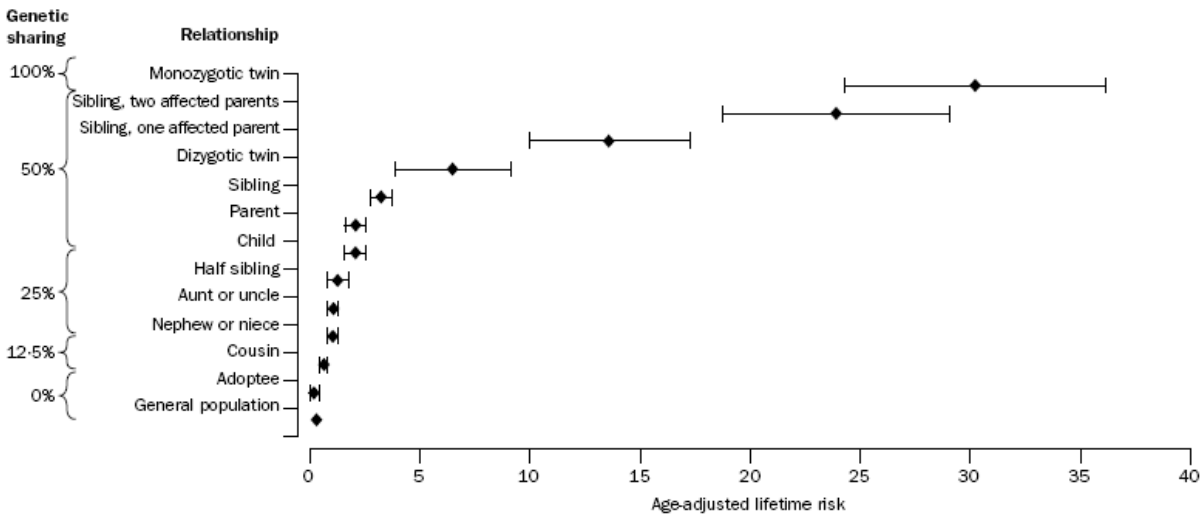

Figure 4 Recurrence risks for multiple sclerosis in families
Age adjusted recurrence risks for different relatives of probands with multiple sclerosis. Pooled data from population based surveys. Estimated 95% Cis are shown. Copied from Compston and Coles, 2002.

Clinical Features of Multiple Sclerosis

Symptoms of MS

In MS, symptoms and signs are variable. Generally, they reflect the extent to which parts of the CNS, which are fulfilling motor and sensory functions, such as the brainstem, cerebellum and spinal cord, are involved. The incidence of initial symptoms, although difficult to find out, are approximately as follows (McAlpine, 1972):

Weakness in one or more limbs	35%
Optic neuritis	20%
Paraesthesia	20%
Diplopia	10%
Vertigo	5%
Disturbance of micturition	5%
Others	5%

Principally, most MS symptoms base on the slowed, or even loss of axonal conduction due to demyelination (McDonald and Sears, 1969). In contrast, by generating ectopic impulses, demyelinated axons can also lead to positive sensations (Baker and Bostock, 1992). A common phenomenon in MS is a dramatic exacerbation of symptoms upon heat. E.g. deterioration during sunbathing, during a hot shower (Waxman and Geschwind, 1983) or a hot bath, which was used for MS diagnosis in the past (Berger and Sheremata, 1982) . Altogether, there exists many symptoms or signs of MS (for review see McDonald, 2006).

Disease course

Generally, the course of MS can be described in terms of relapses, remissions and chronic progression either from onset or after a period of remissions (Confavreux, 2006). For the majority of patients, the usual course of MS is characterized by repeated relapses generally associated with the eventual onset of disease progression.

Generally, the overall disease course was classified in four different categories (Confavreux, 2006) (see Figure 5). In ***relapsing-remitting MS***, clearly defined relapses with full recovery or with sequelae and residual deficits upon recovery are seen. Furthermore, the periods between the disease relapses are characterized by a lack of disease progression. ***Secondary-progressive MS*** is defined as MS with initial relapsing-remitting disease course followed by progression with or without occasional relapses, minor remissions, and plateaus. In ***primary progressive MS***, the disease progression starts from onset with occasional plateaus and temporary minor improvements allowed. The fourth category is the ***progressive relapsing MS***, which is defined as progressive disease from onset, with clear acute relapses, with or without full recovery. The periods between relapses are characterized by continuing progression. A „fifth“ definition would be *transient progressive MS*, which sometimes is used for few patients with a progressive course except for a single relapse at some time (Filippi et al., 1995a; Filippi et al., 1995b; Gayou et al., 1997). Others use this term solely for patients with a progressive course devoid of relapses beginning years after an isolated episode (Gayou et al., 1997). Again other clinicians allow the single attack before or after the onset of the disease (Stevenson and Miller, 1999; Stevenson et al., 2000). Although these different MS patterns were defined, one must be aware that the course of MS in an individual patient is largely unpredictable.

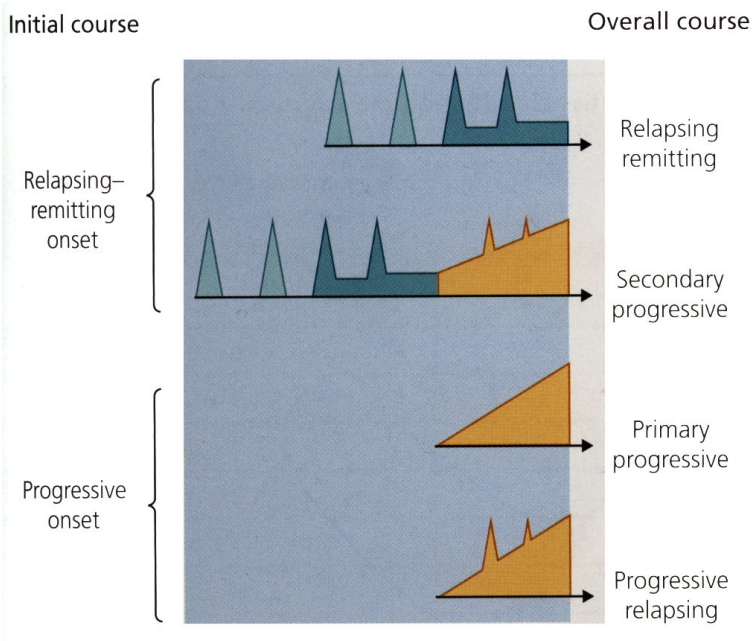

Figure 5 Classification of the course of multiple sclerosis.
Copied from Confavreux and Compston, 2006

Prognosis in MS

As the knowledge of prognosis is a major concern for almost every patient, long-term studies were performed to identify prognostic informations during the clinical course of MS. But, although current prognostic knowledge shows reasonable consistency, this is based on statistical analysis of patient populations, and therefore, extensive individual variations are encountered.

As a first association to the clinical outcome of the disease, men were found to develop a faster clinical progression (Confavreux et al., 2003; Confavreux et al., 2000). As others did not found this effect, this led to the conclusion that, the influence on gender on prognosis is weak (Confavreux, 2006). Another finding was the association of age at onset to disability: the older the age at onset, the shorter the time of disability (Broman et al., 1981; Confavreux et al., 2003; Confavreux et al., 2000; Eriksson et al., 2003; Renoux et al., 2007). Furthermore, presentation with optic neuritis is associated with slower disability progression, while onset with a spinal cord syndrome, or motor and cerebellar features correlated with a faster disability progression (Confavreux et al., 2003; Confavreux et al., 2000; Eriksson et al., 2003). The strongest clinical predictor in MS is the initial course of the disease. A progressive course from onset is associated with a shorter time to reach disability landmarks, if compared with relapsing-remitting MS (Amato and Ponziani, 2000; Broman et al., 1981; Confavreux et al., 2003; Confavreux et al., 2000; Kantarci et al., 1998).

MS variants

MS is a heterogeneous disease with many different features. Today, five „variants“ of MS are defined, although it is not absolutely clear whether these „variants“ might be independent diseases or not. The *chronic MS*, the major variant and issue of this dissertation, was defined by Charcot (Charcot, 1868). Another variant is the *acute MS*, which was first described by Marburg (Marburg, 1906). Clinically, *acute MS* is characterized by rapid progression and an exceptionally severe course (Lassmann, 2006). Another important difference to chronic MS is the additional occurrence of peripheral nerve demyelination (Lassmann et al., 1981; Marburg, 1906). In *Balo's concentric sclerosis,* large demyelinated plaques are found, that show peculiar alternating rims of myelin preservation and loss (Balo, 1928). This gives the lesions

an appearance of onion bulbs. The major feature of *neuromyelitis optica* (*Devic's disease*) is a dominant involvement of the spinal cord and the visual pathway (Devic, 1894). This subform shows a very severe clinical manifestation and is commonest in oriental people (Lassmann, 2006). In *diffuse sclerosis* (*Schilder's disease*), the pathology was characterized by primary demyelination that, unlike in chronic MS, was not restricted to focal plaques but affected large parts of the periventricular white matter (Lassmann, 2006; Schilder, 1912).

Pathology and Pathogenesis of Multiple Sclerosis

Originally, MS has been recognized as a disease of the CNS where an inflammatory process is associated with focal plaques of primary demyelination (Charcot, 1868; Lassmann, 2006; Lassmann et al., 2007). The most characteristic pathological feature of MS is the demyelinated plaque with glial scar formation (see page 34), which can be found anywhere where myelin sheaths are present (Lassmann, 2006; Lassmann et al., 2007). Currently, MS is still considered to be a CD4+ T helper cell type 1 mediated autoimmune disease, most likely targeting myelin (Sospedra and Martin, 2005). Whether this inflammation is the primary event in, or just a consequence of the disease is still not known. This current concept of MS pathogenesis takes issue with immune system mechanisms, which are somewhat special in the brain, and therefore, referred to as immune privilege of the CNS.

Immune system and the brain

The immune system can be roughly divided into the innate and the adaptive part, which can regulate each other but are mostly independent. Both can identify harmful structures and mount a response to neutralize this threat. However, both have to be tightly controlled in order to avoid damage to self tissue. It is speculated, that in MS this control fails in the adaptive immune system, leading to the observed autoimmune reaction in MS.

Innate immunity

The innate immune system forms the first line of defence against pathogens. It's main role is self-protection and maintenance of homeostasis, but can also trigger autoimmunity (Sospedra and Martin, 2005). The innate immunity recognizes evolutionarily conserved structures that are common to invading pathogens (O'Brien et al., 2008), discriminating those from self structures. The recognition of these structures is mediated by pattern-recognition receptors such as Toll-like receptors (TLRs), which are expressed on cells of the innate system. Responses mediated by the innate immune system occur rapidly, and can reach any location of the affected organism. The innate immune system is composed of specialized cells such as

macrophages, dendritic cells, natural killer cells (NK) and CNS-resident cells such as microglia (O'Brien et al., 2008).

Adaptive immunity

In contrast to the innate immune system, immune responses of the adaptive immune system are highly specific and focussed, and earlier immune responses of the adaptive immune system leave an imprint on the immunological memory. This leads to a faster and more vigorous immune reaction on pathogens repeatedly entering the body. The basis for this specificity and memory capacity of the adaptive immune system is the clonal diversity of its immune cells, lymphocytes, which each of which is characterized by diverse surface receptors for (ideally) one antigen. Therefore, pathogens intruding the body are recognized by specific lymphocytes expressing receptors which best fit the antigen. These antigens are presented to the lymphocytes by specialized cells, the antigen presenting cells (APC), via the major histocompatibility complex (MHC). Subsequently, these lymphocytes are activated, multiply and differentiate in order to neutralize the antigen.

The adaptive system relies mainly on two cell types, the T and B lymphocytes. Whereas the main role of B lymphocytes is the generation of antibodies and present antigens to T lymphocytes, T lymphocytes are responsible for cell-mediated immunity. T lymphocytes themselves can be divided into two main groups, the T helper cells (Th), directing and orchestrating immune responses, and the cytotoxic T cells (Tc), inducing death of pathogen infected cells. Most commonly these two types are distinguished according to their expression of surface markers. Therefore, T helper cells are also known as CD4+ cells, whereas cytotoxic T cells are called CD8+ cells (Janeway, 1992).

The Th1/Th2/Th17 trichotomy

The selective activation of CD4+ T helper cell subsets is an important step determining the immune response to pathogens as well as the pathogenesis of allergies and autoimmunity (for review see Rautajoki et al., 2008). Generally, CD4+ T helper cells can be divided in functionally different subsets, such as Th1, Th2 and Th17 cells. This differentiation is made according to their expression of cytokines, which are critical components in immune regulation and signalling, determining the

action of different immune cells. Whereas Th1 and the newly discovered Th17 subset are associated with autoimmune diseases and the clearing of intracellular pathogens (Steinman, 2007; Szabo et al., 2003), the Th2 subset is predominantly linked to atopic diseases and allergies (Mowen and Glimcher, 2004). A major pathway involved in the differentiation and polarization of T helper cell subsets is the JAK/STAT signalling pathway. Members of the signal transduction and activator of transcription (STAT) family like STAT1, STAT3, STAT4 and STAT6 are thought to be critically involved in the polarization of Th1, Th17 or Th2 cells (Harrington et al., 2006; Kaplan et al., 1996; Takeda et al., 1996). Whereas STAT1, STAT3 and STAT4 are involved in Th1 and Th17 cell polarization, Th2 cell polarization depends most exclusively on STAT6 (Rautajoki et al., 2008). Both, Th1 cells as well as Th17 cells are believed to act in a pro-inflammatory way, releasing pro-inflammatory cytokines such as e.g. IL-1, IL-6, IL-12, IL-23 and IFN-γ and therefore promoting autoimmune mechanisms. In contrast, Th2 cells are thought act in an anti-inflammatory way (Sospedra and Martin, 2005), releasing e.g. IL-4 and IL-10 and by that, limiting autoimmune mechanisms.

Cytokines / Chemokines

Immune systems cytokines are small signalling molecules that orchestrate all phases of the immune response (for review see Sospedra and Martin, 2005). Cytokines are organised in highly complex, dynamic networks and have in part redundant functions due to their signalling through multi-component receptors. Generally, cytokines are divided into pro- and anti-inflammatory subsets, according to their effect on the immune response. Under normal conditions, a dynamic balance between pro- and anti-inflammatory cytokines is established to maintain homeostasis (for review see Sospedra and Martin, 2005). Under pathological conditions, cytokine release is shifted towards either pro-inflammatory cytokines (e.g. autoimmunity - IL-1, IL-12, IL-23 and IFN-γ) or anti-inflammatory cytokines (e.g. allergic reactions - IL-4, IL-6 and IL-10).

On the other hand, chemokines are a subset of cytokines with chemoattractant properties. Therefore, chemokines and their receptors play a central role in the recruitment of leukocytes and other cell types (for review see Sospedra and Martin, 2005). They can induce and activate leukocyte adhesion molecules and establish a

chemotactic concentration gradient resulting in leukocyte recruitment. As cytokines, chemokines can be divided into a pro- and anti-inflammatory subset.

The CNS immune privilege

It has been clearly demonstrated, that destructive T-cell responses are much more difficult to be initiated in the CNS parenchyma than in many other non-CNS sites (Barker and Billingham, 1977; Perry, 1998). Furthermore, T-cell receptor (TCR) transgenic mice in which T cells are predominantly specific for CNS myelin antigens do not spontaneously develop T-cell infiltration, demyelinating lesions or clinical signs of EAE (Brabb et al., 1997; Brabb et al., 2000). Still, this privilege does not apply to all regions of the CNS, as pro-inflammatory T-cell responses are readily triggered within the non-parenchymal sites of the CNS (Carson et al., 2006; Perry, 1998). CNS immune privilege was believed to base on the immunologically separation of the CNS from the peripheral immune system, as well as on an immunologically inert CNS (Barker and Billingham, 1977). However, newer studies suggest that CNS immune privilege is not equal to immune isolation, but rather a collection of CNS-driven mechanisms that actively regulate T-cell responses within the CNS (Carson et al., 2006).

In MS, the nature and function of the immune privilege is of high interest, as T-cell responses are thought to play a major role in MS pathogenesis. Therefore, major cells and structures currently known to be involved in the formation of this immune privilege are shortly reviewed hereafter.

The blood-brain-barrier (BBB)

The BBB is a complex structure which is built by specialized cerebrovascular endothelial cells in contact with pericytes and astrocytes (Abbott et al., 2006; Balabanov and Dore-Duffy, 1998; Han and Suk, 2005). Primarily, the BBB is used for the regulation of nutrient movement into the CNS, potentially toxic molecules out from the CNS and limiting the entry uncontrolled blood-borne metabolisms and toxins in the brain (Han and Suk, 2005; Ohtsuki, 2004; Zlokovic, 2005).

In immunological terms, the BBB has been defined to limit leukocyte, in particular lymphocyte, migration into the CNS (Carson et al., 2006). This movement regulation

may be in part under the control of CNS-resident cell such as astrocytes and microglia (Gimenez et al., 2006).

Microglia

Microglia play a major role in homeostatic and reparative functions, and are the earliest sensors of all forms of pathological incursion (Ransohoff, 2007). In immunological terms, microglia possess properties of antigen-presenting-cells and phagocytes (Jack et al., 2005; Sanders and De Keyser, 2007). Microglia are involved in the innate as well as the adaptive immune system and are able to regulate inflammation and cell damage (Chew et al., 2006). Being the APC and phagocyte of the CNS, microglia are believed to be the primary cell type responsible for brain-immune system interactions (Jack et al., 2005). However, microglia never seem to leave the CNS and thus proinflammatory T cell responses are less likely activated (Carson et al., 2006; Carson et al., 1999). Thus, microglia are more likely to play a role in modifying or directing T-cell function rather than in antigen-specific recruitment of lymphocytes into the CNS (Greter et al., 2005). The expression of molecules inhibiting T-cell proliferation and decreasing MHC expression, microglia appear to be specialized to promote a short self-limiting T cell response in the brain (Carson et al., 2006).

Astrocytes

The main task of astrocytes in the CNS is the maintenance of physiological homeostasis of neurons (Gimsa et al., 2004). In CNS-immune system interactions the main role of astrocytes seem to be the limitation of T-cell responses, as they were shown to induce T cell apoptosis (Bechmann et al., 2002). Furthermore, astrocytes may play an anti-inflammatory role during T-cell mediated neuroinflammation (Gimsa et al., 2004), further limiting detrimental, pro-inflammatory T-cell responses. Nevertheless, astrocytes can also act as APCs. Astrocytes were shown to express MHC class I as well as MHC class II molecules (Hamo et al., 2007; Hoftberger et al., 2004) and therefore capable of activating T lymphocytes.

Neurons

Indirectly, neurons are also involved in the formation of the immune privilege. It has been shown, that microglial MHC expression is regulated by neuronal activity (Neumann, 2001; Reinke and Fabry, 2006). Furthermore, it is speculated whether neurons are trying to promote their survival by triggering increased neuroprotective APC function in microglia (Carson et al., 2006). Whether neurons are able to express MHC class I molecules under normal conditions remains controversial, as some have shown an MHC class I expression by neurons (Huh et al., 2000; Lidman et al., 1999; Redwine et al., 2001) whereas others have not (Fujimaki et al., 1996; Lampson, 1995).

Oligodendrocytes

Oligodendrocytes are the cells of the CNS forming myelin, a unit of membranes wrapped around axons allowing fast saltatory conduction. Recently, research has begun to elucidate the extent that oligodendrocytes actively regulate immune responses. Although a direct modification of immune responses by oligodendrocytes could not be demonstrated yet, oligodendrocytes were reported to express several molecules capable of influencing immune responses (Balabanov et al., 2007; Cannella and Raine, 2004; Christians et al., 2002; Stahnke et al., 2007; Zeis et al., 2008). As in MS, oligodendrocytes are the main target of the immune response, the question whether oligodendrocytes are capable of modifying immune responses is of high significance.

The MS lesion

The pathological hallmark of MS is the demyelinated plaque with preserved axons and astrocytic scar formation. The characterization of these lesions is based on the state of demyelination, the nature and appearance of inflammatory infiltrates, their state and the presence or absence of antibodies. By these features, several distinct lesion types were defined (for review see Lassmann, 2006).

The basic feature of plaques in chronic MS consists of a sharply demarcated area with a diameter of <1mm to several centimetres (Lassmann, 2006). These plaques are generally centred around small blood vessels (Rindfleisch, 1863). Microscopically, myelin sheaths are lost whereas axons are relatively spared and embedded in astroglial scar tissue (Lassmann, 2006). Depending on the type of lesions, inflammatory infiltrates, mainly composed of mononuclear cells, are present.

Actively demyelinating lesions

A characteristic of actively demyelinating lesions is the presence of myelin sheaths in the process of dissolution (Figure 6). Additionally, degradation products of myelin are found within macrophages (Bruck et al., 1995; Gay et al., 1997; Lassmann, 2006). In acute plaques, demyelination occurs simultaneously in the whole lesion whereas in chronic active plaques (a late form of actively demyelinating lesions), a zone of active demyelination surrounds the already demyelinated centre of the plaque (Lassmann, 2006).

Inactive demyelinated lesions

Inactive demyelinated lesions are characterized by an absence of ongoing destruction of myelin sheaths. Nevertheless, there can still be some inflammation with activated macrophages (Bruck et al., 1995). In early stages, inactive plaques can still be infiltrated by lymphocytes and macrophages and thus be hypercellular. In contrast, late inactive plaques are characterized by the presence of only few lymphocytes and macrophages. Furthermore, a dense astrocytic scar has been formed, embedding remaining demyelinated axons (Lassmann, 2006).

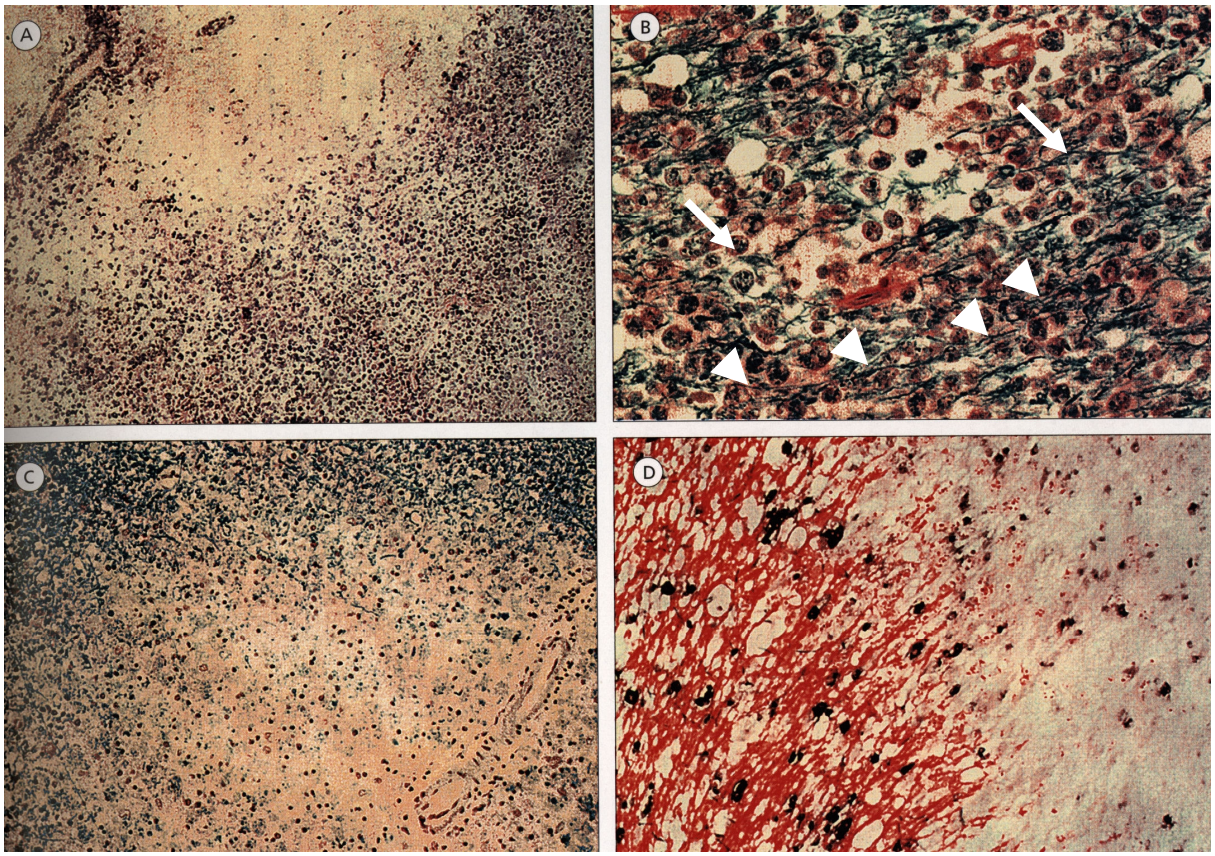

Figure 6 Lesional activity in multiple sclerosis. (A) Acute multiple sclerosis plaque; immunocytochemistry shows numerous macrophages stained with macrophage activation marker MRP14; 80x. (B) Acute multiple sclerosis; luxol fast blue myelin stain; early lesion stage infiltrated by numerous macrophages containing luxol fast blue-positive degradation products (arrow); in between are preserved myelinated fibres (arrowhead); 500x (C) Chronic multiple sclerosis, luxol fast blue myelin stain showing radially expanding lesion; numerous macrophages with luxol fast blue-positive myelin degradation products in the demyelinated area; 80x (D) Chronic multiple sclerosis: *in situ* hybridization for proteolipid protein mRNA (black) and immunocytochemistry for proteolipid protein (red). Edge of a radially expanding lesion showing destruction of proteolipid protein-positive myelin sheaths (red) and proteolipid protein taken up by macrophages (small red granules). Oligodendrocytes with proteolipid protein mRNA (black cells) are reduced at the plaque margin. 300x
Copied from Lassmann, 2006

Shadow plaques

Immunocytochemical as well as ultrastructural data strongly suggest that a complete remyelination of a previously demyelinated plaque leads to the formation of the so-called shadow plaque (Figure 7). During remyelination only thin myelin sheaths are formed, which leads to a decreased density of myelin staining (Lassmann, 1983; Lassmann, 2006).

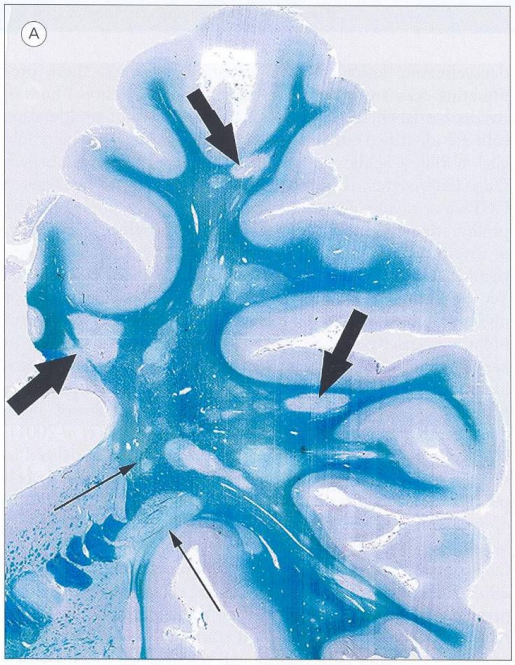

Figure 7 Remyelination in multiple sclerosis
Chronic multiple sclerosis: luxol fast blue myelin stain; multiple lesions in the white matter; some lesions are completely demyelinated (thick arrows), whereas others are shadow plaques (thin arrows)
Copied from Lassmann, 2006

Destructive lesions

Another lesion type are the so-called destructive lesions. These lesions are characterized by an extensive additional tissue destruction affecting astrocytes and axons as well, sometimes giving rise to cystic brain lesions (Lassmann, 2006). Destructive lesions are responsible for very severe and rapid progressing acute and chronic MS (Miller et al., 1991; Sugano et al., 1992).

Heterogeneity of active demyelinating lesions

Recent studies of active demyelinating lesions, have shown interindividual differences in the patterns of oligodendrocyte pathology and myelin destruction, which might possibly reflect different pathogenesis (Figure 8, Lucchinetti et al., 2000). Whether in a single patient, the pattern of demyelination and oligodendrocyte injury are homogeneous or not is currently under heavy discussion (Barnett and Prineas, 2004; Breij et al., 2008; Lucchinetti et al., 1999; Lucchinetti et al., 2000; Lucchinetti et al., 2004; Raine, 2008).

Pattern I – Macrophage mediated lesion formation

In active demyelinating lesions showing a pattern I, active demyelination is associated with a T-lymphocyte and macrophage-dominated inflammation. Macrophages and microglia are found in close contact with degenerating myelin. This pattern shows a relative axonal as well as oligodendrocyte sparing. Furthermore, a reappearance of oligodendrocytes is frequent and often an extensive remyelination can be seen. As a putative mechanism, demyelination induced by macrophage or cytotoxic T-cell toxins is suggested (Lassmann, 2006; Lucchinetti et al., 2000).

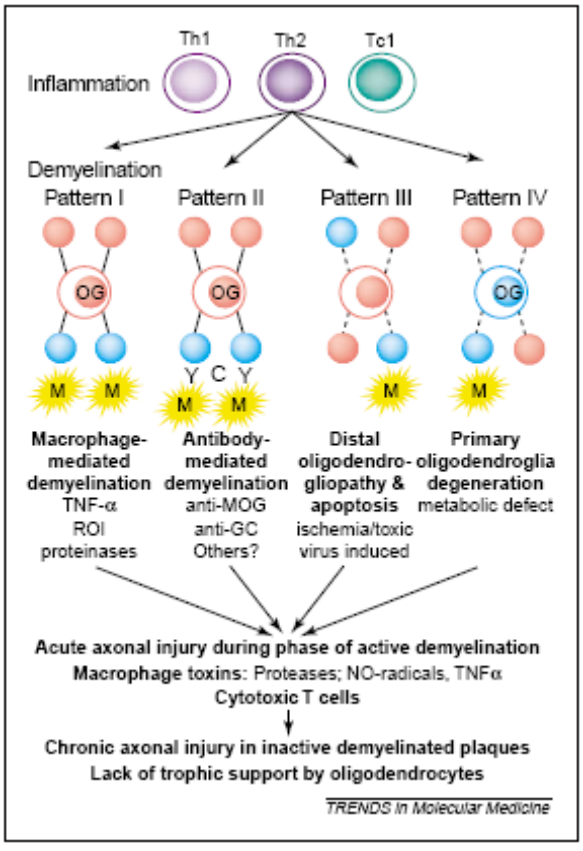

Figure 8 Summary of pathogenetic mechanisms involved in the formation of multiple sclerosis lesions.
Inflammation: evidence indicates that T helper 1 (Th1) cells have a role in inducing inflammatory reactions in the central nervous system. In addition, however, T helper 2 (Th2) cells and cytotoxic, class-I restricted cytotoxic T cells (Tc1) might modify the outcome of the lesions. Demyelination: myelin sheaths and oligodendrocytes (OG) can be destroyed, possibly by different mechanisms in different individuals. This results in distinctly different patterns of demyelination in active lesions. Demyelination may be induced by macrophages (M) and/or their toxic products (resulting in pattern I), by specific demyelinating antibodies and complement (C, resulting in pattern II), by degenerative changes in distal processes, in particular those of periaxonal oligodendrocytes (distal oligodendrogliopathy), followed by apoptosis (resulting in pattern III) or by a primary degeneration of oligodendrocytes followed by myelin destruction (resulting in pattern IV).
Axonal injury: axonal injury follows acute destruction of myelin sheaths. In the active phase of demyelination, axonal injury is likely to be induced by macrophage toxins or by the direct effects of cytotoxic T cells. The chronic axonal injury observed inactive plaques may be caused by a lack of trophic support by glial cells, such as oligodendrocytes, but could also involve inflammatory mediators, produced by macrophages, that persist in most active chronic lesions. Copied from Lassmann et al., 2001

Pattern II – Antibody mediated lesion formation

In contrast to pattern I lesions, in pattern II lesions a deposition of Immunoglobulins and complement C9neo antigen at sites of active myelin destruction can be detected. This is exclusively found in pattern II lesions whereas oligodendrocyte and axon pathology is similar as in pattern I. Therefore, it is suggested that beside of a T-cell mediated inflammation with macrophage and microglia activation, complement mediated lysis of antibody-targeted myelin is occurring (Lassmann, 2006; Lucchinetti et al., 2000).

Pattern III – Distal oligodendrogliopathy

The hallmark of pattern III lesions is the selective loss of MAG and CNPase in early stages of plaque formation. These changes are associated with alterations in the most distal processes of oligodendrocytes, later followed by oligodendrocyte apoptosis and demyelination (Lassmann, 2006; Lucchinetti et al., 2000). In contrast to pattern I and II lesions, edges of pattern III lesions are ill-defined and lesions are not necessarily centred on small vessels (Lassmann, 2006). Pattern III lesions are thought to be formed by a T-cell-mediated small vessel vasculitis with secondary ischemic damage of the white matter (Lassmann et al., 2001).

Pattern IV – Primary oligodendrocyte damage with secondary demyelination

In addition to pattern I and II lesions, pattern IV lesions show a prominent oligodendrocyte degeneration in a small rim of periplaque white matter (Lucchinetti et al., 2000) followed by an inflammatory infiltration of the tissue. Pattern IV lesions are a rather uncommon MS lesion type (<2% of lesions analyzed by Lucchinetti et al., 2000). Whether this lesion type is formed due to a genetic defect or not remains to be determined.

Cellular composition of MS lesions

MS is a disease most probably driven by inflammation. Whether this inflammation is due to immune-mediated processes or is a secondary consequence of tissue injury is currently still not known. Mediated by cells of the adaptive immune system, such as T and B lymphocytes, MS inflammation is thus reflected by their presence within lesions. In addition, cells of the innate immune system such as macrophages as well as microglia and astrocytes are activated and recruited. Most recently, an involvement of oligodendrocytes in these inflammatory mechanisms is discussed.

T lymphocytes

In MS lesions, CD4+ (T helper cells) as well as CD8+ (cytotoxic/suppressor T cells) T lymphocytes are present. Together, these T cells represent the majority of infiltrating lymphocytes (Nyland et al., 1982). Currently, it is not clear whether CD4+ cells are the dominant T cell population in lesions or CD8+ T cells. Some studies report a domination of CD4+ T cells (Traugott et al., 1983a; Traugott et al., 1983b) whereas others show a domination of CD8+ T cells (Booss et al., 1983). In most recent studies, an equal number of CD4+ and CD8+ T cells is reported (Babbe et al., 2000; Gay et al., 1997). Both, CD4+ as well as CD8+ T lymphocytes have been shown to be clonally expanded (Babbe et al., 2000), suggesting an activation by the recognition of their specific antigen (Babbe et al., 2000; Wekerle, 2006).

Whereas the immune response of CD4+ T cells is MHC class II restricted, CD8+ T cells respond to MHC class I presented peptides. MHCII expression is restricted to professional antigen presenting cells such as microglia/macrophages and dendritic cells (Becher et al., 2000; Greter et al., 2005). In the case of CD4+ T cells, three subpopulations have been identified to date. Studies in EAE suggest that CD4+ T cells of the Th1 and Th17 lineage play a major role in disease pathology (Gutcher et al., 2006; Langrish et al., 2005; Langrish et al., 2004; Lassmann and Ransohoff, 2004; Sospedra and Martin, 2005; Weaver et al., 2006). These two lineages are thought to be pro-inflammatory, secreting cytokines like IFN-γ (Th1), IL-17A, IL-17F and IL-22 (Iwakura and Ishigame, 2006; Kreymborg et al., 2007; McGeachy et al., 2007). In contrast, CD4+ T cells of the Th2 type, are thought to be mostly beneficial

and have been associated with remission and recovery from disease (Cannella and Raine, 2004; Sospedra and Martin, 2005).

By the interaction of the CD8+ T cell receptor together with the MHC class I peptide complex, CD8+ T cells are activated and are directly cytotoxic to cells presenting their specific antigen (Parkin and Cohen, 2001). In EAE, an involvement of CD8+ cytotoxic T lymphocytes in autoimmune demyelination was shown (Huseby et al., 2001; Sun et al., 2001). Except for microglia, no resident CNS cells express MHC class II, but an expression of MHC class I is common (Sospedra and Martin, 2005). Therefore, a major involvement of CD8+ T cells is suggested.

Altogether, both CD4+ and CD8+ T cell responses seem to contribute to MS pathogenesis, although probably at different steps and with different roles (Sospedra and Martin, 2005).

B lymphocytes

Beside T lymphocytes, B lymphocytes are found to be present within plaques (Prineas and Wright, 1978). Furthermore, the presence of oligoclonal bands suggests an involvement of B cells in MS pathogenesis. The clonal expansion of B cell populations in lesions has been shown (Gilden et al., 2001), suggesting their activation. It has also been shown that immunoglobulin-containing cells were significantly more numerous in plaques than in non plaques and in recent plaques as compared with old plaques (Esiri, 1977).

Macrophages / Microglia

Macrophages are a type of phagocytes, which continuously migrate through tissues with the primary task to phagocyte and destroy pathogens. Microglia are a heterogeneous population of CNS-specific macrophages that play an important part in maintaining CNS immune privilege (Carson et al., 2006). Within MS lesions, the majority of hematopoietic cells are monocytes and macrophages (Adams and Poston, 1990; Adams et al., 1989). An involvement of macrophages was shown by the presence of myelin degradation products in macrophages (Bruck et al., 1994). Furthermore, macrophages and microglia were shown to express activation markers, e.g. Ki-M1P (Bruck et al., 1995; Ozawa et al., 1994), suggesting their phagocytic

activation. In MS lesions, macrophages and microglia were further shown to express a variety of molecules required for propagation and regulation of the inflammatory response (Wekerle, 2006). These include cytokines and their receptors (Bonetti and Raine, 1997; Ramanathan et al., 2001; Woodroofe and Cuzner, 1993), MHC antigens (Esiri and Reading, 1987), adhesion molecules (Peterson et al., 2002) and others. This suggests a major involvement of microglia and/or macrophages in lesion formation. In EAE, a correlation between disease severity and macrophage infiltration was found, further suggesting a major role for macrophages in lesion pathogenesis (Berger et al., 1997).

Astrocytes and the glial scar

Under normal circumstances, astrocytes are dynamic cells capable of communicating with neurons and other glial cells. They are involved in synaptic functioning, physical structuring of the brain, its metabolism as well as responding to pathological insults (Williams et al., 2007). Upon such insults, astrocytes are activated resulting in a process called astrogliosis (Williams et al., 2007). In MS, a described feature of lesions is the proliferation and hypertrophy of astrocytes. Their abnormality in MS lesions even led to the hypothesis that MS was a primary disease of astrocytes (Müller, 1904). Astrocytes were also reported to express cytokines such as TNF-α and LT-α, which might point to an involvement of astrocytes in lesion formation (Zeis and Schaeren-Wiemers, 2008). In the majority of chronic MS lesions, activated astrocytes form the so-called astrocytic (or glial) scar (Figure 9, Anton, 1912; Jakob, 1915; Williams et al., 2007). This astrocytic scar is characterized by a fibrillary network of astrocytic processes containing high amounts of GFAP, embedding remained axons (Williams et al., 2007). This scarring is known to produce an inhibitory environment which can impede tissue repair (Holley et al., 2003). In particular, migration of oligodendrocyte

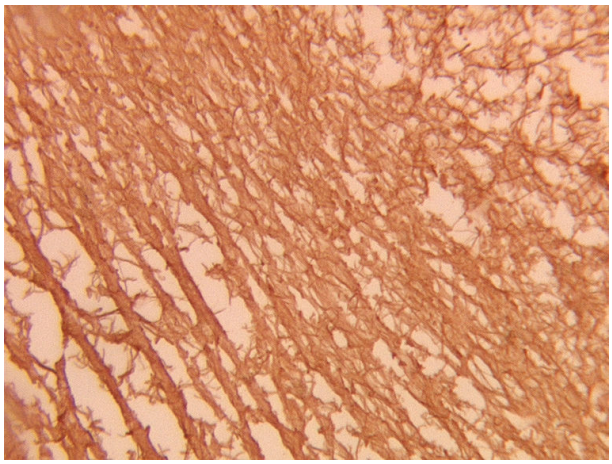

Figure 9 Astrocytic scar in multiple sclerosis
Anti-GFAP stained fibrillary network forming the astrocytic scar in a chronic lesion. (100x) Kindly provided by U.Graumann.

progenitors, remyelination and axonal regeneration was shown to be prevented by the astrocytic scar (Faissner, 1997; Rosen et al., 1989).

Oligodendrocytes

Oligodendrocytes are the cells of the CNS forming myelin, a unit of membranes wrapped around axons allowing fast saltatory conduction (Figure 10). In contrast to the Schwann cells from the PNS, Oligodendrocytes can maintain up to 50 internodes of myelin simultaneously. Due to the fact that oligodendrocytes are highly specialized and have a high metabolic demand maintaining many myelin sheaths, oligodendrocytes are one of the most vulnerable cells in the CNS (Merrill and Scolding, 1999; Zeis and Schaeren-Wiemers, 2008). In MS, myelin appears to be the primary target of the inflammatory reaction. It appears that a T-cell mediated immune reaction against myelin antigens is responsible for the induction of inflammation in MS (Wekerle, 2006). Therefore, the myelin producing oligodendrocytes are the major target cell of the immune reaction in MS. Although oligodendrocyte loss is evident during the chronic disease process, recent studies have shown that oligodendrocytes may successfully protect themselves. In particular, this has been shown during the pathogenesis of Balo's concentric sclerosis (see page 19, Stadelmann et al., 2005). Furthermore, oligodendrocytes have been shown to be capable of expressing a variety of protective molecules (Balabanov et al., 2007; Christians et al., 2002; Stahnke et al., 2007), and have been suggested to have potential immune-modulating capacities (Balabanov et al., 2007; Cannella and Raine, 2004; Christians et al., 2002; Stahnke et al., 2007; Zeis et al., 2008). This suggests a view of oligodendrocytes being at least capable to defend themselves, or even being a part of the immune privilege of the brain (Zeis and Schaeren-Wiemers, 2008).

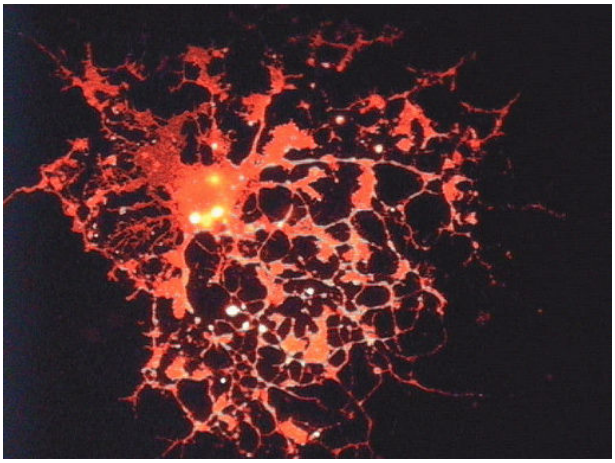

Figure 10 Oligodendrocyte in culture
Oligodendrocyte in culture stained by MBP. Note the many processes extending to attach to and myelinate axons. (500x). Kindly provided by N.Schaeren-Wiemers

The „normal“ white matter in MS

In MS, lesions form predominantly in white matter regions. The reason why lesions form in some parts of the white matter, whereas other parts are spared is still unknown. The knowledge of mechanisms present in the MS white matter facilitating or preventing lesion formation would be highly relevant. Studies of the white matter in MS have revealed accumulating evidences that the white matter in MS is far from being normal. MRI and spectroscopy studies have revealed diffuse abnormalities within the white matter, and therefore called normal appearing white matter (NAWM) of MS patients. Among others, blood-brain-barrier changes, axonal injury and to some extent astro- and microgliosis has been reported to be present in MS NAWM (Aboul-Enein et al., 2003; Fu et al., 1998; Silver et al., 2001). Furthermore, recent MRI studies describe profound alterations in NAWM in whom focal lesion load is small and cannot account for the extent of diffuse changes (Bozzali et al., 2002; Filippi et al., 2003). Microscopically, a mild inflammatory reaction, reflected by some microglia activation, astrocytes scarring as well as increased expression of proteolytic enzymes within astrocytes and microglia has been shown (Allen et al., 1981; Allen and McKeown, 1979; Allen et al., 2001; McKeown and Allen, 1978). An inflammatory reaction is further supported by the finding of diffuse inflammatory damage spreading throughout the whole brain in the chronic phase of the disease associated with slow progressive axonal injury at sites without obvious inflammation (Kutzelnigg et al., 2005). Furthermore, a study from our lab revealed an upregulation of a number of functionally related genes involved in oxidative stress, homeostasis and endogenous neuroprotection (Graumann et al., 2003). In particular, hypoxia inducible factor 1α (HIF-1α) and some of its targets genes such as e.g. vascular endothelial growth factor 1 (VEGFR1) were shown to be upregulated in most MS cases, possibly reflecting an adaptation of cells of the NAWM to the pathophysiology of MS. Although, changes in NAWM are present, this tissue reflects a promising possibility to detect MS specific, pre-lesional changes, which are to some extent independent from possible, secondary induced changes due to the strong inflammatory reaction.

