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Investigation of the astrocytic reaction in different stages of lesion evolution in an animal model of multiple sclerosis and correlation to the remyelination capacity

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Abstract

Multiple Sclerosis (MS) is the most common neurological disease in young adults and often causing permanent disability. It is commonly accepted to be a T-cell-mediated autoimmune disease; the exact disease mechanisms however are not fully understood. The role of the astrocytes in degeneration and regeneration in this disease is discussed controversially. Especially the formation of a dense glial scar is often believed to act as a mechanical barrier for remyelinating oligodendrocytes. In this master thesis we investigated the astrocytic reaction in an animal model of MS induced by active immunization with Myelin Oligodendrocyte Glycoprotein (MOG) in Dark Agouti (DA) rats. This animal model mimics most of the histological features of multiple sclerosis and allows examination of lesion evolution over the full course – from active demyelination up to a successfully completed repair. We correlated the astroglial reaction by immunohistochemistry for glial fibrillary acidic protein (GFAP, a marker for astrocytes) to the remyelination capacity by *in situ* hybridization for mRNA of the myelin protein PLP, which is a marker for remyelinating oligodendrocytes. Quantitative evaluation and statistical analysis was performed for all lesion types. The amount of GFAP positive astrocytes increased during lesion evolution and was highest in remyelinated lesions. In early remyelinating lesions, with high numbers of oligodendrocyte progenitors (OGP), also high numbers of astrocytes were present. In fully remyelinated shadow plaques, with numbers of OGPs nearly equal to the normal appearing white matter (NAWM), extensive astrocytic scarring was detectable. From our data we conclude that extensive remyelination is present despite an abundant glial reaction in this standardized model of autoimmune mediated encephalomyelitis. Our findings thus indicate, that –at least in the inflammatory demyelinating scenario of MOG-EAE in the DA rat– the astroglial reaction does not impede plaque repair.

Zusammenfassung

Multiple Sklerose (MS) ist eine der häufigsten neurologischen Erkrankungen im jungen Erwachsenenalter und die führende, nicht traumatisch bedingte Ursache für bleibende Schäden. Es handelt sich um eine T-Zell-mediierte Autoimmunkrankheit wobei die exakten Krankheitsmechanismen noch nicht vollständig aufgeklärt sind. Die Rolle der Astrozyten in der Degeneration und Regeneration der MS wird kontrovers diskutiert. Insbesondere die Ausbildung einer dichten Glianarbe soll sich als mechanische Barriere für remyelinisierende Oligodendrozyten negativ auf Regenerationsprozesse auswirken. In dieser Masterarbeit wurde die Astrozytenreaktion in einem Tiermodell der MS systematisch untersucht. Dazu wurden Dark Agouti (DA) Ratten aktiv mit Myelin Oligodendrozyten Glykoprotein (MOG) immunisiert. Dieses Tiermodell ähnelt histologisch weitgehend der MS beim Menschen und ermöglicht die Betrachtung der Läsionsentwicklung über den gesamten Verlauf der Erkrankung - von aktiver Demyelinisierung bis hin zur kompletten, erfolgreichen Remyelinisierung, dem sogenannten Markschattenherd.

Die Reaktion der Astrozyten wurde in Läsionen aller Stadien in der Immunhistochemie mittels Antikörper gegen GFAP (glial fibrillary acidic protein), einem üblichen Marker für aktivierte Astrozyten, verfolgt und quantitativ ausgewertet. Außerdem konnten an denselben Läsionen mit Hilfe der *in-situ*-Hybridisierung in Verwendung von anti-PLP (Proteolipid Protein) mRNA Sonden, die vorhandenen remyelinisierenden Oligodendrozyten dargestellt und die Astrozytenreaktion mit der laufenden Remyelinisierungsaktivität korreliert werden.

Die Anzahl der GFAP-positiven Astrozyten stieg während der Läsionsentwicklung an und war in den komplett remyelinisierten und gerade remyelinisierenden Läsionen am höchsten. In Regionen, die frühe Remyelinisierung aufwiesen und damit eine hohe Anzahl von Oligodendrozyten-Vorläuferzellen (OGP) zeigten, war auch eine hohe Anzahl an Astrozyten zu erkennen. Überraschenderweise konnte in fertig remyelinisierten Markschattenherden, in denen die Anzahl der Oligodendrozyten-Vorläuferzellen annähernd der in Normalgewebe (NAWM –

normal aussehende weiße Substanz) entsprach, eine dichte Glianarbenbildung detektiert werden.

Aus unseren Daten schließen wir, dass trotz deutlicher Astrozytenaktivierung und Bildung einer Glianarbe dennoch ausgedehnte Remyelinisierung stattfinden kann. Diese Resultate weisen darauf hin, dass – zumindest im inflammatorisch demyelinisierenden Szenario der MOG-EAE in DA-Ratten – Astrogliose die Plaquereparatur nicht behindert.

Contents

1. Introduction

1.1 Multiple sclerosis

Multiple sclerosis is a chronic neurological disease affecting mostly young adults (mean age of onset 27 years) and affects worldwide about 2.5 million people. It is commonly accepted to be a T-cell-mediated autoimmune disease, and as such females are commonly more often affected than males. The target of the immune reaction is the myelin sheath covering the nerve axons, which leads to attacks of clinical neurological symptoms followed – after repair of the myelin sheath – by remissions of the neurological disturbance.

Although there are certain predilection sites within the central nervous system for these attacks to occur (optic nerves, cervical portion of the spinal cord and brainstem), basically any region of the brain and spinal cord can be affected. This results in a wide variety of neurological symptoms, ranging from vision or sensory disturbance to paralysis and, in later stages, bladder and gait dysfunctions and cognitive impairment.

The clinical course of multiple sclerosis is no less variable. The most common form follows a relapsing course with attacks and subsequent recovery for many years. The average number of such relapses is 1.5 per year. After duration of 25 years, most patients enter a secondary progressive phase of the disease with a more or less steady worsening of existing neurological symptoms. In about 10% of patients the illness is progressive from onset. Approximately one-third of patients are in a quiescent phase of the disease and not significantly disabled, a further third are slowly deteriorating and the remainder are stable in their neurological deficit. Multiple sclerosis is not necessarily a disabling disease although the sum of lesions, that do not cause impairment alone, can lead to handicap. Conversely, some patients may die acutely from massive cerebral demyelination. No symptomatic correlations can be made for the great majority of fleeting cerebral abnormalities seen on MRI and even established chronic demyelination in highly eloquent sites may be symptomless (Compston, 2001).

Multiple sclerosis has long been recognized as a disease of the central nervous system in which an inflammatory process is associated with destruction of myelin sheaths (Charcot 1868a; 1868b). The lesions in brain and spinal cord are characterized by inflammation, demyelination, axonal damage and gliosis (Hagemeier, 2012). Thus, the essential brain lesion is the demyelinated plaque, which may occur at any place where myelin sheaths are present. Demyelination is accompanied by astrocytosis and the formation of scars, reflected in the name multiple sclerosis. The first histological investigations were published by Eduard Rindfleisch (1863). He interpreted the noted plaques and increased density of cells as evidence for a chronic inflammatory process. During the following years, a systematic clinical and neuropathological survey of the disease was provided by Charcot in which he defined in detail the demyelinating process that leads to formation of plaques with glial scar formation (see pathology of multiple sclerosis) (Compston, 2001). The cause for the permanent neurological deficits in MS patients is axonal loss. Demyelinated axons are prone to damage due to the lack of trophic support by myelin sheaths and oligodendrocytes. Remyelination occurs, but is frequently limited to a small rim at the lesion border. Current treatment strategies are based on anti-inflammatory or immunomodulatory drugs to reduce the number of newly evolving lesions. Yet no treatment strategy exists to influence or prevent the progressive disease phase. Therefore, the development of neuroprotective treatment options, such as the promotion of remyelination could be a yield strategy. In order to develop such new treatments, the understanding of the mechanisms leading to remyelination and the reasons for insufficient endogenous repair in MS are necessary (Hagemeier, 2012).

1.1.1 Myelin organization

Myelin has a central role in normal functioning of the central nervous system. In physiological conditions, myelin is the insulating material ensheating CNS axons, conducts action potentials and enables the communication between neurons (Baumann and Pham-Dinh, 2001). Oligodendrocytes are the myelin-maintaining cells of the CNS. The myelin sheath itself is built of different proteins and other macromolecular components; the dry mass consists of

70-85% lipids and about 15-30% proteins (Braun, 2009). Physiological interuptions of the myelin sheaths are called nodes of ranvier, which are responsible for the fast saltatoric (“jumping”) conduction along the axon. Any disturbance of the myelin sheath can lead to either a severely reduced conduction velocity or a conduction block. In MS demyelination and cell death of mature oligodendrocytes affect the ability of axons to conduct electrical signals, which results in the reduction or complete loss of action potential transmission (Noseworthy, 2000).

1.1.2 Axon-glial interactions in the nervous system

Rudolph Virchow (1858) first introduced the term neuroglia (“nerve glue”) and assigned them two functions, firstly the mechanical support of nerve cells and secondly tissue repair. There were further activities proposed such as nutritional support of neurons, engulfment of cellular debris and isolation of nervous conduction. Hortega (1921) later histologically distinguished two different cell types responsible for these functions, which he named microglia and oligodendrocytes. He also proposed a mesodermal derivation for microglia which forms a macrophage-related population within the CNS, but even now their origin remains controversial. Having described glial lineages in the developing nervous system, it is now considered that the interaction of glia and axons produce myelin sheaths. This conclusion is based on *in vitro* studies in the rat. CNS myelination occurs when the processes of mature oligodendrocytes contact and ensheath axons, and compact to form the myelin lamellae. Astrocytes are involved in the formation of internodes. Careful orchestration is needed to juxtapose progenitors alongside axons and to ensure that differentiation does not proceed along only one route. It is expected that several important influences mediated by soluble factors and cell-cell contacts are exchanged between axons and glia. Although it is the oligodendrocyte which synthesizes myelin around short segments of neighbouring axons, debate continues on which cell within the lineage myelination primarily depends. Axons are able to inhibit maturation in the oligodendrocyte lineage. Rosen (1989) has shown that myelination of rat dorsal root ganglion cells by oligodendrocytes is inhibited by type-1 astrocytes and astrocyte-conditioned

medium *in vitro*. They suggested that this effect is due to factors released by reactive astrocytes and could be one explanation for the lack of remyelination seen in the chronic astrocytic lesions of multiple sclerosis. Myelin synthesis is triggered when oligodendrocyte processes make contact with nearby axons. The elongated oligodendrocyte processes form a cup around the axon at the point of contact and extends lengthwise to form the multiple membrane layer or lamellae around the axon (Compston, 2001).

Regeneration of axon-glial interactions

The failure of central axons to regenerate following injury to the adult CNS can be explained in part by their intrinsic growth-limiting properties. One aspect of glial neurobiology which relates to axon regeneration is the inhibitory effect of oligodendrocytes and the mechanical restrictions imposed by astrocytes. In vitro studies indicate that astrocytes, oligodendrocytes and myelin each actively inhibit neurite growth. However, the relationship between axons and glia is reciprocal and complex (Compston, 2001). Since damage to CNS axons will always produce a glial scar, the place where regeneration of axons fails contains a developing glial scar. All the five cell types – astrocytes, oligodendrocyte procursors, oligodendrocytes, meningeal cells and microglia – that make up this scar have inhibitory properties. However, not all of the cells are inhibitory all of the time. Astrocytes in particular are enourmously plastic and can display growth-promoting or growth-inhibiting properties under different circumstances (Fawcett, 1999).

1.1.3 Oligodendrocytes and their role in remyelination

There are different types of oligodendrocytes in the CNS. Precursor cells mature in the spinal cord to OPCs (oligodendrocyte precursor cells) and differenciate to myelin-producing oligodendrocytes (Cai, 2005). Mature oligodendrocytes are glial cells with highly ramified processes that form membrane sheets following the generation of oligodendrocyte-axon contact. The compaction of these primary membrane wraps results in the generation of the multilamellar myelin sheath (Bauer, 2009). Myelinisation is a process that occurs during the embryonic development and needs the activity of neurons

(Connor, 1996). The myelin sheaths form internodes which are separated by Nodes of Ranvier and these are the sections where the action potentials are generated (Bauer, 2009). During development axons are ensheathed by OPCs. Newly generated OPCs contact axons in order to start differentiation and myelination of axons (Miller, 2002). The migration of OPCs is controlled by secreted molecules such as growth factors, guidance molecules and chemokines. The control of these extrinsic and intrinsic factors is a prerequisite for successful differentiation of oligodendroglial lineage cells and myelination (Hagemeier, 2012). Although the stages of oligodendrocyte development are well characterized, the exact factors needed for oligodendrocyte differentiation and myelination are not fully understood.

Completed remyelination after a damage is histologically characterized by shortened internodes and decreased myelin thickness. Remyelination is a process that involves new oligodendrocytes and cannot be accomplished by already existing mature oligodendrocytes (Carroll, 1998). The presence of axons and OPCs is the basic requirement for remyelination as well as the correct interaction of extrinsic signalling pathways. The majority of OPCs generated during development differentiate into mature myelinating oligodendrocytes but a small population of immature OPCs remains as progenitors that respond to demyelination by proliferation, migration and differentation to form new myelin sheaths (Horner, 2000; Franklin, 2002; Dawson, 2003).

Although long being presumed, final proof of the oligodendroglial relevance for normal nerve function occured late in 1965 by investigations through Périer and Grégoire. First, oligodendrocyte precursor cells have to be converted in the regenerative phenotype. Not till then precursor cells develop to remyelinating oligodendrocytes. Following these findings, the multiple sclerosis research focused mainly on preservation and regeneration of oligodendrocytes.

1.1.4 Astrocytes

Astrocytes are specialized glial cells that outnumber neurons by over fivefold and exert many essential functions in the healthy CNS, including formation and

maintainance of the blood brain barrier. Astrocytes respond to all forms of CNS insults through a process called reactive astrogliosis. This process is not a simple all-or-none phenomenon but is a finely graded continuum of changes that occur in context-dependent manners regulated by specific signaling events. These changes range from rather mild reversible alterations to long lasting scar formation with rearrangement of tissue structure. Over the past years it has become clear that astrocytes are responsible for a wide variety of complex and essential functions in the healthy CNS (Sofroniew, 2010).

Molecular markers for Astrocytes

Immunohistochemical techniques that enable the detection of specific molecular markers at the single-cell level are essential for identifying and characterizing cells in tissue. Expression of glial fibrillary acid protein (GFAP) has become a typical marker for immunohistochemical identification of astrocytes. GFAP is a family member of intermediate filament proteins, including vimetin, nestin and others, that serve cyto-architectural functions. GFAP was first isolated as a protein highly concentrated in old demyelinated plaques from multiple sclerosis patients. It was then found that GFAP upregulation is associated with reactive astrocytes also in other pathological contexts. GFAP expression can be regarded as a sensitive and reliable marker that labels most reactive astrocytes, but is often not detectable in astrocytes in healthy CNS tissue. Expression of GFAP is not essential for the normal appearance and function of most astrocytes in the healthy CNS, but is essential for the process of reactive astrogliosis and glial scar formation (Sofroniew, 2010).

Reactive astrogliosis and glial scar formation

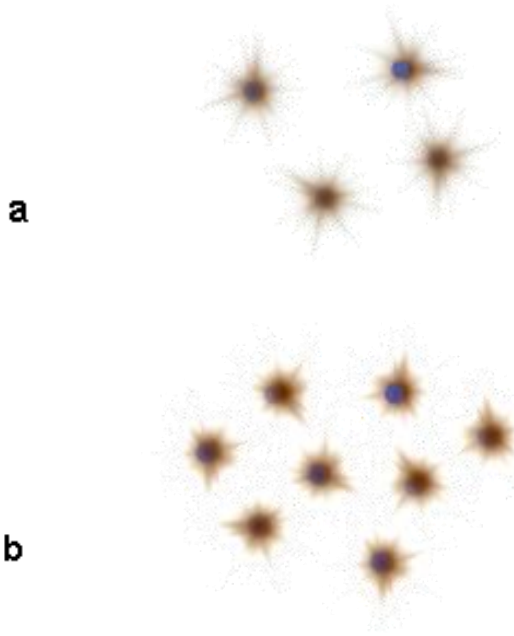
Reactive astrogliosis is a spectrum of potential molecular, cellular and functional changes in astrocytes that occur in response to all forms of CNS injury and disease. These changes undergone by reactive astrocytes, vary with severity of the insult along a graded continuum of progressive alterations in molecular expression, progressive cellular hypertrophy and scar formation. To describe astrogliosis, three broad categories are used:

1. **Mild to moderate reactive astrogliosis:** There is variable upregulation of GFAP expression and other genes. Little or no astrocyte proliferation can be determined and there is little or no reorganization of tissue architecture. This process is fully reversible.

2. **Severe diffuse reactive astrogliosis:** There is pronounced upregulation of expression of GFAP and other genes. Morphologically, this is accompanied with astrocyte proliferation resulting in considerable extension of processes beyond the previous domains of individual astrocytes. The changes can result in long-lasting reorganization of tissue architecture.