Axonal pathology

Although MS seems to be a primary demyelinating disease, acute axonal pathology is seen in active demyelinating lesions, and axonal density is reduced in most chronic MS plaques (Ferguson et al., 1997; Trapp et al., 1998; Zeis and Schaeren-Wiemers, 2008). Generally, active destruction of axons is higher in actively demyelinating lesions and acute multiple sclerosis than in chronic plaques (Barnes et al., 1991). Loss of axonal profiles in established lesions up to 60-70% has been observed (Bjartmar et al., 2000; Bjartmar et al., 2001; Bjartmar and Trapp, 2001). Axonal loss is not restricted to demyelinated plaques but is also found in remote tract systems (Bjartmar et al., 2001; Ganter et al., 1999; Lovas et al., 2000). Furthermore, similar spinal cord atrophy was shown in areas with demyelinating plaques and areas without lesions (Evangelou et al., 2005). Furthermore, a correlation of tract degeneration in the corpus callosum and the degree of axonal damage in adjacent plaques of the white matter was demonstrated (Evangelou et al., 2000). This suggests that secondary Wallerian degeneration is an important feature of MS.

Acute axonal injury is apparent in actively demyelinating lesions of acute MS as well as in active plaques of chronic MS (Bitsch et al., 2000; Marburg, 1906; Trapp et al., 1998). This damage consists of axonal transsection, axonal interruption, swelling with formation of spheroids and regenerative sprouting (Trapp et al., 1998). It was shown that axons are damaged during the process of acute demyelination and shortly after it (Ferguson et al., 1997; Kornek et al., 2000). Therefore, it is widely accepted that the active inflammatory process also affects axons (Kornek and Lassmann, 1999). As oligodendrocytes secrete neurotrophic factors important for neuronal survival, an oligodendrocyte loss might additionally contribute directly to axonal injury and degeneration (Meyer-Franke et al., 1995). Although inflammation is quiescent in completely demyelinated lesions, a process of slow axonal injury was demonstrated in such lesions (Kornek et al., 2000). This slow axonal injury might be functionally significant during the long disease duration of MS (Lassmann, 2006). It has been suggested, that this chronic axonopathy is not due directly to inflammation, but results from loss of trophic support normally provided to axons by myelin or glia, acting directly or through the maintenance of electrical activity, or both (Barres and Raff, 1993; Wilkins et al., 2001). In a study of acute axonal injury in relation to disease duration, it was suggested that axonal injury might be most extensive at

early stages of the disease, decreasing over time (Kuhlmann et al., 2002). Another interpretation of this study suggested that the extent of axonal injury reflects the severity of the disease rather than the stage or duration (Lassmann, 2006). To a lower incidence, axonal injury and loss was also demonstrated to be present in the PPWM, white matter directly adjacent to a lesion, and NAWM (Evangelou et al., 2000a; Evangelou et al., 2000b; Ferguson et al., 1997; Kornek et al., 2000). This overall, chronic axonal degeneration might slowly increase the clinical deficit, decaying a compromised but functioning pathway and leading to disease progression (Figure 11, Compston and Coles, 2002).

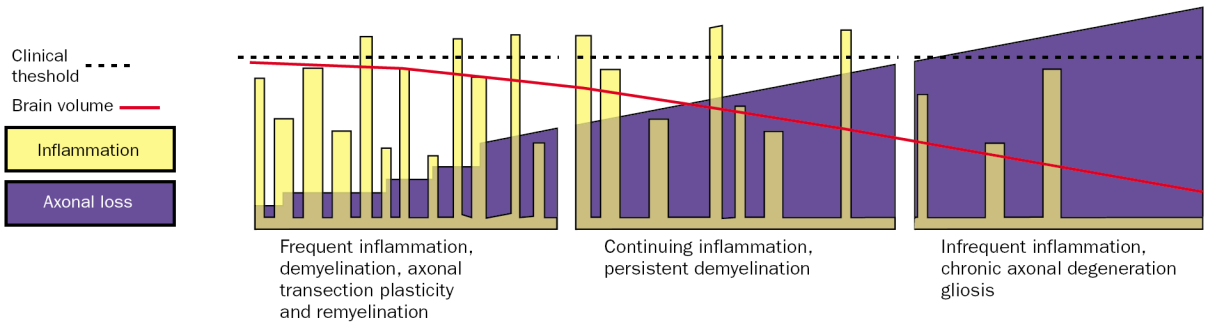

Figure 11 The course of MS correlated with axonal pathology
Inflammatory axonal conduction blocks (Yellow bars) during earlier stages, as well as chronic axonal degeneration (purple area) accumulating over lead to clinical symptoms of MS (dashed line). Copied from Compston and Coles, 2002.

Grey matter pathology

Although being most obvious in the white matter, lesions are also found in all grey matter areas of the CNS of MS patients (Brownell and Hughes, 1962; Sander, 1898). As in the white matter, myelin remains the principal target in grey matter plaques. Three different types of grey matter lesions have been defined: intracortical-, cortico-subcortical- and surface-oriented band-like cortical- lesions (Bo et al., 2003; Kidd et al., 1999; Peterson et al., 2001). Whereas the first two develop, as white matter lesions around small veins and ventricles (Lassmann, 2006), the third type is characterized by demyelination of the outer 3-5 layers of the cortex (Figure 12) (Lassmann, 2006; Peterson et al., 2001). This results in band-like lesions spanning millimetres to centimetres of the cortical surface (Peterson et al., 2001). Although to a

certain degree similar to white matter lesions, grey matter lesions show a limited inflammatory reaction and BBB damage.

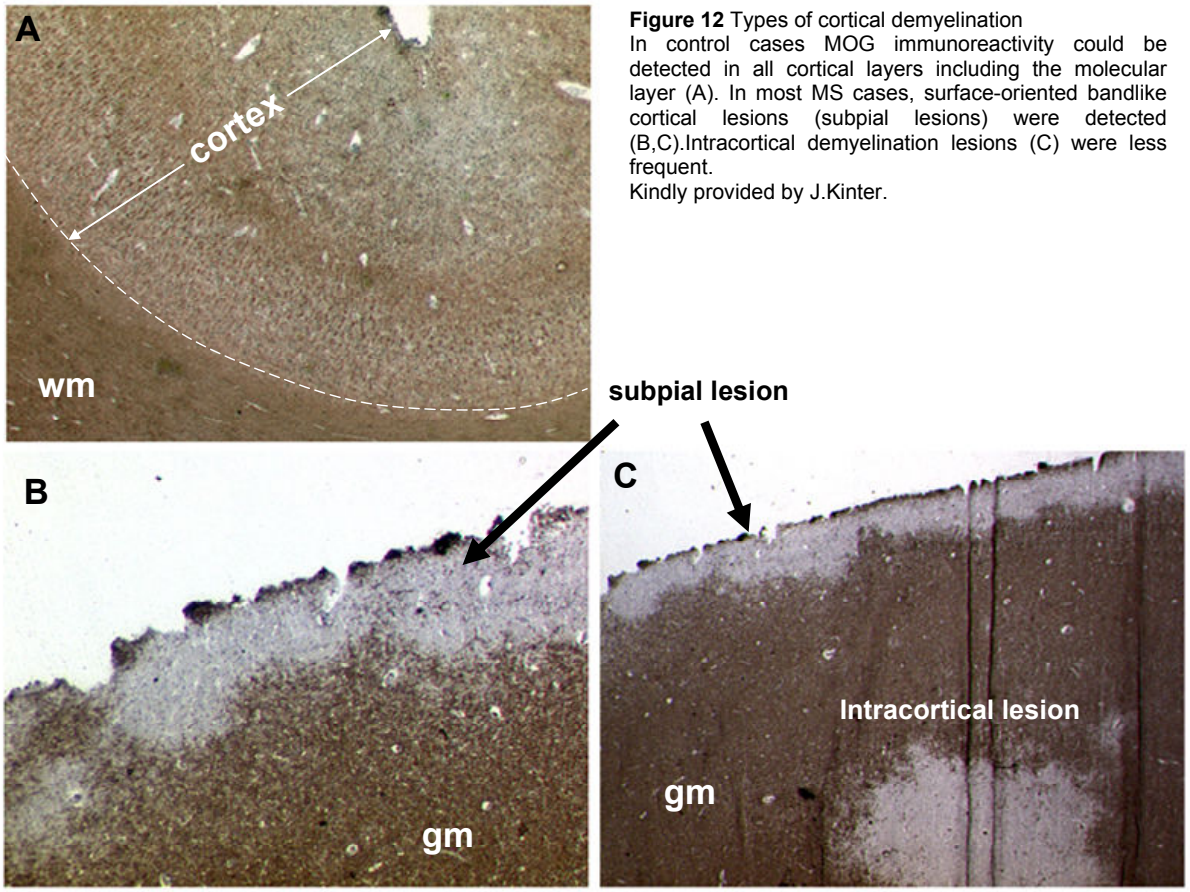

Figure 12 Types of cortical demyelination
In control cases MOG immunoreactivity could be detected in all cortical layers including the molecular layer (A). In most MS cases, surface-oriented bandlike cortical lesions (subpial lesions) were detected (B,C).Intracortical demyelination lesions (C) were less frequent.
Kindly provided by J.Kinter.

Origin or cause of MS

As the cause of MS is still not known, speculations and hypotheses were developed and published. Most serious hypotheses were developed by the study of animal models mimicking certain aspect of the disease. Other speculations evolved by correlating prevalence of MS with other factors, such as e.g. the usage of amalgam fillings. However, except for the two below presented hypotheses, no other hypotheses are really considered by the majority of the scientific community.

MS as an autoimmune disease

Currently, the autoimmune hypothesis is the most generally accepted among MS researchers. This view is based on the cellular composition of brain and CSF infiltrating cells as well as data from EAE, one of the major MS animal model (Sospedra and Martin, 2005; Zamvil and Steinman, 1990). In this concept, MS is a CD4+ T helper cell type 1 mediated autoimmune disease (Hafler, 2004; Martin et al., 1992). This then leads to a secondary activation of macrophages and microglia. Supporting this view, several self antigens correlated with MS have been identified, such as MBP, PLP and MOG (Schmidt et al., 1997; Steinman, 1996). In the EAE model, injection of these and other myelin components into susceptible animals leads to a CD4+ mediated autoimmune disease, sharing similarities with MS (Martin et al., 1992; Zamvil and Steinman, 1990). Furthermore, the disease can be adoptively transferred by encephalitogenic CD4+ T cells into naive animals (Martin et al., 1992; Pettinelli and McFarlin, 1981; Zamvil and Steinman, 1990). A major role of CD4+ T cells in MS is further supported by the fact that HLA class II molecules represent the strongest genetic risk factor for MS (Sospedra and Martin, 2005). Nevertheless, the presence of autoreactive T cells does not fully explain autoimmunity in MS, as these cells were also found in healthy individuals (Steinman et al., 1995; Wucherpfennig et al., 1994).

It is suggested that autoimmunity in MS is triggered by superantigens or molecular mimicry (Marrack and Kappler, 1994; Wucherpfennig and Strominger, 1995a). Superantigens might unspecifically stimulate T cells leading to an autoimmune reaction. It has been shown that such antigens can induce relapses and/or stimulate MBP-specific T cells (Brocke et al., 1993). Alternatively, structural similarities of epitopes from pathogens and epitopes of human tissues lead to the activation of

autoaggressive T cells resulting in their migration into the CNS (Brocke et al., 1993). As viruses such as measles, influenza viruses and adenoviruses have homologies with MBP, this implicated their involvement in MS pathogenesis (Wucherpfennig and Strominger, 1995). Nevertheless, none of these hypothesis were confirmed convincingly.

MS as a virus-induced disease

The involvement of a virus in the pathogenesis of MS is debated since 1884 (Marie, 1884). Viral infections are logical candidates as triggers of MS, as it was shown that the prevalence rate depend to some extent on environmental factors. Furthermore, it has been shown that MS relapses often follow viral infections (Sibley et al., 1985). The phenomenon of „MS epidemics“ as e.g. the one on the Faroe Islands further points to an infectious agent (Kurtzke et al., 1982; Kurtzke and Hyllested, 1975; Sospedra and Martin, 2005). Another evidence for infectious triggers origins from EAE of transgenic mice expressing a TCR specific for an encephalitogenic peptide of MBP. If housed under nonpathogen-free conditions, almost 100% of these mice develop EAE whereas if housed under pathogen-free conditions no disease occurred (Goverman et al., 1993). Furthermore, the TMEV animal model is an example of a virus-induced demyelinating disease and also used as a model for MS (Sospedra and Martin, 2005). In this context, the predominant presence of CD8+ T-cells in MS plaques is suggestive of a viral infection, as activation of CD8+ T-cells is mediated by MHC class I molecules normally responsible for viral antigen presentation (Morrison et al., 1986).

Viruses that are pathogenic in humans, such as HHV-6 and EBV, are suitable candidates and have been widely studied in MS (Sospedra and Martin, 2005). The detection of HHV-6 in oligodendrocytes in MS plaque tissue suggested a role of HHV-6 in MS (Challoner et al., 1995). Nevertheless, HHV-6 was also found in oligodendrocytes of normal brains. Furthermore, HHV-6 DNA and HHV-6 IgG and IgM antibodies were found in serum and CSF of MS patients, which data are, however, controversial (Sospedra and Martin, 2005). A linkage of EBV with MS is suggested by an elevation of Anti-EBV antibodies in patients with MS. A recent finding of B cell follicles in the cerebral meninges of some MS cases as major site of EBV persistence further supported the involvement of EBV in MS pathogenesis

(Serafini et al., 2007). To date, numerous reports claimed the involvement of viruses as MS triggers, but none of these observations withstood scrutiny so far (Soldan and Jacobson, 2001; Sospedra and Martin, 2005).

Animal models for MS

Currently, several models are used to investigate particular aspects observed in MS. Although all these models mimic to some extent pathological features of MS, none of it mimics them all. Two main models are used in the context of modelling pathological features of MS. The most widely used model is experimental autoimmune encephalomyelitis (EAE), followed by the Theiler's virus induced encephalomyelitis model. These two models led to a certain extent to the two hypotheses of MS origin (see page 40, 41). Additionally, there are also other models mimicking aspects of MS such as e.g. toxin-induced demyelination models or transgenic mice.
In this study, we have chosen to use a specific variant of MOG-induced EAE, as this model mimics most closely the clinical disease course of MS and furthermore shows a extended demyelination. However, the EAE models for MS has its potential as well as its limitations.

Experimental autoimmune encephalomyelitis

A common animal model used to study possible pathological mechanisms of MS is experimental autoimmune encephalomyelitis (EAE). Neurological complications due to rabies vaccination, first suggested autoimmunity targeted against nervous tissue elements can induce brain inflammation (Remlinger, 1928). This was then proven in experiments showing an induction of inflammatory demyelinating lesions after active immunization with brain tissue (Rivers, 1933). Since the initial experiments by Rivers, several different models of EAE were developed, which differ in the immunological reaction, inflammatory processes and the neuropathophysiology in the CNS. The various models differ in the choice of species, strain, antigen, and immunization protocol that are used. There exist models for non-human primates like marmosets and rhesus monkeys, as well as for rodents like guinea pigs, rats, and mice. Each model shares similarities to MS but also differs in some aspects from these (Table 1). Furthermore, transgenic mice have been developed which spontaneously develop autoimmune encephalomyelitis (for review see Bettelli, 2007; Krishnamoorthy et al., 2007).

Model	Similarities to human disease	Differences from human disease	Further comments
Lewis rat Active EAE (CNS myelin, MBP, MOG, PLP)	T-cell inflammation and weak antibody response	Monophasic, little demyelination	Reliable model, commonly used for therapy studies. With guinea-pig MBP little demyelination
Adoptive-transfer EAE (MBP, S-100, MOG, GFAP)	Marked T-cell inflammation. Topography of lesions	Monophasic, little demyelination	Homogeneous course, rapid onset. Differential recruitment of T cells/macrophages depending on autoantigen
Active EAE or AT-EAE + co-transfer of anti-MOG antibodies	T-cell inflammation and demyelination	Only transient demyelination	Basic evidence for role of antibodies in demyelination
Congenic Lewis, DA, BN strains Active EAE (recombinant MOG aa 1–125)	Relapsing–remitting disorders, may completely mimic histopathology of multiple sclerosis and subtypes	No spontaneous disease	Chronic disease course, affection of the optic nerve, also axonal damage similar to multiple sclerosis
Murine EAE (SJL, C57BL/6, PL/J, Biozzi ABH) Active EAE (MBP, MOG, PLP and peptides)	Relapsing–remitting (SJL, Biozzi) and chronic-progressive (C57BL/6) disease courses with demyelination and axonal damage	No spontaneous disease	Pertussis (toxin) required for many strains, whilst it is often not needed for SJL and some Biozzi EAE models. Higher variability of disease incidence and course, often cytotoxic demyelination in C57BL/6. With rat MBP inflammatory vasculitis with little demyelination
Murine EAE in transgenic mice or knockout mice (mostly C57BL/6 background)	Specifically addresses role of defined immune molecules/neurotrophic cytokines/ neuroanatomical tracts	Most results obtained with artificial permanent transgenic or knockouts	Extensive backcrossing (>10 times) on C57BL/6 background required. Future work with conditional (cre/loxP) or inducible (e.g. Tet-on) mutants

Table 1 Commonly used rodent EAE models. Copied form Gold et al., 2000.

Also, transgenic mice which overexpress TNF-a were shown to cause myelin damage and apoptosis of oligodendrocytes and their precursors (Probert et al., 1995).

Active EAE

The active induction of EAE is most commonly used for modelling MS (for review see Gold et al., 2006). Active immunization of susceptible animals with CNS tissue or with purified components of CNS myelin, for instance MBP, PLP or MOG results in a high incidence of disease with more or less reproducible clinical course (Gold et al., 2000; Gold et al., 2006). After the sensitization with the antigen, first clinical signs of the disease are generally observed within 9-14 days. However, this depends strongly on the species and mode of sensitization (Gold et al., 2000). Clinical signs reported depend on the position of the active inflammatory infiltrates. Due to a predominant spinal cord infiltration in EAE, weakness of the tail and paraplegia are most commonly observed. Generally, induction of EAE leads to a strong T-cell mediated inflammatory reaction, which, according to the model used, can be mediated by CD4+ or CD8+ T-cells. This inflammatory reaction leads to some extent to

demyelination, axonal conduction block and axonal degeneration and. In some models, inflammation and demyelination is potentiated by the presence of autoantibodies. Recently, it was reported that the induction of EAE in marmosets as well as the MOG-induced EAE in rats clearly reflect the immunopathology of pattern II multiple sclerosis lesion (de Graaf et al., 2008; Merkler et al., 2006). In contrast, MOG-induced EAE in C57/Bl6 mice is thought to reflect pattern I MS lesions. Altogether, the variety of possible EAE pathomechanisms, achieved by different immunization protocols, made active EAE an ideal model for MS (Gold et al., 2006).

Adoptive-transfer EAE

In AT-EAE, *in vitro* propagated, sensitized T-cell lines are injected intravenously into a susceptible recipient. Experiments with AT-EAE conclusively proved for the first time that EAE is induced by an autoimmune reaction. Due to the clarity of the disease mechanism of AT-EAE, this model has become a major experimental tool for investigating T-cell function and regulation in neuroinflammatory and autoimmune disease (Gold et al., 2000). Furthermore, the importance of CD8+ T-cells in autoimmune reactions were demonstrated, as adoptive transfer of CD8+ T-cell were shown to induce severe EAE (Sun et al., 2001). Nevertheless, in the context of MS, AT-EAE shows critical limitations such as the monophasic disease course and little demyelination.

Limitations and potential of EAE as a model for MS

Limitations of EAE

The view of MS being a T-cell mediated autoimmune disease is derived primarily from EAE. Nevertheless, one must be aware that EAE is not MS. The most obvious difference between MS and EAE is that MS develops spontaneously whereas EAE has to be actively induced. Currently, transgenic animals are developed which suffer from spontaneous EAE (Bettelli et al., 2003; Goverman et al., 1993; Waldner et al., 2000). Furthermore, in most EAE models, a strong immune adjuvant is required to induce the disease whereas in MS no comparable immunological trigger is known. A major difference between MS and EAE is the nature of the immune response. Most EAE models are dominated by a CD4+ T cell response whereas in MS lesions, CD8+

T cells seem to be the predominant T cell type present (Sriram and Steiner, 2005). Therefore, the relevance of cytotoxic CD8+ T cells in MS has been underestimated (Gold et al., 2000). Furthermore, an efficient and fast remyelination seen in EAE (Papadopoulos et al., 2006), was suggested to protect axons in inflammatory lesions and prevent the development of chronically demyelinated lesions as in MS. Also, currently no EAE model exists mimicking primary progressive MS.

Potential of EAE

Despite of the many differences between EAE and MS, this model has proven to be most useful to study certain aspects of MS. By using EAE models, T-cell mediated immune damage and inflammatory processes were intensively studied (Table 2). Although this T-cell response differs between EAE and MS, it led to the understanding of T-cell mediated immune damage of the CNS (Berger et al., 1997; Gold et al., 2006). Furthermore, macrophage recruitment into lesions was shown to correlate with disease severity, which was shown to apply for both, MS as well as EAE (Berger et al., 1997; Gold et al., 2006). Therefore, EAE might be a valuable model to study macrophage recruitment or activation during CNS autoimmunity. Although in most EAE models demyelination is rather limited, others have proven to be highly useful to study mechanisms of demyelination and remyelination. Growing evidence further suggests that EAE models might be used to investigate the pathomechanisms of different MS lesion patterns (Merkler et al., 2006) as well as of different MS subsets (Krishnamoorthy et al., 2006). Altogether, several aspects of

Feature of multiple sclerosis lesion	Most suitable EAE Model	References
$CD4^+$ T-cell-mediated inflammation	AT-EAE in Lewis rat	Ben-Nun *et al.* (1981)
$CD8^+$ T-cell-mediated inflammation	Passive transfer of $CD8^+$ T cells in mice	Huseby *et al.* (2001), Cabarrocas *et al.* (2003), Sun *et al.* (2001)
T-cell- and macrophage-mediated demyelination	Chronic EAE in C57BL/6 mice induced by MOG peptide 35-55	Mendel *et al.* (1995)
T-cell- and antibody-mediated demyelination	Chronic EAE in DA and BN rats or in marmosets sensitized with recombinant MOG 1-125	Storch *et al.* (1998), T'Hart *et al.* (2004)
Inflammation-induced hypoxia-like tissue injury	LPS injection into white matter	Felts *et al.* (2005)
T-cell- and macrophage-mediated demyelination with increased oligodendrocyte susceptibility	Chronic EAE in CNTF-deficient mice sensitized with MOG 35-55	Linker *et al.* (2002)
Axonal injury in demyelinated plaques	All chronic EAE models in mice and rats	Kornek *et al.* (2000)
Cortical demyelination	Recombinant MOG 1–125 induced EAE in marmosets or in LEW 1.W and LEW 1.AR1 rats	Pomeroy *et al.* (2005), T. M. Storch *et al.*, unpublished data
Global diffuse axonal injury in the normal appearing white matter	So far no model available	

Table 2 Pathological features of multiple sclerosis and the most suitable EAE models. Copied from Gold et al., 2006

potential pathogenic mechanisms of MS can be mimicked in EAE (Table 2).

Theiler's virus-induced encephalomyelitis

Another model which mimics certain aspect of MS is the Theiler's murine encephalomyelitis. In this model, infection with TMEV leads to demyelination and oligodendrocyte apoptosis (Pender et al., 1991; Tsunoda and Fujinami, 1996). During the acute phase of TMEV-induced demyelination, the virus is eliminated from the gray matter of the CNS, whereas in the chronic phase, TMEV persistently infects glial cells and/or macrophages/microglia in the white matter. The recruitment of T-cells and macrophages during the chronic phase subsequently leads to inflammation and demyelination (Tsunoda and Fujinami, 1996). Apart from a anti-viral immune response, epitope spreading was detected in TMEV-induced demyelination, where virus-induced damage led to activation of autoreactive T cells specific for myelin protein epitopes (Miller et al., 1997). As in EAE, it is suggested that CD4+ T cells are directly involved in the pathogenesis of the disease (Dal Canto et al., 1996). Furthermore, apart from dose and strain of the virus, susceptibility for TMEV-induced demyelination depends, as in EAE, on strain, gender and age of the animals (Dal Canto et al., 1996).

Cuprizone-induced demyelinating models

Another experimental models mimicking in particular demyelination, are toxin-induced demyelinating models such as e.g. cuprizone-induced demyelination. By feeding mice with the copper chelator cuprizone, a massive demyelination can be reproducibly induced in large areas of mouse brain. Demyelination is induced by oligodendrocyte apoptosis, which is then followed by a phagocytosis of myelin by microglia. These models are especially valuable for studying manipulations which may accelerate or repress the process of demyelination and or remyelination.

Impacts on therapy of MS

Animal models for MS are further used for developing therapeutic strategies. For that, EAE is the predominantly used model. From the current therapies planned for phase II and III trials in MS, most were first examined in EAE. But, although EAE is a useful model to study certain aspects of MS, transferring promising therapies beneficial in EAE is difficult. Many agents have been found to successfully ameliorate EAE (Table 3), but many failed finally in subsequent human clinical trials (Sriram and Steiner, 2005). Whereas some showed no beneficial effects in MS, others did, but had strong side effects in addition. Few cases showed even worsening effects (Sriram and Steiner, 2005). Nevertheless, six medications have received approval from the US Food and Drug Administration for treatment of MS. Three of them, glatiramer acetate, mitoxantrone and natalizumab were developed after showing promise in EAE (Steinman and Zamvil, 2006). Additionally, several new medications first developed in EAE are currently being investigated in clinical trials.

Antibodies to T-cell surface antigens	CD3,CD4,T-cellreceptor,CD2,IL-2R,IL-2R,CD24,CD40LCD28
Antibodies directed to antigen-presenting cells	MHC class II antigens, CD40, B7-1 and B7-2, Fc receptor blockade
Antibodies to NK cells	Anti–NK cell antibody, α-Gal ceramide
Antibodies to adhesion molecules	VLA-4, ICAM-1, LFA-1
Antibodies to cytokines	IL-2, IL-6, IL-12, IL-15, TNF-α, IL-1, IL-23
Antibodies to chemokines	Anti–MIP-1—α Rantes
Antiinflammatory cytokines	IL-4, IL-10, TGF-β, IFN-β, IFN-α, ?γ-IFN
Antagonists of signaling molecules	Tyrphostins (inhibitors of JAK-Stat activation), lysofyline, inhibitors of MAP kinase pathway, inhibitors of NF-κB activation, Inhibitors of iNOS activation, amsamycin, cholera toxin, AMPA antagonists, glutamate receptor antagonists, IL-1 receptor antagonists
Activation of nuclear receptors	PPAR-γ retinoic acid
Hormones	Estrogen, progesterone, vitamin D, DHEA, leptin antagonists
Antibiotics	Minocycline, rapamycin
Antimetabolites and immunosuppressants	FK-506, cyclosporin, dyspergualin, corticosteroids, azathioprine, cyclophosphamide, mycophenolate, bone marrow transplantation
Gene therapies	Targeted delivery of IL-4, IL-10
Inhibitors of enzymes	HOMG coreductase inhibitors (statins), COX-2 inhibitors
Peptides/proteins	Oral myelin proteins, glatiramer acetate, myelin peptides (iv)
Food supplements	Essential fatty acid, omega 3 fatty acid, curcumin, padma-28, fish oil
Small organic molecules	Linomide, silica, sodium phenyl acetate, copper chelators (*N*-acetylcysteine aminde), laquinamod, piperazylbutroxide, uric acid, dermatan sulphate, amionoguanidine, cuprizone, roliprim, H-2 receptor antagonists, indoleamine 2-3 deoxygenase, FTY-270, pentoxyfyline
Miscellaneous	Incomplete Fruend's adjuvant, BCG vaccination, Helminthic infections

AMPA = alpha-amino hydroxy methyl propionic acid; BCG = Bacille Calmette Guerin; DHEA = dehydro epi androsterone; EAE = experimental allergic encephalomyelitis; HMG = hydroxymethyl glutaryl coreductase; IFN = interferon; IL = interleukin; iNOS = inducible nitric oxide synthase; MAP = microtubule-associated protein; MHC = major histocompatibity complex; MIP = macrophage inflammatory protein; TGF-β = transforming growth factor-β.

Table 3 Agents Successful in Treating EAE. Copied from Sriram and Steiner, 2005.

Oligodendrocytes and MS

Oligodendrocytes are the cells of the CNS forming myelin, a unit of membranes wrapped around axons allowing fast saltatory conduction. In MS, myelin appears to be the primary target of the inflammatory reaction. It appears that a T-cell mediated immune reaction against myelin antigens is responsible for the induction of inflammation in MS (Lassmann, 2006). Therefore, the myelin producing oligodendrocytes are the major target cell of the immune reaction in MS.

Mechanisms mediating oligodendrocyte damage

Oligodendrocytes can be damaged by various mechanisms (for review see Zeis and Schaeren-Wiemers, 2008). These “attacks” are occurring either direct via lysis or indirect via toxic mediators or via an imbalance of the surrounding environment. Immune cells such as CD8+ cytotoxic T-cells have been shown to be directly cytotoxic to cells presenting their specific antigen (Parkin and Cohen, 2001), which oligodendrocytes are capable to. In contrast, CD4+ T helper cells are capable to induce oligodendrocyte damage by secreting cytokines, such as TNF-α, and promoting activation of nearby macrophages and microglia. Activated macrophages and microglia were shown to have incorporated myelin products, and express a large variety of different for oligodendrocytes deleterious compounds, such as TNF-α, reactive oxygen species (ROS), reactive nitrogen species (RNS) and Fas-ligand (FasL). TNF-α is a potent cytotoxic molecule capable of inducing oligodendrocyte cell death (D'Souza et al., 1996b; Jurewicz et al., 2005; Selmaj and Raine, 1988). Furthermore, the production of ROS and RNS by activated macrophages and microglia can lead to various types of damage such as lipid peroxidation, tyrosine nitrosylation and DNA strand breaks (van der Veen and Roberts, 1999; Willenborg et al., 1999; Zhang et al., 1994). High expression of inducible nitric oxide synthase (iNOS) as well as neuronal nitric oxide synthase (nNOS) has been reported in activated macrophages and microglia within active lesions in MS (De Groot et al., 1997; Hill et al., 2004), and RNS mediated damage in oligodendrocytes has also been demonstrated (Jack et al., 2007; Li et al., 2005; Merrill et al., 1993).

Oxidative stress and oligodendrocytes

Under normal conditions reactive oxygen and nitrogen species (ROS, RNS) are routinely produced in low concentrations, posing little threat to oligodendrocytes as they possess defense and repair mechanisms. However, during the inflammation process of MS, a high expression of inducible nitric oxide synthase (iNOS) as well as neuronal nitric oxide synthase (nNOS) has been reported (De Groot et al., 1997; Hill et al., 2004). Furthermore, RNS-mediated damage in oligodendrocytes has been demonstrated (Jack et al., 2007; Li et al., 2005; Merrill et al., 1993).

As oligodendrocytes are vulnerable to NO-mediated damage (Smith et al., 1999; Smith and Lassmann, 2002), activation of mechanisms protecting oligodendrocytes from oxidative stress-induced damage would be highly beneficial. A recent study of subcortical NAWM from MS cases has shown the upregulation of several genes involved in ischemic preconditioning (Graumann et al., 2003). In particular, HIF-1α has been shown to be an important regulator of hypoxic preconditioning (Bergeron et al., 2000; Bernaudin et al., 2002; Sharp et al., 2001) and is activated by hypoxia, growth factors, NO and others (for review see Brune and Zhou, 2007; Semenza, 2002). HIF-1α and some of its downstream genes were shown to be elevated in MS NAWM (Graumann et al., 2003), and *in-situ* hybridization experiments of MS NAWM (Zeis et al., 2008) as well as examinations of Balo's concentric sclerosis identified oligodendrocytes expressing this transcription factor (Stadelmann et al., 2005), suggesting that oligodendrocytes mount ischemic protective mechanisms during the disease course. Furthermore, oligodendrocytes were also shown to express heat-shock proteins 70 (HSP70) (Stadelmann et al., 2005) as well as HSP32 (Stahnke et al., 2007). In the case of HSP70, a protective role has been shown in brain ischemia (for review see Christians et al., 2002), whereas HSP32 was shown to exert a protective role against oxidative stress in an oligodendroglial cell line (Stahnke et al., 2007).

Interestingly, sub-lethal doses of inflammatory cytokines such as IFN-γ and TNF-α were reported to induce ischemic protective mechanisms in target cells. The induction of HSP70 in oligodendrocytes was shown *in-vitro* by treatment of oligodendrocyte cultures with a mix of cytokines (D'Souza et al., 1994). Further, treatment of oligodendrocyte cultures with IFN-γ led to an increase in the expression of genes involved in protection against oxidative stress (Balabanov et al., 2007). In

line, treatment of mice with IFN-γ before onset of EAE led to an amelioration of the disease through activating the integrated stress response (Lin et al., 2007). Altogether, oligodendrocytes induce and express endogenous protective mechanisms possibly influencing lesion formation in MS.

Oligodendrocytes and growth factors

Changes in growth factors as well as growth factor receptors expression were demonstrated in MS. Several growth factors such as nerve growth factor (NGF), insulin-like growth factor (IGF) and transforming growth factor β (TGF-β) were reported to be expressed by oligodendrocytes (for review see Du and Dreyfus, 2002). By expression of these factors, oligodendrocytes might influence the survival and/or function of neighboring cells. NGF can bind to the tyrosine kinase receptor A (TrkA) as well as to the low-affinity nerve growth factor receptor ($p75^{NTR}$). By binding to TrkA, NGF promotes cell survival whereas binding to $p75^{NTR}$ under some circumstances might also modulate susceptibility to programmed cell death or apoptosis (Casaccia-Bonnefil et al., 1999; Yoon et al., 1998). In EAE, expression of TrkA was detected on neurons, astrocytes and oligodendrocytes (Oderfeld-Nowak et al., 2003; Oderfeld-Nowak et al., 2001), whereas $p75^{NTR}$ was detected on neurons, microglia, astrocytes and oligodendrocytes (Nataf et al., 1998; Villoslada et al., 2000). In EAE, NGF was shown to have beneficial effects, as NGF-deprived rats display more severe neurological deficits during disease course. Further, treatment of marmoset monkeys with NGF prevented the full development of EAE lesions and delayed the onset of clinical EAE (Micera et al., 2000; Villoslada et al., 2000). Another growth factor expressed by oligodendrocytes is IGF-1, which was reported to ameliorate TNF-α induced demyelination in transgenic mice (Ye et al., 2007). Furthermore, IGF-1 was also reported to reduce demyelination in EAE (Liu et al., 1995), although this beneficial effect is still under debate (Cannella et al., 2000). The expression of TGF-β by oligodendrocytes was also reported, which is discussed in the next chapter. Altogether, by expressing several growth factors, oligodendrocytes in MS might influence the function and survival of themselves, but also of the nearby cells, possibly enhancing remyelination and hindering lesion formation.

Immune modulating ability of oligodendrocytes might have an impact on MS

In vitro experiments suggested, that upon stimulation by Interferon-γ, oligodendrocytes express protective genes against oxidative stress as well as a number of chemokines, including CXCL10, CCL2, CCL3 and CCL5 (Fig. 4) (Balabanov et al., 2007). CXCL10, CCL2 and CCL5 were also found to be upregulated in MS NAWM (Graumann et al., 2003). Furthermore, mice with oligodendrocytes with suppressed responsiveness to IFN-γ showed higher oligodendrocyte apoptosis in EAE as well as an accelerated disease onset, but milder perivascular inflammation and minimal parenchymal infiltration and demyelination (Balabanov et al., 2007). This effect of IFN-γ on oligodendrocytes demonstrates that oligodendrocytes are capable to react on external immune challenges by induction of protective mechanisms, and that they possibly modulate inflammatory responses. The expression of different cytokine receptors on oligodendrocytes in active and silent lesions may further suggest an active role in innate immunity of the CNS (Cannella and Raine, 2004). Oligodendrocytes were also shown to express TGF-β *in vitro* (da Cunha et al., 1993; McKinnon et al., 1993), which can suppress immune and inflammatory responses (for review see Pratt and McPherson, 1997), and might promote myelination and remyelination (Setzu et al., 2006).

The expression of cytokine receptors and the possibility of chemokine expression might point to oligodendrocytes playing a role in the innate immunity by actively modulating their environment and interacting with cells of the immune system. Therefore, oligodendrocytes might be capable to influence lesion formation in MS.

Aim of the work

Multiple sclerosis is a chronic, inflammatory and demyelinating disease of the CNS. Although diffuse inflammatory damage as well as progressive axonal injury has been shown in the chronic phase of the disease, little is known about the molecular mechanisms underlying these pathological processes. In previous studies, accumulating evidence suggested that the morphologically normal appearing white matter in MS is far from being normal. A study from our lab revealed an upregulation of a number of functionally related genes involved in endogenous neuroprotection, oxidative stress response and ischemic preconditioning (Graumann et al., 2003). Furthermore, increasing evidence point to an induction of inflammatory mechanisms in MS NAWM. Altogether, this raises several questions:

1. Is an immune response mounted in the NAWM ? And if so, which cells mount this response ?
2. Are these changes observed in late disease course NAWM already induced in NAWM from early disease course ?
3. Are these changes of the MS NAWM also present in an animal model of MS ?

In order to investigate a potential immune response of the NAWM, a differential gene expression analysis as well as immunohistochemistry experiments were performed. Furthermore, a discrimination between early and late pathogenic mechanisms of the MS NAWM was achieved by analyzing a biopsy taken from a patient during early disease course. As tissue derived from MS patients is limited and predominantly origins from older patients with a long-time disease course, a differential gene expression analysis of NAWM of MOG-induced EAE was investigated. The presence of similar molecular and cellular mechanisms in EAE NAWM as in MS NAWM would allow to study these mechanisms in more detail.

Results

The following section consists of work submitted to or published in scientific journals as well as a manuscript being prepared for submission.

Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection

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Abstract

Multiple Sclerosis is a chronic inflammatory disease of the central nervous system (CNS). Although progressive axonal injury and diffuse inflammatory damage has been shown in the chronic phase of the disease, little is known about the molecular mechanisms underlying these pathological processes. In order to identify these mechanisms, we have studied the gene expression profile in non-lesion containing tissue, the so called normal appearing white matter (NAWM). We performed differential gene expression analysis and quantitative RT-PCR on subcortical white matter from 11 MS and 8 control cases. Differentially expressed genes were further analyzed in detail by *in-situ* hybridization and immunofluorescence studies. We show that genes known to be involved in anti-inflammatory and protective mechanisms such as STAT6, JAK1, IL-4R, IL-10, Chromogranin C, and Hif-1α are consistently upregulated in the MS NAWM. On the other hand, genes involved in pro-inflammatory mechanisms, such as STAT4, IL-1β and MCSF, were also upregulated in MS NAWM but less regularly. Immunofluorescence colocalization analysis revealed expression of STAT6, JAK1, IL-4R and IL-13R mainly in oligodendrocytes, whereas STAT4 expression was detected predominantly in microglia. In line with these data, *in-situ* hybridization analysis showed an increased expression in MS NAWM of HIF-1α in oligodendrocytes and HLA-DRα in microglia cells. The consistency of the expression levels of STAT6, JAK1, JAK3 and IL-4R between the MS cases suggests an overall activation of the STAT6 signalling pathway in oligodendrocytes, whereas the expression of STAT4 and HLA-DRα indicates the activation of pro-inflammatory pathways in microglia. The upregulation of genes involved in anti-inflammatory mechanisms driven by oligodendrocytes may protect the CNS environment and thus limit lesion formation, whereas the activation of pro-inflammatory mechanisms in microglia may favour disease progression. Altogether, our data suggests an endogenous inflammatory reaction throughout the whole white matter of MS brain, in which oligodendrocytes actively participate. This reaction might further influence and to some extend facilitate lesion formation.

Keywords: Multiple Sclerosis, Inflammation, Cytokines, Chemokines, Neuroprotection

Introduction

Multiple Sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Studies of the histopathology of the demyelinated lesions characteristic of MS (Lucchinetti et al., 2000; Raine and Scheinberg, 1988) have revealed a great deal of heterogeneity at the cellular and molecular level, which might partially reflect the diversity of the clinical disease course. A major effort in the study of the pathogenesis of MS has been to understand the molecular mechanisms of lesion formation (Lock et al., 2002; Lucchinetti et al., 2000), and in particular the role of various immune-modulating components in the different types of lesions (Lassmann and Ransohoff, 2004).

Pathological similarities to the animal model for Th1 mediated brain inflammation, experimental autoimmune encephalomyelitis (EAE), have suggested that MS is an autoimmune disorder caused by myelin-specific CD4+ T cells of the Th1 (Lassmann and Ransohoff, 2004; Sospedra and Martin, 2005) and/or Th17 type (Gutcher et al., 2006; Weaver et al., 2006). In contrast, other studies have highlighted a major role for CD8+ T cells in EAE and MS (Babbe et al., 2000; Booss et al., 1983; Neumann et al., 2002; Sun et al., 2001). Furthermore, Th2 helper-cell mediated mechanisms have also been suggested to contribute to inflammation in a subset of patients with MS (Lafaille et al., 1997). Cytokines are critical components in immune regulation and signalling, and thus, play a major role in determining the actions of different immune cells. Although still debated, anti-inflammatory cytokines of the Th2 type (e.g. Il-10, IL-4) are thought to be mostly beneficial and have been associated with remission and recovery from disease (Cannella and Raine, 2004; Sospedra and Martin, 2005). In contrast, cytokines of the Th1 and Th17 type (e.g. Il-1, IL-6, IL-12, IL-17, IFN-γ and TNF-β) are thought to be predominantly pro-inflammatory and are likely to play a role in the pathogenesis of MS. In regard to CNS-Immune system communication cytokines are highly relevant as well. As an example CNS targeted expression of IL-3 is sufficient to promote recruitment and activation of macrophage/microglial cells in white matter regions of the brain leading to subsequent demyelination (Chiang et al., 1996), highlighting the crucial role of CNS specific modulation of the immune system.