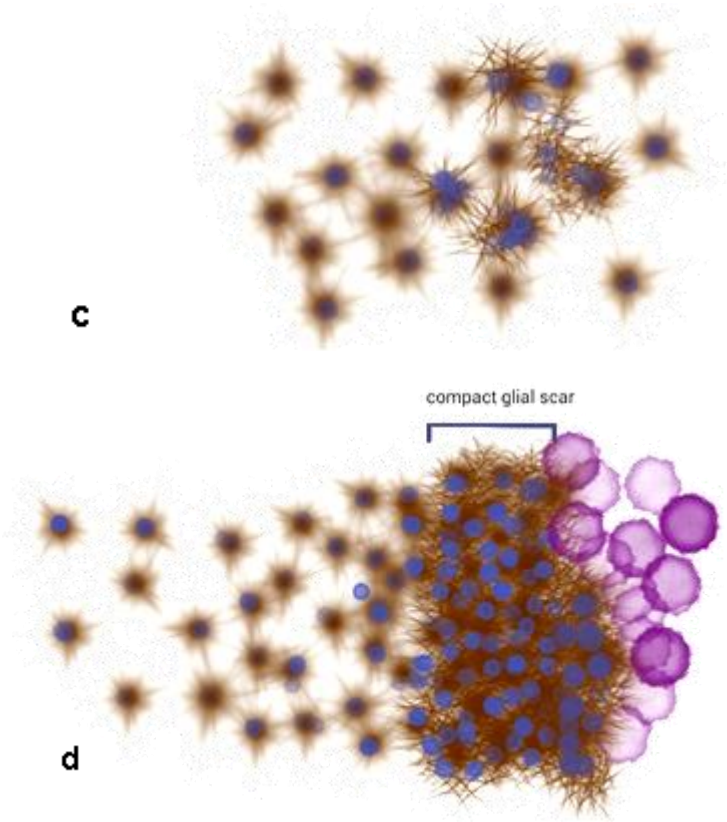
3. **Severe reactive astrogliosis with compact glial scar formation:** In addition to changes associated with milder forms such as upregulation of GFAP and other genes, reactive astrocytes overlap with other reactive astrocytes and a dense, narrow and compact glial scar formation occurs. These astrocyte scar acts as neuroprotective barrier to inflammatory cells and infectious agents and forms along borders to severe tissue damage, necrosis, infection or inflammatory infiltration (Sofroniew, 2010). This scar formation is irreversible.

An overview of the different types is given in figure 1.



Astrocytes in healthy CNS tissue:
Not all astrocytes express detectable levels of GFAP. This astrocytes have non-overlapping domains.

Mild reactive astrogliosis:
Most astrocytes are GFAP positive. Preservation of individual domains.



Severe diffuse reactive astrogliosis:
Most astrocytes are GFAP positive.
Disruption of individual domains.

Severe astrogliosis with compact glial scar formation:
Bordering along regions of tissue damage and inflammation due to Ischemia, Infection, Autoimmune inflammation, Neoplasm, Trauma or Cytotoxicity.

Figure 1: Schematic representation that summarizes different stages of reactive astrogliosis. **a** Astrocytes in healthy CNS tissue. **b** Mild to moderate reactive astrogliosis comprises changes in molecular expression and functional activity together with cellular hypertrophy. These changes vary with insult severity, involve little anatomical overlap of the processes of neighbouring astrocytes and exhibit the potential for structural resolution. **c** Severe diffuse reactive astrogliosis includes changes in molecular expression, functional activity and cellular hypertrophy, as well as newly proliferated astrocytes (represented with intense blue nuclei in the figure). **d** Severe reactive astrogliosis with compact glial scar formation occurs along borders to areas of overt tissue damage and inflammation and includes newly proliferated astrocytes (intense blue nuclei in the figure) and other cell types such as fibromeningeal cells and other glia, as well as deposition of dense collagenous extracellular matrix. In the compact glial scar, astrocytes have densely overlapping processes. Mature glial scars tend to persist for long periods and act as barriers to inflammatory cells, infectious agents and non-CNS cells in a manner that protects healthy tissue from nearby areas of intense inflammation *(Sofroniew, 2010).*

The glial scar, or reactive gliosis, is the environment in which spontaneous CNS repair succeeds or more often fails. It is the environment in which any therapeutic efforts will have to contend. The complexity of this structure and the large number of molecules demands the development of techniques to control the recruitment of the different cell types by influencing cell division, migration or by killing some cells. The active molecules will probably have to be manipulated. An important guide to the way in which the damaged CNS will need to be changed in order to enhance repair must be the processes that

make axon growth and myelination so effective in development (Fawcett, 1999).

The role of astrocytes in multiple sclerosis

MS research focused on preservation and promoting regeneration of oligodendrocytes for a long time. Astrocytes stayed in the background and were only seen as the causer of the dreaded glial scar only inhibiting axon regeneration. They seemed to form a dense impenetrable barrier for remyelinating oligodendrocytes. Furthermore highly active astrocytes were found in subacute MS-lesions, some even with more than one nucleus. These astrocytes contained myelin degradation products. This findings of glial scar formation as an inhibitor of regeneration has often led to a generalized negative view of reactive astrogliosis and the whole process was typecast as a uniformly negative phenomenon that causes neurotoxicity, inflammation or chronic pain. Some of these reactive astrocytes seemed to surround healthy oligodendrocytes (Prineas, 1990; Ghatak 1992; Wu and Raine 1992). This was interpreted as phagocytosis but later refuted: Looking closer at this phenomenon, this interpretation happened because of an intense cell-cell-contact between oligodendrocytes and astrocytes. But this is more a protective function of astrocytes and not at all a phagocytic attack. This absolutely negative viewpoint of reactive astrogliosis is no longer tenable and it is now clear that there is a normal process of reactive astrogliosis that exerts essential benefical functions. Reactive astrocytes protect CNS cells and tissue by uptake of potentially excitotoxic glutamate, protects from oxidative stress, facilitates the blood brain barrier repair, reduces vasogenic edema after trauma or stroke and limits the spread of inflammatory cells or infectious agents from areas of damage or disease into healthy CNS parenchyma (Sofroniew, 2010).

In the most cases of multiple sclerosis, plaques of demyelination are interspersed with and surrounded by reactive astrocytes. There are widespread regions of reactive astrogliosis in varying intensity. Astrocytes in demyelinating disease may exhibit unusual nuclear features – they may contain multiple distinct nuclei. Such enlarged multinucleated astrocytes are also called “Creutzfeldt astrocytes”. This multiple nuclei may be fragmented and appear like

the atypical or granular mitosis often seen in gliomas. Astrocytes in multiple scerosis may also demonstrate the phenomenon of “emperiopolesis”. This term describes an astrocyte engulfing one or more other cells such as oligodendroglia or lymphocytes. The role of this process in the pathogenesis of multiple scerosis is not certain. Both multinucleation and emperipolesis also occur in other disorders. Astrocytes can produce a wide varity of pro- or anti-inflammatory molecules and can exert potent suppressive effects (Sofroniew, 2010).

1.1.5 Pathology of multiple sclerosis

All inflammatory demyelinating diseases depend primarily upon a T-cell-mediated inflammatory reaction, but their clinical course and pathology my be modified by additional immune reactions. The most characteristic feature of multiple sclerosis pathology is the demyelinated plaque, with preserved axons set in a matrix of astrocyte scar formation. In general, plaques are centred on one or several medium-sized vessels and have a tendency to accumulate near the periventricular surfaces of the brain and spinal cord (Oppenheimer 1978). The lesions show extensions in the periphery (Dawson 1916) that follow the path of a vessel (“Dawson’s fingers”). Microscopically, myelin sheaths are completely lost in the plaques and axons are embedded in dense astroglial scar tissue. The demyelinating process is associated with persistent inflammation throughout the central nervous system (Babinski 1885; Lassmann, 1993).

Lesional activity

In MS autoimmunity causes T-cell-mediated inflammation leading to macrophage activation. Products of activated macrophages lead to apoptosis and anti-myelin antibodies destroy myelin. This further leads to oligodendrocyte destruction, demyelination and reactive gliosis.

Definition of lesional staging

Active demyelinating lesion: Very early lesions are infiltrated by numerous macrophages containing myelin degradation products. Macroscopically, active plaques have a pink discolouration – microscopically, they reveal demyelination

with little astroglial scar formation. The lesions are infiltrated by numerous inflammatory cells, in particular macrophages, that contain myelin and tissue debris. This is represented by tiny blue granula within the macrophages infiltrating the active lesion (figure 2, a) in luxol fast blue staining (LFB). In pathology of demyelinating lesions, activity can thus be defined either by the inflammatory reaction or the state of myelin degradation products within macrophages.

Inactive demyelination: In LFB-stained inactive lesions, macrophages contain tiny pink granula (figure 2, b), because the myelin debris was already lysosomal digested (Compston, 2001).

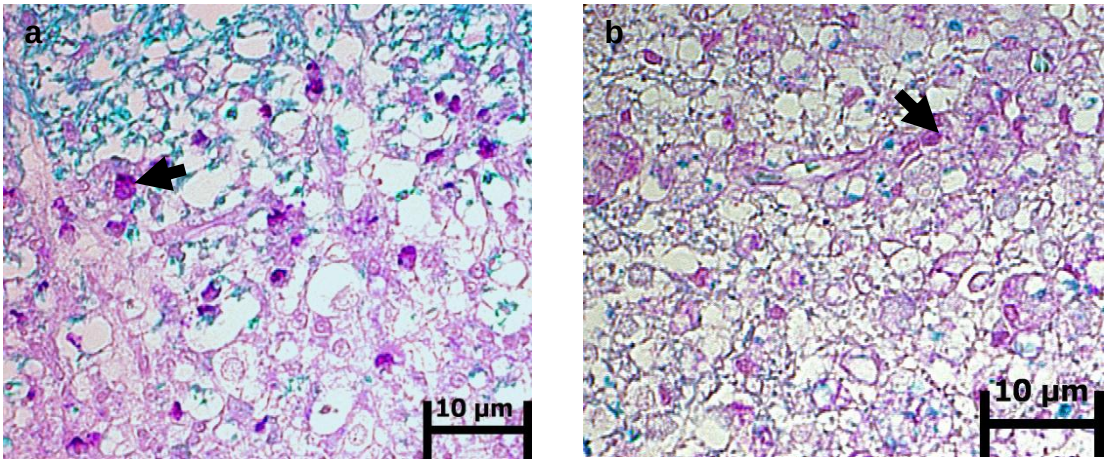


Figure 2 Comparison of an active and an inactive lesion in LFB staining. In **a** an active lesion is represented. The black arrow points on a macrophage. It contains blue granula, which means that this is myelin degradation product. In **b** a photo of an inactive lesion is displayed. The black arrow points here on a macrophage, too. This cell contains pink granula, because in this case, myelin is already lysosomal digested. Both photos were made with a "Zeiss Axiocam MRc5" camera on a light microscope (Zeiss Axioplane2 imaging) with a 400x magnification.

Remyelinating lesion: Understanding the mechanisms of remyelination and the reasons for regeneration failure is one of the major challenges in multiple sclerosis research. Remyelination is a process accompanied by a changing pattern of myelin protein expression. The myelin proteins MBP (myelin basic protein) and PLP (proteolipid protein) are detectable after approximately 4 days of remyelination whereas MOG (myelin oligodendrocyte glycoprotein) is recognized later on. Thus MOG staining will not pick up the early processes of remyelination but is a good marker for myelin formation during the later phase (Lindner, 2008).

Shadow plaque: Shadow plaques were first depicted as a decreased density of myelin in a systematic study of the pathology of acute multiple sclerosis by Marburg (1906) who interpreted them as “incomplete demyelination”. This aspect was later pursued by Schlesinger (1909), who applied this term to plaque-like lesions in which a reduced density of myelinated fibres was associated with unusually thin myelin sheaths. A decreased density of myelin has several different pathological explanations. In active demyelination, some myelin sheaths may already have been destroyed whilst others are still preserved. In other lesions, a decrease in density of myelinated axons can be found in association with pronounced axon loss. Beside the classical plaque-like lesions, it can represent a complete remyelination of previously demyelinated lesions (Lassmann 1983). In this master thesis the term shadow plaque only is used to describe this type of lesion (Compston, 2001).

The destructive lesion: Destructive lesions are responsible for rapidly progressing acute and chronic multiple sclerosis (Sugano, 1992; Youl, 1991). In this case demyelination is accompanied by extensive additional tissue destruction, affecting also astrocytes and axons with no or very limited signs of remyelination.

Molecular markers

For lesional staging there are several markers useful and specific for the different states. Active myelin degradation via macrophages is typical in active lesions. Therefore either immunhistochemical markers against macrophages or myelin components (PLP, MOG, MBP) within these macrophages can help to characterize a lesion as the myelin degradation within the macrophages follows a distinct pattern. Similarly, also the remyelination follows a defined pattern with regenerating myelin fibers regaining positivity for the different myelin components. Because the different myelin proteins PLP, MOG, MBP are detectable at different time states, as described above, they are useful markers to differ between early (PLP and MBP) and late (MOG) remyelination (Lindner, 2008).

The research question in this work focuses on active remyelination. Because the myelin proteins are fluctuating during the demyelinating and remyelinating process (as both processes can appear side by side), it is reasonable to look at the PLP mRNA. Detecting the PLP mRNA can be seen as a valid marker of the remyelinating process.

1.2 Experimental allergic encephalomyelitis: an experimental model of multiple sclerosis

Experimental models have contributed much to our understanding of the pathogenesis in multiple sclerosis. These models not only allow the evolution and pathogenesis of inflammatory demyelinating lesions to be studied directly but they have helped to clarify basic aspects of neuroimmunology, too. The experimental allergic (autoimmune) encephalomyelitis (EAE) became one of the most intensively studied experimental systems in immunology and has contributed significantly to our understanding of basic T-cell immunology and the pathogenesis of inflammation in general. EAE can be induced in virtually all mammalian species, including humans. The disease has a complex pathogenesis and involves both cell-mediated and humoral immune mechanisms and can usefully be divided into several basic components (Lassmann, 1983). T-lymphocytes, reactive against CNS proteins, are essential for induction of the disease (Compston, 2001).

T-cells targeted against different antigens induce diseases that are surprisingly similar in their pathology (Berger, 1997). They are all characterized by perivenous inflammation, inflammatory infiltrates and activated macrophages. The major difference seen in these diseases resides in the topography of lesions in the central and peripheral nervous system. T-cells that recognize antigens present exclusively in the CNS, such as MOG, do not give rise to inflammation in the peripheral nervous system.

Myelin-oligodendrocyte glycoprotein (MOG) is one of the candidate autoantigens in multiple sclerosis. It was first identified (independently by Linington in 1984 and Lebar in 1986) as a minor myelin component exclusively located within the CNS. MOG is an unusual member of the immunoglobulin-

supergene family with a long extracellular domain which can readily be accessed by humoral mediators of immunity (Kroepfl, 1996). MOG-specific humoral antibodies thus can bind myelin membranes and cause large areas of demyelination (Linington, 1988). Also MOG possesses highly autoimmunogenic T- and B-cell epitopes. In rodents, immunization with MOG peptides or recombinant protein causes activation of encephalitogenic T- and B-cells, mediating a disease which is similar to multiple sclerosis (Compston, 2001).

There are three different models of this kind of EAE commonly used. Firstly, passive T-cell transfer is done to induce a mild monophasic disease course, secondly active immunisation with a myelin component preparation for a more severe disease course and thirdly, also a cotransfer of T-cells and autoantibodies is possible. The clinical course and immunological details of the resulting disease depends furthermore on the rat genotype. In this work we chose the active MOG-EAE model in dark agouti (DA) rats. MOG seems to be the only myelin component that elicits B- and T-cell autoimmune responses which together produce histological and neurological changes with many features typical of the human multiple sclerosis lesion (Compston, 2001), and this particular model enabled us to see the full range of lesion evolution up until completely remyelinated shadow plaques.

1.2.1 Astrocytes in EAE

In EAE, the animal model of multiple sclerosis, reactive astrocytes form scar-like barriers around perivascular clusters of inflammatory cells. Transgenically targeted disruption of these astrocyte scars in EAE exacerbates the spread of inflammation, increases axonal degeneration and worses clinical signs. Reactive astrocyte may can exert both pro- and anti-inflammatory roles that are essential in EAE. Reactive astrocytes take part both in attracting inflammatory cells to specific sites and in restricting inflammatory cells to these sites by limiting their spread into adjacent healthy CNS. This findings suggest that the loss of normal functions or the gain of abnormal effects by reactive astrocytes may contribute to disease mechanisms in multiple scerosis (Sofroniew, 2010).

1.3 Aim of the study: Investigation of the astrocytic reaction in different stages of lesion evolution

The role of the astrocytes in degeneration and regeneration in multiple sclerosis is, as described above, discussed controversial with contradicting research data available. To investigate the astrocytic reaction over the full course of the disease from active demyelination to complete remyelination, active MOG-EAE was induced in DA rats. To examine the astroglial reaction, immunoreactivity for GFAP was correlated to the remyelinating capacity obtained by *in situ* hybridization for mRNA of the myelin protein PLP, which is regarded as a marker for remyelinating oligodendrocytes. For statistical analysis of the data all lesion types were quantified.

The aim of our study was to investigate the astrocytic reaction at different stages of lesion evolution and the correlation between the extent of astrocytic gliosis and remyelinating capacity in MOG-EAE in order to gain more information about the role of astrocytes in multiple sclerosis. We tried to figure out whether remyelination can coexist with astrocytic gliosis or if one is really incompatible with the other. Understanding the role of astrocytes and reactive astrogliosis could lead to a better understanding of the disease mechanism.

2. Material and Methods

Several techniques were used to investigate the role of astrocytes in different lesion stages during lesion evolution. At the beginning hematoxylin-eosin (HE) and luxol fast blue (LFB) staining was used to gain the necessary overview over inflammatory infiltrates and to determine the lesion types, respectively. Immunohistochemical staining for the myelin components PLP, MBP and MOG were performed in order to determine the lesion stage. As discussed above, PLP and MBP are markers for early remyelination whereas MOG is an excellent marker to determine late remyelination. This information of the different lesions was part of previous research work and therefore already available at the beginning of this thesis.

For our special research question, furthermore, immunohistochemical stainings were performed using antibodies directed against GFAP (Glial Fibrillary Acidic Protein) in order to detect astrocytes and to investigate the astrocyte behaviour in the different lesion types on the rat tissue.

In order to investigate PLP mRNA occurrence, *in situ* hybridization was performed. Additionally for visualization of the protein, slides were afterwards double-stained against PLP. This protein is a major component of myelin, but differs in that it is an integral membrane component and therefore has marked lipophilic properties (Compston, 2001). The PLP mRNA can be seen as a marker for occuring remyelination and the double-stained PLP shows the restored or residual protein. This is very useful to assess the remyelinating capacity of a particular lesion.

2.1 Experimental tissue

Acute and chronic MOG-EAE was induced in Dark Agouti (DA) rats by active immunization with 50µg rMOG aa 1-125. The CNS tissue was sampled at day 20 to 120 p.i. to give an overview on the full course of the disease from active inflammatory myelin degradation to a successfully completed remyelination. For the histological staining procedure serial brain and spinal cord sections were

investigated with respect to lesion type, expression of myelin antigens and GFAP.