There is accumulating evidence for the deregulation of various aspects of the immune response in the brain parenchyma as well as in the periphery in MS. An important signal transduction pathway involved in immune regulation is the JAK/STAT signalling pathway, which consists of the protein families of Janus tyrosine kinases (JAK) and the signal transducers and activators of transcription (STATs). Other members of the JAK/STAT pathway are the protein tyrosine phosphatase (SH-PTP2) and the growth-factor-receptor-bound protein 2 (GRB2). GRB2 and SHP2 can form a complex together with the IL-6R (gp130) and are known to link the JAK/STAT pathway to the MAPK pathway (Schiemann et al., 1997), which was shown recently to be upregulated in MS (Graumann et al., 2003). Most interestingly in the context of MS, the JAK/STAT signalling pathway plays a critical role in the regulation of the sensitivity to cytokines and in their expression. It is known that the JAK/STAT signalling pathway is a sensitive switch playing a major role in the Th-polarization of the immune system. Members of the STAT family like STAT1, STAT3, STAT4 and STAT6 are thought to be critically involved in the polarization of Th1, Th17 or Th2 cells (Harrington et al., 2006; Kaplan et al., 1996; Takeda et al., 1996). In EAE, it has been shown that STAT4 deficient mice are resistant to the induction of MOG-induced EAE, whereas STAT6 deficient mice show a more severe clinical course (Chitnis et al., 2001). These findings suggest that STAT4 may be involved in the promotion of inflammatory mechanisms, whereas STAT6 may be important in their limitation. Therefore, we concentrated our work on STAT4, STAT6 and their particular downstream genes as markers for either pro- or anti-inflammatory mechanisms in resident cells of the brain.

In addition to the well characterized inflammatory white matter lesions, studies by magnetic transfer imaging (MTI) have suggested that the normal appearing white matter (NAWM) of the majority of MS patients has significant abnormalities, such as blood-brain-barrier changes, axonal injury and to some extent astro- and microgliosis (Aboul-Enein et al., 2003; Fu et al., 1998; Silver et al., 2001). In line with this, our recent microarray analysis of MS NAWM revealed the upregulation of a number of functionally related genes involved in endogenous neuroprotection, as well as in the maintenance of cellular homeostasis (Graumann et al., 2003). In addition to these protective responses, the NAWM also mounts an immune modulating response, which is the subject of this study.

Material and Methods

Tissue collection

MS and control tissue samples were supplied by the UK Multiple Sclerosis Tissue Bank (UK Multicentre Research Ethics Committee, MREC/02/2/39), funded by the Multiple Sclerosis Society of Great Britain and Northern Ireland (registered charity 207495). Additional tissue samples were collected at the Department of Pathology, University Hospital Basel (Ethics Committee of the University Hospital Basel). Tissue samples representing normal control white matter and normal appearing MS white matter were collected and analyzed as described previously (Graumann et al., 2003). All brains were routinely screened by a neuropathologist to confirm diagnosis of MS and to exclude confounding pathologies such as amyloid deposition, metastasis and ischemic lesions.

Table 1 - Summary and characterization of patients

	Patient ID	Age (yr)	Sex	MS Type	Disease Duration (yr)	Postmortem Interval (hr)	Cause of Death	Array, LC
MS patients	MS1*	56	f	SP	31	12	Bilater basal pneumonia	I, II, III, LC
	MS2	58	f	PP	22	16	Peritonitis	I, LC
	MS3	76	f	SP	>14	18	Myocardial infarction	I, II, III, LC
	MS5*	58	f	PP	21	6	Bronchopneumonia	I, II, III, LC
	MS10	69	f	PP	31	11	Acute pyeionephritis	I, II, LC
	MS12	78	f	PP	31	9	Lung infection	I, LC
	MS14	56	f	PR	34	6	not known	LC
	MS18	78	f	SP	42	5	Bronchocarcinoma	I, LC
	MS20	66	f	SP	30	13	Aspiration pneumonia	I, LC
	MS25	54	f	SP	20	22	Bronchopneumonia	LC
	MS26	83	f	Silent	-	6	Myocardial infarction	I, II, III, LC
Controls	CLo3	85	f	-	-	9	not known	LC
	CLo6*	73	m	-	-	21	Heart attack	I, II, III; LC
	CLo7	77	m	-	-	26	Lung cancer	I, LC
	CLo8	64	f	-	-	18	Cardiac failure	I, II, LC
	CBS1	70	m	-	-	15	Myocardial infarct	I, II, III, LC
	CBS2	66	m	-	-	16	Bronchocarcinoma	I, II, III, LC
	CBS4	69	m	-	-	10	Myocardial infarct	I, LC
	CBS5	59	f	-	-	22	Acute pancreatitits	I, II, III, LC

Clinical and pathological information concerning the 11 MS and 8 control cases studied are shown. All MS and all control tissue samples except MS25 and CLo3 were used for microarray analysis. For MS1, MS5 and CLo6 (asterisk) two different tissue blocks were used for the microarray and qRT-PCR analysis. Abbreviations: SP=Secondary-progressive, PP=Primary-progressive, PR=Progressive-Relapsing, I=BD Atlas™ Human 1.2 Array I, II=BD Atlas™ Human 1.2 Array II, III=BD Atlas™ Human 1.2 Array III, LC=Lightcycler

Furthermore, NAWM tissues were screened for T-cell infiltration (CD3), activated microglia and macrophages (CD68), activated astrocytes (GFAP) and signs for demyelination and remyelination (PLP); tissues showing signs of lesion formation such as accumulation of CD3- or CD68-positive cells or abnormal myelin staining were excluded from this study. From the MS cases, five showed a secondary-progressive, four a primary-progressive, one a progressive-relapsing and one a silent disease course. The mean age of the control cases was 70.4 ± 10.7 years with a post-mortem delay time of 17.1 ± 5.9 hours. The mean age of the MS cases was 66.5 ± 10.7 years with a post-mortem delay time of 11.3 ± 5.6 hours (Table 1). From these tissue blocks provided (Fig.1A), about 20-40mg pure subcortical white matter tissue was isolated and used for RNA isolation (hatched area).

Antibodies

For histochemical analysis the following antibodies were used: anti-STAT4 (R&D Systems, Cat.Nr. PA-ST4, 1:250), anti-STAT6 (R&D Systems, Cat.Nr. AF2167, 1:250), anti-Olig2 (Chemicon, Cat.Nr. AB9610, 1:250), anti-JAK1 (Chemicon, Cat.Nr. MAB3700, 1:250), anti-hIL-4R (R&D Systems, Cat.Nr. MAB230), anti-IL13R (Abnova, Cat.Nr. H00003598-MO1), anti-CD68 (DAKO, Cat.Nr.M0814, 1:500), anti-GFAP (SIGMA, Cat.Nr. G-3893), anti-PLP (kindly provided by K.-A. Nave, Göttingen), anti-CD3 (Novocastra Labs, Cat.Nr.NCL-CD3-PS1), anti-MBP (Chemicon, Cat.Nr. MAB386), pan neurofilaments (rabbit pAb cocktail; Biomol, Cat.Nr.NA1297). The secondary antibodies used were: donkey-anti-mouse-biotin (1:500), donkey-anti-rabbit-Cy2 (1:500), donkey-anti-rabbit-Cy3 (1:500), donkey-anti-rabbit-Cy5 (1:500), donkey-anti-mouse-Cy2 (1:500), donkey-anti-mouse-Cy3 (1:500), donkey-anti-rat-Cy5 (1:500), donkey-anti-goat-Cy2 (1:500) and donkey-anti-goat-Cy3 (1:500) (all from Jackson ImmunoResearch).

Immunohistochemistry

All tissue samples were analyzed by immunohistochemistry. Cryostat sections (10μm) used for tissue characterization using anti-CD68, -PLP, -MOG and -CD3 antibodies were fixed for 10min in 10% formalin. Sections used for CD3 staining were then boiled in 10mM sodium citrate buffer, pH 6.0 for 30min. For inactivation of

endogenous peroxidase all sections were treated with 0.3% hydrogen peroxide and blocked with blocking buffer (1% normal donkey serum, 2% Fish skin gelatine, 0.15% Triton). After quenching, sections were incubated with primary antibodies overnight at 4°C. Secondary biotinylated antibodies were applied for 1 hour at room temperature followed by the ABC complex reagent (Vector Labs) for 1 hour. Color reaction was performed with 3-Amino-9-ethylcarbazole (Erne et al., 2002). Counterstaining was performed in haematoxylin for 1min followed by rinsing the slide in running tap water.
For fluorescent colocalization study using anti-STAT4, -STAT6, -MBP, -OLIG2, -JAK1, -Neurofilament, -GFAP, and -CD68 antibodies, cryostat sections (10μm) were air-dried for 20min and fixed for 10min in acetone at -20°C. For MBP/JAK1 colocalization immersed fixed tissue samples were used (tissue blocks were fixed in 4% paraformaldehyde in PBS for seven days). Sections were incubated with the primary antibodies at 4°C overnight. After washing with PBS, sections were incubated for 1 hour with cupric sulphate in ammonium acetate buffer (10mM $CuSO_4$, 50mM CH_3COONH_4, pH 5.0) in order to reduce autofluorescence (Schnell et al., 1999). Secondary antibodies were incubated for 1 hour at room temperature. Slides were mounted with Fluorosave and kept at 4°C.

Total RNA preparation

Total RNA isolation was performed by homogenization in guanidinium thiocyanate followed by a CsCl ultracentrifugation (Bothwell et al., 1990). Freshly isolated RNA was tested for integrity by ethidium-bromide gel electrophoresis and GFAP Northern blot analysis as shown before (Graumann et al., 2003).

Atlas™ cDNA Expression Array hybridization

The Clontech Atlas™ cDNA Expression Array 1.2 (I-III) contains 3'528 selected cDNA sequences arrayed on three different nylon membranes (1'176 per array). Array hybridization was performed according to Graumann et al., (2003). Array I was hybridized with 11 MS and 8 control samples, Array II with 7 MS and 5 control samples and Array III with 6 MS and 4 control samples (Table 1, 3). Quantification of differential hybridization signal intensities was achieved with the AtlasImage™ 2.0 software program. Data analysis was performed according to Graumann et al.,

(2003). Quantitative RT-PCR was performed to verify gene expression data received from the array experiments. As we described previously, qRT-PCR results revealed an even greater fold difference, indicating that differential expression patterns are rather under- than overestimated from the microarray analysis (Graumann et al., 2003). Therefore, median values showing fold changes above 1.5 are indicative to be upregulated in a particular MS case and median values below 0.66 to be downregulated.

Quantitative RT-PCR

Real-time RT-PCR was performed using the LightCycler system (Roche). Primer sequences were either provided by Clontech or designed from unique site over exon-intron junctions to prevent amplification of genomic DNA. Real-time RT-PCR was performed according to the manufacturer's protocol (Roche). RNA amounts were calculated with relative standard curves for all mRNAs of interest and 60s ribosomal protein L13A was used for normalization. Normalization was further evaluated by the 40s ribosomal protein S9. Primer sequences used for qRT-PCR are shown in Table 2.

Table 2 Primer Sequences used for qRT-PCR

Primer Name	Gene Acc.Nr.	Sequence
5' STAT4 3' STAT4	NM_003151.2	5' - TCCGAAGTGATTCAACAGAGCC - 3' 5' - TTCTTGGTGCGTCAGAGTTTATCC - 3'
5' STAT6 3' STAT6	NM_003153.3	5' - CACTGGAAGCAGGAAGAACT - 3' 5' - TCAAGCTGTGCAGAGACACT - 3'
5' SOCS3 3' SOCS3	NM_003955.3	5' - TGTGCCTCCTGACTATGTCT - 3' 5' - CCTGACTGGCCAATACTTAC - 3'
5' Aqp4 3' Aqp4	NM_001650.4	5' - TACTGGTGCCAGCATGAATC - 3' 5' - TTGTCCTCCACCTCCATGTA - 3'
5' HLA-DR 3' HLA-DR	K01171	5' - ATGGCCATAAGTGGAGTCC - 3' 5' - TTCACTGAGGTCAAGGATTG - 3'
5' nNOS 3' nNOS	NM_000620.1	5' - TGGAGGTGCTGGAGGAGTTC - 3' 5' - TGAGCCAGGAGGAGCATACG - 3'
5' MAG 3' MAG	NM_080600.1	5' - CCGCCGAAGACGGCGTCTATGC - 3' 5' - CTCTCGTAGATGACCGTGGACAGG - 3'
5' MOG 3' MOG	NM_002433.3	5' - CCTCCTCCTCCTCCAAGT - 3' 5' - CCATGCCTGTAGCGTTCTTC - 3'
5' PLP 3' PLP	NM_000533.3	5' - TTCTGTGGCTGTGGACATG - 3' 5' - GAGGCAGTTCCATAGATGAC - 3'
5' MBP 3' MBP	M30515	5' - ACCCGGCAAGAACTGCTCACTATGGCTC - 3' 5' - TGAGCCGATTTATAGTCGGACGCAC - 3'
5' GFAP 3' GFAP	J04569	5' - GACGAGATGGCCCGCCACTTGC - 3' 5' - CTCCACGGTCTTCACCACGATGTTC - 3'
5' NSE 3' NSE	NM_001975.2	5' - TGGTGTGCTGAGGTGTTAG - 3' 5' - CCTTATTAGCCAGGCGTGA - 3'
5' Snap25 3' Snap25	XM_009497	5' - TAGTGGACGAACGGGAGCAGATGG - 3' 5' - CGTTGGTTGGCCTCATCAATTCTGG - 3'
5' PDGFR-alpha 3' PDGFR-alpha	NM_006206.2	5' - CCACGCTACCAGTGAAGTCT - 3' 5' - CACAGCAGGATGGTCACTCT - 3'
5' 60s 3' 60s	X56932	5' - TCGTGCGTCTGAAGCCTAC - 3' 5' - TCTTCCGGTAGTGGATCTTG - 3'
5'IL-4 3'IL-4	NM_000589.2	5' - CCGAGTTGACCGTAACAGAC - 3' 5' - CTCTGGTTGGCTTCCTTCAC - 3'
5'IL-4R 3'IL-4R	NM_000418.2	5' - CGTCAGCGTTTCCTGCATTG - 3' 5' - CTCTTTGGCAGCCTTGTGAG - 3'
5'IL-12p40 3'IL-12p40	NM_002187.2	5' - AACCTGACCCACCCAAGAAC - 3' 5' - AGATGCCCATTCGCTCCAAG - 3'
5'IL-13 3'IL-13	NM_002188.2	5' - ATCCGCTCCTCAATCCTCTC - 3' 5' - AGCATCCTCTGGGTCTTCTC - 3'

5' and 3' Primers used for quantitative RT-PCR analysis together with the gene accession numbers are shown. The gene 60s was used as housekeeping gene for normalization.

In situ hybridization

cDNA sequences of STAT6 (U16031, pos. 1248-2522) and HIF-1α (U22431, po. 1215-2394) were cloned from human cerebellum tissue into pBluescript KS II+. cDNA for HLA-DRα was kindly provided by Viktor Steimle (Department of Biology, University of Sherbrooke, Sherbrooke, Canada). Digoxigenin-labelled cRNAs were generated for Hif-1α and HLA-DRα. In situ hybridization was performed on cryosections (10μm thick) of freshly frozen tissue as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993; Spiegel et al., 2002). Hybridization was done overnight at 68°C and color reaction time for the alkaline phosphatase reaction was 48 hours for all probes.

Statistics

Statistical significance is expressed by p-values generated by the non-parametric Mann-Whitney U-Test. For each gene two p-values were calculated (Table 3). A hierarchical cluster analysis was performed using the program “Cluster” and “TreeView” from Michael Eisen (Eisen et al., 1998). Hierarchical cluster analysis revealed two MS case groups based on similarities in their gene expression pattern. Therefore, two p-values were calculated. The first p-value (marked by an asterisk) was calculated using the signal intensities from the cases MS 1, 2, 3, 5, 12 and 26, whereas the second p-value (marked by double-asterisk) was calculated by using all MS cases. To evaluate differential gene expression data from the microarrays in more detail, a boxplot from each gene was generated and analyzed (data are shown for particular genes in Fig.5). For this, the hybridization signal intensities were normalized to one of the control samples according to Graumann et al. (2003). Statistical correlation was calculated using the non-parametric Spearman’s test.

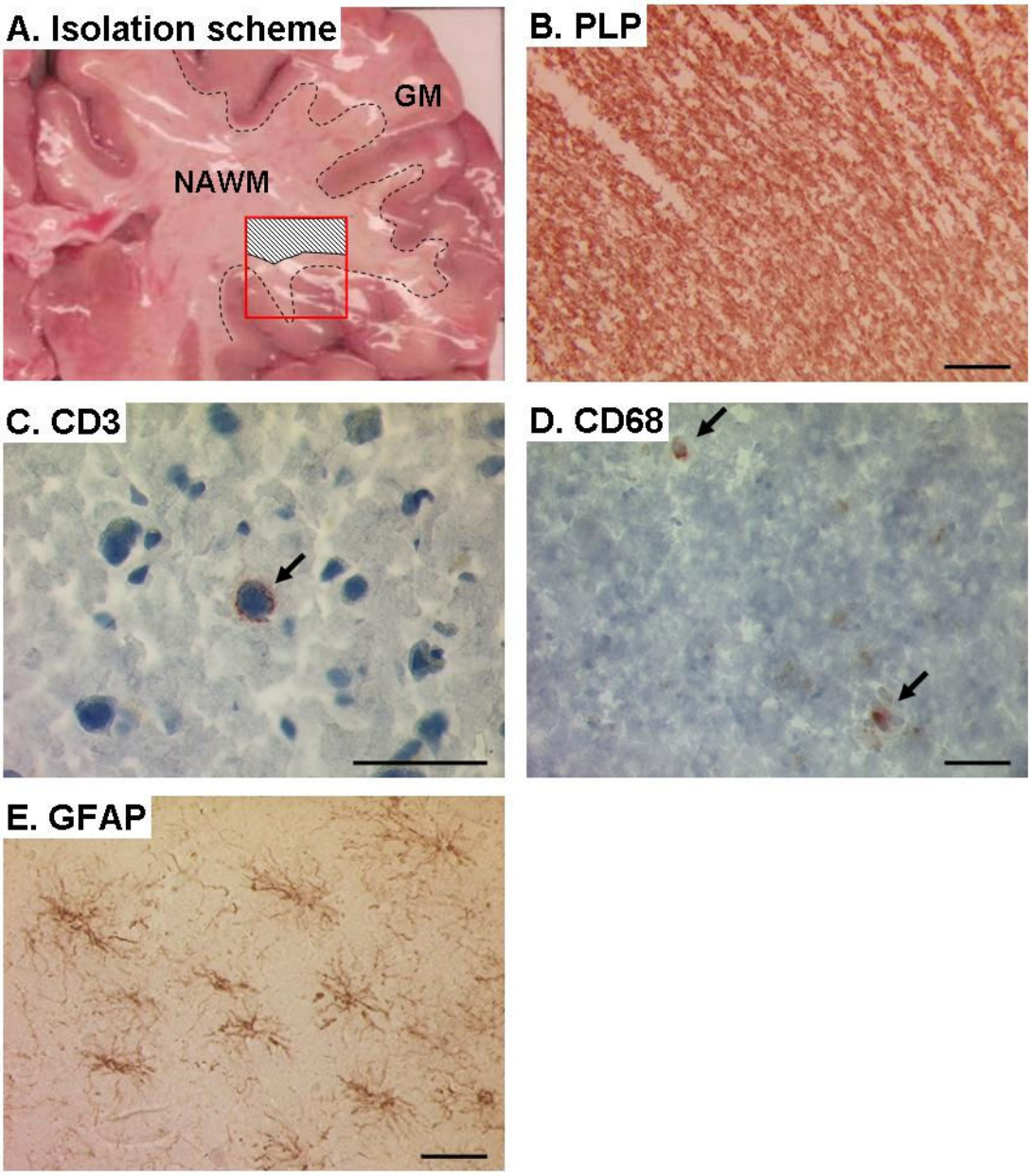

Figure 1: Histopathological examination of subcortical MS NAWM tissue.
To analyze gene expression changes in the NAWM we isolated pure subcortical white matter (hatched area) from tissue blocks (A, red box). Immunohistochemical analyses were made to characterize the NAWM and to exclude possible lesions. NAWM stained by PLP (B) showed normal myelin. CD3 positive cells were detected rarely in the NAWM (C, arrow). We could detect some CD68 positive cells in the NAWM (D, arrows). Furthermore, staining for GFAP revealed weakly activated astrocytes (E). Bars = 50 μm

Results

Histological and molecular examination of subcortical NAWM from MS and control brains

All brains used in this study were routinely screened by a neuropathologist to confirm diagnosis of MS and to exclude confounding pathologies such as for example Alzheimer's disease. The clinical and pathological information on the MS and control cases was collected and is summarized in Table 1. To analyze gene expression changes in the NAWM, we dissected subcortical white matter from cortical grey matter to ensure that only RNA from white matter tissue was isolated (Fig. 1A, hatched area). Isolated RNA was tested for its integrity by ethidium-bromide gel electrophoresis and GFAP Northern blotting, and only samples with high quality RNA were used. In order to confirm normal cellular integrity and composition, and to exclude any possible pathological changes such as demyelination and inflammation, we performed immunohistochemical analysis on the NAWM tissue. Staining for myelinated fibres (Fig. 1B, PLP) showed no signs of demyelination or shadow plaques. We detected almost no CD3-positive cells in subcortical NAWM (Fig. 1C). However, we detected CD68 positive cells distributed throughout the MS NAWM (Fig. 1D). There was no evidence of astrogliosis as revealed by an anti-GFAP staining (Fig. 1E, GFAP). As we have described before, some neurons were found in human subcortical white matter as well (Graumann et al., 2003). In summary, all tissue included in this study showed no histological evidence of demyelinating lesions, remyelination or inflammatory infiltrates.

In a recent study, we performed a differential expression analysis of subcortical NAWM from MS cases and corresponding subcortical white matter from control cases (Graumann et al., 2003). We performed a hierarchical cluster analysis to identify groups of MS cases based on similarities in their gene expression pattern. This analysis revealed a major group within the MS cases (MS1, 2, 3, 5, 12 and 26), which showed strong similarities in their expression patterns (Graumann et al., 2003). A minor group of three MS cases (MS10, 18 and 20) showed an expression pattern with some similarities to the major group such as the upregulation of STAT6, but showed overall fewer alterations compared to the control cases.

Table 3 Genes differentially expressed in NAWM

		MEDIAN								*P*-value	MEDIAN			*P*-value
gene-bank accession	gene name	MS26	MS1 A3B2	MS1 A3D1	MS12 P1A4	MS5 A3C2	MS5 A3C7	MS3 A4B4	MS2 P5C4		MS20 P3B3	MS10 A4D3	MS18 P3B4	
STAT signaling pathway														
U16031	STAT6	2.8	3.8	2.9	2.8	3	1.9	3.1	4.5	**0.005**	1.5	1.5	2	**0.005**
L29277	STAT3	1.4	2	2	1.7	2.4	1.5	1.5	1.5	**0.012**	1	1.2	1.2	**0.032**
M35203	JAK1	1.5	4.3	2.6	1.7	1.9	1.4	2.1	1.9	**0.003**	1.4	0.9	1.3	**0.005**
AF005216	JAK2	3	2.7	2.2	2	1.2	1.6	1	1.3	0.172	1.2	1.5	0.9	0.186
U09607	JAK3	3.3	3.4	2.3	1.1	1.5	1.5	2	8	0.074	3.5	0.6	1.3	0.137
X54637	TYK2	1.8	2.4	1.8	1.4	1.7	1.4	2.3	1	0.141	0.8	0.8	1	0.322
L08807	SH-PTP2	1.6	1.9	1.9	2.3	1.6	1	1.6	2	**0.012**	2.1	1.3	1.4	**0.008**
L29511	GRB2	2.2	2.1	2.5	2	2.7	2.2	2	1.4	**0.002**	1.4	1.1	0.9	**0.01**
M57230	IL-6Rb	2.1	3.2	2.1	1.6	2.7	1.9	1.5	1.1	**0.036**	1.5	0.9	1.8	0.069
L05624	MAPKK 1	3.7	6.3	4.1	2.3	2.1	2	1.9	1.6	**0.002**	0.7	1.3	0.7	**0.013**
X02751	N-ras	3.8	1.8	1.5	2.3	2.1	1.1	1	0.7	0.115	0.9	1.1	1.1	0.248
X03484	c-raf	3	1.7	1.9	2.1	1.3	1.1	1.4	1.8	**0.016**	1.3	1	0.6	0.083
U09579	WAF1	3.5	2.5	2.2	2.1	1.5	1.6	1.4	2	0.172	1.2	0.6	1.6	0.215
L34075	FRAP	2.8	4.1	1.4	2.5	1.7	1.3	2.7	0.4	**0.027**	1.1	2	0.5	0.075
Cytokines														
M57627	IL-10	2.4	2.7	1.3	1.8	2	1.7	1.1	1.3	**0.016**	1.1	0.8	0.8	**0.048**
M19154	TGF-β2	2.5	3.1	1.8	1.5	3	2.2	2.3	1.7	**0.006**	0.9	1	2.6	**0.01**
D49950	IL-18	2.3	2.2	1.5	1.2	1.6	1.6	1.1	1	0.093	0.6	1.5	0.5	0.248
K02770	IL-1β	2.6	2.6	1.7	1.5	2.2	1.9	1.6	1.2	**0.046**	0.9	0.8	1.5	0.117
Cytokine receptors														
X77722	INF-α/βR	1.2	2.4	1.2	2.3	2.6	1.5	1.1	0.9	**0.046**	1.5	0.9	0.8	0.099
M57230	IL-6Rβ	2.1	3.2	2.1	1.6	2.7	1.9	1.5	1.1	**0.036**	1.5	0.9	1.8	0.069
X01057	IL-2Rα/β	3.6	7.1	1	2.1	2.7	2.1	1.1	2.9	**0.021**	1.6	0.6	0.6	0.058
M33294	TNFR1	2.8	3	1.9	1.5	2.3	1.5	2.1	2.1	0.141	1.3	1.2	1.8	0.16
Chemokines / -receptors														
M37435	MCSF	3.8	5.6	1.1	3.1	2.7	2.4	1.9	1.6	**0.009**	1.4	1.9	1	**0.01**
M24545	MCP1	3.7	4.6	2.1	1.6	1.7	1.9	2.2	6.1	0.172	1.3	0.5	1.4	0.215
U10117	EMAP II	3.1	3.1	1.3	2.9	1.9	2.2	1.4	1.9	**0.012**	1.9	1	1.1	**0.017**
M21121	RANTES	3.5	2.9	0.9	1.1	1.3	1.5	1	2	0.059	1.6	0.7	0.7	0.16
D10925	CCR1	1.1	4.5	1.3	1.7	2	1.8	1.1	1.2	0.115	0.9	0.6	1	0.283
M25756	Chromogranin C	2.4	2.5	4.1	2.3	2.7	3.6	5.1	1.9	**0.009**	0.9	1.8	0.9	**0.021**
Adhesion molecules														
J03132	ICAM1	2.2	3.5	2.6	1.8	1.9	1.1	1.2	1.9	**0.046**	1.2	0.7	2.5	**0.048**
X57766	MMP11	1.4	16.9	2.2	1.9	4	3.1	2.1	1.6	0.074	0.9	0.9	1.5	0.137
M15395	LFA-1	1.7	2.9	1.7	2	4.2	2.4	1.1	0.7	0.059	0.3	1.1	1.1	0.283
Hypoxia related genes														
U22431	HIF-1α	1.5	6.5	3.6	2.3	2.2	1.8	2	1	**0.016**	2.3	0.5	1.4	**0.021**

Upregulation is shown in dark grey whereas downregulation is shown in yellow. Medians of ratios against all control samples are shown for each MS sample. Statistical significance is expressed as p-value (non-parametric Mann-Whitney U-Test). P-values were calculated for the major group (*) and for all MS cases (**). P-values below 0.05 are printed in bold numbers, whereas p-values between 0.05 and 0.1 are shown in italic numbers.

Changed expression of genes of the JAK/STAT signalling pathway

One of the major pathways involved in immune regulation is the JAK/STAT signalling pathway (Dell'Albani et al., 2003). By microarray and quantitative RT-PCR analysis of MS NAWM, we observed changes in the regulation of gene expression of several genes known to be involved in the JAK/STAT signalling pathway (Table 3, Fig. 2A-D). One member of the STAT family, STAT6 was upregulated in all MS cases (Table 3, Fig. 2A, p=0.005). Furthermore, we also found STAT3 to be significantly upregulated in these MS NAWM (Table 3). The other members, STAT1, 2, 4, 5a and 5b could not be detected by microarray analysis. However, using the more sensitive qRT-PCR, we revealed that STAT4 was also significantly upregulated in MS cases (Fig.2D, p=0.002). To identify the cell types expressing STAT4 and STAT6, we performed a immunofluorescent colocalization study (Fig. 3 and 4). Most of the STAT4 expressing cells were CD68-positive, suggesting that the main cell population expressing STAT4 are activated microglia (Fig. 3A, arrow). Occasionally, we identified STAT4-positive astrocytes (Fig. 3B, arrow). We did not detect any STAT4-positive oligodendrocytes or neurons in MS NAWM. In contrast, immunofluorescent staining for STAT6 showed that STAT6 was mostly in cells expressing OLIG2 (Fig. 4A, arrows), demonstrating that cells of the oligodendrocyte lineage were the main cell population expressing this transcription factor. The arrangement of STAT6/OLIG2-positive cells in interfascicular rows indicates an expression by myelinating oligodendrocytes. However, STAT6 expression was not limited to oligodendrocytes, as occasionally GFAP/STAT6-positive astrocytes were also detected in the NAWM (Fig. 4B, arrow). Colocalization with neurofilament revealed STAT6/ Neurofilament-positive neurons in the NAWM as well (Fig. 4C, arrow). No colocalization of STAT6 together with CD68 could be detected. Triple immunofluorescence staining showed sporadically astrocytes positive for both STAT4 and STAT6 in the NAWM (Fig. 4D, arrow).

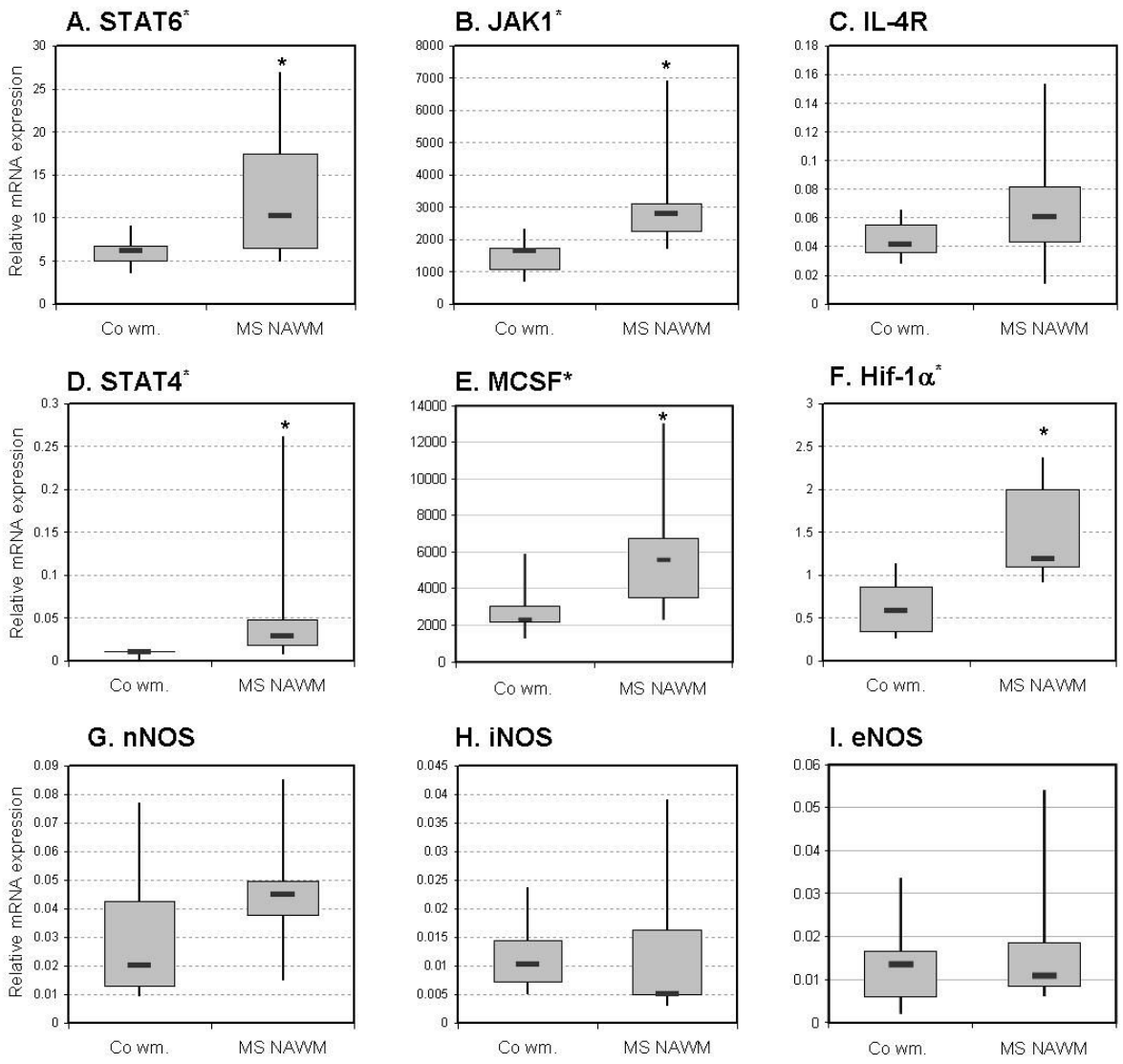

Figure 2: Boxplots of selected differentially expressed genes analyzed by quantitative RT-PCR and microarray. qRT-PCR was performed for STAT6 (A), IL-4R (C), STAT4 (D), HIF-1α (F), nNOS (G), iNOS (H) and eNOS (I). JAK1 (B) and MCSF (E) were analyzed by microarray. Boxplots show differential gene expression in control white matter and MS NAWM. Genes significantly upregulated in MS NAWM are marked with an asterisk.

The expression of all four members of the JAK family could be detected by microarray analysis. Whereas JAK2 and TYK2 were not statistically significant changed, JAK1 was significantly upregulated in the NAWM of MS cases (Table 3, Fig. 2B). Another member of the JAK tyrosine kinase family, JAK3, was also upregulated in MS NAWM but with a weaker significance (Table 3). To identify the cell types expressing JAK1, we performed a colocalization immunofluorescent study, which revealed the expression of JAK1 in oligodendrocytes expressing MBP (Fig. 4E, arrow). Interestingly, we could show a positive correlation of JAK1 (r=0.505, p=0.027)

and JAK3 (r=0.530, p=0.020) expression levels to the expression levels of STAT6 within the different MS cases. Although an overall increase in expression of JAK2 and TYK2 in MS could also be observed, the upregulation was not statistically significant. Other genes belonging to the JAK/STAT signalling pathway, such as SH-PTP2 and GRB2 were also significantly upregulated in MS NAWM (Table 3). Furthermore, we found the upregulation of neuroblastoma RAS viral (v-ras) oncogene homolog (N-RAS), v-raf-1 murine leukemia viral oncogene homolog 1 (c-raf) in some and mitogen-activated protein kinase kinase 1 (MAPKK1) in most MS cases. Also, cyclin-dependent kinase inhibitor 1A (p21, WAF1) and FK506 binding protein 12-rapamycin associated protein 1 (FRAP1) were upregulated in the majority of the MS cases. In summary, our results show the upregulation of genes from the Interleukin-4/Interleukin-13 signalling pathway expressed by oligodendrocytes and genes of other JAK/STAT signalling pathways in resident brain cells in a majority of the MS cases studied.

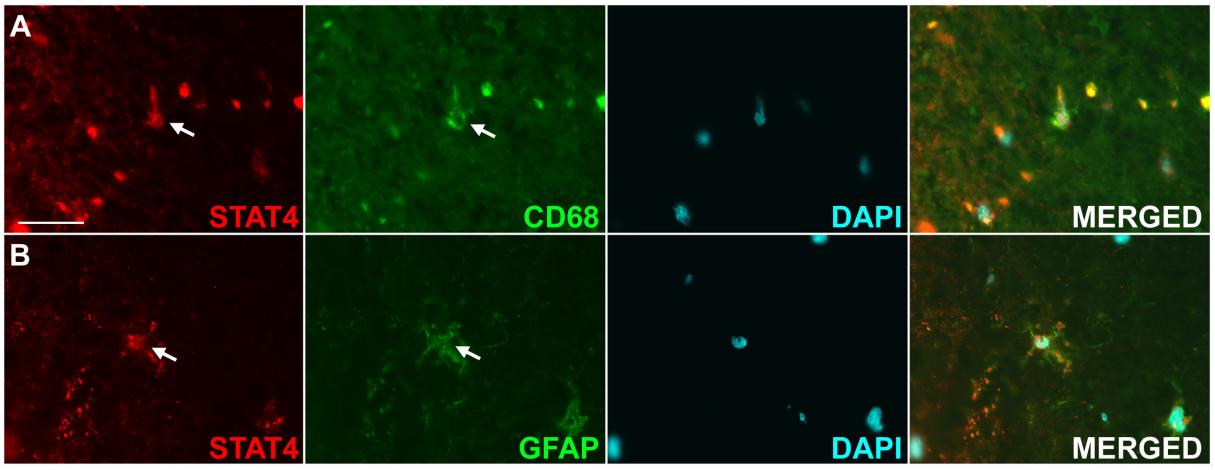

Figure 3: Immunofluorescence localization of STAT4 in MS NAWM. Colocalization analysis showed STAT4 being expressed in CD68 positive cells (A, arrow). Furthermore, we could show some astrocytes positive for STAT4 (B, arrow). We did not detect any neurons or oligodendrocytes positive for STAT4 in the NAWM. Bar = 20μm

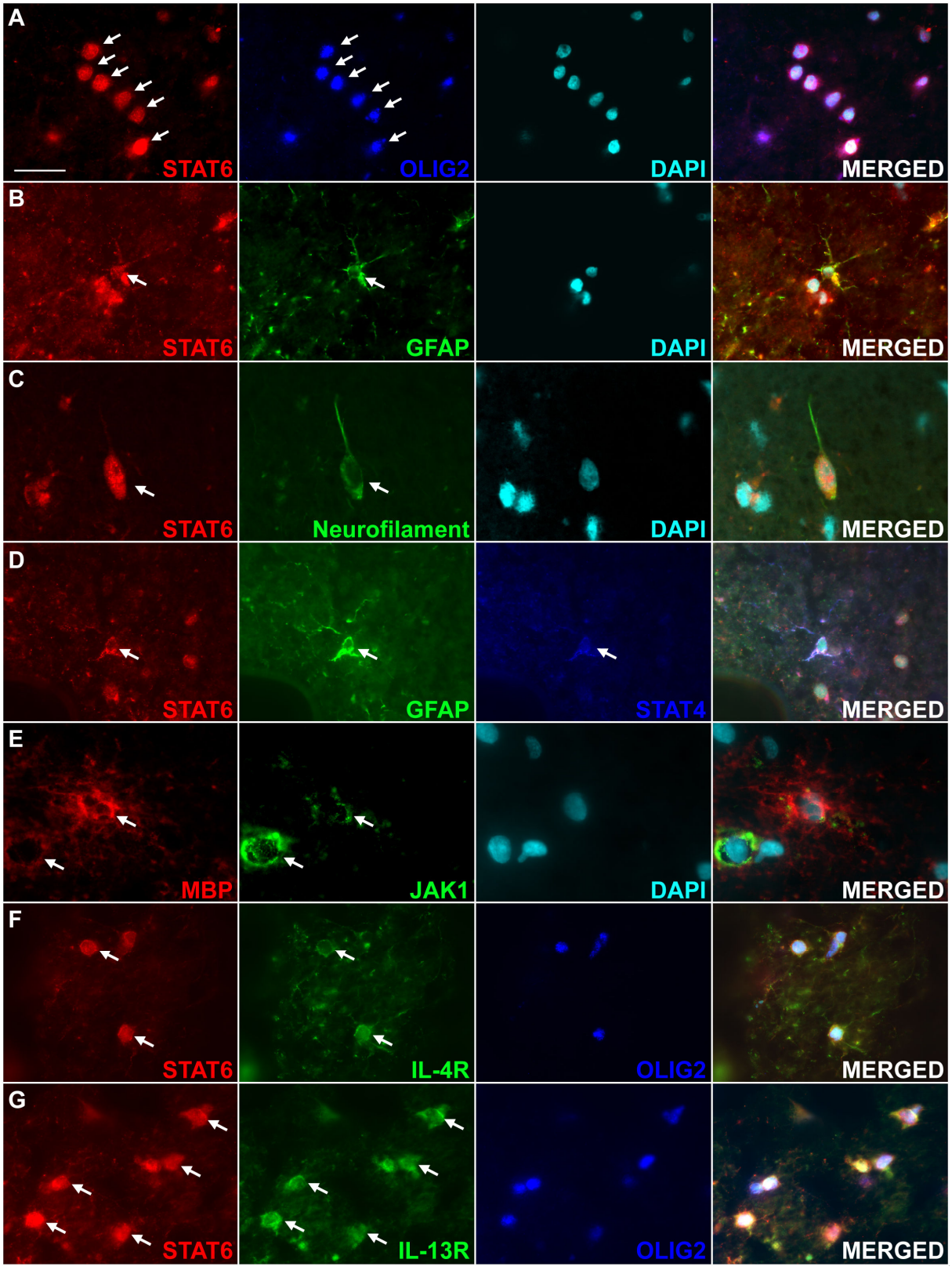

Figure 4: Immunofluorescence localization of STAT6, JAK1, IL-4R and IL-13R in MS NAWM. STAT6 was mostly colocalized together with the oligodendrocytes marker OLIG2 (A, arrows). In few cases colocalization with astrocytes (B, arrow) and subcortical white matter neurons (C, arrow) could be observed. Sporadically, we could detect some astrocytes positive for both, STAT4 and STAT6 (D, arrow). Additionally, we could show that JAK1 mostly colocalized together with the myelin protein MBP (E, arrows). Furthermore, IL-4R (F, arrows) and Il-13R (G, arrows), the main receptors leading to STAT6 activation, colocalized with STAT6 and OLIG2. Bar = 20μm

Upregulated cytokine and cytokine-receptor expression in NAWM of MS patients

Changes in the balance between pro- and anti-inflammatory cytokines and their receptors have been implicated in the pathogenesis of MS. Therefore, we investigated the expression pattern of cytokines and their receptors in the NAWM of MS patients (Table 3). Most strikingly, we observed a significant upregulation of the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor beta 2 (TGF-β2) in the MS cases. On the other hand, IL-18 and IL-1β also showed elevated expression levels in the majority of MS cases. Other cytokines such as TGF-α and TGF-β3 showed only minor changes or were even decreased in some MS cases (data not shown). An endogenous expression of most of the cytokines, in particular IL-4, IL-6, IL-12, IL-13, IL-17, IL-23 and IFN-γ could not be detected by microarray analysis or by quantitative RT-PCR.

Microarray analysis of cytokine receptors revealed detectable expression of IL-2 receptor subunit alpha (IL-2Rα), IFN-γ receptor, IFN-alpha/beta receptor, IL-6 receptor (IL-6R) and tumor necrosis factor receptor 1 (TNFR1; Table 3). We detected an upregulation of the IL-6Rβ subunit (gp130) and TNFR1 in 9 out of 11 MS samples. In contrast to IL-6Rβ, which was significantly upregulated in MS NAWM, upregulation of TNFR1 was not statistically significant due to the fact that several control samples had also relative high TNFR1 expression levels (Table 3). One of the most consistently upregulated cytokine receptors was the interleukin-2 receptor alpha (IL-2Rα). Its co-receptors IL-2Rβ and IL-2Rγ, however, could not be detected by microarray (data not shown). Significant upregulation was also detected for the IFN-α/β receptor, whereas the IFN-γ receptor was only upregulated in 2 of 11 MS samples (Table 3).

To investigate the anti-inflammatory signalling pathway of STAT6, JAK1 and JAK3 in more detail, we examined the expression pattern of one of its main receptors, the IL-4 receptor (IL-4R), by quantitative RT-PCR. We detected elevated expression levels of IL-4R in MS NAWM (Fig. 2C). Even though we could detect an overall higher expression of IL-4R in MS NAWM samples this was not statistically significant. The reason for this is a relative high expression of one of the controls (CLo6), and in

addition, a moderate expression of IL-4R in the three cases of the minor group. However, IL-4R expression levels correlated well with the expression levels of STAT6 (r=0.657, p=0.008) within the analyzed tissue samples. Immunofluorescent colocalization study with STAT6 revealed STAT6/IL-4R positive oligodendrocytes throughout the NAWM (Fig.4F). Furthermore, immunofluorescent colocalization study for IL-13 receptor (IL-13R), which is another STAT6-activating receptor, revealed STAT6/IL-13R positive oligodendrocytes in the MS NAWM as well (Fig.4G). In summary, our results show that oligodendrocytes in MS NAWM are expressing the necessary components for STAT6/JAK1 signalling.