2.1.1 About the rat strain and tissue harvesting

The animals were sacrificed and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS phosphate buffered saline. Brains and spinal cords were dissected and then postfixed in the same fixative for 24 h at 4 °C. After washing in PBS the tissue was routinely embedded in paraffin. The tissue was then cut into 1.5-2 µm paraffin sections, for the *in situ* hybridization this was done RNase-free. LFB staining was done on the 2 µm sections, *in situ* hybridization and immunohistochemistry on the 1.5 µm sections.

2.2 Histological staining protocols

2.2.1 Luxol fast blue staining (LFB)

This staining is routinely used for staining of myelin. Myelin contains a high number of phospholipids and basic proteins. The dye luxol fast blue shows a strong affinity to phospholipids and choline bases. Staining of lipoproteins occurs via ionic bond. First these structures are overdyed followed by differentiation in lithiumcarbonate to make the gray and white matter visible (Lang, 2013). Intact myelin appears in dark blue, demyelinated regions are pink. Shadow plaques appear in light blue. Besides, a differentiation between active and inactive demyelination is possible because of the different coloured granula within macrophages.

Solutions:

Luxol fast blue solution: 1 g Luxol fast blue in 1000 mL 96% ethanol

Perjodic acid solution: 4 g perjodic acid in 500 mL distilled water

Lithiumcarbonate solution: 1 g lithiumcarbonate in 1000 mL distilled water

1. Deparaffinise paraffin embedded tissue sections to 96% ethanol:

a. Incubate the slides for 10 min in xylene two times

b. Rinse with 96% ethanol

2. Incubate the sections in LFB solution at 56° C over night (make sure the sections are fully covered with the solution)
3. Rinse with 96% ethanol
4. Rinse in distilled water
5. Incubate the slides in lithiumcarbonate solution for 5 minutes
6. Differentiate with 70% ethanol until myelin sheaths appear blue
7. Rinse in distilled water
8. Incubate the slides for 10 min in periodic acid
9. Rinse in distilled water
10. Incubate the slides for 20 min in Schiff's reagent
11. Rinse the slides for 10 min in warm tap water
12. Dehydrate the tissue through a graded ethanol line
13. Apply mounting medium and coverslip

2.3 Immunohistochemistry

2.3.1 Theory of Immunohistochemistry (IHC)

The entry of immunohistochemical methods in histology succeeded in the late 1980s. Using specific antigen-antibody reaction to determine tissue properties was first limited on frozen sections. By intense research, this technique could finally be established on formalin fixed, paraffin embedded tissue, too. This went along with an increase of detectable tissue properties. Because of the high

specificity of this method many differentiated diagnosis in various fields became possible (Lang, 2013).

Immunohistochemistry is a method to determine the localization of antigens in tissue sections. These antigens are recognized specifically by the antibody. The antibodies are coupled with a detection system and are visualized by a marker e.g. fluorescent dye or an enzyme. This main system goes mostly hand in hand with signal amplification. Therefore it is possible to make a tiny epitope with many antibodies and many secondary antibodies and markers visible. There are different methods common; either the primary antibody or the secondary antibody is marked. The principle of this method sounds easy, but in fact there are some difficulties that can make the staining tricky. The stability of the epitope, an optimal reaction condition for the antigen-antibody binding and the possibility of unspecific binding on the tissue are just a few possible troubles (Lang, 2013).

Fixation:

One of the most important steps in IHC is the fixation process. Many publications originated in the 1980s, engaging with the question, whether formalin can influence or even destroy certain epitopes. The used tissue in our study was fixed in 4% PFA/PBS solution. The tissue stayed in this solution for 24h after perfusion. The embedding in paraffin was performed with Thermo Shandon Citadel 1000 with the following program:

1. 50% ethanol for 30 minutes
2. 70% ethanol for 60 minutes
3. 70 % ethanol for 90 minutes
4. 80% ethanol for 60 minutes
5. 80% ethanol for 90 minutes
6. 96% ethanol for 60 minutes
7. 96% ethanol for 90 minutes

8. 96% ethanol for 120 minutes
9. Xylene for 30 minutes
10. Xylene for 60 minutes
11. 2 times paraffin for 120 minutes each

Pretreatment:

During the fixation process the proteins get cross linked. In order to detect the antigen this linkage has to break up ("demasked") again. In this antigen retrieval step it is important to find the right pretreatment method. Too harsh pretreatment could even destroy the epitope, and too mild pretreatment could break too little cross-linked bonds, so that the antibody is still not able to detect the corresponding antigen.

There are many techniques for successful pretreatment depending on the used tissue, the antibody and the fixation. Most common is the pretreatment with citrate (pH 6) ore EDTA buffer (pH 5.5 or 8.5) in a steamer or a microwave. There is also the possibility to use enzymes like proteinase k or trypsin to digest the crosslinks. But in this case a careful, accurate dosage of the enzyme is necessary because a digestion of the epitope happens easily when overvaluing the concentration. Which of the various pretreatment methods should be used has to be determined experimentally. For the GFAP staining the sections were steamed in EDTA buffer (pH 5.5) for 1 hour in a commercially available food steamer since this gave the best specificity and the fewest background.

Blocking:

In order to avoid background staining the unspecific binding sites have to be blocked. For the GFAP-IHC 10% fetal calf serum in DAKO antibody diluent was used to perform this blocking step. Another possible cause of background staining is the activity of the endogenous peroxidase; therefore sections are pre-treated with hydrogen peroxide after the deparaffinising step.

Avidin-Biotin Complex (ABC) and labeled Avidin-Biotin (LAB) technique:

Avidin is a large glycoprotein with a very high affinity for biotin. It can be labelled with peroxidase. Biotin can easily bind covalently to antibodies because of its low molecular weight. Both methods use three “layers” of antibodies. The primary, unlabelled antibody first binds to the specific antigen on the tissue (in case of GFAP-IHC this primary antibody was produced in mouse). The secondary antibody (anti-mouse immunoglobulin) can than bind the primary antibody. This second antibody is biotinylated. The third “layer” is either a complex of avidin-biotin with an enzyme (ABC) or enzyme-labelled avidin (LAB). This enzyme-labelled avidin complex is smaller than the ABC-complex. Therefore a better permeation in the tissue is provided while less background staining occurs. This is only one of many possible methods in immunohistochemistry but the LAB method is due to its signal amplification very powerful and has therefore been preferred in this work. A scheme of the LAB method is shown in figure 3.

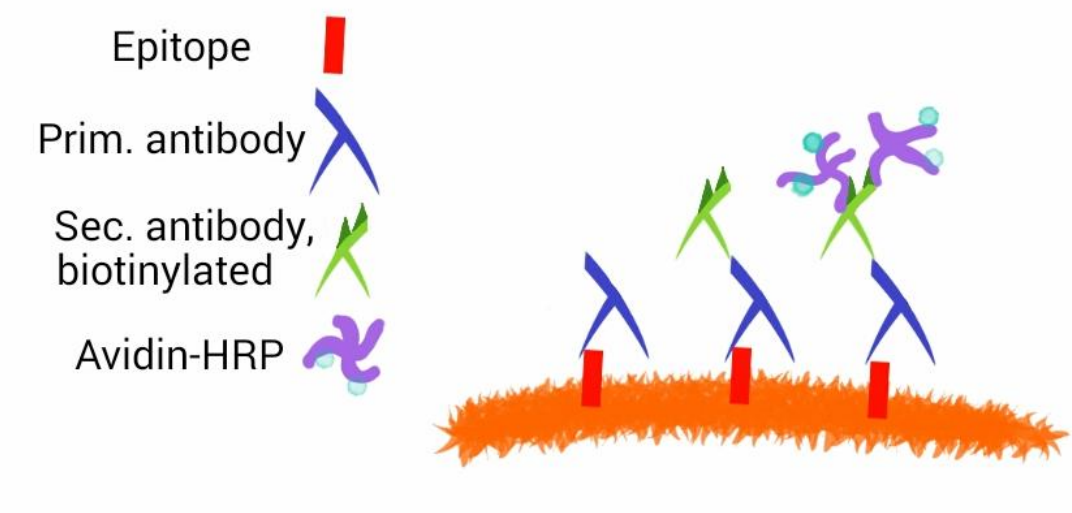


Figure 3 Schema of the LAB-method. The orange bar represents the tissue and the red bars are the epitops on this tissue. The primary antibody (blue in this figure) binds that epitope specifically whereas the secondary antibody (green) binds the primary antibody specifically. This secondary antibody is biotinylated (represented in dark green in this figure) which has a high affinity to avidin. Avidin has structurally 4 arms and each of them can bind biotin. Avidin is conjugated with an enzyme; in this case HRP (horse-radish peroxidase, in this figure represented by light blue dots) and this enzyme can catalyze the colour reaction.