Upregulated chemokines and chemokine receptor expression in NAWM of MS patients

Chemokines and chemokine receptors play a critical role in the recruitment of lymphocytes and other inflammatory cells into the CNS (Sospedra and Martin, 2005). Microarray analysis revealed significant upregulation of several chemokines in MS NAWM (Table 3), in particular chromogranin C, monocyte colony-stimulating factor (MCSF) and endothelial-monocyte-activating polypeptide (EMAP-II). Boxplot analysis for MCSF shows an overall low-level expression in control cases and upregulation in almost all MS cases (Fig. 2E). Although monocyte chemotactic protein (MCP-1) showed an upregulation in all MS cases of the major group (Table 3), this was not statistically significant because MCP-1 expression among control cases was highly variable (data not shown). Other chemokines such as RANTES and its receptor CCR1 also showed elevated expression levels in particular MS cases, but for the same reason as for MCP-1 these were not statistically significant. Altogether, we could demonstrate that the expression levels of several chemokines involved in the attraction of peripheral immune cells into the CNS were increased.

Differential gene expression of cell adhesion molecules

Cell adhesion molecules play an essential role in many inflammatory processes, such as blood-brain-barrier changes, cell migration and differentiation. Therefore, we investigated the gene expression profile of different cell adhesion molecules (Table 3). Most of these genes did not show altered expression levels with the exception of

ICAM-1, which was significantly upregulated in the majority of the MS cases (Table 3). Others such as LFA-1β had a more heterogeneous expression pattern (Table 3). Matrix metalloproteinases (MMP) are also implicated in the pathogenesis of the inflammatory process in MS, but our microarray study revealed that only MMP-11 (stromelysin-3) was upregulated in MS NAWM. MMP-7, MMP-9 and MMP-16 were found constitutively expressed in all control and MS cases, but their expression levels were not altered.

Upregulation of HLA-DRα in MS NAWM

The expression of the major histocompatibility complex HLA-DR α-chain, crucial for antigen presentation, was upregulated in the majority of MS cases, whereas upregulation of HLA II DP α-chain was less evident and only detected in a subset of MS cases (data not shown). To identify cells expressing HLA-DRα, we performed in situ hybridization analysis (performed by Sacha Zaugg during his master thesis). This revealed the expression of HLA-DRα mRNA throughout the NAWM of the majority of MS cases (Fig. 5A), whereas in control white matter HLA-DRα was not detectable (Fig. 5B). Due to the morphology of the HLA-DRα expressing cells - small cells with thin processes - we identified most of the HLA-DRα expressing cells as microglia (Fig. 5C). Colocalization with anti-GFAP immunohistochemistry revealed that some astrocytes might also express HLA-DRα (data not shown). In summary, our data indicate that a part of the HLA Class II complex, especially HLA-DRα, is upregulated in MS NAWM.

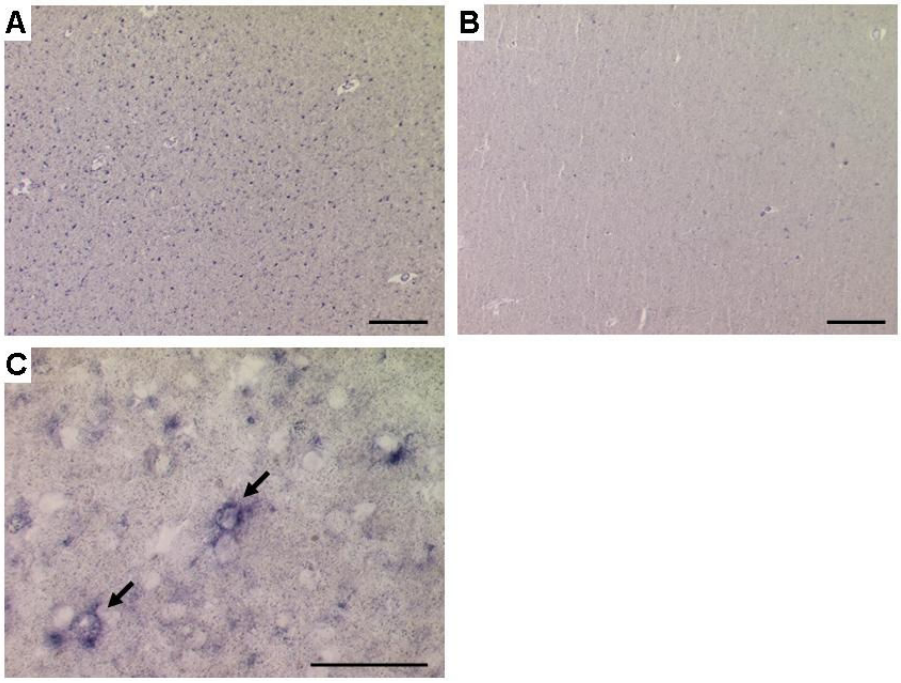

Figure 5: *In situ* hybridization of HLA-DR mRNA in MS and control white matter.
Many HLA-DRα positive cells were detected in the NAWM of MS cases (A) compared with white matter from control cases where the hybridization signal was below background levels (B). *In situ* hybridization shows that HLA-DRα mRNA expression in cells of the NAWM

Upregulation of the neuronal- but not of the inducible and endothelial nitric oxide synthase

Nitric oxide (NO), and thus nitric oxide synthases (NOS) play an important role under physiological as well as pathological conditions (Smith and Lassmann, 2002). Therefore, we performed qRT-PCR analysis and detected upregulation of the neuronal form of NOS (nNOS), in most of the MS NAWM tissue samples (Fig. 2G). In contrast, the inducible NOS (iNOS) showed comparable expression levels in MS NAWM as in controls cases (Fig. 2H). Also the expression of endothelial NOS (eNOS) in MS NAWM showed no difference to the control cases (Fig. 2H, I).

Upregulation of HIF-1α in NAWM

In our previous microarray study we showed that HIF-1α, a key regulator of hypoxia-induced gene regulation, and its downstream genes were significantly upregulated in MS NAWM (Graumann et al., 2003). Quantitative RT-PCR analysis in 11 MS and 8

control cases for HIF-1α revealed a consistent upregulation of HIF-1α in MS NAWM (Fig. 2F, p=0.002), verifying our microarray data (Graumann et al., 2003). In order to identify the cell types expressing HIF-1α, we performed in-situ hybridization analysis (Fig. 6, performed by Ralf Brunner during his master thesis). We found that in subcortical white matter of control cases HIF-1α mRNA could not be detected (Fig. 6A), indicating an overall weak expression in white matter. However, in MS NAWM HIF-1α hybridization signals became detectable in a number of cells throughout the NAWM (Fig. 6B, arrows). Colocalization analysis for GFAP revealed that a small subpopulation of astrocytes expressed HIF-1α (Fig. 6C). Other HIF-1α positive cells were arranged in interfascicular rows, suggestive of myelinating oligodendrocytes (Fig. 6D, arrows).

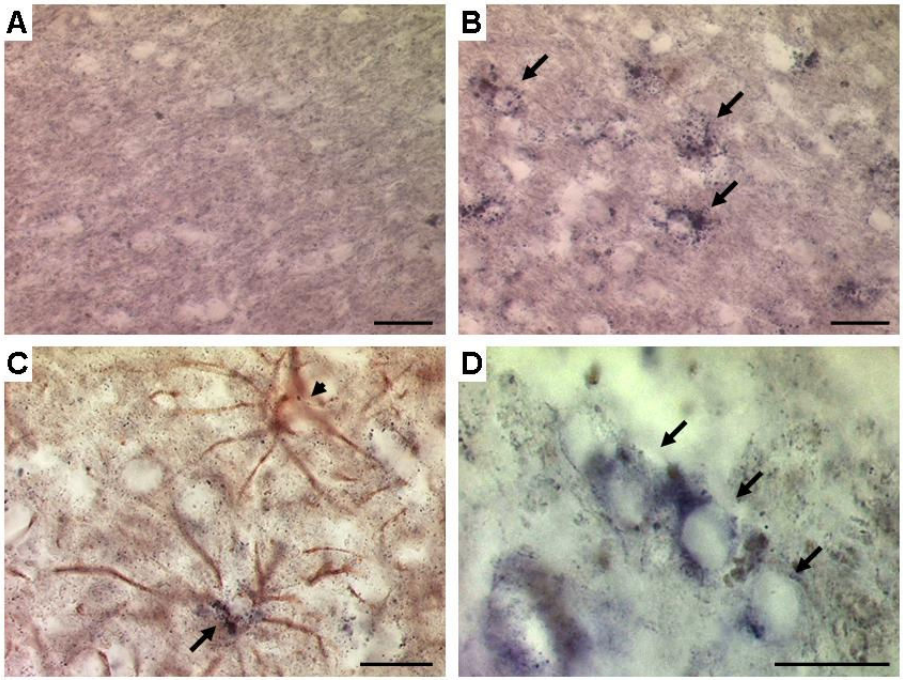

Figure 6: *In situ* hybridization of HIF-1α mRNA in MS and control white matter.
In subcortical white matter of control cases HIF-1α could not be detected (A). In MS NAWM, however, we could detect a hybridization signal for HIF-1α mRNA in a number of cells (B). Colocalization with anti-GFAP immunohistochemistry revealed a subpopulation of astrocytes expressing HIF-1α (C, arrow), whereas other astrocytes were negative for HIF-1α (C, arrowhead). Due to the chain-like arrangement and their morphology, we suggest that Hif-1α is also expressed by oligodendrocytes (D, arrows). Bars = 25μm

Discussion

Very little is known about the earliest intrinsic changes to the CNS occurring in MS before the appearance of overt inflammatory and demyelinating plaques. The normal appearing non-lesional white matter looks normal from a morphological and cellular point of view. However, MRI and gene expression studies have shown significant changes in NAWM that might give indications concerning both, the early changes occurring before lesion formation and the attempts by the CNS to prevent the same (Aboul-Enein et al., 2003; Fu et al., 1998; Graumann et al., 2003; Silver et al., 2001). Furthermore, most recent data suggests a diffuse inflammatory damage spreading throughout the whole brain in the chronic phase of the disease associated with slow progressive axonal injury at sites without inflammation (Kutzelnigg et al., 2005). In order to identify pre-lesional changes and immunological regulators, we analyzed the gene expression profile in the NAWM of 11 MS cases. All tissue included in this study was routinely screened by a neuropathologist and showed no signs of additional neurological diseases. Tissue from MS cases with confounding pathologies such as hypoxic neurons, fibrillary tangles or metastasis as well as accumulation of inflammatory cells or demyelination were excluded from this study. In particular, subcortical NAWM tissues revealed almost no T-cell infiltration. In line, T-cell specific transcripts were rarely detectable in single MS cases and no correlation of genes from the STAT signalling pathways and T-cell specific transcripts could be made. In addition, we could not detect any CD3/STAT6-expressing cells in MS NAWM. As gene expression changes were obtained by comparing MS cases with age-matched control cases, detection of differential gene expression due to age-related mechanisms was avoided. This so-defined NAWM tissue is ideal to study the intrinsic changes of brain cells during the long-lasting disease course of MS.

Hierarchical cluster analysis of the MS cases based on the similarities of their expression pattern revealed two possible groups. But, no grouping according to the MS type could be identified. Possibly, gene expression changes in the MS NAWM from different MS types might equalize during the long-lasting progressive disease course. As gene expression changes taking place in the MS NAWM do not depend upon the MS type, this allowed us to combine gene expression data from all MS types.

Altogether, our analysis revealed that genes involved at different levels of the inflammatory response, such as signalling, transcription, cell adhesion and antigen presentation, were upregulated in the MS NAWM. Most interestingly, we detected upregulation of genes linked to both anti- as well as pro-inflammatory mechanisms. Two central players involved in these two mechanisms are the transcription factors STAT6 and STAT4 (Pfitzner et al., 2004).

Diffuse damage of the CNS might be due to pro-inflammatory microglia in the MS NAWM

We found that in the NAWM, STAT4, a characteristic marker for pro-inflammatory mechanisms, was generally expressed by CD68-positive cells suggestive for activated microglia; occasionally astrocytes as well. In line, an upregulation of HLA-DRα, known to be generally expressed by microglia, was detected in the NAWM of MS cases. STAT4 is involved in IL-12 and IL-23 mediated signalling and as a consequence in Th1 cell differentiation (Watford et al., 2004). The upregulation of STAT4 found in the NAWM tissue is strikingly prominent and might point to the development of a pro-inflammatory environment allowing or facilitating the infiltration of peripheral immune cells into the CNS. This is supported by a study investigating the role of STAT4 in the development of local allergic airway response. In this study, STAT4 knockout mice showed a significant decrease in airway hyperactivity via local alteration of chemokine production, such as CCL5, CCL6, CCL11 and CCL17 (Raman et al., 2003). Furthermore STAT4 is known to play a major role in IFN-γ regulation and is involved in the induction of IL-2R (Watford et al., 2004). Also involved in IL-12 and IL-23 signalling are the Janus kinases JAK2 and TYK2, which were upregulated in some MS cases. Furthermore, IL-1β, which is described as a pro-inflammatory cytokine primarily secreted by activated microglia cells and infiltrating macrophages (Correale and Villa, 2004) was significantly upregulated in MS NAWM. An enhanced expression of IL-1β might also be one cause of the reported diffuse damage of the CNS (Kutzelnigg et al., 2005), as intracerebral microinjections of IL-1β induced death of intrinsic CNS cells (Holmin and Mathiesen, 2000). The view of microglia playing a pro-inflammatory role in MS NAWM is further supported by a study showing that a blockage of microglial release of nitrite oxide,

pro-inflammatory cytokines and chemokines, resulted in a strong reduction of CNS inflammation and an amelioration of the clinical signs in EAE (Heppner et al., 2005). Furthermore, in MS NAWM, we could detect the upregulation of several chemokines, such as MCSF, EMAP II, MCP-1 (CCL2), ICAM-1 and RANTES (CCL5). This is inline with a study which showed an increased MCSF expression in the periplaque white matter of MS cases (Werner et al., 2002). Altogether, we speculate that the alteration of the pro-inflammatory gene expression pattern in resident cells of the NAWM, most likely microglia, could prepare or later contribute to the enhancement and facilitation of the infiltration of active immune cells into the CNS. At this point, the production of these pro-inflammatory signals might still not be enough to promote T-cell recruitment but they might be already deleterious for the CNS, as the expression of one specific cytokine under certain circumstances can already lead to an infiltration of immune cells into the CNS (Chiang et al., 1996).

Are oligodendrocytes playing an active role in immune regulation in the CNS?

A major finding of this study was the upregulation of genes involved in anti-inflammatory mechanisms in MS NAWM, illustrated by the upregulation of IL-10, a potent anti-inflammatory cytokine known to inhibit the activation of monocytes, dendritic cells and macrophages (Beebe et al., 2002). Furthermore, TGF-β2, which was reported to reduce demyelination and macrophage recruitment in a viral model of MS (Drescher et al., 2000), was strongly upregulated in NAWM of most of the MS cases. However, most interesting was the finding that STAT6 was upregulated in the NAWM of all MS cases. Although the function of STAT6 is not yet fully determined, this transcription factor is known to be involved in anti-inflammatory pathways. STAT6 is one of the major steps in IL-4 and IL-13 signalling (Takeda et al., 1997) and this is further demonstrated by the exacerbation of EAE in STAT6-deficient mice (Chitnis et al., 2001). The main cells expressing STAT6 in the MS NAWM were found to be oligodendrocytes. Furthermore, JAK1, IL-4R and IL-13R, all belonging to the STAT6 signalling pathway (Hebenstreit et al., 2006), were also expressed by oligodendrocytes. In the case of JAK1 and IL-4R, this is in agreement with the findings of Cannella and Raine (2004). Comparison of the expression pattern of STAT6, IL-4R, JAK1 and JAK3 showed a strong correlation within the different cases. This suggests an overall upregulation of the STAT6 signalling pathway in

oligodendrocytes. Additionally, we show that HIF-1α, a transcription factor inducing hypoxic preconditioning (Bergeron et al., 2000), is also expressed in oligodendrocytes in MS NAWM. Therefore, we hypothesize that in contrast to the pro-inflammatory response, the protective, anti-inflammatory response in the NAWM is mounted predominantly by oligodendrocytes. This conclusion is supported by a recent study showing that oligodendrocytes are capable of mounting protective mechanisms preventing demyelination (Lin et al., 2007). Furthermore, it was recently reported that mice with suppressed oligodendrocyte responsiveness to IFN-γ developed EAE with an accelerated onset and markedly increased oligodendrocyte apoptosis (Balabanov et al., 2007). In the same study, oligodendrocytes were shown to be capable of expressing several chemokines. This supports the view of oligodendrocytes participating in the regulation of CNS intrinsic immunity. Therefore, we speculate that the exacerbation of EAE in STAT6 knockout mice (Chitnis et al., 2001) might also result from the deficiency in activating the STAT6 signalling pathway in oligodendrocytes. Because oligodendrocytes are highly susceptible to inflammation mediated damage it may be crucial for them to compensate for the upregulated pro-inflammatory environment and to limit the inflammatory response and damage. The upregulation of an anti-inflammatory, and therefore also neuroprotective, environment in the MS NAWM is further supported by the activation of other neuroprotective pathways shown in our previous NAWM study (Graumann et al., 2003).

Are signals from lesions leading to an imbalance of inflammatory mechanisms in NAWM or vice versa?

We hypothesize that there are two possible reasons for the differential regulation of immune modulating genes in the cells of the NAWM. One possibility could be that diffusing, soluble factors released by activated inflammatory cells found in active MS lesions might activate and modulate inflammatory gene expression in resident cells of the distant NAWM. This idea is supported by a study using quantitative magnetic resonance imaging (MRI) techniques, where the authors suggest that axonal damage and demyelination in NAWM mainly arise as a secondary result of visible lesions with the largest effect close to these lesions (Vrenken et al., 2006). With the exception of IL-1β, IL-10, IL-18 and TGF-β2, most cytokines could not be detected by microarray

in MS NAWM, which suggests that these cytokines are not, or very weakly, expressed in NAWM tissue. Because we observed STAT4 and STAT6 upregulation in MS NAWM, we performed quantitative RT-PCR to identify the possible expression of their main activating cytokines, IL-4, IL-13, IL12 and IL-23. The absence of IL-4, IL-12, IL-13 and IL-23 expression on one hand, and the simultaneous upregulation of genes from the STAT4 and STAT6 pathway in MS NAWM on the other hand, further imply that extrinsic signals might influence the expression of immune modulating genes in the resident cells of the CNS. Additionally, this might be supported by the fact that in different tissue samples from the same MS case some genes are differently regulated, such as MCSF or CCR1 in the case of MS1.

Another possibility could be that brain intrinsic events such as impairment of oligodendrocyte and/or neuronal function, and subsequent astrocyte and microglia activation may be the initial cause for the differential gene regulation observed in NAWM. This hypothesis is supported by the upregulation of the endogenous neuronal NOS (nNOS), but not of the inducible NOS (iNOS) and endothelial NOS (eNOS), suggesting a parenchymal deregulation. Immune modulating signals from the periphery would first activate microglia inducing iNOS or endothelial cells of the blood-brain-barrier activating eNOS. Moreover, the upregulation of HIF-1α in oligodendrocytes and neurons supports the view of oligodendrocyte and/or neuronal dysfunction in the NAWM as a possible primary cause. This idea is supported by a study of relapsing-remitting MS cases, showing widespread oligodendrocyte apoptosis as the earliest change in lesions in which other cells appeared normal (Barnett and Prineas, 2004). It could be that in certain regions of the NAWM activated endogenous neuroprotective mechanisms may gradually fail. This may lead to an imbalance between protective and pro-inflammatory mechanisms facilitating lesion formation.

Is MS NAWM in a subtle balance between inflammation and neuroprotection?

Overall our results suggest that although the NAWM of MS patients shows no visible signs of active inflammation, many different genes are expressed in the tissue, which are known to be involved in the regulation and activation of the immune response that are normally not expressed in the CNS. The expression of pro- as well as anti-

inflammatory genes in the NAWM of the MS brain suggests that the CNS is in a state of low-level inflammation and an unsteady balance between protection and inflammation. This is further reflected by the heterogeneous regulation of cell adhesion molecules, matrix metalloproteinases and genes of the HLA complex. Yet, this combination of inflammatory factors seems not to be enough to promote T-cell recruitment. Possibly, these immune modulating genes are expressed below an active threshold or not in the required combination. The expression of the different subunits of the interleukin 2 receptor may also suggest that some of these mechanisms are not yet functionally active. Furthermore, the presence of competing anti-inflammatory mechanisms may inhibit infiltration of peripheral immune cells. Nevertheless, the activation of pro-inflammatory components might contribute to the reported diffuse damage of the CNS in MS (Kutzelnigg et al., 2005).

In summary, we show that a substantial set of genes involved in inflammation is expressed in resident cells of the NAWM. These genes are specifically regulated in the NAWM of MS patients compared with healthy controls, indicating an activation of the intrinsic immune regulation of the CNS, whereat oligodendrocytes actively participate. Therefore, the MS NAWM may be in a state of subtle balance between inflammation and neuroprotection, leading to an immune preconditioning of the non-infiltrated NAWM.

Acknowledgements

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Molecular changes in white matter adjacent to demyelinating lesions in early Multiple Sclerosis

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Abstract

A stereotactic biopsy of a seventeen year-old woman revealed an acute inflammatory demyelinating lesion compatible with pattern III multiple sclerosis according to Lucchinetti et al.(Lucchinetti et al., 2000) The biopsy included a region distant from the active inflammatory demyelinating lesion with abnormal MRI signal, however lacking histopathological signs of demyelination and/or oligodendrocyte apoptosis. Expression analysis of this area revealed a strong upregulation of neuronal nitric oxide synthase (nNOS). Furthermore, detection of nitrotyrosine provided evidence for reactive nitrogen species (RNS) mediated damage to oligodendrocytes. Concomitantly, genes involved in neuroprotection against oxidative stress such as heme oxygenase 1 were upregulated. This study shows for the first time earliest molecular changes in white matter distant to actively demyelinating lesions during the first manifestation of MS, pointing towards a more widespread pathological process. Therapeutic targeting of the identified mechanisms of tissue injury might be crucial to prevent further lesion formation as well as secondary tissue damage.

Introduction

Multiple sclerosis (MS) is a chronic, inflammatory demyelinating disease of the central nervous system (CNS) with great heterogeneity in clinical course, response to therapy and lesion pathogenesis. In 2000, four different patterns of demyelination were suggested.(Lucchinetti et al., 2000) One of these, pattern III, is characterized by an early selective loss of myelin-associated glycoprotein (MAG) associated with oligodendrocyte apoptosis. Oligodendrocyte apoptosis in the absence of significant inflammatory cell infiltrates has also been described as a possible initial event of lesion formation, and has been designated as `pre-phagocytic` stage of demyelinating MS lesions.(Barnett and Prineas, 2004) As a potential cause of oligodendrocyte apoptosis in MS nitric oxide (NO) was suggested.(Smith and Lassmann, 2002) Recently, one isoform of NO synthase, neuronal nitric oxide synthase (nNOS), was reported to play a key role in CNS demyelination.(Linares et al., 2006) In our study, we analyzed white matter without signs of demyelination or oligodendrocyte apoptosis (from a region with abnormal T1 and T2 weighted MRI signal) distant from the actively demyelinating pattern III lesion. Gene expression

analysis revealed strong upregulation of nNOS, but only minor upregulation of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS). This and the finding of nitrotyrosine-immunoreactive oligodendrocytes, indicative of NO-mediated damage, support the view of early, widespread damage to oligodendrocytes preceding active demyelination with oligodendrocyte apoptosis seen in pattern III MS.

Materials and Methods

Stereotactic brain biopsy and histopathological examination

Biopsy specimens were taken from three different target regions in the frontal subcortical white matter (Figure 1B and D). Four biopsy specimens were formalin-fixed and embedded in paraffin (3) or epon (1) for diagnostic purposes; two others were snap-frozen for RNA extraction and transferred to our lab for molecular analysis. Conventional myelin staining was performed with Holmes/Luxol. Immunohistochemical staining was performed as described before.(Graumann et al., 2003; Lucchinetti et al., 1998; Stadelmann et al., 2005) Detection of reactive nitrogen species (RNS), in particular peroxynitrite mediated damage was performed by immunofluorescent staining with anti-nitrotyrosine antibody as described before.(Jack et al., 2007) For detection of fragmented DNA, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was used as described.(Stadelmann et al., 1998)

RNA isolation and quantitative RT-PCR analysis

Total RNA isolation and quantitative RT-PCR analysis was performed as previously described.(Graumann et al., 2003; Zeis et al., 2008) Expression data obtained from the biopsy of the patient were compared to expression levels of the subcortical white matter from brain tissue obtained from post-mortem control cases and normal appearing white matter (NAWM) of MS cases as previously described in detail.(Graumann et al., 2003; Zeis et al., 2008) In addition, expression data were also compared to a control autopsy from a 36 year old patient without neurological disease (C25, male, cause of death: carcinoma of the tongue). As the amount of

RNA derived from the biopsied patient was very limited, qRT-PCR analysis was performed at least in duplicates and for particular genes (e.g. nNOS, iNOS) in triplicates.

Results

Case Report

A previously healthy, seventeen-year-old woman was hospitalized with subacute progressive global aphasia, right-sided hemiparesis, difficulty in walking and dysphagia. Neurological examination revealed right sided hemianopsia and right hemiplegia with hemihypaesthesia with extensor plantar response. Laboratory tests for rheumatic factors, antinuclear antibodies, c-ANCA, p-ANCA as well as CNS serology for HIV, HSV, VSZ, measles, mumps, FSME, syphilis and Lyme disease were negative. Urine sediment, as well as vitamins B12, B1 and E were normal. Analysis of the cerebrospinal fluid (CSF) revealed a pleocytosis (43 leukocytes $x10^6$/l; normal value <5), increased protein (548mg/l; normal value 180-480mg/l) and more than 5 oligoclonal bands. T1-weighted MRI showed a large hypointense lesion of the subcortical white matter extending throughout the left hemisphere, hyperintense on T2-weighted scans (Figure 1A). Gadolinium enhanced T1 MRI revealed a punctate garland-like enhancement in the fronto-parietal subcortical white matter (Figure 1B, arrow), which was only seen on the second MRI, 10 days after admission. Because of the pseudotumoral lesion characteristics, stereotactic biopsy from three different target regions in the frontal subcortical white matter was undertaken on day 15 of hospitalization (Figure 1B and D). Initial high dose steroid treatment followed by a course of i.v. immunoglobulins did not lead to a therapeutic response, whereas in subsequent months there was a slow improvement of the neurological deficits. No relapses occurred in the following 5 years. In a routine follow-up MRI investigation 5 years after the initial presentation, two new lesions were observed in the contralateral hemisphere (Figure 1C, arrows). Two months later a relapse occurred with increased ataxia, aphasia and sensory-motor impairment on the right side. Brain MRI displayed new lesions with diffusion restriction and gadolinium enhancement in the left brachium pontis (not shown). The patient underwent steroid and mitoxantrone therapy. After further 5 months she suffered from another relapse with

hyperaesthesia of the left leg. Spinal MRI showed intramedullar hyperintense lesions in the cervical (C2) and thoracic (Th5/6 and Th10/11) spinal cord. Thus, she fulfilled the diagnostic criteria for MS.(McDonald et al., 2001; Poser, 2006)

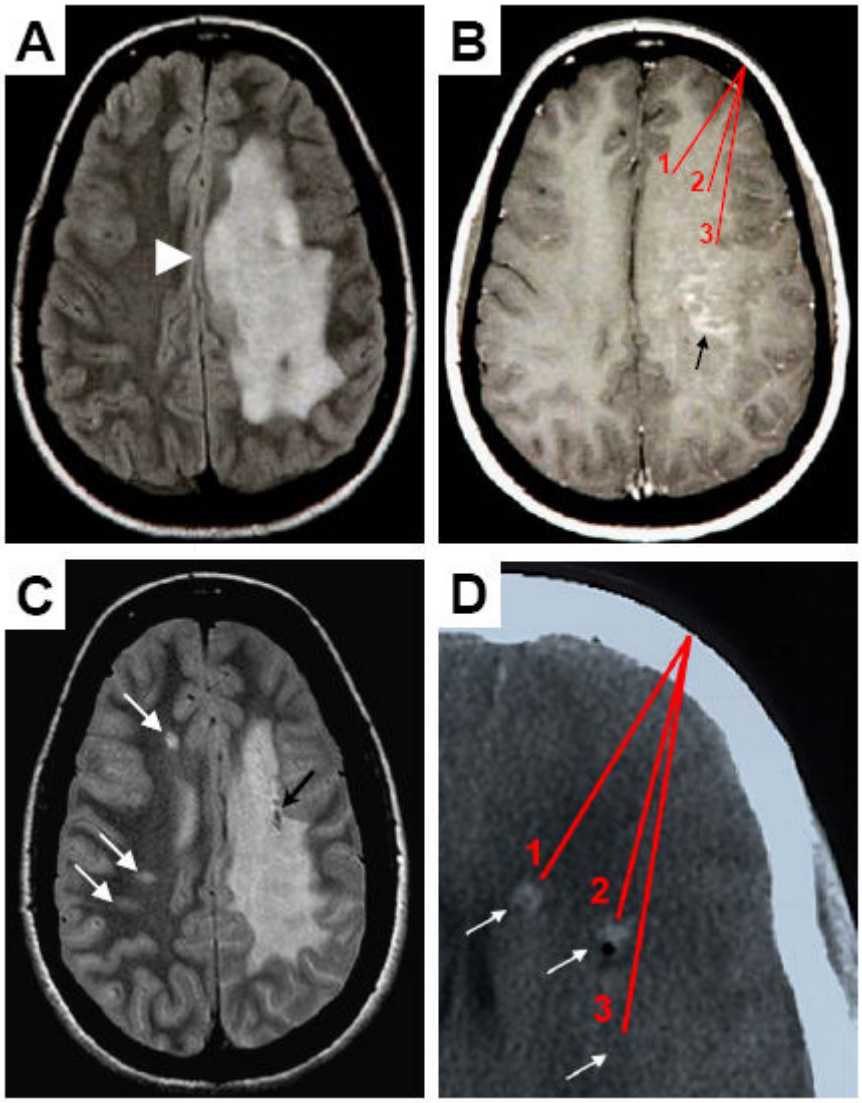

Figure 1: MRI analysis of the index patient

A T2-weighted MRI during the first manifestation displays a large confluent hyperintense lesion in the subcortical white matter of left hemisphere (A) with mass effect (arrowhead). Gadolinium enhanced T1-weighted MRI revealed a punctate, garland-like enhancement in the subcortical white matter (B, arrow). Red lines mark schematically the three target regions from which the stereotactic biopsy specimens were taken (B). A routine follow-up MRI investigation after 5 years revealed three new white matter lesions in the contralateral hemisphere (C, white arrows). Black arrow points to tissue destruction by one of the stereotactic brain biopsies (C). A CT picture (D) illustrates the three target regions of the biopsy specimens taken from the frontal subcortical white matter (arrows). Note, CT signal enhancement by bleedings caused by the excision of tissue is visible (D, arrows 1 and 2). Note, that the origin of all biopsy specimens was in regions of abnormal T1, T2 and CT signals, but only some biopsy specimens contained inflammatory demyelinating lesion, whereas others contained histomorphologically normal appearing white matter only.

The active demyelinating lesion reveals MS pattern III-like pathology

In one of the three formalin-fixed and paraffin-embedded biopsy specimens taken from target region 1 (Figure 1B, D), a demyelinating lesion was seen (Figure 2A) with perivascular lymphocytic cuffs (Figure 2A, inset arrow) and dense infiltrates of foamy macrophages (Figure 2B) containing MOG-positive myelin debris (Figure 2E, arrows) indicating early active demyelination. In contrast to myelin oligodendrocyte glycoprotein (MOG) and PLP (Figure 2C), the specific absence of myelin-associated glycoprotein (MAG, Figure 2D) was apparent. In addition, oligodendrocytes with dark and fragmented nuclei suggestive of apoptosis were found within the lesion, which was confirmed by TUNEL staining (Figure 2F, arrow). Altogether, this pathology is in agreement with a pattern III lesion type according to Lucchinetti et al.(Lucchinetti et al., 2000)

White matter with no signs of demyelination or inflammation was included in the biopsy

Histopathology of another formalin-fixed biopsy specimen, taken from the target region 2 (Figure 1B, D), showed normally myelinated white matter with signs of slight microglia activation, but no evidence of inflammatory infiltrates or active demyelination (data not shown). One additional fixed biopsy specimen of target region 3 showed a small very focal, sharp bordered lesion alternating with histopathologically normal appearing tissue without any inflammatory infiltrates (data not shown).

The two fresh snap frozen biopsy tissue specimens, taken from target regions 2 and 3 (Figure 1B,D), revealed white matter with no signs of inflammation or demyelination (Figure 2G,H). This was evidenced by homogenous expression of proteolipid protein (PLP, Figure 2G) and other myelin proteins. Perivascular lymphocytes were absent (Figure 2I, arrow points to a blood vessel), however, a number of CD68 positive microglia were detected throughout the tissue (Figure 2K). In addition, activated astrocytes were evidenced by increased immunoreactivity for glial fibrillary acid protein (GFAP, Figure 2L). Signs of microglia and astrocyte activation may correspond to hypointense alterations observed on T1-weigthed MRI as well as hyperintense alterations on T2-weighted MRI (Figure 1A).(Bruck et al., 1997; Fisher et al., 2007) These white matter tissues, revealing no signs of demyelination or

inflammation (Figure 2G-L) - although abnormal in MRI - were used for the qRT-PCR analysis.

Characterization of the non demyelinated white matter reveals high levels of nNOS expression and nitric oxide mediated damage of oligodendrocytes

To characterize earliest molecular changes in the non demyelinated white matter, we performed quantitative RT-PCR for selected genes (Table). As NO is suggested to play an important role in MS pathogenesis and lesion formation, we investigated the expression pattern of the three NO producing enzymes: nNOS, iNOS and eNOS. The most striking result was the high expression level of nNOS in the non demyelinated white matter of the biopsied patient compared to the low nNOS expression levels in subcortical white matter of control and NAWM of MS post-mortem brains. We could also detect slightly elevated levels of the expression of iNOS and eNOS (Table). Immunohistochemical analysis for iNOS revealed moderate expression in the non demyelinated white matter of the biopsied patient, most probably in microglia or astrocytes (Figure 2M, arrow). The low induction of iNOS expression was also evident by qRT-PCR (Table). In contrast, a high expression of iNOS was detected within the inflammatory lesion, where macrophages and activated microglia were strongly positive for iNOS (data not shown).

Staining of the non demyelinated white matter with an anti-nitrotyrosine antibody revealed an accumulation of nitrotyrosine on myelin and oligodendrocytes far-away from the actively demyelinating lesion (Figure 2N), pinpointing to a peroxynitrite-mediated damage to oligodendrocytes . However, apoptotic oligodendrocytes were only detected in the actively demyelinating lesion (Figure 2F).

Molecular analysis of the non demyelinated white matter suggests early changes in oligodendrocytes

Since MS pattern III pathology was postulated to reflect an oligodendrogliopathy, we analyzed oligodendrocyte specific genes involved in myelin maintenance. MOG and myelin basic protein (MBP) displayed comparable expression levels in the biopsied patient and in the subcortical white matter of post mortem brains from control and MS cases (Table). However, much higher expression levels of MAG and PLP were

detected in the non demyelinated white matter of the biopsied patient. Compared with the expression levels of both, old and young (C25) post-mortem control tissue, an upregulation of about four times was observed (Table). Furthermore, platelet-derived growth factor B (PDGFB), known to influence oligodendrocyte development,(Silberstein et al., 1996) was 9-fold upregulated in the biopsied patient. Analysis of genes implicated in oligodendrocyte development and known to be expressed by oligodendrocyte precursor cells such as platelet-derived growth factor receptor alpha (PDGFRα) and early growth response protein 1 (EGR1/Krox24), revealed also increased expression levels in the biopsied patient. However, a comparable expression level of PDGFRα was detected in the young control autopsy (C25) suggesting age-related regulation (Prof. Dr. R. Franklin, University of Cambridge, personal communication).

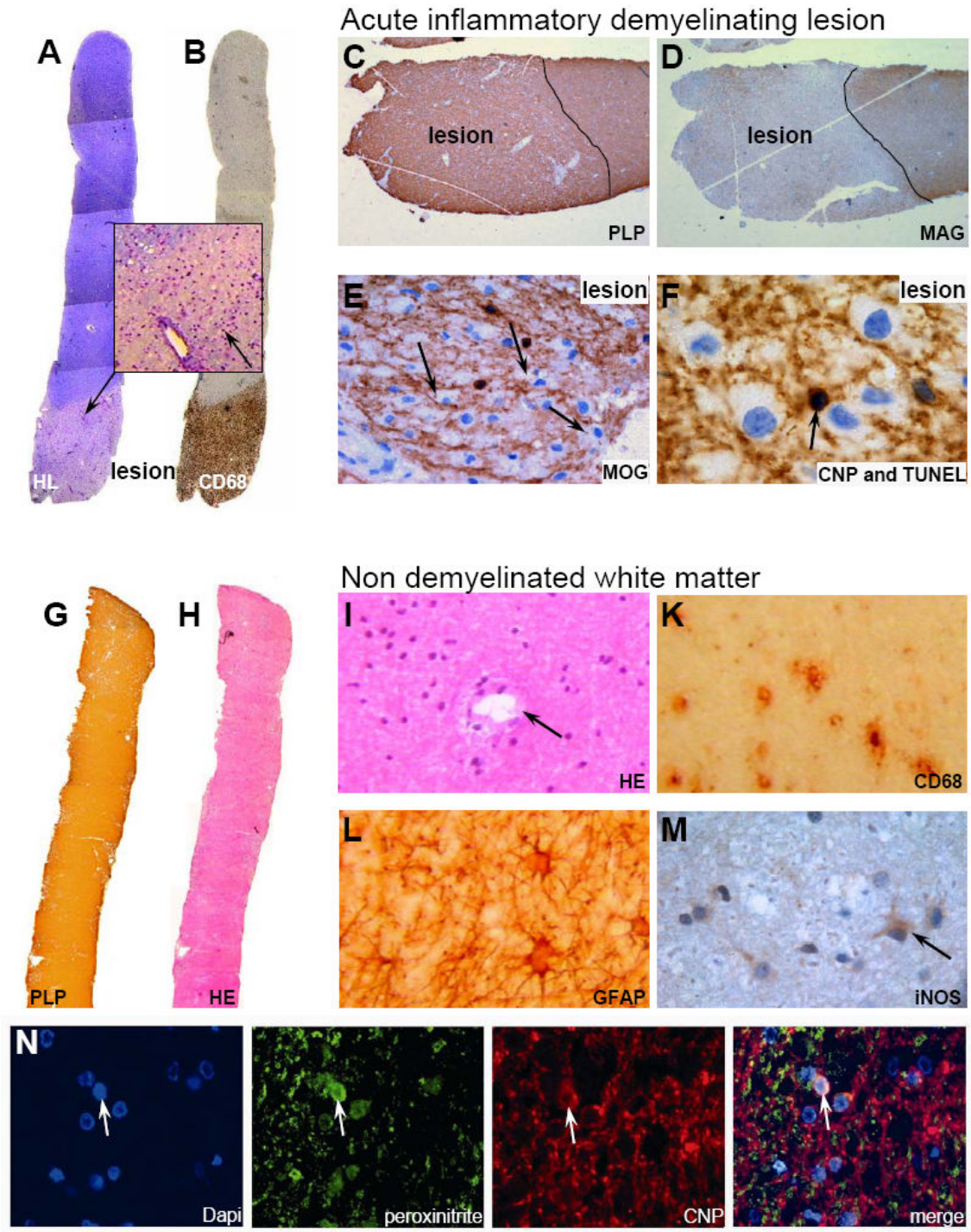

Figure 2: Immunohistochemical analysis of lesion and non demyelinated white matter tissue

Immunopathological examination of paraffin-embedded biopsies containing lesion tissue (A-F) revealed a relatively sharp bordered lesion by Holmes/Luxol staining (A) with a dense infiltrate of CD68-positive macrophages (B). Inset of (A) shows dense infiltrating cells around a blood vessel (arrow). Staining for PLP in the acute lesion still appeared normal (C), whereas a specific absence of MAG was detected (D). Furthermore, MOG-positive myelin debris was detected within macrophages (E, arrows). TUNEL-staining revealed apoptotic oligodendrocytes, co-stained by CNPase, within the lesion (F, arrow).

Examination of the fresh-frozen biopsies used for RNA analysis did not show any signs of infiltrates or lesion formation (G-M). Staining by anti-PLP showed normal myelin (G), haematoxilin-staining revealed normal cellularity (H) and blood vessels without infiltrating cells (I, arrow). However, a number of CD68-positive cells, histopathological microglia, were detected (K). Furthermore, activated astrocytes could be detected throughout the white matter (L). Staining for iNOS in non-demyelinated white matter distant to lesion revealed low expression levels in cells resembling microglia or astrocytes (M, arrow). Immunofluorescent colocalization for nitrotyrosine showed an accumulation in CNPase-positive oligodendrocytes (N, arrow).

High expression of Aquaporin 4 in non demyelinated white matter

As the MRI analysis showed alterations in the white matter of the left hemisphere, and immunohistochemical staining for GFAP revealed activated astrocytes, we investigated astrocyte-specific genes such as GFAP, Aquaporin 4 (AQP4) as well as the taurine transporter (TAUT) by qRT-PCR. Expression levels of AQP4 as well as GFAP were about 5 times higher than in the control cases (Table), further reflecting astrocyte activation and edema formation.

Induction of endogenous neuroprotective mechanisms in non demyelinated white matter

In an earlier publication we showed the upregulation of endogenous neuroprotective genes in the normal-appearing white matter of MS autopsy cases.(Graumann et al., 2003)[,](Zeis et al., 2008) Therefore, we selected specific protective genes for our analysis of the white matter of the present patient. In contrast to our previous study on post-mortem brain tissue, heme oxygenase 1 (HO-1) - protective against oxidative stress and known to be expressed in oligodendrocytes as well as astrocytes and microglia(Stahnke et al., 2007) - showed a six-fold upregulation in the white matter of the biopsy. On the other hand, the expression of HSP70.1, another protective protein which is induced mainly by heat-shock and not by oxidative stress,(Goldbaum and Richter-Landsberg, 2001) was not changed. Also, in the case of hypoxia-inducible transcription factor 1α (HIF1α), a key regulator in hypoxic preconditioning and upregulated in NAWM in chronic MS,(Graumann et al., 2003; Zeis et al., 2008) expression levels in the non demyelinated white matter of the biopsied patient were comparable to those from the control cases. Nevertheless, under acute hypoxic conditions, activation of HIF1α is fulfilled by stabilizing the protein rather than mRNA upregulation.(Dery et al., 2005) Indeed, the upregulation of vascular endothelial growth factor receptor (VEGFR), a downstream gene of HIF1α, indicates possible HIF1α activation.(Semenza, 2001) Altogether, the upregulation of HO-1 as well as VEGFR suggests an activation of protective mechanisms against oxidative stress in the non demyelinated white matter in pattern III MS.