2.3.2 Immunohistochemical staining protocol

1. Deparaffinising of paraffin embedded tissue sections:
    a. Incubate sections for 10 min in xylene two times
    b. Rinse the sections with 96% ethanol for two times
    c. Incubate for 30 min in H_2O_2-Methanol (180 ml Methanol + 1.2 mL H_2O_2)
    d. Rinse with 96% Ethanol
    e. Rinse with 70% Ethanol
    f. Rinse with 50% Ethanol
    g. Rinse with deionized water
2. Pretreatment: steam the sections (1 hour) in citric acid buffer (pH 6.0)
3. Wash the slides 3 times in PBS
4. Bordering the tissue with DAKO-Pen
5. Blocking: 20 min in 10% fetal calf serum in DAKO diluent (FCS/DAKO)
6. Get rid of the Blocking solution by tipping the slide-side gently against pulp
7. Apply primary antibody: GFAP (monoclonal mouse anti GFAP antibody) 1:100 diluted in FCS/DAKO. Incubate at 4° C overnight
8. Wash 3 times in PBS
9. Apply secondary (biotinylated anti-mouse) antibody 1:200 diluted in FCS/DAKO and incubate at room temperature for 60 min.
10. Wash 3 times in PBS

11. Apply avidin peroxidase labelled 1:100 diluted in FCS/DAKO and incubate at room temperature for 60 min

12. Get rid of the avidin solution by tipping the slide-side gently against pulp

13. Visualization of the labelling with diaminobenzidine (DAB) – 2 mL DAB-Solution (1 g DAB in 40 mL PBS) in 100 mL PBS + 33 µL H_2O_2, filtered

14. Stop the reaction with tap water

15. Counterstain with hematoxylin:

    a. Incubate sections for 1 min in filtered hematoxylin

    b. Rinse in tap water 2 times

    c. Differentiate in 70% ethanol

    d. Rinse in distilled water 2 times

    e. Incubate slides for 5 min in bluing reagent

    f. Rinse in distilled water

    g. Dehydration through graded ethanol line

16. Apply mounting medium and coverslip

2.4 *In situ* hybridization

2.4.1 Theory of in situ hybridization (ISH)

In situ hybridization enables detection of mRNA on tissue by labelled probes. A probe is a labelled DNA molecule which binds to its complementary strand and therefore makes cells expressing the corresponding mRNA visible. Hybridization is a sequence specific clustering between two complementary single stranded nucleic acids to one double strand. Hybridization between DNA and DNA, RNA and RNA as well as DNA and RNA is possible. In this case, RNA hybridizes with an RNA probe. After an initial attachment between the two

nucleic acids, a fixation step follows. This step is important to denaturize the secondary RNA structure. Pre-hybridization is important for blocking unspecific binding sites.

This technique was first used with radioactive labelling. In the course of time non-radioactive methods gained more and more importance. Nowadays non-radioactive staining methods are preferred because of its easier handling and various advantages:

1. Good structural assignment of the signal, because the signal occurs in the cytoplasm and not in form of silver deposit on the cell surface.
2. Better possibilities of double staining via immunohistochemistry.
3. Continuous control of the developing-status of the section and therefore decision possibility whether the section should be developed further or not.
4. A good signal-background ratio.
5. Non-radioactive staining can be transposed in every lab and is more ecological.

2.4.2 In situ hybridization preparation

For ISH RNase free slides are necessary. RNases are ubiquitary occurring enzymes, destroying RNA. These enzymes are able to digest RNA on the tissue slide. Thus it is very important to take care in preparation for *in situ* hybridization and avoid RNase contamination. This can be obtained by observing several preventive measures:

- always use cloves
- clean the microtom, the water bath and the brushes with alcohol or RNase ZAP Spray
- use a new microtom blade

- use DEPC-water in the water bath
- use a new slide pack

DEPC-water: 1 mL DEPC (diethylpyrocarbonate) solved in 1 L distilled water. This mixture stands for 24 h by room temperature followed by autoclavation to destroy the DEPC. All solutions are made with this DEPC-water; also the water bath for microtom section uses this water.

TBS-stocksolution 1 M, pH 7.5: 500 mL DEPC-water, 61 g TRIS 0.5 M, 400 mL HCl 1 M and 180 g NaCl mixed together and filled up to 1 L with DEPC water. Afterwards the pH is adjusted to 7.5.

TBS to use: 50 mL TBS-stocksolution mixed with 950 mL DEPC-water.

PBS-stocksolution 0.2 M: 5.5 g NaH_2PO_4 and 28.5 g NA_2HPO_4 mixed together and solved in 1 L DEPC water. Afterwards the pH is adjusted to 7.4.

PBS to use: 250 mL PBS-stocksolution added to 750 mL DEPC-water and mixed with 8.7 g NaCl.

4% Paraformaldehyde: 40 g Paraformaldehyde solved in 500 mL PBS to use at 60° C and filled up with DEPC-water to 1 L

Proteinase K: 100 µg/mL proteinase K solution mixed with 50 mL TBS and 2 mM $CaCl_2$.

TRIS 1M pH 8 stocksolution: 500 mL DEPC-water mixed with 60.6 g 5.5 M TRIS and added to 350 mL 1 M HCl. The pH is than adjusted to 8.

TRIS 0.1 M solution to use: 100 mL stocksolution mixed with 900 mL DEPC-water.

50% Dextransulfate: 10 g solved in 20 mL DEPC-water.

2% SDS: 200 µg solved in 10 mL DEPC-water.

0.5% sheart DNA: 50 mg Salmon Sperm DNA in 10 mL water mixed for 4 h. This mix is than pushed through a needle and in boiling water denaturised.

Blocking reagent: 0.5% blocking reagent in corresponding buffer solved.

Buffer for NBT/BCIP: 12 g 100 mM TRIS buffer mixed with 6 g 100 mM NaCl and 10 g 50 mM $MgCl_2$ and filled up to 1 L with DEPC water.

NBT: 500 mg solved in 5 mL 70% dimethylformamide.

BCIP: 250 mg solved in 5 mL 100% dimethylformamide

NBT/BCIP to use: 100 mL buffer mixed with 450 µL NBT and 350 µL BCIP.

RNA-Probe: kindly provided by the brain research institute of Vienna.

APAAP-substrate: Mix 920 ml TRIS with 60 mL HCl (0.1 M pH8.2), dissolve 200 mg Naphtol-AS-MX-phosphate in 20 mL dimethylformamide and give 1 mL levamisole in this mix.

Fast red: dissolve 50 mg fast red TR salt in 50 mL APAAP-substrate.

2.4.3 In situ hybridization staining protocol

The following protocol uses a digoxigenin labelled probe visualized by alkaline phosphatase.

1. Deparaffinise paraffin embedded tissue sections: same protocol like in the immunohistochemistry but all with DEPC-water.
2. 20 min 4% Paraformaldehyde to fix the intersection
3. Wash 3 times with TBS
4. Incubate the sections in 0.2 M HCl for 10 min
5. Wash 3 times with TBS
6. Incubate the sections in the proteinase K solution for 20 min at 37° C
7. Wash 3 times with TBS
8. In order to stop the enzyme activity, incubate the sections in TBS at 4° C

9. Incubate the sections in 0.5% acetic anhydride under stirring for 10 min
10. Wash 3 times with TBS
11. Follow the ascending alcohol line (30%, 50%, 75%, 3 times 96%) and end in chloroform.
12. Incubate the slides in a wet chamber by 55° C for 30 min
13. Apply the full length probes (depending on the tissue size approximately 40 µL), coverslip and incubate for exactly 4 min on a 95° C heat plate.
14. Hybridization occurs over night at 65° C
15. Let the coverslip swim away in buffer
16. Wash the slides in 50% formamide in buffer 3 times for 20 min at 55° C
17. Wash the slides with buffer
18. Put the sections in Boehringer blocking reagents with 10% FCS for 15 min
19. Anti-digoxigenin and alkaline phosphatase 1:500 is applied in blocking reagents with 10% FCS for 1 h.
20. Wash 5 times with TBS
21. Develop the sections with NBT/BCIP at 4° C and make microscopic controls from time to time. This can take up to 140 h.
22. Wash the sections in water
23. Apply primary antibody (PLP 1:1000) in 10% FCS/TBS over night at 4° C
24. Wash the sections in TBS
25. Apply biotinylated secondary antibody (1:200) in 10% FCS/TBS 1 h at room temperature
26. Wash the sections in TBS

27. Incubate with avidin-AP for 1 h at room temperature
28. Develop the sections with fast red
29. Counterstain with hematoxylin:
    a. Incubate the slides for 3 sec in filtered hematoxylin
    b. Rinse in tap water 2 times
    c. Differentiation with 70% ethanol
    d. Rinse in distilled water 2 times
    e. Incubate the slides for 5 min in bluing reagent
    f. Rinse in distilled water
30. Apply watersoluble mounting medium and coverslip

(Protocol according to Breitschopf, 1992)

2.4.4 Explanations to the protocol

Note that all steps are using DEPC water, including the deparaffination steps. Paraformaldehyde is necessary to fix the RNA again, because during the microtome sectioning process new nuclei get sliced and therefore new RNA runs free. Although the HCl step is using a very low molarity to denature proteins, experience showed a better result when doing this step. Proteinase K is used to free mRNA and break the fixation bonds. It is very important to find the right middle way to free enough mRNA but avoid digestion. Acetic anhydride changes the cell charge in the tissue to avoid unspecific binding. After the descending alcohol line, the slides are dried with chloroform. In the hybridization mix, the deionised formamide is very important to decrease the hybridization temperature to 65°C. Dextransulfate has an important spacer function – the full length probes can differentiate better under the viscous space between tissue and coverslip. SDS simply works as a detergent. Because of the following

4 minutes step on the 95° C heat plate, the RNA gets linearized. This avoids unspecific bindings (Breitschopf et al., 1992).