Upregulation of anti- as well as pro-inflammatory genes in non demyelinated white matter

Autoimmunity plays a major role in the pathology of MS. We and others have recently suggested an involvement of innate immune mechanisms in MS.(Cannella and Raine, 2004; Zeis et al., 2008) We thus examined the expression of selected genes involved in innate inflammatory mechanisms, which we had already studied in post-mortem NAWM (Table).(Zeis et al., 2008) A major finding was the 18-fold increase in STAT6, an anti-inflammatory transcription factor which we found to be expressed by oligodendrocytes in the MS NAWM.(Zeis et al., 2008) The expression level of STAT4, a major pro-inflammatory transcription factor was also elevated in the biopsied patient, but the young control case showed even higher levels. HLA-DRA showed a seven-fold upregulation compared to the post-mortem control cases, and was not detectable in the young control case, which may correlate with the increased expression of iNOS, and possibly reflects the activation status of CD68-positive microglia (Figure 2D).

Discussion

This study reports for the first time earliest molecular changes in non demyelinated white matter during the first manifestation of an actively demyelinating pattern III MS case. Although all white matter tissue specimens were taken from brain areas showing abnormal MRI signals, histopathological examination of the specimens used for RNA extraction revealed white matter without any signs of inflammation or demyelination. As a major finding, we show that nNOS is strongly upregulated in non demyelinated white matter during a very early, acute phase of MS. As nNOS gene expression level of a young control autopsy case (C25) was comparable to that of post-mortem control cases, this upregulation most likely represents a disease specific mechanism and not an age-related artifact.

Recent data showed that nNOS plays a key role in mediating CNS demyelination in a toxin-induced demyelinating animal model.(Linares et al., 2006) The presence of NO is further supported by the finding of nitrotyrosine-positive oligodendrocytes, suggesting early NO-mediated oligodendrocyte damage. Thus, upregulation of nNOS in the non demyelinated white matter may reflect early brain intrinsic changes in pattern III MS preceding active demyelination. Although nNOS was highly expressed,

overt apoptotic oligodendrocytes were not detectable in the non demyelinated white matter, suggesting sublethal damage to oligodendrocytes not reaching the apoptotic threshold. Alternatively, oligodendrocyte death might be prevented by simultaneously induced protective mechanisms.(Zeis et al., 2008) This is consistent with our observation of the upregulation of heme oxygenase HO-1, which is known to be protective against NO-mediated damage.(Reiter et al., 2006) Ongoing alterations in oligodendrocytes were further indicated by the upregulation of PLP and MAG mRNA, which could reflect stress of oligodendrocytes, or might be specific for pattern III MS. The upregulation of particular genes in oligodendrocytes, such as STAT6, MAG or PLP, might further strengthen the assumption of brain intrinsic events preceding lesion formation; e.g. impairment of oligodendrocyte and/or neuronal dysfunction, because these alterations in gene expression are present in the white matter distant from actively inflammatory, demyelinating lesions. Eventually, these alterations might then lead to, facilitate or impede lesion formation. Barnett and Prineas suggested widespread oligodendrocyte damage and apoptosis as one of the earliest change in lesion formation,(Barnett and Prineas, 2004), which would fit our observation. Whether this is specific for a subset of MS patients (pattern III lesions) or a general phenomenon in MS pathology needs to be further elucidated.

Another explanation for nNOS expression in the non demyelinated white matter might be activated astrocytes, as these cells were shown to increase expression of nNOS under pathological conditions.(Catania et al., 2001; Kim et al., 2000) Activation of astrocytes is also evident by the upregulation of GFAP, AQP4 and TAUT. Nevertheless, the inducible isoform of NOS (iNOS) was only moderately upregulated, additionally supporting the concept that rather brain intrinsic changes than primary immune reactions are causative for of the observed differential gene expression in the non demyelinated white matter,

Although abnormalities in a large area of the left hemisphere have been detected on T1 and T2 weighted MRI, only a small part of the biopsy specimens contained areas of inflammatory demyelination. We conclude from our study that even in large T1 and T2 abnormalities there remains non demyelinated white matter without overt signs of inflammation . This is in agreement with a study from Fisher et al., 2007 showing that T1 and T2 MRI abnormalities do not necessarily imply demyelination and lesion formation.(Fisher et al., 2007) Still, our data indicate activated microglia, astrogliosis

and edema formation, which may not yet reach a pathological level leading to tissue destruction, but disclose intrinsic molecular alterations.

Taken together our study suggests that earliest molecular changes are present in the non demyelinated white matter distant from an active inflammatory demyelinating lesion. The changes in the non demyelinated white matter might be crucial for lesion initiation and the further development of the disease, determining lesion progression or limitation. Therefore, therapeutical modulation of these alterations in the white matter might be an important target for the prevention of tissue damage in MS.

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Molecular Changes in normal appearing tissue in an animal model of MS

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Several studies suggest that additional to the focal lesions occurring in MS there are diffuse inflammatory and degenerative alterations in the CNS. We investigated whether in an animal model for MS, namely EAE similar changes take place. We isolated normal appearing white and grey matter from the corpus callosum and the somatosensory cortex of DA rats with rMOG-induced EAE and performed gene expression analysis. Analysis after 60 days of clinical course revealed only minor changes in the corpus callosum of EAE rats. But, in the somatosensory cortex a number of gene expression alterations could be identified, although on the morphological and cellular alterations were not evident. One of the most striking observations is the downregulation of genes involved in mitochondrial function as well as a whole set of genes coding for different glutamate receptors. Our data suggest that molecular alterations in neurons far distant to inflammatory demyelinating lesions are evident; some may reflect degenerative processes induced by lesion mediated axonal injury in the spinal cord. These results indicate that the MOG-induced EAE in DA rats is a valuable model to analyze neuronal alterations due to axonal impairment in an acute phase of a MS-like disease, and could be use for development of neuroprotective strategies.

Keywords: EAE, neuroprotection, inflammation, MS, NAWM, NAGM

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Currently, it is thought that MS is an autoimmune disorder directed against CNS antigens which leads to inflammation and demyelination (Hemmer et al., 2002; Noseworthy et al., 2000). A major characteristic of MS is the formation of the so-called plaques or lesions, areas of neuronal damage and demyelination (Lassmann, 1998). Studies of the histopathology of the demyelinated lesions revealed a great deal of heterogeneity in the mechanism of their formation (Lassmann et al., 2001; Lucchinetti et al., 2000; Raine and Scheinberg, 1988) for review see Lassmann et al., 2001), which might to some extent reflect the diversity of clinical disease courses of MS. Studies of the so-called normal appearing white matter (NAWM) by magnetic transfer imaging (MTI) revealed indeed abnormalities already occurring before lesion formation (Filippi et al., 1998). Further imaging studies reported early changes in the NAWM of MS patients which include axonal damage or loss (Filippi et al., 2003; Fu et al., 1998) and blood-brain-barrier (BBB) alterations (Silver et al., 2001; Werring et al., 2000). Other studies reported diffuse axonal injury with microglia activation (Kutzelnigg et al., 2005), or differential regulation of specific proteins or enzymes (Allen and McKeown, 1979; Sinclair et al., 2005). In line with these findings, recent studies from our lab revealed an upregulation of a number of functionally related genes involved in endogenous neuroprotection (Graumann et al., 2003). In particular, hypoxia inducible factor 1α (HIF-1α) and some of its targets genes such as e.g. vascular endothelial growth factor 1 (VEGFR1) were shown to be upregulated in most MS cases, possibly reflecting an adaptation of cells to the chronic progressive pathophysiology of MS. In another study, we have shown the upregulation of the STAT6 signaling pathway and an overall inflammatory reaction through the NAWM of MS brains (Zeis et al., 2008). As the majority of cells expressing STAT6 in NAWM were oligodendrocytes, we suggested that oligodendrocytes are actively participating in endogenous inflammatory reactions of MS brains (Zeis et al., 2008; Zeis and Schaeren-Wiemers, 2008). In summary, normal appearing white matter in MS shows a wide range of abnormalities which might influence the pathogenesis of MS.

One of the most widely used models to mimic certain aspects of MS is experimental autoimmune encephalomyelitis (EAE). There are several forms of EAE which can be

induced in different species and strains by immunization with different antigens and by using various immunization protocols (Gold et al., 2000). One of the models sharing major features of MS such as a relapsing-remitting disease course and demyelination is the MOG-induced EAE in DA rats (Storch et al., 1998b). Depending on the mode of induction of EAE in DA rats, a variable quantity of different disease courses such as chronic relapsing, chronic progressive, relapsing remitting or acute progressive can develop (Kinter et al., 2008; Storch et al., 1998b). Unlike in other EAE models, inflammatory demyelinating lesions are not only induced by T-cell mediated immune reactions, but also mediated by anti-MOG antibody response. Therefore, EAE in DA rats is controlled by genetic factors, regulating T- and B- cell responses, and by sensitization, differentially stimulating T- or B-cell responses (Storch et al., 1998b).

Many features of MS can be mimicked in EAE. We investigated whether in EAE similar diffuse alterations in normal appearing tissue as seen in MS take place. Therefore, we isolated normal appearing white and grey matter from the corpus callosum and the somatosensory cortex of EAE and control rats and performed gene expression analysis.

2. Materials and Methods

2.1 Expression of Recombinant Mouse MOG

For the expression of recombinant rat MOG, the bacterial expression vector pRSET A (Invitrogen Corp.) was used containing the amino acids 1-125 of the mature rat protein fused to several histidine residues. An overnight culture of a transformed E.coli Bl21 strain was used for inoculation of a large expression culture (SOB, ampicillin, kanamycin). The OD600 was measured until it reaches 0.5 and expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside at 1 mM final concentration. After 4 h the bacteria were harvested by centrifugation (15 min, 4,000 g). The pellet was then frozen and stored until purification was performed.

2.2 Purification of HIS-Tagged MOG

For immobilized metal ion affinity chromatography, the Talon purification system (Clontech) was used. The bacterial pellet was resuspended in lysis buffer (8 M urea, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 8) and sonicated to disrupt the bacteria. After a further centrifugation (20 min, 10,000 g), the pellet was again resuspended in lysis buffer and the centrifugation step was repeated. Both supernatants were pooled and subjected on the immobilized metal ion affinity chromatography column for purification at room temperature. After loading, the column was washed with 2 vol lysis buffer and 2 vol washing buffer (8 M urea, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 6.3). The purified recombinant protein was collected by eluting the column with elution buffer (8 M urea, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 4.5). To obtain soluble recombinant MOG, the purified protein was dialyzed 4 times (dilution factor 1:200 each) against 20 mM sodium acetate buffer (pH 3.6) at 4°C. Finally, the purified and soluble protein was concentrated (Centricon, 10,000 MWCO) until the protein concentration was at least 2 mg/ml. The protein was aliquoted and stored at -80°C.

2.3 Induction of EAE

EAE induction was made as described before (Storch et al., 1998b). For induction of EAE 50µg of rMOG emulsified with incomplete Freud's adjuvant (IFA) (Difco Laboratories, Detroit, MI) was injected into inbred adult female DA rats (10-12 weeks, from Harlan, Netherlands). Injections were given intradermally in the dorsal aspect of the base of the tail. A group of control rats were injected with saline, emulsified with

an equal volume of IFA. The clinical progress of the disease was monitored daily. Rats were weighted and their neurological deficits were scored according the standard protocol (Storch et al., 1998b).

2.4 Immunohistochemistry

Cryostat sections (10μm) used for tissue characterization using anti-CD68, -PLP and -GFAP antibodies were fixed for 10min in 10% formalin. For inactivation of endogenous peroxidase all sections were treated with 0.3% hydrogen peroxide and blocked with blocking buffer (1% normal donkey serum, 2% Fish skin gelatin, 0.15% Triton). After quenching, sections were incubated with primary antibodies overnight at 4°C. Secondary biotinylated antibodies were applied for 1 hour at room temperature followed by the ABC complex reagent (Vector Labs) for 1 hour. Color reaction was performed with 3-Amino-9-ethylcarbazole (Erne et al., 2002). Counterstaining was performed in haematoxylin for 1min followed by rinsing the slide in running tap water. For histochemical analysis the following antibodies were used: anti-PLP (AbD serotec Cat.Nr. MCA839G), anti-Monocytes/Macrophages (CD68; Chemicon, Cat.Nr. MAB1435) and anti-GFAP (Sigma, Cat.Nr. G3893).

Table 1: Primer sequences used for qRT-PCR

Primer Name	Gene accession number	Sequence
5' Rpl13a	NM_173340.2	5'-GCCATTGTGGCCAAGCAGGT-3'
3' Rpl13a		5'-GTAGGCTTCAGCCGCACAAC-3'
5' Rpl19	NM_031103.1	5'-TCGCCAATGCCAACTCTC-3'
3' Rpl19		5'-ACCCTTCCTCTTCCCTATGC-3'
5' Rps9	NM_031108.1	5'-CCGCACGATGCCTGGAGTTA-3'
3' Rps9		5'-TGCACCACCACGGAGGTACA-3'
5' Hif1a	NM_024359.1	5'-CTGTCACTGCCACCGCAACT-3'
3' Hif1a		5'-TGGTGAGGCTGTCCGACTGT-3'
5' Hmox1	NM_012580.2	5'-CTGGTGATGGCCTCCTTGTA-3'
3' Hmox1		5'-ACCAGCAGCTCAGGATGAGT-3'
5' Ogg1	NM_030870.1	5'-AGGTGTGAGGCTGCTGAGAC-3'
3' Ogg1		5'-AGGCCCAACTTCCTGAGGTG-3'
5' Flt1	NM_019306.1	5'-GAGATGCACAGTGACATACC-3'
3' Flt1		5'-TTCTCTATCCTGACGACTGG-3'
5' Ywhah	NM_013052.1	5'-TTGAGAAGGAGCTGGAGACA-3'
3' Ywhah		5'-CCTTATACGCTGCCTCAGAA-3'
5' Aqp4	NM_012825.1	5'-GCATGAATCCAGCTCGATCC-3'
3' Aqp4		5'-TGTCCTCCACCTCCATGTAG-3'
5' Bcl2l2	NM_021850.2	5'-TTCCGGCGCACCTTCTCTGA-3'
3' Bcl2l2		5'-GCCAAGCGTGTCTCCAGGTA-3'
5' Ctgf	NM_022266.2	5'-CAGGCTGGAGAAGCAGAGTC-3'
3' Ctgf		5'-GGCAGGCACAGGTCTTGATG-3'
5' Sod1	NM_017050.1	5'-TTTCTCGTGGACCACCATAG-3'
3' Sod1		5'-TACACAAGGCTGTACCACTG-3'
5' Nos1	NM_052799.1	5'-AGTCATGTTTGCCGTCAGTC-3'
3' Nos1		5'-TTGAAAGCACCAGCACCTAC-3'
5' Nos2	NM_012611.2	5'-TTCTGTGCTAATGCGGAAGGTC-3'
3' Nos2		5'-TTCTTCAGAGTCTGCCCATTGC-3'
5' C2ta	NM_053529.1	5'-GCCTGAGCAAGGACCTCTTC-3'
3' C2ta		5'-GGCATCTCACCGTGGTAGAC-3'
5' Eno2	NM_139325.1	5'-CCTCTATCGCCACATTGCTC-3'
3' Eno2		5'-CATCCTTGCCGTACTTGTCC-3'
5' Pik3c3	NM_022958.2	5'-GGAGACCGGCACCTGGATAA-3'
3' Pik3c3		5'-AATGTGCCGGAAGGAGGTGG-3'
5' Slc6a6	NM_017206.1	5'-TCGCGCTCTGCCTCCTCTTA-3'
3' Slc6a6		5'-TTCCAGCGTCGATCCACACC-3'
5' Stat4	NM_001012226.1	5'-AGGTCGGGTTTCCAAAGAGA-3'
3' Stat4		5'-ATGGCAGCCACTTTGTGTTC-3'
5' Stat6	XM_343223.3	5'-GGTGTCCTGGACCTCACTAA-3'
3' Stat6		5'-TCTATCTCTTGTAGGTCGGC-3'

Table shows the 5' and 3' primers used for quantitative RT-PCR analysis. The genes 40s S9, 60s L19 and 60s L13A were used for normalization.

2.5 Total RNA purification and quantitative RT-PCR analysis

To achieve the highest tissue comparability between our analysis of human subcortical NAWM (Graumann et al., 2003), and this analysis, we isolated brain tissue far away from inflammatory lesions, that are predominantly located in the spinal cord. As a corresponding tissue to MS NAWM we dissected white matter from the corpus callosum (Fig.1A, cc). Additionally, cortical grey matter from the somatosensory cortex which contains pyramidal neurons projecting into the spinal cord was isolated (Fig.1A, cx). Total RNA was isolated using the RNeasy lipid tissue kit (Qiagen). cDNA was generated using the Superscript II reverse transcriptase (Invitrogen). Real-time RT-PCR was performed using the LightCycler system (Roche). Primer sequences were designed from unique site over exon-intron junctions to prevent amplification of genomic DNA. Real-time RT-PCR was performed according to the manufacturer's protocol (Roche). RNA amounts were calculated with relative standard curves for all mRNAs of interest and 60s ribosomal protein L13A, 40s ribosomal protein S9 and ribosomal protein L19 were used for normalization. Primer sequences used for qRT-PCR are shown in Table 1.

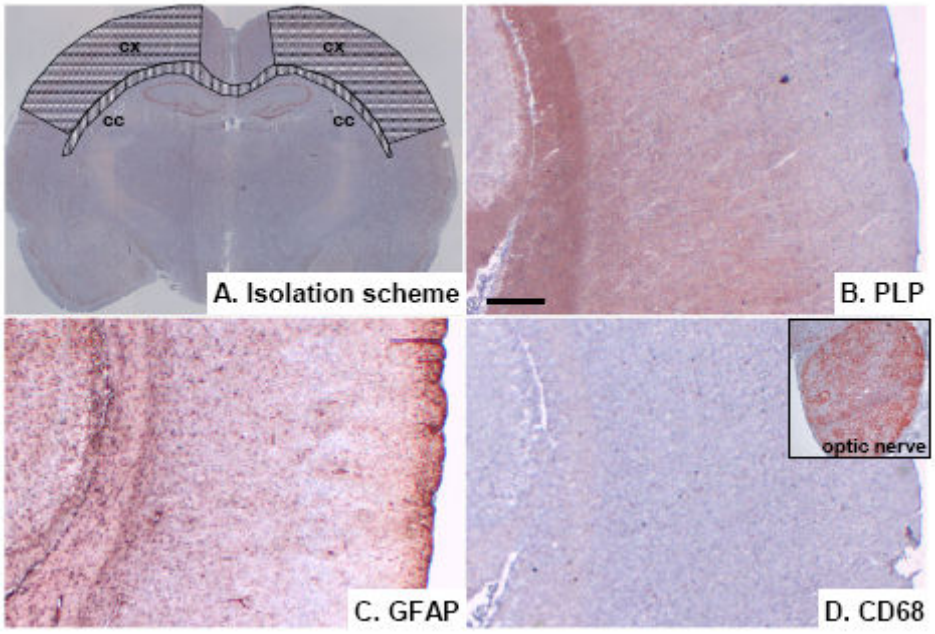

Figure 1: Histopathological examination of corpus callosum and cortex tissue.

To analyse gene expression changes in EAE NAWM from corpus callosum (cc) as well as EAE NAGM from the somatosensory cortex (cx) we isolated NAWM and NAGM tissue from DA rat brains (A, hatched areas). Immunohistochemical analyses were made to exclude possible inflammatory infiltrates or demyelination. Tissue stained by PLP (B) showed normal myelin. Stainings for GFAP (C) revealed normal astrocytes. No inflammatory infiltrates were detected by staining for CD68-positive cells (D). Bar=200μm

2.6 cDNA Expression Array hybridization

The Clontech Atlas™ Rat 1.2 cDNA Expression Array contains 1'176 selected cDNA sequences arrayed on a nylon membrane. Array hybridization was performed according to Graumann et al., (2003). Array was hybridized with 3 control and 7 EAE grey matter samples. Quantification of differential hybridization signal intensities was achieved with the AtlasImage™ 2.0 software program. Hybridization signals which in the average were below 2500 pixels were excluded from our analysis as background values range from about 700 to about 2000 pixels. Therefore, 580 cDNA sequences were included in our analysis.

2.7 Animal grouping

In a first step, the gene expression of all diseased animals was compared to the control group. Afterwards, as the human cases analyzed in Graumann et al., 2003 were suffering from MS in average for over 25 years, the animal samples were divided into two groups for analysis. Animals were sorted according to their clinical course. Animals showing a disease course characterized by a primary relapse with a secondary chronic phase were assigned to the "Chronic" group, which is most reflecting the situation of MS patients analyzed. All other animals showing miscellaneous clinical courses such as short acute attacks with death in a few days or primary relapse with secondary long-term remission were assigned to the "Variable" group (Table 2, Fig. 1). This was done as no correlation of gene expression was detected if compared with disease onset, duration, severity and type.

2.8 Normalization and Statistics

Normalization of calculated RNA amounts by qRT-PCR was done by using 60s ribosomal protein L13 (NM_031101.1), 40s ribosomal protein S9 (NM_031108.1) and ribosomal protein L19 (NM_031103.1). Using these three ribosomal genes, a geometric mean was calculated to which all target genes were normalized. Such a normalization strategy, recently proposed, showed to be more accurate for qRT-PCR normalization rather than using single-gene normalization (Vandesompele et al., 2002). Statistical significance was then calculated by comparison of the normalized raw data from control animals to EAE animals, and is expressed by *P*-values generated by the non-parametric Mann-Whitney U-Test.

Normalization of the Clontech Atlas™ Rat 1.2 cDNA Expression Array was performed by using 60s ribosomal protein L13 and ribosomal protein L19 but not 40s ribosomal protein S9 as 40s ribosomal protein S9 was not present on the array. The normalized hybridization intensities from the EAE animals were then compared to the corresponding ones from the control animals to provide fold changes. Statistical significance was calculated by comparison of the normalized raw data from control animals to EAE animals, and is expressed by *P*-values generated by the non-parametric Mann-Whitney U-Test.

Table 2: Analyzed animals

Animal	Disease Type	Disease Duration	Avg. Disease Score	Group
EAE - 1	Chronic	46d	2.91	Chronic
EAE - 2	Chronic	15d	2.67	Chronic
EAE - 3	Chronic	31d	2.81	Chronic
EAE - 4	Chronic	14d	2.73	Chronic
EAE - 5	Chronic	21d	3.00	Chronic
EAE - 6	Chronic	47d	1.90	Chronic
EAE - 7	Chronic	47d	2.56	Chronic
EAE - 8	Chronic	44d	2.66	Chronic
EAE - 9	Chronic	28d	2.79	Chronic
EAE - 10	Chronic	22d	2.80	Chronic
EAE - 11	RR	46d	1.54	Variable
EAE - 12	RR	33d	1.34	Variable
EAE - 13	RR, Acute	16d	2.37	Variable
EAE - 14	Acute	8d	2.87	Variable
EAE - 15	Acute	6d	3.25	Variable
EAE - 16	no disease	-	-	Variable
EAE - 17	no disease	-	-	Variable

Animals were grouped according to their clinical course. A majority of animals analyzed showed a chronic disease course. These animals were grouped together in the “Chronic” group. Additionally, two animals with an acute progressive and three animals with a relapsing-remitting disease course were analyzed. Furthermore, two animals without any detectable clinical disease were analyzed. As animals showing acute progressive, relapsing-remitting or no disease showed no consistent gene expression changes if compared with the control group and no correlations could be found, these animals were grouped together in the “Variable” group. Additionally, the table shows the average clinical score and the average disease duration of the animals until they were sacrificed.

3. Results

3.1 Clinical course of EAE

DA rats induced with EAE developed different clinical courses (Fig. 2). About 20% of the animals never developed obvious neurological signs. 40% of all animals showed a chronic course, sometimes with one relapse. 20% of the animals develop a relapsing-remitting clinical course, whereas the remaining 20% show a short, acute disease course (Fig. 2). Inflammatory infiltrates and demyelination are predominantly located in the spinal cord and the optical nerve. Nevertheless, inflammatory infiltrates can also develop in brain tissue such as corpus callosum. Therefore, immunohistochemical analysis for PLP, GFAP and CD68 were made to exclude possible inflammatory lesions present in the isolated tissue (Fig.1B, C and D).

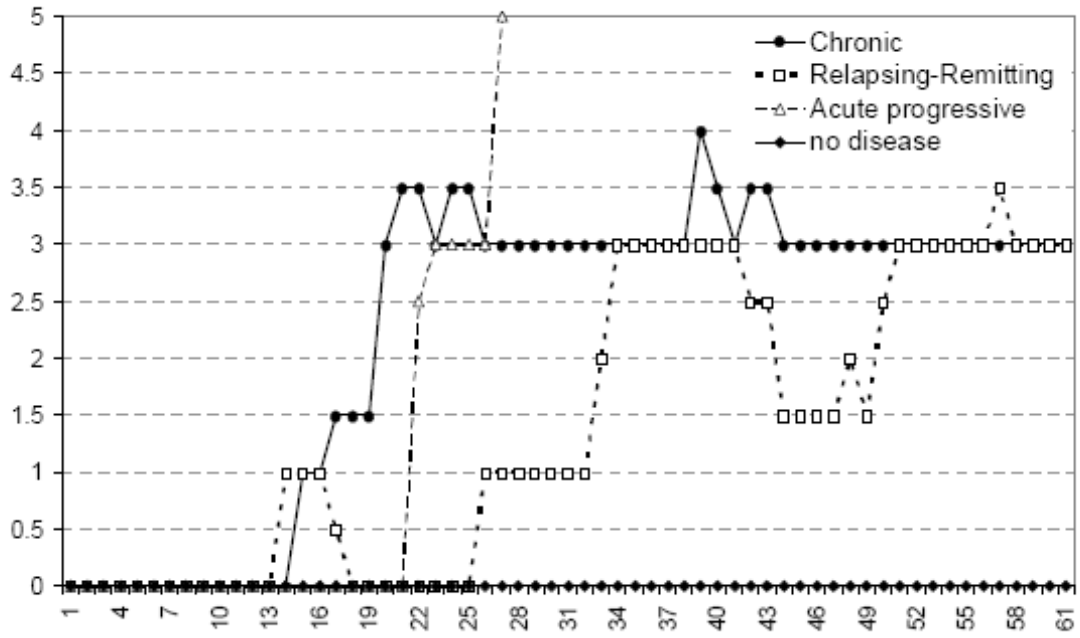

Figure 2: Different clinical disease courses observed in MOG-induced EAE in DA rats

Examples of different clinical courses observed. Most animals showed a chronic disease course mostly similar to the one showed. Other disease courses observed were acute progressive, relapsing-remitting. In some animals no disease could be scored.

3.2 Most of the selected genes related to ischemic preconditioning show no expression changes in the normal appearing white and grey matter

To test the hypothesis, if in experimental autoimmune encephalomyelitis comparable mechanisms of ischemic preconditioning take place as in MS, the expression of a set of genes involved in ischemic preconditioning and reported to be elevated in MS NAWM (Graumann et al., 2003) were investigated. In a first step, we have analyzed differential gene expression of hypoxia inducible factor 1α (HIF-1α), heme oxygenase 1 (HO-1), 8-oxoguanine DNA glycosylase (OGG1) and vascular endothelial factor receptor (VEGFR, Fig. 3). HIF-1α, a key regulator of hypoxia-induced gene regulation, showed no significant expression changes, neither in NAWM nor in NAGM. In contrast, the expression of HO-1, which can be induced among others by oxidative stress or cytokines, was changed. Whereas HO-1 was not significantly changed in the NAWM, there was a significant downregulation of HO-1 in the NAGM of all EAE animals ($p=0.035$), when compared to controls. If animals were sorted according to their disease pattern, a significant downregulation of HO-1 was detected in the “Chronic” group ($p=0.045$) but not in the “Variable” group ($p=0.151$). OGG1, a DNA repair enzyme, which was reported to be activated upon oxidative stress, showed high variations in the white matter of control as well as EAE animals. In the grey matter of control animals, OGG1 was rather homogeneously expressed, whereas in the NAGM OGG1 expression varied strongly from animal to animal. In the case of VEGFR, a downstream gene of HIF-1α and also known to be involved in ischemic preconditioning, no significant changes in gene expression of the NAWM or NAGM could be detected.

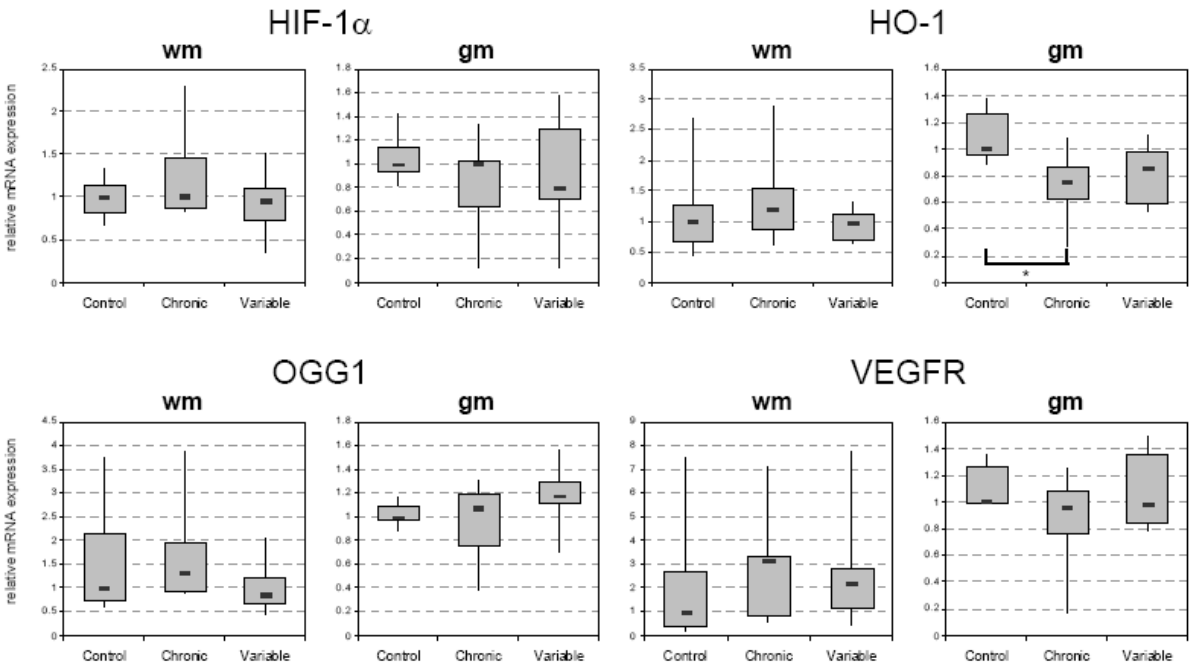

Figure 3: Boxplots of selected genes known to be involved in ischemic preconditioning mechanisms
Boxplots of selected differentially expressed genes analyzed by quantitative RT-PCR. Boxplots show differential gene expression in Control, "Chronic" and "Variable" groups. Values are shown proportionally to the control group. Genes showing a significant changed expression ($p<0.05$) was marked by asterisks.

In Graumann et al., 2003 we also showed to upregulation of BCL2-like 2 (BCL2L2) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein eta (YWHAH) which are both involved in cellular processes such as apoptosis and survival. In contrast to human NAWM, Ywhah shows no significant changes in EAE NAWM and NAGM (Table 3). Nevertheless, in the EAE NAWM, Ywhah shows a tendency to be downregulated in the „Variable" group ($p=0.051$). In the case of Bcl2l2, also no changes could be detected in the EAE NAWM (Table 3). Another gene which is involved in ischemic preconditioning is connective tissue growth factor (Ctgf). Therefore, we have checked whether the expression of Ctgf is changed in the NAWM and NAGM tissue, but no significant changes could be detected (Table 3). In order to detect blood-brain-barrier changes possibly induced by ischemic mechanisms, we have investigated the expression of Aquaporin4 (Aqp4), a major water channel in the brain. Though, no significant changes in Aqp4 could be detected, neither in EAE NAWM nor in NAGM tissue. Taurine transporter (Solute carrier family (neurotransmitter transporter, taurine), member 6), which we

investigated as an indicator of hypoxic insults and which was also upregulated in human NAWM, showed also no significant changes in EAE NAGM (Table 3). To investigate neuronal reactions upon the induction of EAE, we have analyzed the expression of enolase 2, gamma (Eno2). We found a slight tendency of Eno2 to be downregulated in the EAE NAGM of the „chronic“ group (p=0.065). Nevertheless, no significant changes could be detected in the „variable“ group and neither if all EAE animals were compared with controls (Table 3). In our previous study from Graumann et al., 2003, we showed an upregulation of several genes from the PI-3 Kinase/Akt pathway, which are also involved in ischemic preconditioning. Therefore, we investigated the expression of the phosphoinositide-3-kinase, class 3 (Pik3c3) as a marker for this pathway, but no significant gene expression changes have been detected (Table 3). We also analyzed Superoxide dismutase 1 (Sod1), shown to be protective after transient focal ischemia. We could detect a significant downregulation of Sod1 in the EAE NAWM of the „variable“ group (p=0.035, Table 3). However, no significant changes were found if comparing all animals. Also, no significant expression changes were detected in the „chronic“ group (Table 3). In another study (Zeis et al., 2008, *submitted*), we have detected a strong upregulation of the neuronal nitric oxide synthase (nNOS) in non demyelinated white matter in a very early case of MS. Therefore, we have investigated the expression of nNOS in EAE NAWM as well as NAGM. Though, no significant gene expression changes were detected (Table 3).
In summary, we detected only minor changes in gene expression of ischemic preconditioning-related genes in the NAWM of the corpus callosum and the NAGM of the somatosensory cortex.

Table 3 Gene expression changes of additional genes analyzed

Normal appearing white matter

Gene name	Chronic				Variable			
	Median	1st Quartile	3rd Quartile	p-value	Median	1st Quartile	3rd Quartile	p-value
14-3-3e	0.93	0.86	1.20	0.428	0.80	0.53	0.85	**0.051 ***
AQP4	1.35	0.83	1.80	0.368	1.19	0.84	1.66	0.628
bcl-w	0.89	0.85	1.02	0.792	0.87	0.80	0.90	0.181
CTGF	0.93	0.73	1.17	0.635	0.69	0.62	0.98	0.295
nNOS	1.03	0.79	1.39	0.635	0.95	0.66	1.16	0.628
SOD	0.77	0.70	1.06	0.492	0.65	0.53	0.86	***0.035 ****

Normal appearing grey matter

Gene name	Chronic				Variable			
	Median	1st Quartile	3rd Quartile	p-value	Median	1st Quartile	3rd Quartile	p-value
14-3-3e	0.74	0.57	1.02	0.171	0.91	0.88	0.91	0.548
AQP4	0.92	0.73	1.06	0.284	1.12	0.86	1.28	1.000
CTGF	0.86	0.68	1.20	0.622	0.89	0.86	1.23	0.841
nNOS	1.08	0.69	1.21	0.724	1.36	0.84	1.40	1.000
NSE	0.91	0.77	1.00	**0.065 ***	1.01	0.88	1.17	0.841
PI3K	1.02	0.83	1.15	0.354	1.11	1.05	1.36	0.421
SOD	0.95	0.82	1.06	0.524	1.11	1.05	1.11	0.690
TAUT	0.87	0.59	1.01	0.354	0.95	0.88	1.01	0.841

Table shows the median of gene expression from NAWM and NAGM compared to control tissue of genes not presented by Boxplots. Grade of variance is shown by the 1st and 3rd quartile. Values are shown proportionally to the control group. Statistical significance is expressed as p-values generated by the non-parametric Mann-Whitney U-test. P-values below 0.05 are printed in bold, italic numbers, whereas P-values between 0.05 and 0.1 are shown in bold numbers.

3.3 Quantitative RT-PCR analysis of selected genes related to inflammation

In our studies of MS NAWM tissue, we found differential regulation of genes related to inflammation, in particular of the JAK/STAT signaling pathway. To identify whether these alterations of inflammatory related genes also occur in EAE normal appearing white and grey matter distant to the acute inflammatory lesion, we investigated the expression pattern of CIITA, iNOS, STAT4 and STAT6 (Fig. 4). To detect an overall immune-related activation in the white matter of the corpus callosum as well as in the grey matter of the somatosensory cortex, we investigated the expression of CIITA.

CIITA, a major regulator of MHC class II gene expression, showed a slight elevation in its expression in the corpus callosum whereas in grey matter the expression of CIITA was a little lower in chronic EAE animals than in controls (p=0.127, Fig. 4). Recent publications showed that inhibition of nitric oxide (NO) production by blocking inducible nitric oxide synthase (iNOS) has a positive effect on the disease course of EAE. Therefore, we investigated the expression of iNOS as a marker of possible low-level microglia and/or astrocyte activation. In white as well as grey matter, we could not detect any significant changes in iNOS expression. Nevertheless, in both tissues there was a slight increase of iNOS expression (Fig. 4). As overall markers for brain intrinsic pro- or anti-inflammatory changes, we decided to investigate the expression of STAT4 and STAT6. STAT4, a pro-inflammatory transcription factor, among others involved in Th1 cell differentiation, was not significantly changed in the white matter of the corpus callosum (Fig.4). In contrast, a significant decrease of STAT4 was detected in the NAGM if expression of all animals was compared to controls (p=0.007, Fig. 4). Furthermore, there was a significant downregulation of STAT4 in the chronic group (p=0.002). In contrast, no significant downregulation could be detected in the variable EAE group (p=0.222). STAT6, an anti-inflammatory transcription factor among others involved in Th2 cell differentiation, showed also no significant changes in normal appearing white matter (Fig. 4). Again, a significant decrease in STAT6 expression was present in the NAGM; but, only in the chronic group (p=0.045, Fig. 4). If the expression of all EAE animals were compared to those of controls, no significant change could be shown (p=0.075). In the variable group there was an even less significant downregulation of STAT6 (p=0.421). Altogether, we show that neither gene expression analyzed is changed in the EAE NAWM of the corpus callosum, whereas STAT4 and STAT6 are downregulated in the NAGM of the somatosensory cortex.

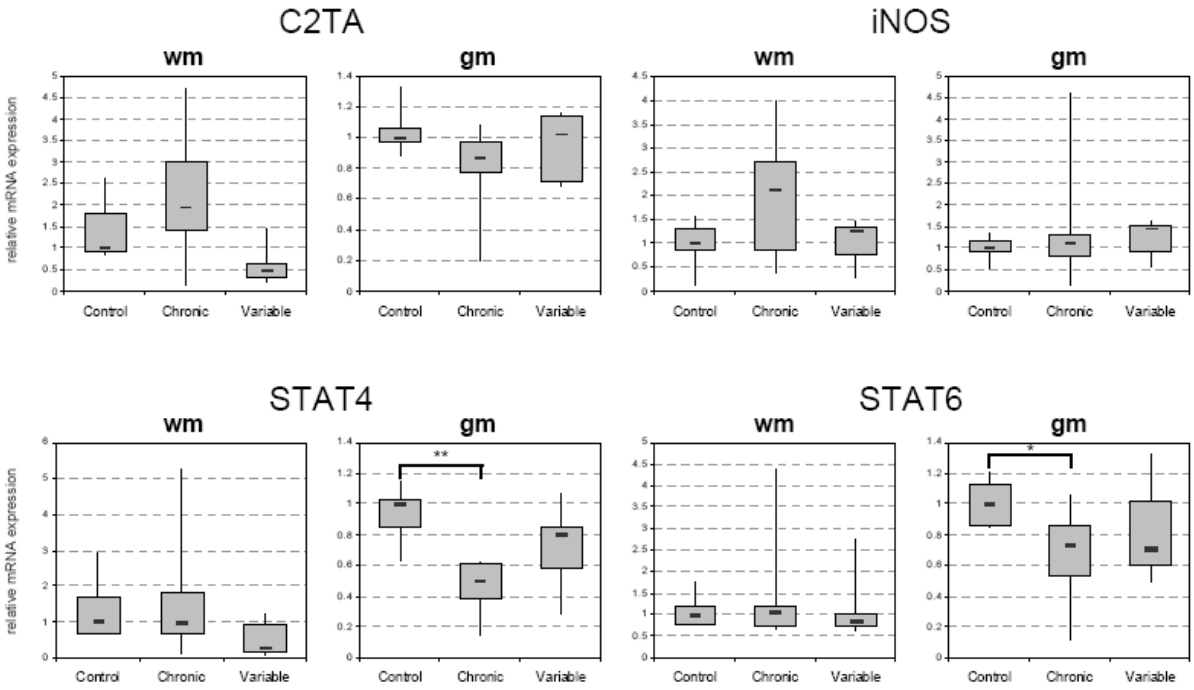

Figure 4: Boxplots of selected genes known to be involved in inflammatory mechanisms

Boxplots of selected differentially expressed genes analyzed by quantitative RT-PCR. Boxplots show differential gene expression in Control, "Chronic" and "Variable" groups. Values are shown proportionally to the control group. Genes showing a significant changed expression ($p<0.05$) was marked by asterisks.

3.4 Microarray study of gene expression in cortical grey matter

To identify altered gene expression in the normal appearing grey matter in more detail, we performed gene expression analysis of the somatosensory cortex using microarray technology. The somatosensory cortex of control and EAE rats were dissected, RNA was isolated and differential gene expression analysis was performed with Clontech Atlas™ Rat 1.2 cDNA Expression Array with 1176 spotted cDNA sequences. We identified genes, which were significantly up- or downregulated in the motor cortex of EAE rats. From 1176 spotted cDNA sequences, 580 were detectable of which were 32 differentially regulated. All of the significant differentially expressed genes of the somatosensory cortex were downregulated. In Table 4 all differentially expressed genes are listed with a p-value lower than 0.05 obtained by the non-parametric Mann-Whitney U-Test. Significant differentially expressed genes with a fold change lower than 0.66 (corresponds an 1.5 fold upregulation) are marked in bold. Among the differentially expressed genes 6 transcripts are encoding mitochondrial proteins.

Table 4 Genes significantly changed in NAGM

Acc. number	Symbol	Gene name	fc	pvalue
J04022	ATP2A2	Atpase, Ca++ Transporting, Cardiac Muscle, Slow Twitch 2	**0.40**	**0.030**
J03933	THRB	Thyroid Hormone Receptor Beta	**0.42**	**0.017**
AF020777	PTK2	Ptk2 Protein Tyrosine Kinase 2	**0.42**	**0.017**
U23443	PAK1	P21 (Cdkn1a)-Activated Kinase 1	**0.43**	**0.017**
X59737	CKMT1	Creatine Kinase, Mitochondrial 1, Ubiquitous	**0.44**	**0.030**
M92905	CACNA1B	Calcium Channel, Voltage-Dependent, N Type, Alpha 1b Subunit	**0.45**	**0.017**
J02646	EIF2S1	Eukaryotic Translation Initiation Factor 2, Subunit 1 Alpha	**0.49**	**0.017**
L27129	MAPK8	Mitogen-Activated Protein Kinase 8	**0.49**	**0.030**
AF001423	GRIN2A	Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate 2a	**0.50**	**0.017**
L27843	PTP4A1	Protein Tyrosine Phosphatase 4a1	**0.52**	**0.017**
J04526	HK1	Hexokinase 1	**0.52**	**0.017**
L07925	RALGDS	Ral Guanine Nucleotide Dissociation Stimulator	**0.53**	**0.017**
D44481	CRK	V-Crk Sarcoma Virus Ct10 Oncogene Homolog (Avian)	**0.54**	**0.017**
L14851	NRXN3	Neurexin 3	**0.54**	**0.030**
D17445	YWHAH	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Eta	**0.58**	**0.030**
M20636	PLCB1	Phospholipase C, Beta 1	**0.58**	**0.030**
U07683	UGT8	Udp Galactosyltransferase 8	**0.59**	**0.017**
S59158	SLC1A3	Solute Carrier Family 1 (Glial High Affinity Glutamate Transporter), Member 3	**0.59**	**0.030**
D85183	PTPNS1	Protein Tyrosine Phosphatase, Non-Receptor Type Substrate 1	**0.60**	**0.030**
U62897	CPD	Carboxypeptidase D	**0.60**	**0.017**
L27513	TR4	Tr4 Orphan Receptor	**0.62**	**0.030**
D50696	PSMC1	Peptidase (Prosome, Macropain) 26s Subunit, Atpase 1	**0.64**	**0.030**
M86621	CACNA2D1	Calcium Channel, Voltage-Dependent, Alpha2/Delta Subunit 1	0.66	**0.030**
D10854	AKR1A1	Aldo-Keto Reductase Family 1, Member A1	0.68	**0.030**
D13062	AK3	Adenylate Kinase 3	0.69	**0.017**
M91590	ARRB2	Arrestin, Beta 2	0.70	**0.030**
M84416	YWHAE	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Epsilon	0.73	**0.030**
M18331	PRKCE	Protein Kinase C, Epsilon	0.74	**0.017**
D64045	PIK3R1	Phosphatidylinositol 3-Kinase, Regulatory Subunit, Polypeptide 1	0.74	**0.017**
L14684	GFM	G Elongation Factor	0.76	**0.030**
M19044	ATP5B	Atp Synthase, H+ Transporting, Mitochondrial F1 Complex, Beta Polypeptide	0.76	**0.017**
M11185	PLP	Proteolipid Protein	0.80	**0.030**

Normal Appearing Grey Matter from the somatosensory cortex of EAE and control rats was dissected to isolate RNA for gene expression profiling using microarrays. 32 of 580 detectable genes were significantly differentially expressed. Statistical significance is expressed as p-values generated by the non-parametric Mann-Whitney U-test. Fold changes (fc) lower than 0.66 (1.5 fold downregulation) are printed in bold.

Table 5 Gene Expression of Glutamate Receptors and Transporters

Acc. number	Symbol	Gene name	fc	pvalue
X17184	GRIA1	Glutamate Receptor, Ionotropic, AMPA1	**0.38**	**0.087**
M85035	GRIA2	Glutamate Receptor, Ionotropic, AMPA2	**0.53**	**0.087**
M85036	GRIA3	Glutamate Receptor, Ionotropic, AMPA3	**0.48**	**0.087**
M85037	GRIA4	Glutamate Receptor, Ionotropic, AMPA4	**0.51**	**0.087**
M83561	GRIK1	Glutamate Receptor, Ionotropic, Kainate1	0.79	0.425
X63255	GRIN1	Glutamate Receptor, Ionotropic, NMDA1	**0.62**	**0.053**
AF001423	GRIN2A	Glutamate Receptor, Ionotropic, NMDA2A	**0.50**	***0.017***
M91562	GRIN2B	Glutamate Receptor, Ionotropic, NMDA2B	0.72	0.305
M92075	GRM2	Glutamate Receptor, Metabotropic, 2	1.02	0.732
M92076	GRM3	Glutamate Receptor, Metabotropic, 3	**0.61**	**0.053**
M90518	GRM4	Glutamate Receptor, Metabotropic, 4	0.69	0.305
D10891	GRM5	Glutamate Receptor, Metabotropic, 5	0.71	0.138
D16817	GRM7	Glutamate Receptor, Metabotropic, 6	0.80	**0.087**

All detectable ionotropic and metabotropic glutamate receptor genes including their fold changes (fc) and their p-values are listed. Statistical significance is expressed as p-values generated by the non-parametric Mann-Whitney U-test. P-values below 0.05 are printed in bold, italic numbers, whereas p-values between 0.05 and 0.1 are shown in bold numbers. Fold changes (fc) lower than 0.66 (1.5 fold downregulation) are printed in bold.

The mitochondrial creatine kinase 1 (CKMT1) and hexokinase 1 (HK1) were significantly lower expressed in the cortex of EAE rats compared to control rats with a fold change lower than 0.66 (0.44 and 0.52 respectively). Whereas the ATP Synthase beta subunit of the mitochondrial F1 complex (ATP5B), G Elongation factor (GFM), 14-3-3 Epsilon (YWHAE) and adenylate kinase 3 (AK3) show only a slight but significant decreased expression in EAE rats. Additional to the three mitochondrial kinases we could further detect six kinases to be downregulated in the cortex of EAE rats. Kinases with more than a twofold decrease of expression were the focal adhesion kinase (PTK2; fc=0.42), p21 activated kinase 1 (PAK1; fc=0.43) and janus kinase 1 (MAPK8, fc=0.49). Interestingly, we could detect a twofold decreased expression of the 2A subtype of N-methyl-D-aspartate (NMDA) receptor (GRIN2A) and a significant reduction of the glutamate transporter GLAST (SLC1A3). Therefore we had a closer look on the expression of ionotropic and metabotropic glutamate receptors (Table 5). In the somatosensory cortex of EAE rats there is reduced expression of AMPA and NMDA ionotropic glutamate receptors. All four AMPA subunits show a reduction more than twofold, although failing slightly the level of

significance (p=0.083). Additional, there was a reduced expression of the NDMA Receptor 1 subunit (p=0.053) and the metabotrobic Glutamate receptor (mGlur3, GRM3). Neither significant nor near significant changes could be detected for the type 2B NMDA receptor, the metabotrobic receptors mGlur2, mGlur4, mGlur5, and mGlur6 (GRM2,4,5,7). In summary, we could detect a downregulation of genes mainly involved in mitochondrial energy metabolism and genes encoding for glutamate receptors.

4. Discussion

In the last years, thorough studies of the NAWM of MS patients, have revealed abnormalities occurring before lesion formation such as axonal damage or loss, BBB alterations, to some extent astro- and microgliosis and also differential regulation of specific proteins or enzymes (Allen and McKeown, 1979; Filippi et al., 2003; Filippi et al., 1998; Fu et al., 1998; Kutzelnigg et al., 2005; Silver et al., 2001; Sinclair et al., 2005; Werring et al., 2000). In line with these findings, recent studies from our lab revealed differentially regulated gene expression in NAWM from MS cases in comparison to control cases (Graumann et al., 2003; Zeis et al., 2008). In particular, we have shown changes in genes related to endogenous neuroprotection such as the induction of genes involved in ischemic preconditioning (e.g. HIF-1α and VEGFR), or genes that reflect a higher energy metabolism (Graumann et al., 2003). This data showed that in MS, the brain might mount a global defense against oxidative stress, which takes place also in areas remote from active inflammation or demyelination (Graumann et al., 2003). In another study, we could show an upregulation of genes involved in anti- as well as pro-inflammatory mechanisms (Zeis et al., 2008). In particular, we found an upregulation of members of the anti-inflammatory STAT6 signaling pathway (Cannella and Raine, 2004; Zeis et al., 2008). As the majority of cells expressing STAT6 in NAWM were oligodendrocytes, we suggested that oligodendrocytes are actively participating in endogenous inflammatory reactions of MS brains (Zeis et al., 2008; Zeis and Schaeren-Wiemers, 2008). On the other hand, an upregulation of STAT4, a pro-inflammatory transcription factor, was also detected, which might mainly take place in microglia (Zeis et al., 2008). Altogether, we postulated that MS NAWM is in a subtle balance between inflammation and neuroprotection (Zeis et al., 2008). In summary, all these publications demonstrated that normal appearing white matter in MS shows a wide range of abnormalities which could influence the pathogenesis of MS.

Therefore, the aim of this study was to investigate whether in a model of MS comparable mechanisms are activated in tissue far away from lesions. To mimic the situation in MS NAWM we have chosen to study differential gene expression in MOG-induced EAE in DA rats, as this model shows many clinical as well as pathological similarities to MS (Kornek et al., 2000; Storch et al., 1998b). Using DA rats, several different clinical courses could be observed such as primary-

progressive, secondary-progressive and relapsing-remitting as well as acute progressive disease courses. We dissected normal appearing white matter from corpus callosum from rats suffering from a chronic disease course which would corresponding best to the situation of previously analyzed subcortical MS NAWM,. To simultaneously analyze possible grey matter changes, we dissected normal appearing grey matter from the somatosensory cortex.

Our analysis revealed only minor changes in the gene expression of the white matter of the corpus callosum. Neither genes known to be involved in ischemic preconditioning mechanisms nor selected genes involved in pro- and anti-inflammatory mechanisms were significantly changed in the NAWM of the corpus callosum. Therefore, we were not able to reproduce changes revealed in MS NAWM in experimental autoimmune encephalomyelitis. This might be due to several reasons. Although we selected animals with a long disease course, we cannot exclude the possibility that gene expression changes in MS are a cause of chronic stimulation over years and thus not yet detectable in EAE corpus callosum tissue. A study of the temporal course of $Na_V1.8$ in Purkinje Neurons in EAE showed that in this case, first significant changes in $Na_V1.8$ protein, which were paralleled by changes in $Na_V1.8$ mRNA, were observed at between 51 and 75 days (Craner et al., 2003). In contrast, EAE NAWM tissue in this study is not directly affected by inflammatory, demyelinating infiltrates, therefore, first changes might even appear later in time. Another difference possibly leading to diverse gene expression changes in EAE and MS NAWM might be the kind of inflammation and the position of its occurrence. Unlike in chronic MS, lesions in MOG-induced EAE in DA rats are predominantly localized in the spinal cord and/or in the optic nerve (Kinter et al., 2008; Storch et al., 1998b). As ischemic preconditioning mechanisms in MS NAWM might be induced by lesions present nearby, in EAE NAWM tissue of the corpus callosum, ischemic preconditioning mechanisms might not be induced due to a higher distance to active inflammatory lesions. Furthermore, the kind of inflammation is different differs between MS and MOG-induced EAE. In our study we have chosen to use an EAE model in which not only T cells are determinant for the immune reactions, but also anti-MOG antibodies (Storch et al., 1998b). This model would most closely reflect the so-called “pattern II” (Storch et al., 1998a), one of the four distinct pathological MS lesion patterns which were proposed by immunopathological classification of lesions (Lucchinetti et al., 2000). Nevertheless, the immune reaction

in MOG-induced DA rat EAE is, as most EAE models, predominantly determined by CD4+ T cells. In contrast, in MS lesions, CD8+ T cells are dominating over CD4+ lymphocytes (Booss et al., 1983) and additionally, clonal expansion of CD8+ T cells was reported (Babbe et al., 2000). Therefore, these immunological differences between EAE and MS might lead to another or even no reaction of the NAWM. Altogether, we speculate that ischemic preconditioning genes or genes involved in intrinsic immune responses of the white matter might not have been induced yet, or are not induced in this EAE model. Therefore, MOG-induced DA rat EAE might be an unsuitable model to study aspects of ischemic preconditioning mechanisms present in MS NAWM.

In recent years, damage to neurons and axons, as well as grey matter abnormalities gained increasing attention in MS research. The extent of axonal pathology has emerged as an important correlate of the clinical deficit in MS patients (Dutta and Trapp, 2007). Total amount of neuro-/axonal damage accumulating in the MS brain may further be augmented by the formation of grey matter lesions. Several abnormalities have been identified in the grey matter of MS patients by modern imaging techniques (Chen et al., 2004; Newcombe et al., 1991) as well as immunohistological methods (Brownell and Hughes, 1962; Schwab and McGeer, 2002). These pathological alterations have been linked to several clinical manifestations, such as seizures, fatigue and cognitive dysfunction (Amato et al., 2004; Benedict et al., 2004; Blinkenberg et al., 2000; Houtchens et al., 2007; Lazeron et al., 2000; Morgen et al., 2006; Sanfilipo et al., 2006). However, there is very little known about the molecular changes in grey matter of MS patients. A major cause of the irreversible neurological disability that occurs in the chronic stages of MS is the degeneration of chronically demyelinated axons. Recent studies identified gene expression changes in the primary motor cortex of MS in post mortem tissue from severely disabled chronic MS patients (Dutta et al., 2007; Dutta et al., 2006). Therefore, we addressed the question whether in EAE there are similar neuronal changes in cortical regions caused by axonal degeneration in the spinal cord. We found several genes encoding mitochondrial proteins to be downregulated suggesting a reduced capacity of the upper motor neurons in producing ATP. These findings are in accordance with a recent study performed with post mortem tissue form MS patients and is consistent with the hypothesis that chronically demyelinated

axons are degenerating due to a misbalance between reduced supply of ATP and energy demand.

A further observation in our array study was the downregulation of ionotropic glutamate receptors of the NMDA and AMPA subclass. All four subunits of the AMPA receptors showed a decreased expression in the somatosensory cortex of EAE rats. In several studies a downregulation of glutamate receptors as result of axonal transsection in axotomy experiments has been reported (Ginsberg et al., 1996; Wang and Tseng, 2004).It was hypothesized that downregulation of glutamate receptors in injured neurons is a mechanism to protect itself against excitotoxicity. It has been suggested that one important mechanism by which neurodegeneration occurs in MS is excitotoxicity caused by imbalanced glutamate metabolism and consequent increased extracellular glutamate concentration (Groom et al., 2003). Indeed, it has been demonstrated that glutamate receptor antagonists and inhibitors of glutamate transmission were efficient in reducing axonal damage, death of oligodendrocytes, and ameliorating clinical EAE (Basso et al., 2008; Groom et al., 2003; Paul and Bolton, 2002; Pitt et al., 2000; Smith et al., 2000; Wallstrom et al., 1996). Therefore, the reduced glutamate receptor expression in the cortex may be a protective mechanism against excitotoxicity induced by axonal transsection in the spinal cord.

The most changes observed in the microarray experiment analysing gene expression in the normal appearing grey matter seem to reflect either neurodegenerative or neuroprotective mechanisms. We couldn’t detect a diffuse inflammatory reaction within the somatosensory cortex of EAE rats. Hence, the detected changes in this study may reflect the neuronal degeneration induced by lesion mediated axonal transsection in the spinal cord.

In summary, in the normal appearing white matter far distant form lesion sites only minor molecular changes could be observed, whereas in the normal appearing grey matter we identified several gene expression alterations. Downregulation of genes encoding mitochondrial as well as glutamate receptor proteins may reflect the neuronal degeneration induced by lesion mediated axonal injury in the spinal cord. A diffuse inflammatory reaction in white matter as reported in MS could not be detected. This indicates that the MOG-induced EAE in DA rats is valuable model for MS to analyze acute damage but is not suitable to investigate several chronic alterations in MS.

Lame Ducks or Fierce Creatures? - The Role of Oligodendrocytes in Multiple Sclerosis

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Abstract

In the pathogenesis of multiple sclerosis, oligodendrocytes and its myelin sheaths are thought to be the primary target of destruction. The mechanism leading to oligodendrocyte injury and demyelination is still elusive. Oligodendrocytes are maintaining up to 50 internodes of myelin, which is an extraordinary metabolic demand. This makes them one of the most vulnerable cell type in the central nervous system, and even small insults can lead to oligodendrocyte impairment, demyelination and axonal dysfunction. For this reason, oligodendrocytes are viewed as more or less the “lame ducks” of the CNS who can easily become victims. However, recent data demonstrate that this perception needs possibly to be revised. Latest data suggest that oligodendrocytes may also act as “fierce creatures”, influencing the surrounding cells in many ways to preserve its own, as well as their function, allowing sustained functionality of the CNS upon an attack. In this review, the concept of “reactive or activated oligodendrocyte” is introduced, describing alterations in oligodendrocytes which are either protective mechanisms allowing survival in otherwise lethal environment, or influencing and possibly modulating the ongoing inflammation. Though, “harnished” oligodendrocytes might actively modulate and shape their environment and be part of the immune privilege of the brain.

Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS). The pathological hallmark of the disease is the inflammatory plaque. Studies of its histopathology have revealed a wide heterogeneity at the cellular and molecular level, which might partially reflect the diversity of the clinical disease course (Lucchinetti et al., 2000; Raine and Scheinberg, 1988). There are several hypotheses to explain the immunological injury in MS. In the most prominent and most widely accepted hypothesis, MS is driven by a T-cell mediated immune response leading to secondary macrophage and microglia activation and demyelination (Compston et al., 2006). In a majority of MS cases, this immune response is further accompanied by antibodies or complement deposition (Lucchinetti et al., 2000). Other hypotheses implicate a viral pathogenesis to be the

origin of MS (Kennedy and Steiner, 1994), or intrinsic oligodendrocyte damage leading to subsequent MS disease (Lucchinetti et al., 2000).

One of the major features of an inflammatory plaque is demyelination and the loss of oligodendrocytes (Ozawa et al., 1994). Due to the fact that oligodendrocytes are highly specialized and have a high metabolic demand maintaining many myelin sheaths, oligodendrocytes are one of the most vulnerable cells in the CNS. There are many ways which lead to oligodendrocyte impairment and injury (for review see Ludwin, 1997; Raine, 1997; Merrill and Scolding, 1999). Still, oligodendrocyte apoptosis and loss is not the major feature in MS, implicating that the major target of the destructive process is the myelin sheath (Ozawa et al., 1994). In particular cases, however, oligodendrocyte apoptosis might be a primary cause (Lucchinetti et al., 2000); (Barnett and Prineas, 2004). Studies from animal models showed that T cell infiltration and subsequent inflammation in the CNS per se does not necessarily lead to extensive demyelination (for review see Gold et al., 2000; Gold et al., 2006). Furthermore, oligodendrocytes are able to resist at least to some extent to autoimmune-mediated demyelination (Ozawa et al., 1994). An important question arises: which mechanisms lead to or protect from potential harmful oligodendrocyte injury?

Oligodendrocytes - lame ducks?

Until now, many cell types have been shown to be potentially able to damage oligodendrocytes. In the first part of this review, we discuss some of these cell types and their mediators leading to oligodendrocyte injury or death. Figure 1 shows a schematic view of these cells and their possible oligodendrocyte harming mediators.

Oligodendrocyte injury mediated by immune cells

In acute MS lesions, CD4+ as well as CD8+ T lymphocytes are present. These cells can recognize their antigen if presented by MHC molecules expressed on target cells, and be subsequently activated. Under normal conditions, MHC expression in the CNS does either not occur or is below detection levels (Redwine et al., 2001). *In vitro* experiments showed, however, that oligodendrocytes can be induced to express

MHC class I molecules (Grenier et al., 1989; Kim, 1985) as well as MHC class II molecules (Bergsteindottir et al., 1992). Also *in vivo*, it has been shown that oligodendrocytes are expressing MHC class I molecules in a murine model of CNS inflammation and demyelination (Redwine et al., 2001) as well as in multiple sclerosis lesions (Hoftberger et al., 2004). This suggests that under pathological conditions, oligodendrocytes induce MHC I expression and can thereby directly activate T cells and consequently be damaged by them.

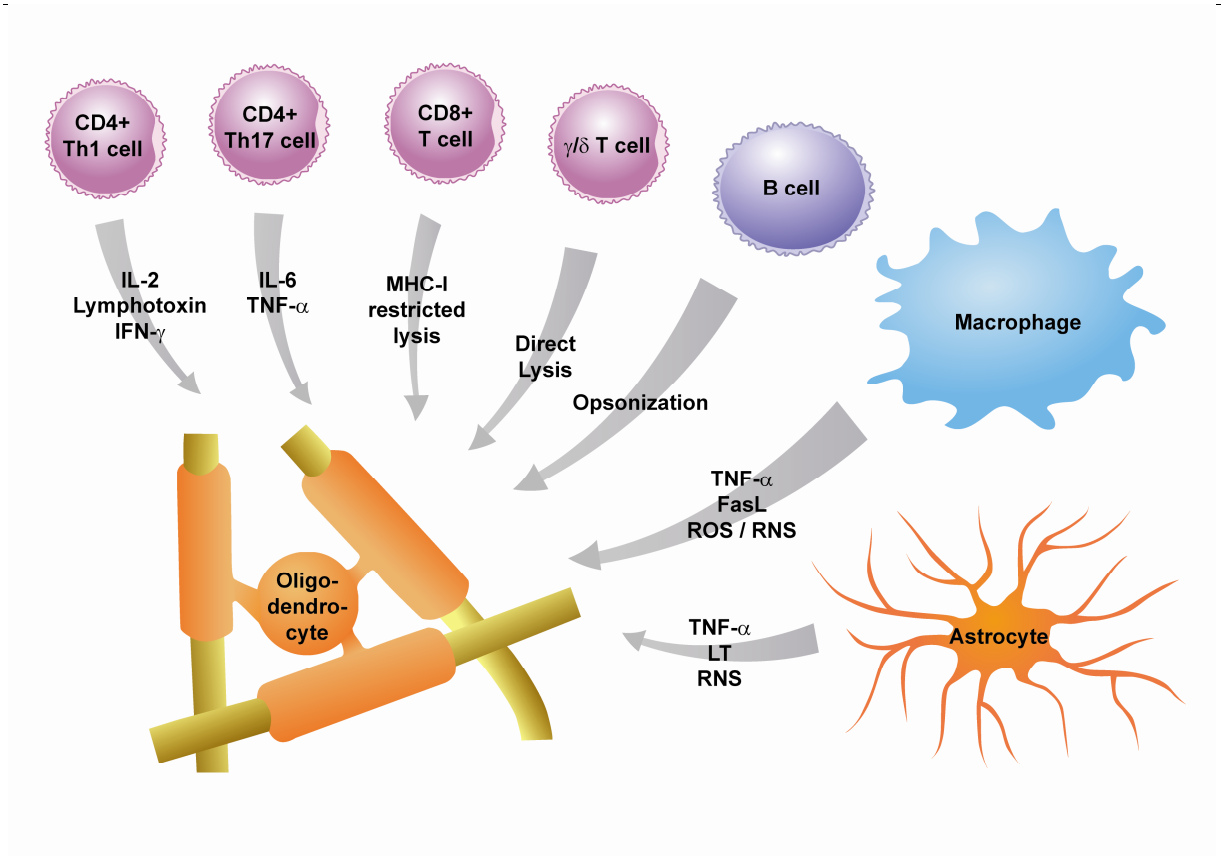

Figure 1: Cells mediating oligodendrocyte injury in the course of multiple sclerosis

Many different cell types have the potential to damage oligodendrocytes. Here, some of these cells and their potential oligodendrocyte damaging mediators are summarized. CD4+ Th1 T cells have been shown to induce oligodendrocyte damage among others through IL-2, LT and IFN-γ, whereas oligodendrocyte damaging mechanisms of Th17 T cells involve IL-6 and TNF-α. CD8+ T cells can induce oligodendrocyte damage directly by MHC-I restricted cell lysis. Furthermore, γ/δ T cells were also shown to have the potential of damaging oligodendrocytes by direct lysis. By secreting antibodies, B cell mediated damage to oligodendrocytes through opsonization was demonstrated. Macrophages are one of the main cell types inducing oligodendrocyte damage by TNF-α, FasL, ROS/RNS and other mechanisms. Furthermore, astrocytes were shown to be potential harmful to oligodendrocytes by mechanisms involving TNF-α, LT and RNS.

CD8+ T lymphocytes

By the interaction of the CD8+ T cell receptor together with the MHC class I peptide complex, CD8+ T cells are activated and are directly cytotoxic to cells presenting their specific antigen (Parkin and Cohen, 2001). Activation of CD8+ T cell by recognition of their specific antigen is then followed by clonal expansion. In MS, this was shown by analyzing lesions as well as blood and cerebral spinal fluid (CSF) for clonal composition and T cell receptor repertoire (Babbe et al., 2000; Skulina et al., 2004). These results suggested that CD8+ T cells might have recognized their specific antigen within the lesion and might have been activated. It has been shown that oligodendrocytes are susceptible to cytolysis by CD8+ T lymphocytes (Jurewicz et al., 1998; Ruijs et al., 1990). Further, an involvement of CD8+ cytotoxic T lymphocytes in autoimmune demyelination was shown in experimental autoimmune encephalomyelitis (EAE) (Huseby et al., 2001; Sun et al., 2001). Altogether, this suggests that CD8+ T lymphocytes might contribute to oligodendrocyte injury in MS.

CD4+ T lymphocytes

CD4+ Th cells recognize their cognate antigen exclusively in the context of MHCII molecules. In contrast to MHC class I molecules, the expression of MHC class II molecules by oligodendrocytes could not be demonstrated in MS (Lee and Raine, 1989). MHCII expression is restricted to professional antigen presenting cells such as microglia/macrophages and dendritic cells (Becher et al., 2000; Greter et al., 2005). It is easily conceivable that CD4+ T helper cells induce oligodendrocyte damage by secreting cytokines and promoting activation of nearby macrophages and microglia. Studies in EAE suggest that CD4+ T cells of the Th1 and Th17 lineage play a major role in disease pathology (Gutcher et al., 2006; Langrish et al., 2005; Langrish et al., 2004; Lassmann and Ransohoff, 2004; Sospedra and Martin, 2005; Weaver et al., 2006). Th1 cells are characterized by the predominant secretion of IFN-γ whereas Th17 cells are shown to secrete IL-17A, IL-17F and IL-22 (Iwakura and Ishigame, 2006; Kreymborg et al., 2007; McGeachy et al., 2007). It was shown that oligodendrocytes express TNF-α receptors (Cannella et al., 2007; Raine et al., 1998) as well as other cytokine receptors such as IFN-γ receptor (Cannella and Raine, 2004), and treatment of oligodendroglial cell lines with IFN-γ induces apoptosis

(Buntinx et al., 2004). Oligodendrocytes were also shown to be susceptible to TNF-α induced cell death (D'Souza et al., 1996a; Jurewicz et al., 2005; Selmaj and Raine, 1988). Taken together, activated CD4+ T lymphocytes do contribute to some extent directly or indirectly to oligodendrocyte injury in MS.

γ/δ T lymphocytes

Another cell type found in MS lesions are γ/δ T lymphocytes (Wucherpfennig et al., 1992). γ/δ T cells are a T cell subpopulation showing a different T cell receptor structure than α/β T cells (Li et al., 1998). The role of γ/δ T lymphocytes in MS is still unclear. Nevertheless, depletion of γ/δ T lymphocytes during EAE has been shown to ameliorate disease severity during the acute phases of the disease (Rajan et al., 1996). Furthermore, γ/δ T cells were shown to enhance adoptive-transfer EAE by promoting antigen presentation and IL-12 production (Odyniec et al., 2004). As lysis of oligodendrocytes by γ/δ T cells has been demonstrated *in vitro* (Freedman et al., 1991), a possible impact on oligodendrocyte injury in MS might not be ruled out.

B-cells and antibodies

In cerebrospinal fluid (CSF) of MS patients, abnormal oligoclonal immunoglobulin bands are detected, which supports clinical diagnosis of MS (Compston et al., 2006). Autoantibodies against myelin components were reported to be present in serum and CSF as well as lesions of MS patients (Genain et al., 1999; Reindl et al., 1999). In-line, IgG isolated from inflamed CNS tissue from MS patients were shown to recognize MOG (O'Connor et al., 2005). Recently, meningeal B-cell follicles were reported to associate with early onset of disease and severe cortical pathology in secondary progressive MS (Magliozzi et al., 2007). Therefore, antibody producing B-cells may have potential impact on oligodendrocyte injury and demyelination. For example, injection of antibodies augmented demyelination during the course of a T-cell mediated transfer EAE (Linington et al., 1988). Further, it has been shown that by opsonizing the myelin-oligodendrocyte surface, antibodies can stimulate oligodendrocyte lysis of macrophages through their Fc receptors (Scolding and Compston, 1991). Also, another demyelinating mechanism by antibodies was shown to involve membrane attack complex (MAC) deposition, which finally leads to

complement mediated cytolysis (Mead et al., 2002; for review see Sospedra and Martin, 2005). Altogether, direct antibody-mediated injury of oligodendrocytes in multiple sclerosis might play an important role, although its impact on MS pathogenesis could not be determined yet.

Oligodendrocyte injury mediated by activated macrophages/microglia

Activated macrophages and microglia may play an important role in inducing oligodendrocyte injury during acute inflammation in MS. It has been shown that disease severity in EAE correlates best with macrophage infiltration (Berger et al., 1997). Activated macrophages and microglia were shown to have incorporated myelin products, and express a large variety of different for oligodendrocytes deleterious compounds, such as TNF-α, reactive oxygen species (ROS), reactive nitrogen species (RNS) and Fas-ligand (FasL). TNF-α is a potent cytotoxic molecule capable of inducing oligodendrocyte cell death (D'Souza et al., 1996b; Jurewicz et al., 2005; Selmaj and Raine, 1988). The production of ROS and RNS by activated macrophages and microglia can lead to various types of damage such as lipid peroxidation, tyrosine nitrosylation and DNA strand breaks (van der Veen and Roberts, 1999; Willenborg et al., 1999; Zhang et al., 1994). High expression of inducible nitric oxide synthase (iNOS) as well as neuronal nitric oxide synthase (nNOS) has been reported in activated macrophages and microglia within active lesions in MS (De Groot et al., 1997; Hill et al., 2004), and RNS mediated damage in oligodendrocytes has also been demonstrated (Jack et al., 2007; Li et al., 2005; Merrill et al., 1993). Oligodendrocytes were also reported to express Fas in MS lesions (D'Souza et al., 1996). FasL was shown to induce oligodendrocyte damage (Li et al., 2002) and as microglia express FasL in MS lesions (Becher et al., 1998), they might therefore induce oligodendrocyte apoptosis. Furthermore, it has been shown that activated macrophages and microglia are capable of damaging oligodendrocytes in an antibody-dependent mechanism (Griot-Wenk et al., 1991). Altogether, activated macrophages and microglia might be one of the major mediators of oligodendrocyte injury in MS.

Oligodendrocyte injury mediated by astrocytes

Astrocytes are known to maintain physiological glutamate levels in the brain. Therefore, malfunctioning or too slow glutamate uptake might lead to an enhancement of oligodendrocyte excitotoxic damage (Newcombe et al., 2007). Additionally, astrocytes are also known to express TNF-α and LT-α. Thus, astrocytes might also be potential inducer of oligodendrocyte injury via TNF-α and LT-α dependent mechanisms (for review see Williams et al., 2007). An expression of all three isoforms of NOS by astrocytes was also reported (for review see Gibson et al., 2005). In MS plaques, high levels of constitutively expressed NOS were detected to be expressed by astrocytes and macrophages (De Groot et al., 1997). In contrast to astrocytes, oligodendrocytes are shown to be much more susceptible to NO induced oxidative stress (Mitrovic et al., 1995). This is explained by the high iron load stored in oligodendrocytes (Connor and Menzies, 1995; Roskams and Connor, 1994; Thorburne and Juurlink, 1996) as well as their low content of reduced-glutathione (GSH) (Juurlink et al., 1998; Thorburne and Juurlink, 1996). Iron (Fe2+) was reported to be involved in the formation of hydroxyl radicals (Gutteridge and Halliwell, 1989), whereas glutathione peroxidase activity, using GSH as an electron donor, scavenges hydrogen peroxide and thus inhibits hydroxyl radical formation (Juurlink et al., 1998). A production of NO through NOS expressed by astrocytes might, therefore, lead to oxidative stress and damage in oligodendrocytes. Taken together, activated astrocytes might also be involved in damaging oligodendrocytes during the disease course of MS.

Reactive or activated oligodendrocytes – pure defensive or even fierce creatures?

As discussed before, immune cells as well as brain resident cells are able to produce a variety of potentially harmful factors for oligodendrocytes. These “attacks” are occurring either direct via lysis or indirect via toxic mediators or via an imbalance of the surrounding environment. As demyelination is a major feature in MS and also loss of oligodendrocyte during the chronic disease process is evident, oligodendrocytes can be regarded as “poor victims” in the pathogenic process of MS. Still, the question arises if oligodendrocytes are really “lame ducks” allowing

passively disease progression, or if they attempt to defend themselves in one way or the other, which could even influence disease progression?

Studies characterizing oligodendrocytes in MS lesion and in primary oligodendrocyte cultures as well as analysis of normal appearing white matter (NAWM) MS tissue, which is mostly devoid of immune infiltrates - therefore suitable to study pre-lesional activities of oligodendrocytes - have recently disclosed a view of oligodendrocytes being potential immune-modulating in MS. Further, oligodendrocytes were shown to successfully protect themselves during pathogenesis of Balo's concentric sclerosis (Stadelmann et al., 2005). Altogether, these findings might lead to a view of oligodendrocytes being at least capable to defend themselves, or even be a reactive - to some extent active cell type - being part of the immune privilege of the brain. Here, we discuss the capacity of oligodendrocytes to react against certain insults for their own protection, and how they might modulate their environment by influencing disease progression.

Activation of endogenous protective mechanisms

In the last few years, growing evidence suggest an involvement of hypoxia-like pathogenic mechanisms in MS (Lassmann, 2003). Especially, in the so-called pattern III of the lesion patterns identified recently (Lucchinetti et al., 2000), hypoxia-like tissue injury may play a pathogenetic role (Aboul-Enein et al., 2003). Hypoxic tissue injury can be induced in many ways. As already mentioned above, reactive oxygen and nitrogen species (ROS, RNS) are known to induce cellular damages, and were proposed to be involved in the demyelinating processes (Smith et al., 1999). For example, NO can impair respiratory chain function in mitochondria, and by that can cause axon conduction block (Redford et al., 1997). In particular, oligodendrocytes are vulnerable to NO-mediated damage (Smith et al., 1999; Smith and Lassmann, 2002), and therefore, activation of mechanisms protecting oligodendrocytes from oxidative stress inducing damage would be highly beneficial. A recent study of subcortical NAWM from MS cases has shown the upregulation of several genes involved in ischemic preconditioning (Graumann et al., 2003). In particular, HIF-1α has been shown to be an important regulator of hypoxic preconditioning (Bergeron et al., 2000; Bernaudin et al., 2002; Sharp et al., 2001) and is activated by hypoxia,

growth factors, NO and others (for review see Brune and Zhou, 2007; Semenza, 2002). HIF-1α and some of its downstream genes were shown to be elevated in MS NAWM (Graumann et al., 2003), and *in-situ* hybridization experiments of MS NAWM (Zeis et al., 2008) as well as examinations of Balo's concentric sclerosis identified oligodendrocytes expressing this transcription factor (Stadelmann et al., 2005), suggesting that oligodendrocytes mount ischemic protective mechanisms during the disease course (Fig. 2). Furthermore, oligodendrocytes were also shown to express heat-shock proteins 70 (HSP70) (Stadelmann et al., 2005) as well as HSP32 (Stahnke et al., 2007). In the case of HSP70, a protective role has been shown in brain ischemia (for review see Christians et al., 2002), whereas HSP32 was shown to exert a protective role against oxidative stress in an oligodendroglial cell line (Stahnke et al., 2007).

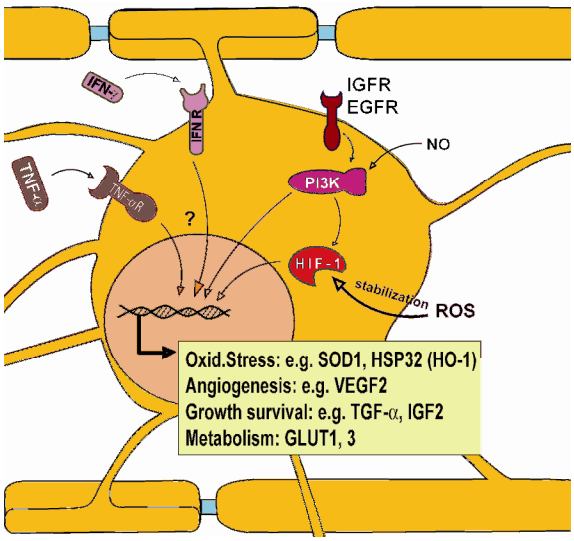

Figure 2: Ischemic preconditioning pathways in oligodendrocytes

Recent studies showed that oligodendrocytes can mount ischemic preconditioning mechanisms upon different stimuli. Treatment of oligodendrocytes with sub-lethal doses of IFN-γ and TNF-α led to the upregulation of genes involved in ischemic tolerance. Protective genes were also shown to be upregulated in oligodendrocytes after stimulation with growth factors. Furthermore, low-levels of RNS/ROS were reported to lead to a stabilization of HIF-1α which in turn activates the transcription of protective genes such as for example VEGFR, GLUT1 and 3.

Interestingly, sub-lethal doses of inflammatory cytokines such as IFN-γ and TNF-α were reported to induce protective mechanisms in target cells (Fig. 2). The induction of HSP70 in oligodendrocytes was shown *in-vitro* by treatment of oligodendrocyte cultures with a mix of cytokines (D'Souza et al., 1994). Further, treatment of oligodendrocyte cultures with IFN-γ led to an increase in the expression of genes involved in protection against oxidative stress (Balabanov et al., 2007). In line, treatment of mice with IFN-γ before onset of EAE led to an amelioration of the

disease through activating the integrated stress response (Lin et al., 2007). Altogether, oligodendrocytes are able to induce and express endogenous protective mechanisms allowing them to survive in an otherwise potentially lethal environment.

Growth factors

Changes in growth factors as well as growth factor receptors expression were demonstrated in MS. Several growth factors such as nerve growth factor (NGF), insulin-like growth factor (IGF) and transforming growth factor β (TGF-β) were reported to be expressed by oligodendrocytes (for review see Du and Dreyfus, 2002). By expression of these factors, oligodendrocytes might influence the survival and/or function of neighboring cells. NGF can bind to the tyrosine kinase receptor A (TrkA) as well as to the low-affinity nerve growth factor receptor ($p75^{NTR}$). By binding to TrkA, NGF promotes cell survival whereas binding to $p75^{NTR}$ under some circumstances might also modulate susceptibility to programmed cell death or apoptosis (Casaccia-Bonnefil et al., 1999; Yoon et al., 1998). In EAE, expression of TrkA was detected on neurons, astrocytes and oligodendrocytes (Oderfeld-Nowak et al., 2003; Oderfeld-Nowak et al., 2001), whereas $p75^{NTR}$ was detected on neurons, microglia, astrocytes and oligodendrocytes (Nataf et al., 1998; Villoslada et al., 2000). In EAE, NGF was shown to have beneficial effects, as NGF-deprived rats display more severe neurological deficits during disease course. Further, treatment of marmoset monkeys with NGF prevented the full development of EAE lesions and delayed the onset of clinical EAE (Micera et al., 2000; Villoslada et al., 2000). Another growth factor expressed by oligodendrocytes is IGF-1, which was reported to ameliorate TNF-α induced demyelination in transgenic mice (Ye et al., 2007). Furthermore, IGF-1 was also reported to reduce demyelination in EAE (Liu et al., 1995), although this beneficial effect is still under debate (Cannella et al., 2000). The expression of TGF-β by oligodendrocytes was also reported, which is discussed in the next chapter. Altogether, by expressing several growth factors, oligodendrocytes are able to influence the function and survival of themselves, but also of the nearby cells.

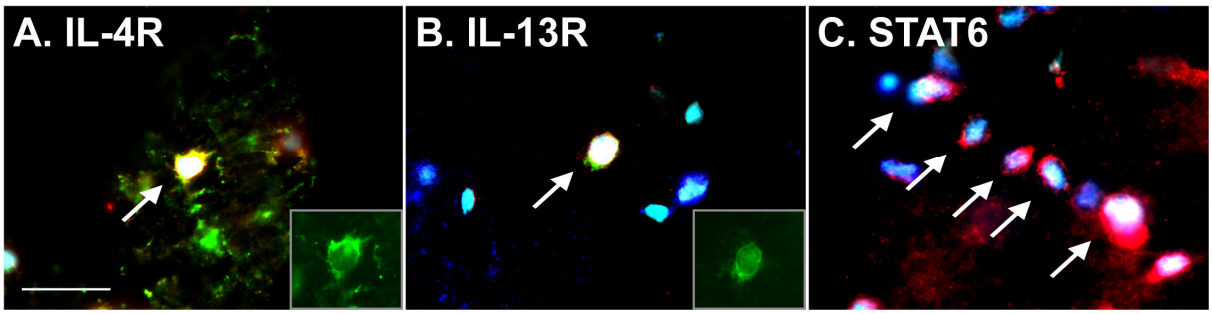

Figure 3: STAT6 signaling pathway expression in oligodendrocytes

Recently, oligodendrocytes were shown to be able to express immune-mechanism relevant genes. Immunofluorescence colocalization analysis of proteins from the STAT6 signaling pathway in multiple sclerosis patients revealed the expression of IL-4R (A), IL-13R (B) and STAT6 (C) in oligodendrocytes (Olig2 positive) in subcortical normal appearing white matter brain tissue. A: Colocalization of STAT6 (red), IL-4R (green, inset), OLIG2 (blue) and DAPI (cyan); B: Colocalization of STAT6 (red), IL-13R (green, inset), OLIG2 (blue) and DAPI (cyan); C: Colocalization of STAT6 (red), OLIG2 (blue) and DAPI (cyan) in oligodendrocytes arranged in interfascicular rows, which is typical for myelinating oligodendrocytes (for more detailed pictures see Zeis et al., 2008). Scale bar= 25μm.

Potential immune modulating ability of oligodendrocytes

Immunohistochemical analysis of proteins expressed by oligodendrocytes revealed that oligodendrocytes are able to express cytokine receptors as well as members from the JAK/STAT family (Cannella and Raine, 2004; Zeis et al., 2008). In a recent study, we have shown that genes from the STAT6 signaling pathway are upregulated in MS NAWM, and that STAT6 and its members JAK1/ 3, IL-4R and IL-13R are expressed by oligodendrocytes (Fig. 3 and 4) (Zeis et al., 2008). The STAT6 signaling pathway is known from CD4+ T helper cells type 2, and it has been shown that STAT6 is critically required for differentiation into Th2 cells (Kaplan et al., 1996). Although still debated, cytokines of the Th2 type such as IL-4 and IL-10 are thought to be mostly beneficial in MS and EAE (Cannella and Raine, 2004; Sospedra and Martin, 2005). In EAE it has been shown that STAT6 knock-out mice develop a more severe disease than wild-type mice (Chitnis et al., 2001). This might be due to the lack of Th2 cells, but may also be due to the inability of oligodendrocytes to modulate their environment in an anti-inflammatory way. The expression and activation of an anti-inflammatory response by oligodendrocytes might be crucial for them to compensate for the upregulated pro-inflammatory environment, and to limit the inflammatory response and damage (Zeis et al., 2008). The expression of different cytokine receptors on oligodendrocytes in active and silent lesions may further

suggest an active role in innate immunity of the CNS (Cannella and Raine, 2004). Oligodendrocytes were also shown to express TGF-β *in vitro* (da Cunha et al., 1993; McKinnon et al., 1993), which can suppress immune and inflammatory responses (for review see Pratt and McPherson, 1997), and might promote myelination and remyelination (Setzu et al., 2006).

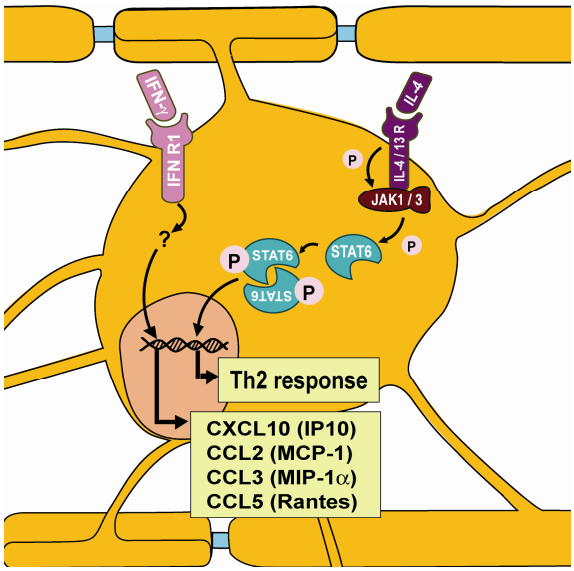

Figure 4: Immune response-mediating pathways in oligodendrocytes

Analysis of proteins expressed by oligodendrocytes revealed that oligodendrocytes are able to express immune mechanisms-related proteins. Members of the STAT6 signaling pathway, such as IL-4R, IL13R, JAK1 and STAT6, were shown to be expressed by oligodendrocytes. This might indicate an anti-inflammatory "Th-2"-like response by oligodendrocytes. Furthermore, treatment of oligodendrocytes with a sub-lethal dose of IFN-γ and TNF-α led to the secretion of chemokines such as CXCL10 (IP-10), CCL2 (MCP-1), CCL3 (MIP-1α) and CCL5 (Rantes). Altogether, this indicates that oligodendrocytes might play an immune-modulating role MS.