3. Results

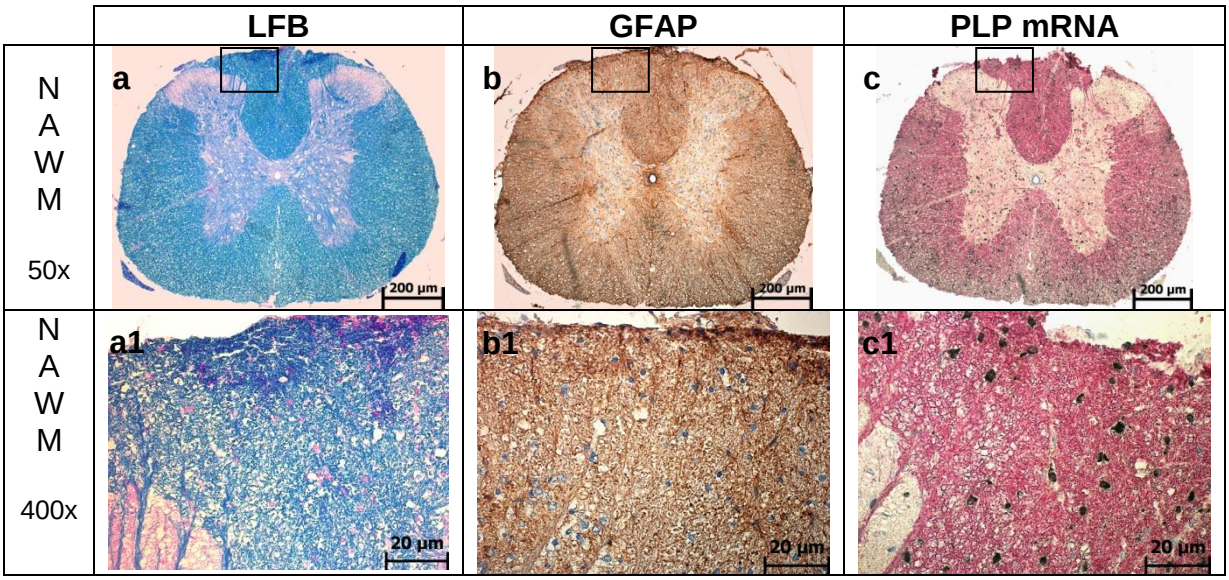
3.1 Light microscopy

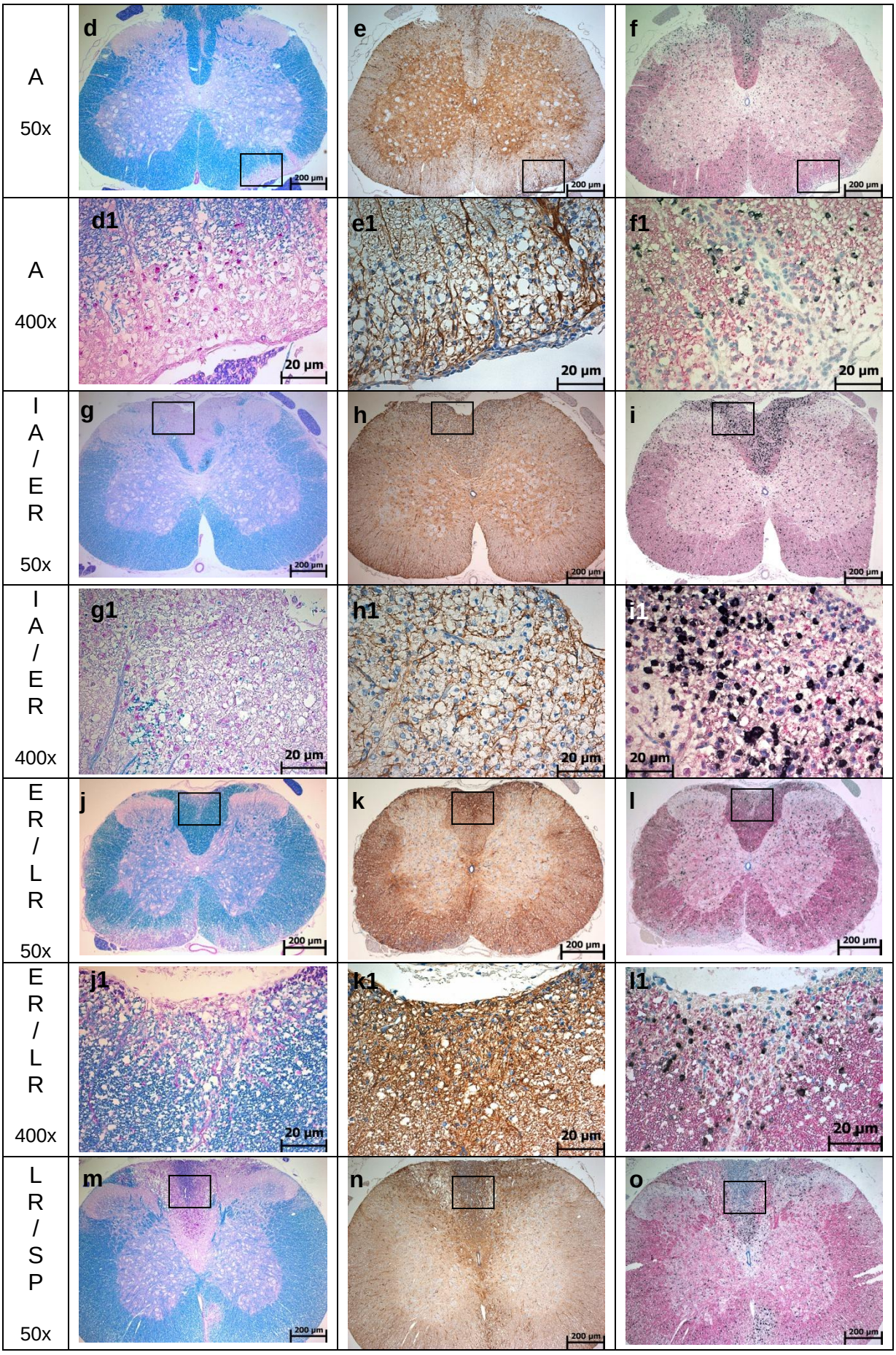
In order to be able to categorize the results, a determination of lesion staging was done. According to previously published criteria (Brück, 1995; Kornek, 2000; Lucchinetti, 1999) the following categories for demyelinated plaques were defined: Actively demyelinating lesions are defined by the presence of LFB-positive degradation products within macrophages. In early active lesions these degradation products are immunoreactive for all myelin proteins including MOG. In comparison, late active lesions contain degradation products immunoreactive for MBP and PLP, but not for MOG. In inactive lesions, macrophages do not contain myelin degradation products anymore because they are already lysosomal digested (Brück, 1995; Lucchinetti, 1996; Ozawa, 1994) and therefore in LFB staining macrophage contents appear pink. Early remyelinating lesions are similar to inactive demyelinated plaques, but already contain some fibers positive for certain myelin proteins. In shadow plaques, the myelin integrity is fully restored and all myelin proteins are detectable again, but LFB staining shows a reduced staining intensity of the plaque compared to the NAWM due to thin myelin sheaths in this area.

Microscopy was done on the “Zeiss light microscope Axioplan2 imaging” and all photos were made with the “Zeiss AxioCam MRc5” camera. In figure 4 are the various lesion types represented in the staining LFB, GFAP and PLP. In general, LFB represents demyelination in pink, myelin in blue and remyelinated regions in lighter blue or sometimes in light violet if these regions are overlapping.

The PLP-ISH/PLP-IHC (PLP mRNA *in situ* hybridization double-stained with PLP immunohistochemically) represents the PLP mRNA in form of black dots and the double stained myelin protein in pink. Lack of myelin can therefore also be determined on the PLP-ISH/PLP-IHC; these areas appear white instead of pink. GFAP is a marker for astrocytes and displays them in dark brown. If there is a strong branched network of astrocytes, individual astrocytes may be difficult to distinguish except from their cell nuclei.

There are not more GFAP positive astrocytes present in active lesions (A) than in the NAWM in the immunohistochemical staining, but the astrocytes differ in their form according to their activation state. In the activated state they appear bigger and darker with respect to GFAP immunoreactivity in comparison to those found in the NAWM. There are significantly less PLP mRNA positive cells present in the active lesion (A) than in the NAWM. The section detail in figure 4 g and g1, representing the IA/ER (inactive/early remyelination) state, appears in LFB more pink than in the A state (figure 4 d and d1). This is due to the degradation products in the stained macrophages. In the GFAP staining, the astrocytes are now organized in a more branched network than in the active state. On the PLP-ISH/PLP-IHC there are significantly more positive cells in the IA/ER state than in the NAWM. The detail of LR/SP (late remyelination/shadow plaque) shows on the LFB staining both, the late remyelination in pink and a beginning shadow plaque in light blue. Now there appears such a big and strong branched network of astrocytes in the GFAP staining so that only the nuclei of the cells (in blue) can be seen as individuals in the shadow plaque (SP) region. In the LR region there appear slightly fewer nuclei than in the SP region whereas the PLP-ISH/PLP-IHC shows a relative homogeneous area with positive cells across both states, LR and SP. The black arrow in figure 4 p1 points to the SP region. This part appears darker blue because of remyelination.