In vitro experiments suggested, that upon stimulation by Interferon-γ, oligodendrocytes express protective genes against oxidative stress as well as a number of chemokines, including CXCL10, CCL2, CCL3 and CCL5 (Fig. 4) (Balabanov et al., 2007). CXCL10, CCL2 and CCL5 were also found to be upregulated in MS NAWM (Graumann et al., 2003). Furthermore, mice with oligodendrocytes with suppressed responsiveness to IFN-γ showed higher oligodendrocyte apoptosis in EAE as well as an accelerated disease onset, but milder perivascular inflammation and minimal parenchymal infiltration and demyelination (Balabanov et al., 2007). This effect of IFN-γ on oligodendrocytes demonstrates that oligodendrocytes are capable to react on external immune challenges by induction of protective mechanisms, and that they can modulate inflammatory responses. The expression of cytokine receptors as well as members from the anti-inflammatory STAT6 signaling pathway, and the possibility of

chemokine expression might point to oligodendrocytes playing a role in the innate immunity by actively modulating their environment and interacting with cells of the immune system.

Conclusions

Oligodendrocytes as the myelinating cell type in the CNS are the major targets in MS. Many studies have shown that oligodendrocytes are easily damaged by various mechanisms. Therefore, oligodendrocytes might be seen as “lame ducks” of the CNS. However, growing evidence indicate that oligodendrocyte are far more than a passive presence in the CNS during MS. Oligodendrocytes are either constitutively expressing or inducing various molecules able to influence inflammatory reactions, and prevent cell death in order to conserve the functionality of the CNS. It seems that oligodendrocytes in MS have a rather active or reactive phenotype, preventing fatal damage as well as modulating their surrounding. Therefore, oligodendrocytes may even act as “fierce creatures”, influencing innate immunity and being an active part in the formation of the immune privilege of the brain.

RNA Profiling of MS Brain Tissues

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Abstract

Recently, the introduction of RNA profiling using microarray technology has helped to elucidate gene expression changes in diseased tissue samples from post-mortem human brains. Especially, in the field of Multiple sclerosis (MS) research, microarray-based RNA profiling has been applied in the hope to identify disease specific alterations. The lack of good biomarkers for diagnostic as well as for prognostic purposes, but also the need for new drug targets and for a better understanding of the pathophysiology makes this technique a valuable tool. Different RNA profiling approaches have been used addressing distinct scientific questions. MS brain tissue samples have been proven to be an appropriate source for RNA profiling to investigate molecular pathomechanisms. This work discusses the critical parameters for RNA profiling of MS brain tissues, and reviews the results obtained by microarray studies analysing differential gene expression in MS brain tissues.

Introduction

Despite the recent progress made in Multiple sclerosis (MS) research the aetiology of the disease remains still unsolved. Reliable and clinically useful diagnostic and prognostic markers for MS are still not available. Recent studies expanded the number of susceptibility genes, underscoring the complexity of the genetic trait of MS (Ebers et al., 1996; Gregory et al., 2007; Hafler et al., 2007; Haines et al., 1996; Kuokkanen et al., 1997; Lundmark et al., 2007; Sawcer et al., 1996). Additionally, environmental influences such as geographical factors as well as viral infections are discussed as important risk factors (Ebers, 2008; Hogancamp et al., 1997). The development and improvement of new experimental and technical tools measuring gene expression regulation made it possible to analyze changes in MS tissue samples by high-throughput technologies like DNA microarrays, also called gene chips.

There are several different platforms available for RNA profiling (Lee and Saeed, 2007). They differ in the number of gene sequences covered by the array, in the length of the cDNA probes, and the way the source RNA is processed for probe labelling. The most commonly used microarrays use short oligonucleotide sequences (25 to 60-mers), which are covalently bound to a small glass surface (e.g. Affimetrix,

Illumina) (Kuo et al., 2006). These arrays cover the whole genome sequence of the species to be analyzed. Total or poly A^+ RNA is extracted from a selected biological sample, converted into cDNA, from which a fluorescently labelled cRNA is generated. Another type of arrays is using radioactive labelled cDNA as a high sensitive probe for detecting expressed gene sequences (e.g. Clontech ATLAS arrays) (Bowtell, 1999; Duggan et al., 1999). The combination of long cDNA fragments (300-500bp) attached to a nylon membrane and the use of radioactivity for labelling cDNA probes makes this type of microarrays very specific and in particular sensitive. A disadvantage of this system is the lower number of gene sequences (500-5000) covered by one array. After hybridization of the labelled cDNA probe, the hybridization signal of each sequence spot is determined either by laser scanning of the fluorescent or by autoradiography of the radioactive signal (phosphor-imager). Routinely, with 1μg of total RNA (down to 10 ng RNA) the gene expression of the whole genome can be analyzed at the same time.

Microarrays in MS

The first study applying microarray technology on MS brain tissue was published in 1999 by Whitney and colleagues (Whitney et al., 1999). Since then, several studies using microarrays were performed to identify differential gene expression in MS tissue as well as in tissues from experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Comabella and Martin, 2007). These studies partially overlap, concerning experimental setup as well as the obtained result. Nevertheless, the heterogeneity of the experimental setups makes it difficult to compare these studies retrospectively (Comabella and Martin, 2007). Several points have to be considered when a microarray study is planned (Figure 1). At the beginning of each gene expression study, the scientific question determines the experimental setup. In MS research, a wide range of scientific questions were addressed using microarray experiments. Several studies aimed to identify biomarkers for diagnostic purposes (Bomprezzi et al., 2003; Mandel et al., 2004), whereas other studies were designed to identify molecular mechanisms of the pathophysiology or to discover new drug targets (Dutta et al., 2006; Graumann et al., 2003; Lindberg et al., 2004; Lock et al., 2002; Mycko et al., 2003; Mycko et al., 2004; Tajouri et al., 2003; Whitney et al., 1999; Whitney et al., 2001). The diversity in experimental design used in the

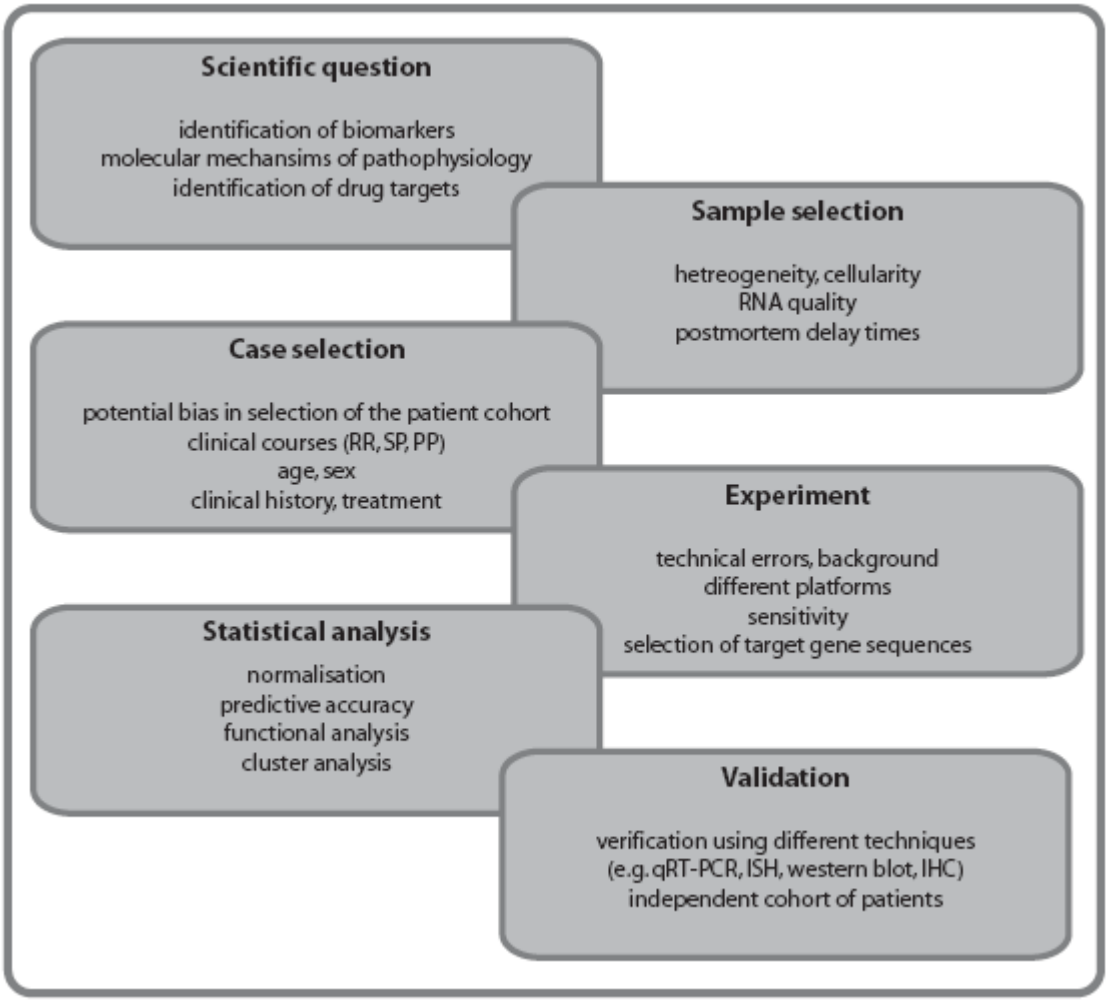

Figure 1 The main issues to be considered when performing RNA profiling of MS brains using microarray analysis. The first step consists of defining the scientific question that should be answered by the output data. The working hypothesis will help to design a study and to select the patient cohort as well as the type of tissue samples. The next step includes the actual microarray experiment by which the gene expression data are obtained. After statistical analysis and generation of an expression profile the data should be verified by alternative methods and optimally validated using an independent cohort of patients.

past, partially reflects the different approaches addressing distinct scientific questions.

An important issue in using microarray technology in MS research is the tissue selection and sampling. This includes different populations of peripheral blood cells (PBMC and CD3+ cells) (Goertsches and Zettl, 2007), different types of lesioned white matter (e.g. acute, active and silent lesions) and normal appearing white matter (NAWM) (Table) (Lindberg and Kappos, 2006). Microarray studies in MS further differ concerning the number of MS cases as well as the type of MS disease course (relapsing remitting, primary progressive or secondary progressive). As already

mentioned, different expression array platforms are nowadays available (Lee and Saeed, 2007) from which most of them have been used in MS research, which further increases difficulties in comparability. Another major step is the analysis of the hybridization signals. Today, there are a large variety of software tools available for statistical analysis used for the identification of differentially expressed genes. A major issue for the correct interpretation of microarray data is the choice of the normalization parameters. Currently, this largely depends on the system used; e.g. “global normalization” is often applied to microarrays covering thousands of sequences, whereas hybridizations signals from microarrays which cover a chosen selection of sequences will rather be normalized with one or a set of house keeping genes. Still, the final interpretation of the obtained results remains one of the most challenging steps in analyzing data from gene chips. Currently, the need in developing new tools converting the enormous amount of generated microarray data into biological significant interpretations is discussed (Comabella and Martin, 2007). Finally, identified differentially expressed genes have to be verified using other techniques such as quantitative RT-PCR, in situ hybridization, Western blot analysis and/or immunohistochemistry. For that, validation by using an independent cohort of patients would be ideal. Among all these issues, sample and case selection is one of the major criteria for successful analysis of gene expression alterations in MS brain tissues, and are therefore discussed in more detail.

Sample and Case Selection

In neurological diseases affecting the central nervous system (CNS), like MS, the availability of the affected tissue is limited. The studies are mostly limited to post mortem tissue because brain biopsies for analytical purpose are only rarely taken. RNA degrades relatively fast after death, and thus cases with a short post mortem time are most likely the best source for RNA that can be used for expression profiling. Still, full length total RNA can be isolated from post-mortem times up to 20 hours; whereas a short post-mortem time does not always guarantee intact RNA (Graumann et al., 2003). Nevertheless, post mortem tissue is a useful source for gene expression studies, used for the analysis of the pathomechanisms, and to identify possible drug targets. A further selection of the tissue samples is an essential issue in the experimental setup. To ensure that the identified changes in gene expression are due to the parameter to be analyzed (e.g. diseased vs. control) it is important to

avoid false results due to a bias in case and tissue selection. Possible bias in case selection are sex, age, treatment, disease course, additional diseases and post mortem time of the collected tissue. Aging effects such as higher expression of anti-oxidative stress genes, low level gliosis and reduced expression of neurotransmitter receptor genes (Lu et al., 2004) can be excluded by using appropriate control brain tissue from the corresponding region, corresponding age, comparable cause of death, and comparable post mortem delay time.

For MS research there is a variety of sources of biological samples when analyzing gene expression in the brain. Most of the studies used post mortem brains from MS and healthy control patients (Figure 2A, Table), but also CNS tissues of EAE animals have been used (Figure 2B). As already mentioned, the maintenance of comparability between MS cases and controls is crucial. Therefore, selected brain areas (e.g. subcortical white matter, periventricular white matter) used for the analysis should be analogous. The sample heterogeneity can be reduced by isolating tissue from defined brain regions to avoid false results due to regional distinctions. Another important issue in identifying differential expressed genes is the cellular composition of the tissue to be compared (Figure 2C-F). To identify genes which are up- or down regulated by a specific cell type, a monotypic cell population as a source for microarray experiments is optimal. Unfortunately, this is not the case for most heterogeneous tissues like the CNS.

White matter is mainly populated by myelinating oligodendrocytes, astrocytes and microglia (Figure 2C). Additionally, interstitial subcortical neurons, endothelial cells forming blood vessel walls, and even axonal transported transcripts can be sources of RNA. Therefore, the obtained expression profile of white matter tissue is a mixture of all these cell types. When using lesioned tissue as source for RNA, the cellular composition is even more complex due to infiltrating immune cells, like T-cells, B-cells and peripheral macrophages (Figure 2D, E, F). Therefore, the comparison of tissue with different cellular composition (e.g. lesioned MS tissue vs. tissue from control cases) must be interpreted with this special focus and awareness. This as differences in the amount of specific RNA transcripts might be either due to an up- or downregulation in resident cells, or due to differences in cellularity, e.g. infiltrating cells, or a combination of both. The challenge but also difficulty of analyzing lesioned brain tissue is to discriminate between these possibilities and to identify truly differentially regulated genes.

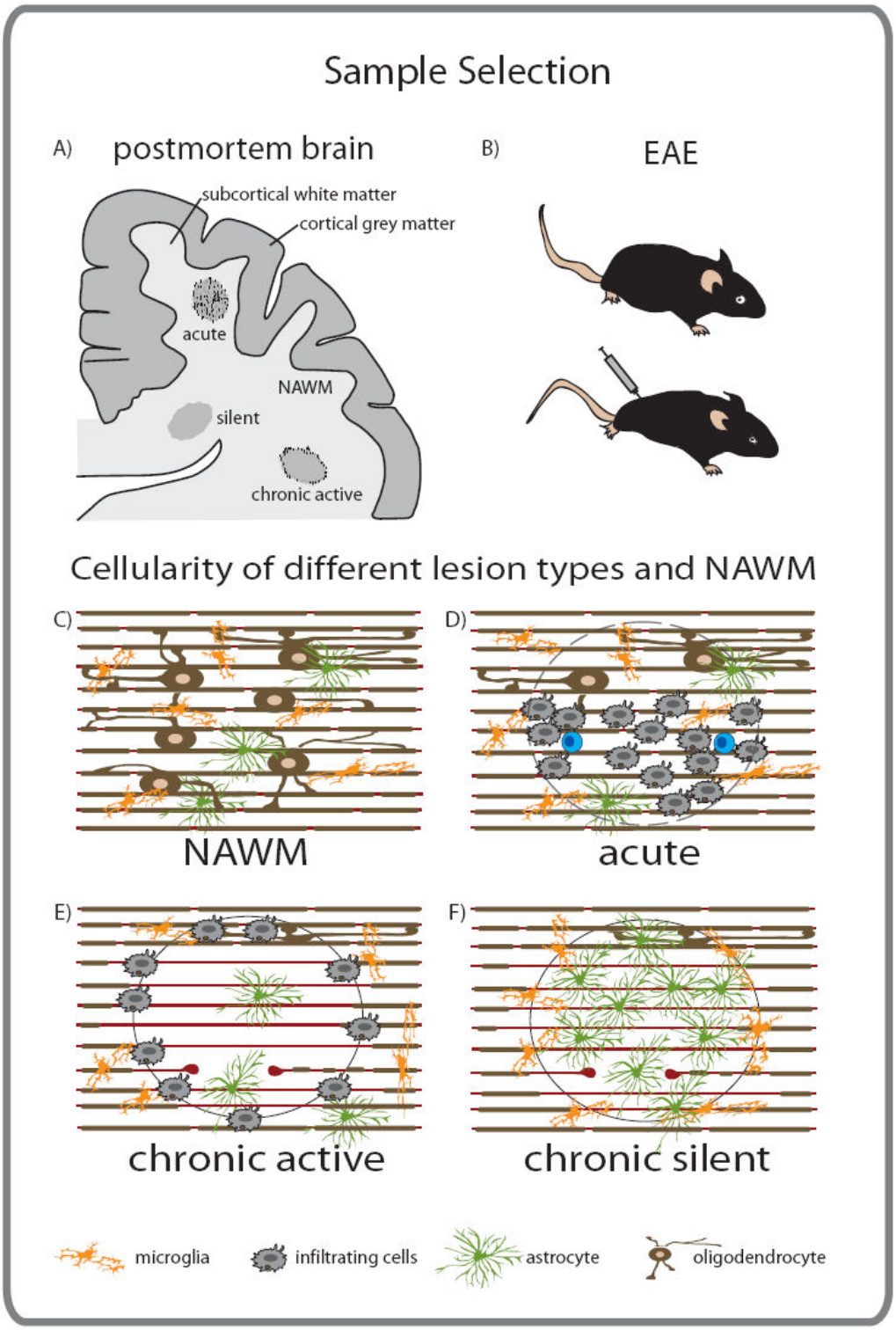

Figure 2 Different type of brain tissues analyzed in MS research.

Different types of brain tissue have been used for microarray studies in the field of MS research (A). Additional, an animal model for MS, namely EAE, has been used to isolate tissue from the central nervous system for microarray experiments (B). An important issue in identifying differential expressed genes is the cellular composition of the tissue to be compared. White matter is mainly populated by myelinating oligodendrocytes, astrocytes and microglia (C). In lesions the cellular composition is even more complex due to infiltrating immune cells, like T-cells, B-cells and peripheral macrophages (D-F). Therefore, the comparison of tissue with different cellular composition (e.g. lesioned MS tissue vs. tissue from control cases) must be interpreted with this special focus and awareness.

Due to the above mentioned points a careful characterization of the tissue that will be finally used for RNA extraction is important. Immunohistological characterization with markers for demyelination, infiltration, and gliosis are necessary and a prerequisite for the interpretation of the data. Certainly, it is nearly impossible to fulfil all these criteria, but they have to be considered when analyzing and interpreting the results. Microarray studies using brain tissue published so far differ in most of the points mentioned above, and are therefore difficult to compare with each other. In the next chapter we summarize these studies using various MS brain tissues as source for RNA.

Results from RNA profiling in MS brain tissue

White matter lesions are the hallmark of MS, and therefore analyzed extensively. Several studies investigated the gene expression pattern in lesions by comparing them with either tissue from control cases, NAWM, or other types of lesions (an overview of the different studies is provided in the Table). More recently, first studies on molecular alterations in cortical grey matter were performed investigating changes in neurons (Allen and McKeown, 1979; Dutta et al., 2006). All of these studies are based on a small number of cases (max. 10), which most of them were in a progressive disease stage. As all these studies based on post-mortem tissue, they represent a snap-shot of the situation at late stages of the disease course. Therefore, gene expression changes observed in studies using post mortem brain tissue are usually reflecting the chronic disease stage plus the bias of aging, which can be excluded by using appropriate control tissue as discussed above. This is in contrast to studies using peripheral blood, where large numbers of patients at different disease stages can be sample.

Studying gene expression in MS lesions

After an initial study comparing an acute lesion with NAWM of one MS case, Whitney et al. published a second study using several lesions from two MS cases and white matter from control cases (Whitney et al., 1999; Whitney et al., 2001). This study combined the use of cDNA microarray technology and EAE, to identify gene transcripts that are more abundant in either disease states, but not in normal white matter or normal mouse brains. One gene, 5-lipoxygenase, could be identified to fulfil

these criteria. Immunohistochemistry showed that this gene is mainly expressed by infiltrating macrophages, and only weakly expressed in normal control brains. Lock et al. detected an increased expression of inflammatory cytokines (e.g., IL-6 and IL-17), complement genes and other genes involved in immunity (Lock et al., 2002). They also analyzed the role of the immunoglobulin (Ig) Fc receptor common gamma chain and the granulocyte colony-stimulating factor (G-CSF) in EAE. Tajouri and colleagues investigated expression profiles in acute and chronic active MS lesions and compared those with patient-matched white matter (Tajouri et al., 2003). In two separate reports, Mycko and colleagues studied gene expression between margins and centres of chronic active and chronic inactive lesions from autopsy samples of four patients (Mycko et al., 2003; Mycko et al., 2004). Significant differences in the transcriptional profiles of these two lesion types were found. Lindberg et al. investigated gene expression in active lesions and NAWM from brain autopsy samples of patients with secondary progressive MS by comparison with aged-matched controls (Lindberg et al., 2004). Genes involved in the humoral immune response showed the highest change in expression underscoring the important role of a humoral immune response in lesions.

Studying gene expression in NAWM

Other studies focused on identification of differential expressed genes in the normal appearing white matter (Dutta et al., 2006; Graumann et al., 2003; Lindberg et al., 2004; Zeis et al., 2008). In 1979 Allen et al described several histological abnormalities in NAWM that was defined as white matter devoid of macroscopic visible plaques (Allen and McKeown, 1979). Microscopically normal white matter was therefore described as white matter tissue without any pathological signs, like gliosis, demyelination, or infiltration. This definition depends strongly on the methodology available and can vary from study to study, especially over time when techniques and knowledge improves. Analyzing NAWM would address the question whether specific changes exist in the NAWM, which either lead to lesion formation or are protective and prevent tissue damage. The number of infiltrating cells in NAWM is very low and can be neglected (Figure 2). Therefore, the cellular composition in NAWM is similar as in the white matter of normal control cases. This makes the NAWM an ideal tissue for analyzing differential expressed genes in the MS brain.

Only a few studies analyzed differential gene expression in NAWM (Dutta et al., 2006; Graumann et al., 2003; Lindberg et al., 2004; Zeis et al., 2008). The study of Graumann et al. compared 12 tissue samples from NAWM of 10 MS cases with 8 white matter tissue samples from 7 control cases (Graumann et al., 2003). To further reduce tissue heterogeneity subcortical white matter was specifically chosen for all tissue samples and used for RNA isolation. Selected results obtained by the microarray experiment were verified using in-situ hybridization and immunohistochemistry (Graumann et al., 2003; Zeis et al., 2008). As a result of this particular selection of well defined brain areas - and by exclusion of tissue samples with different cellular composition - endogenous expression changes could be detected. Several alterations have been identified, in which the molecular footprints pointed towards changes that are characteristic of neuroprotective mechanisms against hypoxic insults (Graumann et al., 2003). This study further revealed upregulation of a number of functionally related genes known to be involved in endogenous neuroprotection as well as in maintenance of cellular homeostasis. The results demonstrated that in MS brain a global defence against oxidative stress is mounted, probably in order to preserve cellular function. This is based on the observation that whole signalling pathways involved in long-term ischemic tolerance were significantly upregulated in MS NAWM, best illustrated by the upregulation of one of the key transcription factors HIF1α and its downstream genes (Graumann et al., 2003).

In a follow-up study, Zeis et al. could demonstrate an alteration of several genes involved in inflammatory mechanisms (Zeis et al., 2008). Using immunohistochemistry, they could show that the anti-inflammatory STAT6 signalling pathway is expressed mainly by oligodendrocytes, whereas the pro-inflammatory transcription factor STAT4 is expressed by microglia. The identification that oligodendrocytes in MS express “anti-inflammatory” genes led to the conclusion that oligodendrocytes might be capable to modulate its environment and by that can influence the progression of the disease (Zeis et al., 2008). An additional study comparing NAWM from MS cases with white matter from age-matched controls provided evidence of dysfunctional homeostasis and changes related to immune-mediated mechanisms, supporting the concept of MS pathogenesis being a generalized process that involves the entire CNS (Lindberg et al., 2004).

Although, MS is a primary demyelinating disease, progressive neuronal dysfunction due to axonal degeneration is occurring. Therefore, studies of molecular alterations in neurons might reveal changes allowing a better understanding of the ongoing neurodegenerative process observed in MS. In a microarray analysis of grey matter from the motor cortex a decreased expression of mitochondrial genes as well as of particular components of the GABA-ergic neurotransmission system was identified (Dutta et al., 2006). In a second study, an upregulation of a set of genes involved in the ciliary neurotrophic factor (CNTF) signalling pathway was observed (Allen and McKeown, 1979). These results suggest that a mitochondrial dysfunction could contribute to progressive neurological disability in MS patients, whereas induction of CNTF signalling might represent a compensatory response to disease pathogenesis.

All the studies used post mortem tissue, and therefore, investigated the expression of genes mostly in a chronic state of the disease. The question arises which of the observed changes are disease specific and which are due to the long disease progression. To analyze the pathophysiology in MS in earlier states of the disease, brain biopsies would be the tissue of choice, but the disadvantage is the rare availability of brain biopsies from MS cases. Still, brain tissue of single MS cases who passed away at a very early time point during the disease course exist, and would be a valuable source for comparing molecular changes during early and acute phase with the one from later and chronic stage (Barnett and Prineas, 2004). Another drawback is the fact that most biopsies are formalin fixed and embedded in paraffin. Improving the technologies in using RNA from fixed and paraffin embedded biopsy samples for gene expression studies would allow the investigation of molecular alterations in the early phase of MS. Alternatively, comparison with other brain disorders in which to some degree inflammatory processes are activated, might help to identify disease specific changes. Microarray and expression profiling in diseases such as ischemia, epilepsy, and Alzheimer have been performed (Majores et al., 2004; Reddy and McWeeney, 2006; Vikman and Edvinsson, 2006), but for the same reason as described before, comparison of the data is difficult. Nevertheless, common molecular changes such as microglia and astrocyte activation could be identified.

Summary

The use of cDNA microarrays in RNA expression profiling studies provided new insights in the pathomechanism of MS. The identification of endogenous protective mechanisms in MS brain may lead to the development of new therapeutics drugs, which can help to impede the progressive loss of neurological function seen in MS. Future expression studies with increased case numbers and better defined MS cases may give more insights in the molecular basis of the heterogeneity seen in the MS disease course. Hopefully, such RNA profiling studies of MS brain tissues will help to design more specific treatments. The potential of studying gene expression using brain biopsies is promising and will hopefully give more insights in the molecular changes occurring in early stages of MS.

Acknowledgment

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A Chronic Relapsing Animal Model for Multiple Sclerosis

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Abstract

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). One of the most used animal models for MS is experimental autoimmune encephalomyelitis (EAE). Until now several different EAE models were developed, differing in the immunological reaction, inflammatory processes and the neuropathophysiology in the CNS. Here, we present a model induced in DA rats by immunization with the N-terminal fragment of myelin oligodendrocyte glycoprotein. This specific model shows several similarities to MS such as a relapsing-remitting disease course, demyelination and axonal degeneration. By immunohistochemical characterization, lesions could be detected mostly in the spinal cord, but also in optic nerve and tract, brainstem, cerebellum and in different areas of the forebrain. The mimicking of particular features of MS and the occurrence of special disease entities like optic neuritis, Devic's disease and the acute MS form of Marburg's type makes this EAE type a excellent model for investigating certain aspects of the pathophysiology seen in MS.

Introduction

Multiple sclerosis (MS) is the most common neurological disorder of young adults in the Western countries. The hallmarks of the disease are demyelinating lesion in the central nervous system (CNS). The current prevailing hypothesis is that MS is an autoimmune disorder directed against CNS antigens leading to inflammation and demyelination (for review see Hemmer et al., 2002; Lassmann et al., 2001; Noseworthy et al., 2000; Wekerle, 1998). The primary cause and the pathogenesis of MS are still unknown. A common animal model used to study possible pathological mechanisms of MS is experimental autoimmune encephalomyelitis (EAE). Since the initial experiments by Rivers et al. (Rivers, 1933), several different models of EAE were developed, which differ in the immunological reaction, inflammatory processes and the neuropathophysiology in the CNS (for review see Gold et al., 2006; Steinman and Zamvil, 2005; Wekerle, 1993). Each model shares similarities to MS but also differs in some aspects from these. Therefore, the proper selection of the most valuable model is essential and depends strongly on the scientific question being addressed.

The various models differ in the choice of species, strain, antigen, and immunization protocol that are used. There exist models for non-human primates like marmosets and rhesus monkeys, as well as for rodents like guinea pigs, rats, and mice. The latter has become the most used animal with the advantage of the availability of genetically modified mice and the good knowledge about the mouse genome. In rats as well as in other animals the susceptibility and the type of EAE observed is strongly strain dependent. Further, the antigen used for induction of the autoimmune response is an important issue. There exist a large variety of antigens to induce EAE: whole spinal cord preparation, purified myelin proteins, recombinant myelin proteins or synthetic peptides. The known CNS antigens that can induce an autoimmune response and EAE are myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein (MAG), proteolipid protein (PLP), and S-100 protein. Generally, there are two different ways to induce EAE: it can be induced by active immunization with neuro-antigens or by passive transfer of neuro-antigen specific T-cells, which were stimulated in vitro with the antigen for 3-4 days. Even the immunization protocols necessary for induction of EAE vary. For example, DA rats develop severe paralytic EAE after immunization with MBP in incomplete Freud's adjuvant (IFA), which lacks mycobacteria, whereas mice may require multiple injections of antigen as well as additional pertussis toxin injections for EAE induction (Lenz et al., 1999; Martin et al., 1992).

As MOG-induced EAE in particular rat strains shares the major features of MS such as a relapsing-remitting disease course and demyelination (Adelmann et al., 1995; Johns et al., 1995), we describe here a protocol for MOG-induced EAE in DA Rats. The inbreeding of these rats was initiated by Odell at the Oak Ridge National Laboratory and completed at the Wistar Institute in 1965. This strain is susceptible to the induction of autoimmune thyroiditis (Rose, 1975) as well as collagen-induced arthritis following immunisation with type II collagens. Protocols were established for MOG-induced EAE in DA rats using either recombinant rat (Weissert et al., 1998) or mouse MOG (Storch et al., 1998b) as antigen. In this chronic relapsing (CR) EAE model, which is presented here, animals are actively immunized with a recombinant N-terminal fragment of mouse MOG, which leads to the formation of inflammatory demyelinating lesions depending on both T-cells and anti-MOG antibodies (Linington et al., 1988).

The pathology in the described MOG-EAE model reflects the spectrum seen in MS in many ways. Not only a relapsing-remitting disease courses like in classical MS can be observed, but also special disease entities like optic neuritis, Devic's disease and the acute MS form of Marburg's type are observed in MOG immunized DA rats (Storch et al., 1998b). Inflammatory demyelinating lesions and axonal degeneration are both typical characteristics seen in MS as well as in the described EAE model. For these reasons, the MOG-induced CR-EAE model in DA rat represents a suitable model for studying the pathophysiological mechanisms in MS.

Materials and Methods

All protocols for animal experimentations must first be reviewed and approved by an Institutional animal Care and Use Committee, or must conform to governmental regulations regarding the care and use of laboratory animals.

Purification and Expression of recombinant MOG

Expression of recombinant mouse MOG. For expression of recombinant mouse MOG the bacterial expression vector pRSET A (Invitrogen Corp) containing the amino acids 1 to 116 of the mature mouse protein fused to a several histidine residues (Pham-Dinh et al., 1993; Reynolds et al., 2002). An overnight culture was used for inoculation of a large expression culture (SOB, ampicillin, kanamycin). The OD_{600} was measured until it reaches 0.5 and expression was induced by the addition of IPTG at 1mM final concentration. After 4 hours the bacteria were harvested by centrifugation (15min, 4000g). The pellet was then frozen and stored until purification was performed.

Purification of HIS-tagged MOG. For immobilized metal ion affinity chromatography (IMAC) the Talon purification system (Clontech) was used. The bacteria pellet was resuspended in lysis buffer (8M Urea, 100 mM NaH_2PO_4, 10 mM Tris HCl, pH 8) and sonicated to disrupt the bacteria. After a further centrifugation (20min; 10000g) the pellet was again resuspended in lysis buffer and the centrifugation step was repeated. Both supernatants were pooled and subjected on the IMAC column for purification at room temperature. After loading, the column was washed with 2 volumes of lysis buffer and 2 volumes of washing buffer (8M Urea, 100 mM

NaH_2PO_4, 10 mM Tris HCl, pH 6.3). The purified recombinant protein was collected by eluting the column with Elution buffer (8M Urea, 100 mM NaH_2PO_4, 10 mM Tris HCl, pH 4.5). To obtain soluble (not refolded) recombinant MOG the purified protein was dialysed 4 times (dilution factor 1:200 each) against 20 mM sodium acetate buffer (pH3.6) at 4°C. Finally, the purified and soluble protein was concentrated (Centricon, 10,000 MWCO) until the protein concentration was at least 2mg/ml. The protein was aliquoted and stored at -80°C. Aliquots once thawed may not frozen again.

Induction of EAE in DA Rats

Animals. 10-12 weeks old female DA/OlaHsd rats were purchased from Harlan (Netherlands). The animals were housed in light- and temperature-regulated rooms under specific pathogen-free conditions with free access to water and food. Note, that the housing conditions can also influence the clinical course of EAE (Goverman et al., 1993).

Antigen Preparation and Immunization. An 1:1 emulsion of 2mg/ml recombinant MOG solution and IFA was prepared. The IFA/antigen mixture was drawn up into a glass syringe with an 18-G needle. The needle was removed and a syringe was attached to a double-ended locking hub connector (Luer-Lok, Becton Dickinson) or plastic 3-way stopcock. At the other end an empty glass syringe was attached and the mixture was forced back and forth from one syringe to the other repeatedly. When the mixture was homogeneous and white the connecter was disconnected, a 22-G needle was attached, and air bubbles were removed. (When extruding a small drop on the surface of water, a good oil-in-water emulsion should hold together as a droplet and not disperse). The emulsion was prepared just before the immunization and kept on ice. The DA rats were first anesthetized with isoflurane, then the base of the tail was shaved and disinfected. Immunization occurred with application of 100µl of the emulsion subcutaneously at the base of the tail. The tail of each animal was marked for identification. Rats were weighted daily and the clinical score was monitored as described below.

Monitoring the Clinical Score

One week after immunization the rats were monitored every day for neurological deficits, which start about 14 days after immunization. Each rat was graded daily and assigned a score from 0 to 5 as shown in table1. Rats were sacrificed after 60 days and brains as well as spinal cords were removed for further analysis. When they had a clinical score of more than 3 for two days in a row they had to be killed for ethical reasons.

The clinical stages of the disease are defined as follows: The *acute phase* is the period of the first clinical signs, in which rats show ascending paralysis following active disease induction. The phase of clinical improvement that follows a clinical episode was described as *remission*. A remission was defined as a reduction of the clinical score by a minimum of one grade for at least 2 days after the peak of the acute phase or a disease relapse has been reached. A *relapse* is the phase of increasing neurological deficits seen after remission. This is normally defined as an increase of at least one grade in clinical score maintained for at minimum of 2 days after remission has occurred. The animals were grouped in four different categories depending on the disease course obtained by the clinical score recorded during the experiment. The animals without any obvious neurological deficits represented one group. A second group was composed of animals with an acute or progressive disease course showing no remissions and did not establish a stable chronic phase. Animals showing a stable chronic state after an initial acute phase represented a third group and a fourth group consisted of rats with a relapsing-remitting disease course, which was defined by at least one relapse of 1 score for a minimum of 2 days.

Table 1. Clinical score

Score	Clinical signs
0	no clinical signs
1	tail weakness
2	monoparesis or monoplegia
2.5	mild paraparesis
3	paraparesis or paraplegia
3.5	paraplegia with spasticity
4	hemiplegia, quadriparesis
5	quadriplegia, moribund state

Immunohistochemistry

Animals were anaesthetized by inhalation with 2-3 vol% isoflurane (Abbott, Switzerland) and sacrificed by decapitation. The brain and the spinal cord were removed and either fixed for 24 h in 4% paraformaldehyde (PFA in PBS, pH 7.5), cryoprotected and embedded, or directly embedded in OCT-Compound and fresh frozen using dry ice. Cryostat sections (12µm) were either mounted on gelatin-coated slides or processed as free floating sections for further analysis. Tissue sections were fixed in 4% PFA for 30 min or in acetone for 1min depending on the antibody used. For staining of PLP or neurofilament the tissue sections were incubated in 70% ethanol over night at room temperature. After blocking the sections in blocking solution (PBS pH 7.5, 2% fish gelatine, 2% normal goat serum, 0.2% Triton X-100) for 1 h, sections were incubated with the appropriate primary antibodies over night at 4 °C in blocking solution (Schaeren-Wiemers et al., 2004). When peroxidase was used, endogenous peroxidase was quenched by incubation of slides for 20 min in methanol plus 0.3% H_2O_2. Incubation of secondary antibodies was performed either with fluorescence labeled or biotinylated antibodies in blocking solution. When biotinylated secondary antibodies were used, further incubations were performed with premixed avidin and biotylinated peroxidase complex (Vecta-stain ABC kit; Vector Laboratories) according to the manufacturer's instructions. The immunohistochemical signal was revealed by a color reaction with AEC (3-Amino-9-ethylcarbazole). Counterstaining was either performed with hematoxylin for 1 min

followed by rinsing the slide in running tap water or with DAPI when using fluorescence labeled antibodies.

Antibodies. Following primary antibodies were used: CD68 (ED1; Serotec; 1:500); PLP (MCA839G; Serotec; 1:500); CD11b (MCA275R; Serotec; 1:500); GFAP (G-A-5; Sigma; 1:2000). As a secondary antibody a biotinylated Goat Anti-Mouse IgG (115-065-166; Jackson ImmunoResearch; 1:500) was used.

Results and Discussion

Clinical Course of disease

The first clinical signs, typically the loss of tail tonicity, were observed about two weeks after the induction of EAE. In the MOG induced EAE model in DA rats the animals show a broad spectrum of different disease courses. By varying the amount or the solubility of the antigen the spectrum of disease courses can be influenced. Immunization of precipitated recombinant MOG leads to an increase of animals that develop an optic neuritis but fail to develop clinical signs (Storch et al., 1998b). Increasing the amount of antigen leads to a larger fraction of animals showing a relapsing-remitting disease course (Papadopoulos et al., 2006). In our study, we used 100 µm MOG resulting in four groups of animals defined in respect of the clinical disease course. Typical EAE disease courses are shown in table 2 and figure 1. One group of up to 30% was composed of animals showing no obvious neurological deficits (fig. 1a). A second group of rats displayed an acute progressive form without a remission or a stable chronic phase (fig. 1b). These animals have to be sacrificed early after the disease onset because of the lack of disease amelioration or stabilisation. After the initial acute phase a third group of animals stabilize in a chronic phase and neither show any detectable disease progression nor amelioration of the disease (fig. 1c). Although, it cannot be excluded that there is still a slow disease progression that is not detectable by the grading system, these rats resemble a group of animals with chronic EAE. The last group is defined by rats developing a relapsing-remitting course (fig. 1d) mimicking the typical disease course seen in MS patients during early disease phase. Although the classification into four groups is a simplification, it demonstrates the spectrum of disease courses seen in MOG-induced EAE in DA rats, which to some extend corresponds to the different

disease courses observed in MS patients. An experiment with a more homogenous disease outcome can be obtained by varying dose and solubility of recombinant MOG used for immunization.

Table 2. Spectrum of disease course

Disease course	Number	Percent of total
No clinical signs	18	33
Acute	15	27
Chronic	8	15
Relapsing-remitting	14	25

EAE type distribution: clinical scores of the animals were analyzed over the experiment time. Animals which had to be sacrificed after less than 7 days after disease onset were counted as acute EAE. An improvement of at least 1 score point for more than 1 day was counted as remission. Animals having a clinical course with a steady worsening and no improvement over at least 20 days were counted as chronic-progressive EAE. In total, we analyzed 70 animals from which 15 were control and 55 were EAE animals.

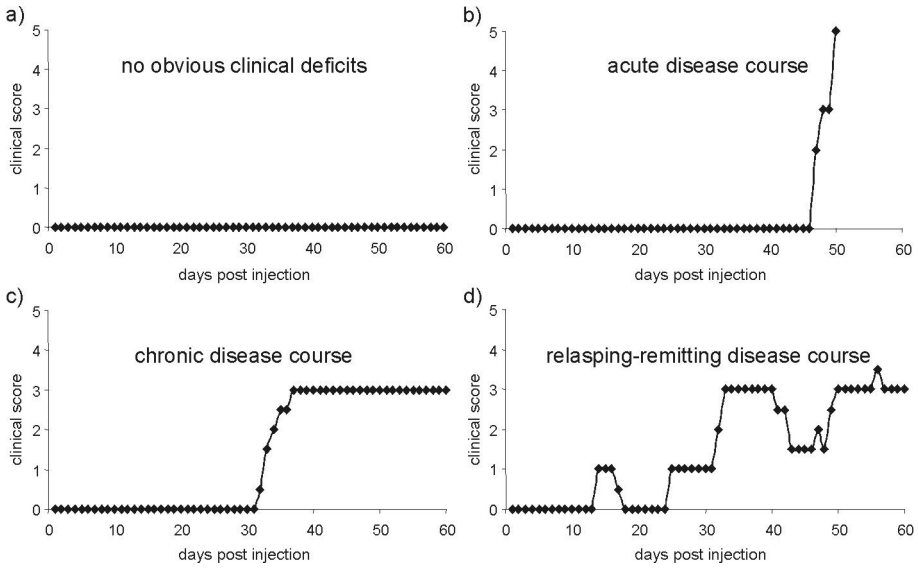

Fig. 1. Different disease courses in MOG-induced EAE in DA rats. Four typical disease courses can be observed in an EAE experiment induced by immunization with 100 µg recombinant MOG. Whereas some animals do not show any clinical signs (***a***), others develop an acute disease onset without remission (***b***). In some cases, animals stabilize after an initial acute phase and show a chronic disease course without further relapses or remissions (***c***). The fourth group includes animals which have a relapsing-remitting disease course characterized by at least 1 relapse (***d***).

Lesion characterization

Lesion identification and characterization within the CNS were done by immunohistological analysis with several antibodies to identify inflammation (CD68), demyelination (PLP), astrogliosis (GFAP), or neuronal degeneration (neurofilament). The pathology of MOG induced EAE was characterized by large inflammatory demyelinating lesions within the CNS. The highest incidence of lesions was found in the spinal cord (fig. 2), optic nerve and tract, brainstem, and cerebellum. In some animals lesions can also be detected in regions of the forebrain (fig. 3). The anatomical localisation of inflammatory infiltrates influences the clinical score, which is based mainly on motor deficits caused by spinal cord lesions. Additionally, ataxia can be observed primary due to lesions in the brainstem or cerebellum. In addition, the anatomical distribution of lesion within the central nervous system is influenced by the antigen specificity of T-cells (Berger et al., 1997). For example, adoptive transfer of MBP-specific T-cells in Lewis rats results in widespread inflammation of the spinal cord and only minor involvement of the forebrain, whereas MOG-specific T-cells induce lesions also within the forebrain and the optical nerve.

Cellular composition of lesions

In most models of EAE the cellular composition consists of infiltrating T-cells, macrophages and activated microglia. To identify infiltrating phagocytotic cells, like activated macrophages and microglia, an antibody against CD68 was used (fig. 2d, g, j, 3b). High density of activated macrophages/microglia could be detected in demyelinated areas within the spinal cord (fig. 2d, g, arrows) and the brain (fig. 3e). Even in areas adjacent to the demyelinated lesions, which were apparently normal, were often populated by CD68 positive cells (fig. 2d, arrowhead). These might be signs of ongoing white matter destruction. Activation of macrophages/microglia could be confirmed by using CD11b as an additional marker (fig. 2f, i, arrows). Strong immunoreactivity of antibodies against GFAP demonstrated pronounced astrogliosis in and around the lesions (fig 3e). In actively demyelinating lesions signs of antibody mediated myelin destruction were observed, which was evident by the presence of complement components and IgG (Linington et al., 1989). In some cases, widespread demyelination, inflammation and astrogliosis were observed throughout the whole width of the spinal cord (fig. 2j, k, l).

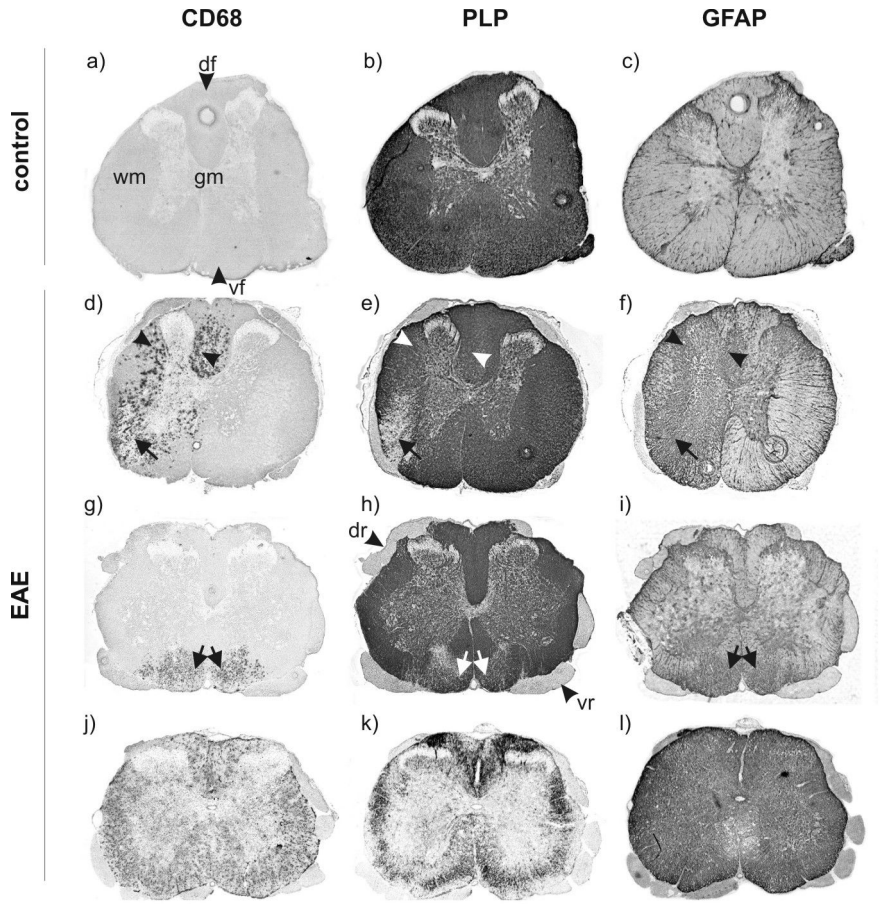

Fig. 2. Examples of inflammatory demyelinating lesions in the spinal cord. Lesion characterization was performed by immunohistological analysis to identify inflammation (CD68; ***a***, ***d***, ***g***, ***j***), demyelination (PLP; ***b***, ***e***, ***h***, ***k***) and astrogliosis (GFAP; ***c***, ***f***, ***i***, ***l***). In contrast to control animals (***a--c***), inflammatory demyelinating lesions were present in animals developing EAE (***d--l***). Inflammation in the white matter (***d*** and ***g***, arrows) was leading to focal demyelinating lesions (***e*** and ***h***, arrows) and astrogliosis (***f*** and ***i***). Interestingly, demyelination did not always correlate with infiltration (***d*** and ***e***, arrowheads). Note that astrocytes are already activated in areas where demyelination was not yet evident (***f***, arrowheads). In some animals, a widespread infiltration as well as an extensive demyelination could be observed (***j*** and ***k***). As a consequence, a strong activation of astrocytes could be detected throughout the whole width of the spinal cord (***l***). df = Dorsal funiculus; dr = dorsal root; gm = gray matter; vf = ventral funiculus; vr = ventral root; wm = white matter.

Demyelination

The extent of demyelination within spinal cord (fig. 2e, h, k) and brain (fig. 3a-c) was visualized by using an antibody recognizing the major myelin protein PLP. MOG-EAE induces not only an encephalitogenic T-cell response but also an autoantibody response which initiates demyelination and enhances disease severity in animals

actively immunized with MOG. Passive transfer of activated MOG specific T-cells only lead to inflammation and additional administration of antibodies is necessary to obtain demyelination (Linington et al., 1988). The strong demyelinating activity seen in animals actively immunized with MOG is unique among the available EAE models. Demyelination is mediated through a combination of complement and antibody-dependent mechanism, while the local production of pro-inflammatory factors enhances the inflammatory response (Linington et al., 1988; Weissert et al., 1998). Remyelination by oligodendrocytes and Schwann cells are observable by tightly compacted abnormally thin myelin sheaths. Myelin repair is already evident after 10 days from onset of disease in DA rats (Papadopoulos et al., 2006).

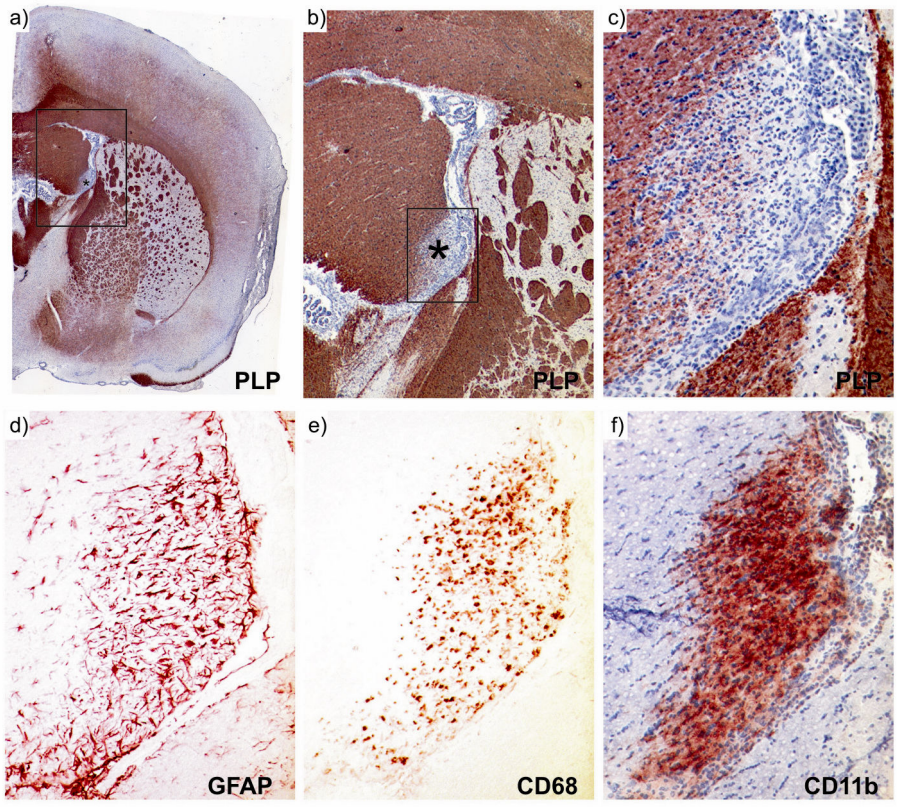

Fig. 3. Demyelinating lesions in the brain. The MOG-induced EAE model in DA rats also induces demyelinating lesions in the brain. Lesions are observed often in the brainstem and the cerebellum, but also in periventricular regions of the forebrain, e.g. the septum (***a***). The lesions are characterized by a loss of myelin proteins like PLP (***b*** and ***c***) and the presence of reactive astrocytes (***d***). In the lesion, numerous CD11b-positive cells (***f***) can be seen, which are composed of activated microglia and macrophages showing also phagocytotic activity identified by the marker CD68 (***e***).

Axonal pathology

The rediscovery of axonal damage as an important component of Multiple sclerosis has lead to new insights in the pathology of Multiple sclerosis lesions (Ferguson et al., 1997; Trapp et al., 1998). Axonal injury and loss is thought to be responsible for the progressive exacerbation of the disease and the need for neuroprotective therapies has become apparent (for review see Bjartmar and Trapp, 2001). Experimental studies soon showed parallel findings in EAE (Kornek et al., 2000; Wujek et al., 2002). Furthermore, specific molecular abnormalities such as redistribution of ion channels on chronically demyelinated axons were identified that may play an important role in the axonal pathology of Multiple sclerosis (Craner et al., 2004a; Craner et al., 2004b; Kornek et al., 2000). The degree of axonal loss correlates with clinical severity in progressive as well as relapsing-remitting forms of MOG-EAE (Papadopoulos et al., 2006). In contrast, demyelination and inflammation does not show any significant correlation with the clinical severity scores in animals having a relapsing-remitting disease course (Papadopoulos et al., 2006).

Conclusions

There are several EAE protocols available and the choice which model should be applied depends strongly on the scientific question to be addressed. Compared to Multiple sclerosis, where the primary cause of the disease remains still unknown, the EAE models are autoimmune mediated diseases. The MOG-induced EAE in DA rat closely mimics some of the main clinical features of Multiple sclerosis, which makes it an attractive model to study the pathophysiology of the disease. A spectrum of disease courses can be observed in MOG-EAE in DA rats ranging from animals showing no clinical sign to severe fatal forms. An advantage is the possibility to influence the disease outcome in favour of a specific disease course, such as relapsing-remitting EAE. The presence of demyelinating lesions and remyelinating activity makes the model a viable tool for investigations of the mechanisms involved in remyelination. In MS patients axonal loss has been observed which correlates with disease progression. Axonal degeneration and loss is also present in the CNS of DA rats immunized with recombinant MOG and this axonal pathology in inflammatory demyelinating lesions closely reflects that observed in MS.

In summary, the MOG-induced CR-EAE in DA rats may serve as a good model for investigating certain aspects of the pathobiology seen in MS.

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Discussion

General discussion

Very little is known about the earliest intrinsic changes in the brain of MS patients before the appearance of overt inflammation and demyelinating lesions. However, MRI and gene expression studies have shown significant changes in NAWM that might give indications concerning both, the early changes occurring before lesion formation and the attempts by the CNS to prevent the same (Aboul-Enein et al., 2003; Filippi et al., 1998; Fu et al., 1998; Graumann et al., 2003; Silver et al., 2001). Most recent data also suggest molecular changes and diffuse inflammatory damage spreading throughout the whole brain in the chronic phase of the disease associated with slow progressive axonal injury at sites without inflammation (Graumann et al., 2003; Kutzelnigg et al., 2005).

Although earliest changes are already present in the NAWM, this tissue bears the most promising possibility to detect primary, MS specific pre-lesional changes, which are independent from secondary inflammatory reactions due to lesion formation. Furthermore, NAWM tissue from chronic MS cases is ideal to study the intrinsic changes of brain cells during the long-lasting disease course of MS, whereas the NAWM of early disease course tissue additionally allows the identification of mechanisms crucial for lesion initiation and further development of the disease.

Anti-inflammatory oligodendrocytes might play a major role in the CNS immune-privilege limiting lesion formation during MS relapses

In order to identify these pre-lesional changes, a gene expression profile study was performed in the NAWM of 11 MS cases suffering from chronic MS. In addition, the non-lesional white matter of a female MS case during her first clinical relapse was analyzed. In both studies, differential gene expression of genes involved in different mechanisms of the inflammatory cascade, such as signalling, transcription, cell adhesion and antigen presentation was detected. Most prominent was the upregulation of STAT6 in all MS NAWM tissues from early as well as late disease course. The function of STAT6 is not yet known, but it is known that this transcription factor plays a central role in anti-inflammatory mechanisms (Pfitzner et al., 2004).

STAT6 is the major transcription factor of IL-4 and IL-13 signalling (Takeda et al., 1997). IL-4 and IL-13 signalling over STAT6 leads to the differentiation of T cells into the anti-inflammatory T helper cell type 2, releasing anti-inflammatory cytokines. The involvement of STAT6 in anti-inflammatory mechanisms is further demonstrated by the exacerbation of the pro-inflammatory autoimmune response in EAE-induced STAT6-deficient mice (Chitnis et al., 2001).

In our post mortem study the main cells expressing STAT6 in the MS NAWM were oligodendrocytes. Furthermore, JAK1, IL-4R and IL-13R, all belonging to the STAT6 signalling pathway (Hebenstreit et al., 2006), were also upregulated in MS NAWM and expressed by oligodendrocytes. JAK1 and IL-4R have been shown to be expressed in oligodendrocytes in MS brains, which is in agreement with the findings of Cannella and Raine (2004). Comparison of the expression of STAT6, IL-4R, JAK1 and another member of the STAT6-signalling pathway, JAK3, revealed a strong correlation in expression intensity within the different cases. This suggests an overall upregulation of the STAT6 signalling pathway in oligodendrocytes in MS NAWM.

From our data, we hypothesize that a protective, anti-inflammatory response can not only be mounted by T cells but also by oligodendrocytes in the MS NAWM. The involvement of oligodendrocytes in the immune response of the brain is further supported by two recent studies showing that oligodendrocytes are mounting protective mechanisms in order to prevent demyelination (Lin et al., 2007), and by the findings that oligodendrocytes are responding to IFN-γ in a protective way and capable of expressing chemokines (Balabanov et al., 2007). These data give rise to a new view of oligodendrocytes participating in the regulation of CNS intrinsic immunity, the so-called CNS immune privilege. As oligodendrocytes are highly susceptible to inflammation mediated damage, it may be crucial for them to compensate for the induced “pro-inflammatory environment” and to limit the inflammatory response and subsequent damage. The upregulation of STAT6 was detected in all MS cases and to a much higher extent in the non-lesional white matter of the MS case during early disease course. A high induction in white matter tissue together with extent MRI abnormalities in a early MS case during the first relapse further highlights an important functional role of STAT6 in MS. Our explanation could be that the induction of anti-inflammatory mechanisms in oligodendrocytes is crucial for limiting immune responses during relapses of MS in order to prevent extensive lesion formation.

Are signals from MS lesions leading to changes in the NAWM or are changes in the NAWM leading to MS lesions?

The cause of differential gene expression in the MS NAWM might either be a consequence of diffusing signalling molecules (e.g. cytokines, chemokines or growth factors) released from nearby lesions and/or peripheral immune cells or due to regulation by NAWM intrinsic events. An involvement of signalling from the nearby lesions of the NAWM is supported by a study using quantitative MRI techniques, where the authors suggest that axonal damage and demyelination in NAWM mainly arise as a secondary result of visible lesions with the largest effect close to these lesions (Vrenken et al., 2006). This is further supported by the fact that an expression of the main cytokines, regulating pro- and anti-inflammatory responses (e.g. IL-4, IL-13, IL-12 and IL.-23), was not detected in our MS NAWM gene expression study, whereas their downstream genes STAT4 and STAT6 as well as other inflammatory genes were shown to be differentially regulated. This absence of IL-4, IL-12, IL-13 and IL-23 expression on one hand, and the simultaneous upregulation of their target genes from the STAT4 and STAT6 pathway in MS NAWM on the other hand, further imply that extrinsic signals might influence the expression of immune modulating genes in the resident cells of the CNS.

A differential gene expression due to intrinsic events is supported by the upregulation of the endogenous nNOS, but not of iNOS and eNOS, suggesting a parenchymal disregulation. Recent data showed that nNOS plays a key role in mediating CNS demyelination in a toxin-induced demyelinating animal model (Linares et al., 2006). Immune modulating signals from the periphery would first activate microglia, the main immune cell of the CNS and by that induce iNOS (Shen et al., 2005). Therefore, an intrinsic deregulation of nNOS, uninfluenced by extrinsic signals, is most likely. The phenomenon of nNOS upregulation while iNOS expression is not induced was also detected in the non-lesional white matter of the very early MS case. As the acute lesion of an early MS case during the first relapse was identified to correspond to pattern III MS lesions, a strong upregulation of nNOS in this case supports the hypothesis that intrinsic changes could be the origin of MS lesions. Whether these changes might be specific for a subset of MS patients (Pattern III lesions) or a general phenomenon in MS pathology cannot be determined yet and needs to be further elucidated. As an induction of nNOS was detected in all chronic MS cases, a

general mechanism rather than a pattern III specific mechanisms is suggested. This view is supported by Barnett and Prineas who suggested widespread oligodendrocyte damage and apoptosis to be one of the earliest change in general lesion formation (Barnett and Prineas, 2004). Still, it is unclear if these alterations might lead to, facilitate or impede lesion formation.

The view of intrinsic events leading to a changed gene expression pattern in MS NAWM is further supported by our differential gene expression study of corpus callosum tissue of MOG-induced EAE in DA rats. Although a high expression of cytokines and chemokines is reported to be induced in EAE lesions (Chitnis et al., 2001; Gold et al., 2000; Greter et al., 2005), no changes in immune-related genes of the corpus callosum were found. Therefore, diffusion of signalling molecules such as cytokines and chemokines across the BBB into the NAWM seems to be very limited, which in consequence favours the view of gene expression changes found in the MS NAWM being of intrinsic origin.

Are molecular mechanisms detected in MS NAWM also present in MOG-induced EAE?

Prominent gene expression changes suggesting an induction of ischemic, neuroprotective mechanisms and anti-inflammatory mechanisms have been revealed by a differential gene expression analysis of the MS NAWM (Graumann et al., 2003; Zeis et al., 2008). Generally, tissue derived from MS patients is limited, and functional experiments (e.g. siRNA, cell culture) are almost impossible to perform. Therefore, it would be highly useful if pathogenic mechanisms identified in MS NAWM can also be observed in a MS animal model. For this reason, we investigated whether in a particular animal model for MS, similar mechanisms are taking place. The MOG-induced EAE in DA rats was chosen since it shows many clinical as well as pathological similarities to MS (Kornek et al., 2000; Storch et al., 1998). EAE diseased DA rats were sacrificed during the chronic disease course at day 60. Corpus callosum was dissected, which would correspond best to the situation of previously analyzed subcortical MS NAWM as rats do not have subcortical white matter. To simultaneously analyze possible grey matter changes reported from MS, normal appearing grey matter from the somatosensory cortex was dissected and analyzed in parallel.

Our analysis revealed only minor changes in the gene expression in the white matter of the corpus callosum in EAE. In the grey matter of the somatosensory cortex in EAE, we found a downregulation of glutamate channels as well as genes encoding for mitochondrial proteins. Neither genes known to be involved in ischemic preconditioning mechanisms nor selected genes involved in pro- and anti-inflammatory mechanisms, shown to be changed in MS NAWM, were significantly changed in the NAWM of the corpus callosum in EAE animals. From these data we conclude that the corpus callosum is not influenced by the encephalitic autoimmune reaction in EAE. This might be due to several reasons. Although we selected animals with a long disease course (60days), we cannot exclude the possibility that gene expression changes in MS are a cause of chronic stimulation over years and thus are not yet detectable in EAE corpus callosum tissue. A study of the temporal course of $Na_V1.8$ in Purkinje neurons in EAE showed that first significant changes in $Na_V1.8$ protein as well as mRNA expression, were observed between day 51-75 (Craner et al., 2003). In contrast, EAE NAWM tissue in this study, taken after 60 days, was not directly affected by inflammatory, demyelinating infiltrates. Therefore, first changes might even appear later in time. Another difference possibly leading to diverse gene expression changes in EAE and MS NAWM might be kind of inflammation and place of its occurrence. Unlike in most chronic MS cases, lesions in MOG-induced EAE in DA rats are predominantly localized in the spinal cord and/or in the optic nerve, and much less frequently encountered in the brain (Kinter et al., 2008; Storch et al., 1998). As ischemic preconditioning mechanisms in MS NAWM might be induced by lesions located nearby, ischemic preconditioning mechanisms might not be induced in EAE NAWM tissue of the corpus callosum, where the distance to active inflammatory lesions is much higher. Additionally, corpus callosum tissue in MS is less affected by lesion formation than other regions such as the periventricular white matter and the cortico-subcortical white matter (Steiner, 1931). This suggests that corpus callosum is less involved by the inflammatory response in autoimmune disorders. Furthermore, the autoimmune response type is different between MS and MOG-induced EAE. In our study we have chosen to use an EAE model in which not only T cells are determinant for the immune reactions, but also anti-MOG antibodies (Storch et al., 1998b). Nevertheless, the immune reaction in MOG-induced DA rat EAE, as in most EAE models, is predominantly determined by CD4+ T cells. In contrast, in MS lesions, CD8+ T cells are dominating over CD4+ lymphocytes (Booss

et al., 1983) and additionally, clonal expansion of CD8+ T cells was reported (Babbe et al., 2000). Therefore, immunological differences between EAE and MS might lead to another or even no reaction of the NAWM.

Alternatively, the absence of brain lesions in MOG-induced EAE might be a consequence of unchanged gene expression in the EAE NAWM. Assuming that the differential gene expression detected in the MS NAWM might facilitate lesion formation, the absent induction of these genes might make EAE NAWM more resistant to inflammatory infiltration and lesion formation.

Ischemic preconditioning genes or genes involved in intrinsic immune responses of the white matter were not induced in the corpus callosum of this EAE model. Whether these mechanisms are induced in the white matter of the spinal cord, adjacent to lesions was not determined yet and has to be elucidated. Nevertheless, MOG-induced DA rat EAE might be an unsuitable model to study aspects of ischemic preconditioning and intrinsic immune-response mechanisms present in MS NAWM far away from lesions. In contrast, a downregulation of glutamate receptors as well as a downregulation of genes encoding for mitochondrial proteins was detected. These findings correspond to some extent to findings of the MS NAGM, possibly reflecting neuronal degeneration due to axonal injury in the spinal cord (Dutta et al., 2006; Ginsberg et al., 1996; Wang and Tseng, 2004).

Final summary

In summary, in this dissertation, the expression of immune-response mechanisms in NAWM oligodendrocytes from early and late disease course was demonstrated. In particular, an upregulation of genes from the anti-inflammatory STAT6 signaling pathway, namely STAT6, JAK1, IL-4R and IL-13R was identified. This suggests an involvement of oligodendrocytes in the intrinsic immune-response of the CNS, and therefore in being part of the CNS immune-privilege. Furthermore, a very high induction of nNOS in MRI-abnormal NAWM tissue of an MS case during early disease course was identified, suggestive of earliest changes taking place in MS, capable of supporting demyelination. Finally, it was demonstrated that NAWM tissue from MOG-induced EAE in DA rats does not necessarily induce changes found in the MS NAWM. This highlights the need of another MS animal model, where similar NAWM gene disregulation can be observed, in order to investigate earliest, intrinsic

NAWM changes possibly leading to or protecting from lesion formation. As in NAWM changes in gene expression occur, this has to be taken into consideration during the development of new therapeutic strategies for MS. Therefore, a new MS animal model which includes NAWM gene disregulation might be of great value in the development of new therapeutic strategies or even a cure for MS.

Outlook

The finding of oligodendrocytes being part of the CNS immune-privilege, is a new concept in brain immunology. The induction of the anti-inflammatory transcription factor STAT6 in oligodendrocytes might be crucial for limiting immune responses and subsequent damage in MS. Therefore, the immunological role of STAT6 in oligodendrocytes has to be further elucidated. For that, an investigation of primary, highly pure oligodendrocyte cultures from wild-type as well as STAT6 knock-out mice is suitable. Differential gene expression analysis of these cultures, untreated or treated with the main STAT6 activator cytokine IL-4, would allow to identify genes expressed in oligodendrocytes which are under the control of STAT6. Furthermore, analysis of the cell culture supernatants for cytokine and chemokine expression would reveal the anti-inflammatory immune response capacity of oligodendrocytes. Finally, treatment of T-cell as well as macrophage cultures with the supernatant of treated oligodendrocyte cultures would reveal whether oligodendrocytes have the capacity to influence the major cell types promoting lesion formation in MS.

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Appendix A : Main Methods

In this section, a more detailed presentation of the main experimental procedures is provided. Methods for qRT-PCR, STAT6 immunohistochemistry and EAE induction are desribed in detail, since they were specifically adapted.

RNA isolation

Guanidium-thiocyanate - Cesium Chloride method

RNA isolation using the Guanidium-thiocyanate (GTC)-Cesium Chloride method was used for isolation of RNA from human NAWM. This method relies on the strong chaotropic nature of GTC to denature any Rnase present in the sample. RNA is then isolated by the centrifugation of the samples on a cushion of 5.7M CsCl, which is passed by the RNA whereas the DNA and other cellular macromolecules remain above the cushion.

- take up to 1g of tissue and transfer it into 50ml Falcon tube on dry ice
- homogenate tissue in 6ml 4M Guanidium-thiocyanate solution
    - 2-3x ~1min with high speed (break in between)
- leave 30-60min on ice
- Pasteur test (Viscosity)
- Add 2ml 4M Guanidium-thiocyanate solution and homogenat again
    - 2x ~1min with high speed (break in between)
- Pasteur test (Viscosity)
- Leave 60min on ice
- Centrifuge 3min at about 600g at room temperature
- fill Ultracentrifuge tube (14ml, Beckmann) with 3.5ml 5.7M CsCl, pH 5.5
- layer homogenate
- fill up and balance tubes/samples with 4M GTC solution
- Ultracentrifuge at 32000rpm, 20h, 20°C
- Carefully remove all of GTC layer with Pateur pipette and vaccuum
- Carefully remove CsCl layer until about 0.5-1cm over bottom
    - → change pasteur pipette frequently, as pipettes could carry RNAses through the layer and contaminate the sample
    - → try to suck liquids from the tube wall (minimizes turbulences)
    - → take care NOT TO SUCK THE SAMPLE from the bottom
- quickly invert UZ-tube to drain (catch remaining ~1ml liquid with a falcon tube)
    - → prepare Eppendorf tubes 1.5ml on ice

→ prepare Phenol(25) /Chloroform(24) /Isoamylalcohol(1) mix
→ Precool tabletop centrifuge to 4°C (14000g)

- cut UZ-tube about 1cm over the bottom with the sample with a razor blade
  → take care, UZ-tube parts might flip away
- rinse pellet with 95% EtOH (4°C) at RT
- Dissolve Pellet in TE pH 8.0 (about 200µl)
- transfer in eppi on ice
- Mix Phe/Chlo/Iso mix to TE (1:1), vortex thoroughly
- leave 30min on ice
- centrifuge 25min at 4°C, 14000g
- take away watery phase in new eppi
- Mix watery phase with: (e.g. for 100µl watery phase)
  → 1/10 Vol NaCl (10µl)
  → 2.5x Vol 100% EtOH, -20°C (250µl)
  → 1-2µl glycogen
- Precipitate RNA on dry ice (~1h) or in -70°C freezer (overnight)
- Spin Eppi with RNA (from dry ice or -70°C freezer) for 45min, 4°C, 15000g
- Wash pellet with 75% EtOh (-20°C)
- Centrifuge 10min, 4°C, 15000g
- take away supernatant (RNA is sometimes visible as a small, clear dot)
- air-dry pellet
- Dissolve pellet in wateror 10mM TE pH. 6.5 (c.a. 20µl)
- measure OD
- Store at -80°C

RNeasy lipid tissue kit

The RNeasy lipid tissue kit (QIAGEN) was used for the isolation of RNA from the corpus callosum as well as the somatosensory cortex of rats suffering from EAE.

- take up to 100mg of tissue and transfer it into a 2ml tube
- Add 1 ml QIAzol Lysis Reagent
- homogenate tissue
  - 2-3x ~1min with high speed (break in between)
- Place the tube containing the homogenate on the benchtop at room temperature (15-25°C) for 5min
- Add 200ml chloroform. Shake it vigorously for 15sec.
- Place the tube containing the homogenate on the benchtop at room temperature for 2-3min
- Centrifuge at 12'000g for 15min at 4°C.
- Transfer the upper, aqueous phase to a new tube.
- Add 1Vol. of 70% ethanol and mix thouroughly by vortexing
- Transfer up to 700µl of the sample to an RNeasy Mini spin column placed in a 2ml collection tube.
- Centrifuge for 15s at 8000g at room temperature
- Discard the flow-through
- Repeat using the remainder of the sample

- Discard the flow-through
- Add 2ml Buffer RW1 to RNeasy column
- Centrifuge 5min at 5000g
- Discard flow-through
- Add 20µl Dnase I stock solution to 140µl Buffer RDD
- Mix by gently inverting the tube
- Add this Dnase incubation mix to the RNeasy column membrane
- Place on benchtop (20-30°C) for 15min
- Add 2ml Buffer RW1 to the column
- Centrifuge 5min at 5000g
- Add 700µl Buffer RW1 to the column
- Centrifuge 15s at 8000g
- Discard flow-through
- Add 500µl Buffer RPE to the column
- Centrifuge 15s at 8000g
- Place the RNeasy column in a new 1.5ml tube
- Add 30ml H2O to the spin column membrane
- Centrifuge 1min at 8000g
- Add another 30ml H2O to the spin column membrane
- Centrifuge 1min at 8000g
- Measure OD
- Store at -80°C

Reverse transcription

Routinely, we used 1µg total RNA for a reverse transcriptase reaction.

- Add 1µl (250ng) of random hexamer primers to a 1.5ml tube
- Add 1µg total RNA to the tube
- Add 1µl 10mM dNTP Mix (10mM of each dATP, dGTP, dCTP and dTTP)
- Add H_2O to 13µl total volume
- Heat mixture for 5min at 65°C
- Incubate 1min at 4°C on ice
- Centrifuge briefly
- Add 4µl 5X First-Strand Buffer
- Add 1µl 0.1M DTT
- Add 1µl H2O
- Add 1µl Superscript III RT (200 units / ml, Invitrogen)
- Mix by pipetting
- Incubate at 25°c for 5min
- Incubate at 50°C for 60min
- Inactivate the reaction by heating at 70°C for 15min
- Store at -20°C

Quantitative RT-PCR / Lightcycler

For quantitative RT-PCR, the Lightcycler system from Roche was used. Primers for this system are designed from unique sites of the expressed gene over the exon-intron junctions to avoid amplification of genomic DNA. Generally, a standard curve for a specific gene is created using a dilution series of the according plasmid as cDNA template. Furthermore, include one reaction without any template in order to see whether there is primer-dimer formation. To spare cDNA template, the standard total reaction volume of 20µl was reduced to 5µl.

For each reaction pipett the following into a 20µl glas capillary:

0.5µl	specific 5' Primer
0.5µl	specific 3' Primer
0.5µl	H_2O
1.0µl	SYBR Green Master mix (Prepared according to the manufacturer)
2.5µl	cDNA template

Insert the glas capillaries into the Lightcycler system and perform a run with the according parameters. By default, Annealing is performed at 65°C for 5sec.

Statistics

Normalization of calculated RNA amounts by qRT-PCR was done by using 60s ribosomal protein L13 (NM_031101.1), 40s ribosomal protein S9 (NM_031108.1) and ribosomal protein L19 (NM_031103.1). Using these three ribosomal genes, a geometric mean was calculated to which all target genes were normalized. Such a normalization strategy, recently proposed, showed to be more accurate for qRT-PCR normalization rather than using single-gene normalization (Vandesompele et al., 2002). Statistical significance was then calculated by comparison of the normalized raw data from control animals to EAE animals, and is expressed by *P*-values generated by the non-parametric Mann-Whitney U-Test.

STAT immunofluorescence staining

For STAT immunofluorescence, tissue had to be fixed with acetone (-20°C), whereas fixation with PFA didn't reveal any signal. Furthermore, autofluorescence was quenched by the incubation in 10mM cupric sulfate.

- Cut 10μm cryostat sections from tissue and mount on a glass slide
- Air-dry for 20min
- Fix for 10min in 100% Acetone (-20°C)
- Wash 3 times in PBS pH 7.0 for 5min
- Incubate for 1h in 2% normal donkey serum, 2% Fish-Skin Gelatine, 0.1% Triton and 0.05% Tween 20
- Incubate 48h with the primary antibody at 4°C (anti-STAT4 (R&D Systems, Cat.Nr. PA-ST4, 1:250), anti-STAT6 (R&D Systems, Cat.Nr. AF2167, 1:250)
- Wash 3 times in PBS pH 7.0
- Incubate for 1h in 10mM $CuSO_4$, 50mM CH_3COONH_4, pH 5.0 to reduce autofluorscence
- Wash 3 times in PBS pH 7.0
- Incubate with 2nd antibody at room temperature (15-25°C) for 1h
- Mount slides with fluorosave
- Store slides at 4°C

EAE induction

EAE induction was made as described before (Storch et al., 1998b). For induction of EAE 50µg of rMOG emulsified with incomplete Freud's adjuvant (IFA) (Difco Laboratories, Detroit, MI) was injected into inbred adult female DA rats (10-12 weeks, from Harlan, Netherlands). Injections were given intradermally in the dorsal aspect of the base of the tail. A group of control rats were injected with saline, emulsified with an equal volume of IFA. The clinical progress of the disease was monitored daily. Rats were weighted and their neurological deficits were scored according the standard protocol.

Appendix B : Curriculum Vitae

Thomas Zeis

June 6, 2008

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Personal information

Birth Date:	5. October 1975
Birthplace:	Zurich
Hometown:	Fällanden ZH
Gender:	male
Family status:	single
Nationality:	Swiss and Czech

Education

2003 - 2008 *University of Basel, Basel, Switzerland*

Ph.D. in Neurobiology, Department of Biomedicine and Neurology, Pharmacenter / Biocenter

Advisor: Prof. N. Schaeren-Wiemers

„Differential gene expression in Multiple Sclerosis and its animal model Experimental Autoimmune Encephalomyelitis“

Degree: Ph.D. (estimated in july 2008)

2001 - 2002 *University of Basel, Basel, Switzerland*

Master thesis work in Neurobiology at the Pharmacenter /

Biocenter

Advisor: Prof. N. Schaeren-Wiemers

„Purification and characterization of new anti-MAL antisera and immunoprecipiation of MAL from human brain myelin membranes”

Degree: Diploma in biology

1996 - 2002 *University Basel, Basel, Switzerland*

Student of biology at the University of Basel

Subjects:

- Biochemistry
- Biophysical chemistry
- Molecular genetics
- Cell biology

Special subject:

- Neurobiology

1991 - 1996 *High school Gymnasium St. Antonius, Appenzell, AI, Switzerland*

Degree: Matura Typus E

1989 - 1991 *High school Heerbrugg, SG, Switzerland*

1987 - 1989 *Secondary school Mittelrheintal, Heerbrugg, SG, Switzerland*

1984 - 1987 *Primary school Berneck, SG, Switzerland*

1981 - 1984 *Primary school Fällanden, ZH, Switzerland*

Professional experience

2003 - 2008 **Scientist**

University of Basel, Basel, Switzerland

1999 - 2003 **Cassier and storekeeper**

Coop (Schweiz) AG, Standort Basel Bahnhof, Basel, Switzerland

1991 - 1996 **Miscellaneous vacation jobs**

WMB AG, Balgach, Switzerland

Languages

Language	Speaking	Writing and reading
English	excellent	excellent
French	good	good
Italian	basic knowledge	basic knowledge
Czech	excellent	good
Japanese	basic knowledge	basic knowledge

Skills

MS Windows	good
MS Excel, Word and PowerPoint	good
Adobe Photoshop and Illustrator	good
Hardware in general	good

Interesses and activities

- Computer in general (Hard- and software)
- Ballroom dancing
- Playing table games with friends
- Strategy games of all kinds
- Reading
- Playing the piano
- Cooking

Publications and talks

Manuscripts

Zeis, T., U. Graumann, R. Reynolds, and N. Schaeren-Wiemers. 2008. Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection. *Brain*. 131:288-303.

Zeis, T., and N. Schaeren-Wiemers. 2008. Lame Ducks or Fierce Creatures? - The Role of Oligodendrocytes in Multiple Sclerosis. *J Mol Neurosci*.

Zeis, T., A. Probst, A.J. Steck, C. Stadelmann, W. Brück, and N. Schaeren-Wiemers. Molecular changes in white matter adjacent to demyelinating lesions in early Multiple Sclerosis. *Submitted*

Kinter, J., **T. Zeis**, and N. Schaeren-Wiemers. RNA profiling in MS brain tissue. *Int.MS.J. In press*

Zeis, T., J. Kinter, E. Herrero-Herranz, and N. Schaeren-Wiemers. Characterization of autoprotective mechanisms in an animal model of Multiple Sclerosis. *Manuscript in preparation*

Zeis, T., O. Howell, E. Herrero-Herranz, J. Kinter, R. Reynolds, and N. Schaeren-Wiemers. Gene expression analysis of active, remyelinating and silent lesions of multiple sclerosis patients. *Manuscript in preparation*

Zeis, T., J. Kinter, C. Stadelmann, and N. Schaeren-Wiemers. Amelioration of experimental autoimmune encephalomyelitis with an orally deliverable iron chelator. *Manuscript in preparation*

Book chapters

Kinter, J., **Zeis, T.**, Schaeren-Wiemers, N. 2008. A chronic relapsing model of Multiple sclerosis: experimental autoimmune encephalomyelitis in DA rats. *BioValley Monogr. Basel, Karger Verlag.*

Talks

Zeis, T., A. Probst, A.J. Steck, W. Brück, and N. Schaeren-Wiemers. *Oral Presentation*. Joint meeting of the Swiss Society for Neuroscience and the Swiss Society for Neuroradiology, Basel, Switzerland, January 28, 2006. „Molecular changes in the normal appearing white matter of a multiple sclerosis patient during an acute phase – a developing lesion ?“

Zeis, T., U. Graumann, R.Reynolds, and N. Schaeren-Wiemers. *Oral Presentation*. U45 Multiple Sclerosis Meeting, Grindelwald, Switzerland, March 24, 2007. „Normal appearing white matter in MS is in a subtle balance between inflammation and neuroprotection”

Zeis, T., U. Graumann, R. Reynolds, and N. Schaeren-Wiemers. *Oral Presentation*. 100 years Neurology in Basel; Symposium for retirement of Prof. Andreas Steck, Basel, Switzerland, June 9, 2007. „Endogeneous neuroprotective mechanisms in Multiple Sclerosis"

Scientific presentations

Graumann U., R. Reynolds, **T. Zeis**, N. Schaeren-Wiemers. Joint Meeting of the Swiss Society for Neuroscience and the Swiss Society for Behavioral Neurology, Lausanne, Switzerland, January 17, 2004. „Molecular Changes in Normal Appearing White Matter in Multiple Sclerosis are Characteristic of Neuroprotective Mechanisms Against Hypoxic Insults"

Zeis T., R. Brunner, O. Howell, R. Reynols, A. Probst, A.J. Steck, W. Brück, and N. Schaeren-Wiemers. Joint Annual Meeting of the Union of the Swiss Societies for Experimental Research, the Swiss Society for Neuroscience and the Swiss Society of Biological Psychiatry, Zürich, Switzerland, February 17-19, 2005. „Differential expression analysis in Multiple Sclerosis"

Zeis T., R. Brunner, O. Howell, W. Brück, and N. Schaeren-Wiemers. 14th annual meeting of ARSEP, Paris, France, March 10, 2005. „Differential expression analysis in Multiple Sclerosis"

Zeis T., R. Brunner, C. Stadelmann, A. Probst, A.J. Steck, W. Brück, and N.Schaeren-Wiemers. Seventh Bi-annual Satellite Meeting of the International Society for Neurochemistry on Myelin Biology, Irsee, Germany, August 17-21, 2005. „Molecular Alterations in a NAWM Biopsy of a Pattern III MS Patient"

Zeis T., U. Graumann, R. Brunner, N. Schaeren-Wiemers. Scientific Presentation. Joint meeting of ARSEP and the Swiss MS Society, Paris, France, March 10, 2006. „Activation of pro- and anti-inflammatory modulating mechanisms in normal appearing white matter in chronic multiple sclerosis"

Zeis T., A. Probst, A. Steck, W. Brück, N. Schaeren-Wiemers. Scientific Presentation. Satellite Meeting of the 20th Biennial Meeting of the ISN, Irsee, Germany, August 17-21, 2005. „Molecular changes in the NAWM of a patient with acute inflammatory demyelinating encephalitis - early stage alterations ?"

Zeis T., R. Brunner, A. Probst, W. Brück, A.J. Steck, N. Schaeren-Wiemers. Scientific Presentation, Joint meeting of ARSEP and the Swiss MS society, Paris, France, March 10, 2006. „Strong upregulation of iNOS and peroxinitrite formation in normal-appearing white matter of a MS patient during an acute phase".

J. Kinter, **T. Zeis**, L. Nobs, R. Reynolds, N. Schaeren-Wiemers. Scientific Presentation, Joint meeting of ARSEP and the Swiss MS society, Paris, France, March 10, 2006. „Characterization of an Animal Model for Chronic Progressive Multiple Sclerosis to Analyze Molecular Changes in Normal Appearing White and Grey Matter."

Zeis T., U. Graumann, R. Reynolds, and N. Schaeren-Wiemers. Scientific Presentation, Joint meeting of the Swiss Society of Neuroscience, NCCR Neuro and the Swiss Multiple Sclerosis Society,

Bern, Switzerland, March 9-10, 2007. „Normal appearing white matter in MS is in a subtle balance between inflammation and neuroprotection"

Zeis T., U. Graumann, R. Reynolds, and N. Schaeren-Wiemers. Scientific Presentation. MS Frontiers 2007 - MS Society Flagship Research Event, London, England, June 14-15, 2007. „Normal appearing white matter in MS is in a subtle balance between inflammation and neuroprotection"

Zeis T., U. Graumann, R. Reynolds, and N. Schaeren-Wiemers. Scientific Presentation. VIII European Meeting on Glial Cells in Health and Disease. London, England, September 4-8, 2007. „Normal appearing white matter in MS is in a subtle balance between inflammation and neuroprotection"

Zeis T., U. Graumann, A.J. Steck, W. Brück, and N. Schaeren-Wiemers. Scientific Presentation. MS Frontiers 2007 - MS Society Flagship Research Event, London, England, June 14-15, 2007. „Strong upregulation of nitric oxide synthase and peroxinitrite formation in normal appearing white matter in MS"

Zeis T., W. Brück, and N. Schaeren-Wiemers. Scientific Presentation. Berichtssymposium der Hertie Stiftung, Frankfurt, Germany. September 18, 2007. „Early molecular changes of the normal appearing white matter in Multiple Sclerosis. Inhibiting or preceding lesion formation?"

Kinter, J., **T. Zeis**, and N. Schaeren-Wiemers. Scientific Presentation. Joint meeting of the Swiss Society of Neuroscience, NCCR Neuro and the Swiss Multiple Sclerosis Society, Bern, Switzerland, March 9-10, 2007. „Molecular and cellular alterations in cortical grey matter of multiple sclerosis patients"

Kinter, J., **T. Zeis**, O. Howell, R. Reynolds and N. Schaeren-Wiemers. Scientific Presentation. MS Frontiers 2007 - MS Society Flagship Research Event, London, England, June 14-15, 2007. „Molecular and cellular alterations in cortical grey matter of multiple sclerosis patients"

Kinter, J., **T. Zeis**, O. Howell, R. Reynolds and N. Schaeren-Wiemers. Scientific Presentation. VIII European Meeting on Glial Cells in Health and Disease. London, England, September 4-8, 2007. „Molecular and cellular alterations in cortical grey matter of multiple sclerosis patients"

Zeis T., W. Brück, and N. Schaeren-Wiemers. Scientific Presentation. 17th scientific meeting of ARSEP, Paris, France. March 28, 2008. „Early molecular changes of the normal appearing white matter in Multiple Sclerosis. Inhibiting or preceding lesion formation?"

Herrero-Herranz, E., **T. Zeis**, and N. Schaeren-Wiemers. Scientific Presentation. 17th scientific meeting of ARSEP, Paris, France. March 28, 2008. „RNA extraction of paraffin embedded biopsies for differential gene expression analysis in early MS cases"