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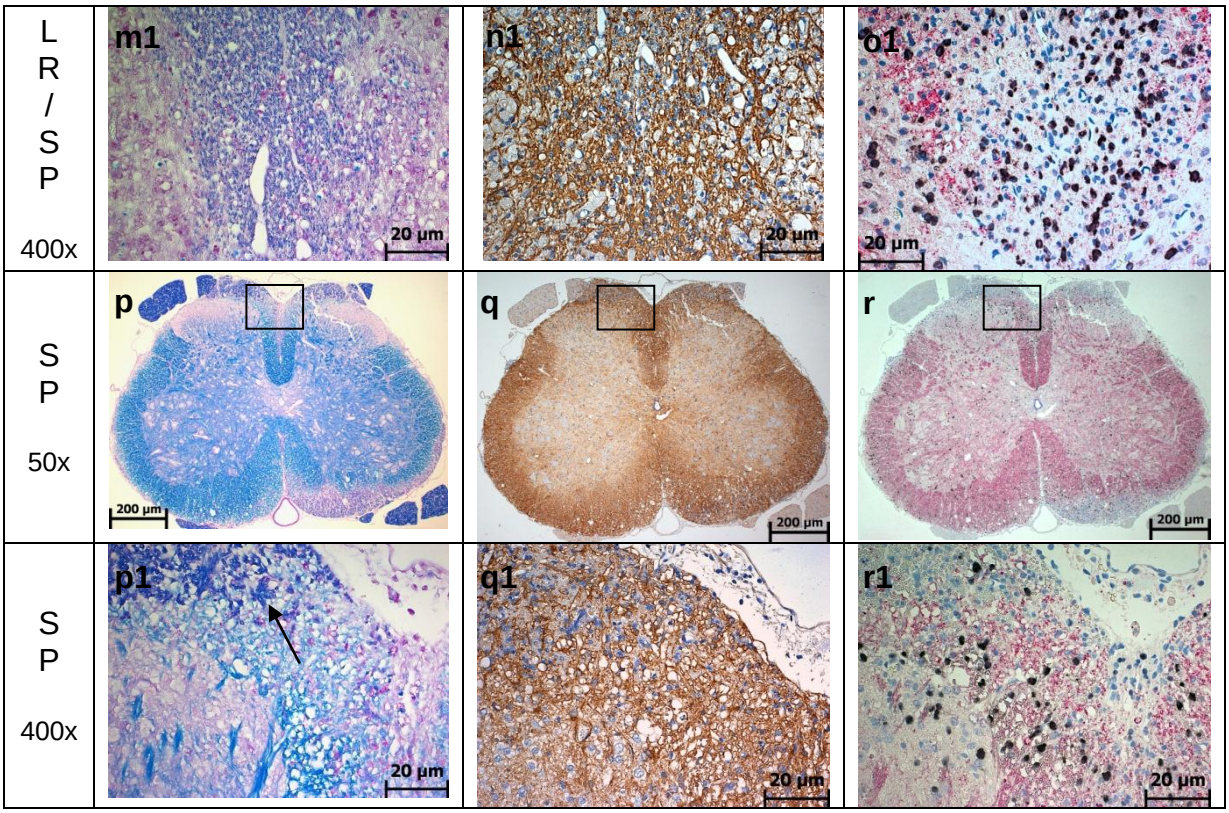

Figure 4 Comparison of representative tissue slides of each lesion type and applied staining. On the left side, the lesion classification is displayed: NAWM (normal appearing white matter) a, b, c, a1, b1, c1; A (active lesion) d, e, f, d1, e1, f1; IA/ER (inactive lesion/early remyelination) g, h, i, g1, h1, i1; ER/LR (early remyelination/late remyelination) j, k, l, j1, k1, l1; LR/SP (late remyelination/shadow plaque) m, n, o, m1, n1, o1; SP (shadow plaque) p, q, r, p1, q1, r1. The number under the lesion description represents the used microscope magnification. All photos were made with a Zeiss Camera (AxioCam MRc5) on a Zeiss light microscope (Axioplan2 imaging). Lightness and contrast were adjusted via Adobe Photoshop Elements 6.0. The first line names the represented staining in the particular column. LFB means luxol fast blue staining, GFAP the immunhistochemical staining with anti-GFAP antibody and PLP the *in situ* hybridization results with the anti-PLP-mRNA-probe and the PLP double-stain. In the LFB column, demyelinated regions are pink, myelin occurs blue and remyelinated areas are light blue or violet. The astrocyte marker GFAP shows astrocytes in brown or dark brown according to the lesion state, and the counterstained nuclei in blue. In the PLP staining, the mRNA is represented as black dots, the stained myelin protein appears pink and the counterstained nuclei are coloured blue. The thin black rectangles in each of the 50x pictures represent the area shown in the corresponding 400x magnification.

3.2 Quantitative evaluation

Each lesion was carefully quantitatively assessed on a light microscope (Zeiss Axioplan2 imaging). Labeled cells within the lesions were counted by using an ocular morphometric grid under a 40x objective on position 6, which corresponds to a 400x magnification. In each lesion region all positive cells in one full square grid were counted. The selected lesions were quantitatively

assessed completely and the counts were then transformed to cells/mm^2. In total 492 lesions of GFAP and 424 lesions of PLP-ISH were quantified. With respect to lesion stage, 128 (GFAP) and 70 (PLP-ISH) were classified as active, 84 (GFAP) and 73 (PLP-ISH) as inactive lesion/early remyelinating, 88 (GFAP) and 84 (PLP-ISH) as early remyelinating/late remyelinating, 90 (GFAP) and 93 (PLP-ISH) as late remyelinating/shadow plaque as well as 85 (GFAP) and 82 (PLP-ISH) as shadow plaque. In total 45 DA rats were analyzed and predominantly spinal cords were used for this quantification. In case the number of assessed lesions differs between the different staining, this is due to technical problems that have appeared during the staining process. Some stained sections haven't shown specific reactions and sometimes tissue got lost in the specific lesion during tissue preparation and handling.

Table 1 shows the total numbers of the quantitatively assessed lesions with respect to lesion type as well as the calculated mean values and standard deviations for GFAP and PLP mRNA, respectively within these lesions.

Table 1 Number of lesions, mean values and standard deviations used for the quantification. First line represents the lesion classes: NAWM (normal appearing white matter), A (active lesion), IA/ER (inactive lesion to early remyelination), ER/LR (early remyelination to late remyelination), LR/SP (late remyelination to shadow plaque), SP (shadow plaque). Mean values refer to cells per mm^2.

	NAWM	**A**	**IA/ER**	**ER/LR**	**LR/SP**	**SP**
GFAP: number of lesions	17	116	84	88	90	85
Mean value	379	361	2661	4092	6179	9824
Standard deviation	296	528	1200	1884	1771	2927
PLP: number of lesions	22	70	76	79	85	79
Mean value	689	39	1631	773	690	693
Standard deviation	264	65	773	341	271	304

Statistical significance was tested via t-test with the corresponding p-values listed in table 2.

Table 2 Statistical significance between different lesion types represented via p-values: A (active lesion), IA/ER (inactive lesion to early remyelination), SP (shadow plaque), NAWM (normal appearing white matter). All p-values except ER/LR to LR/SP (PLP) are under 0.05 and therefore representing a statistically significant result.

GFAP p-values	**SP to NAWM**	**SP to A**	**SP to IA/ER**	**IA/ER to NAWM**	**ER/LR to LR/SP**
	4.4E-48	1.6E-47	2.2E-40	1.9E-27	1.6E-12
PLP p-values	**A to NAWM**	**A to IA/ER**	**SP to IA/ER**	**IA/ER to NAWM**	**ER/LR to LR/SP**
	9.9E-11	5.7E-29	2.4E-16	3.0E-14	8.5E-02

The diagrams in figure 5 and 6 show the calculated mean values of the cell amount in columns, each representing one of the different lesion classes. The bars on the columns represent the standard deviation for each class.

The raw data is based on several lesion types, including active lesion, late active lesion, inactive lesion, late remyelination, destructive lesion and shadow plaque. On closer examination, we found out that a strict separation of one lesion type to the other is not always possible as there is often a smooth transition and one lesion may also display partial features of the following. Therefore, we decided to make the following groups for statistic evaluation: Active lesion (A), inactive lesion/early remyelination (IA/ER), early remyelination/late remyelination (ER/LR), late remyelination/shadow plaque (LR/SP), shadow plaque (SP). The group NAWM represents the “normal appearing white matter”, an area some distance away from any lesion activity in histologically normal white matter. In figure 5 the quantitative results of the GFAP counts are presented. In the normal appearing white matter, there were in average not more than 600 astrocytes per mm^2 GFAP positive. In active lesions there were less cells GFAP positive than in the normal appearing white matter. From inactive lesions to shadow plaques, the GFAP positive astrocyte number increases to approximately 10,000 cells per mm^2. A significant difference can be determined between inactive lesion/early remyelination and late remyelination: The IA/ER class showed not more than 3,000 GFAP positive astrocytes per mm^2 in the lesion region and the ER/LR group had its maximum

by more than 4,000 cells per mm^2. Another big difference can be mentioned between the LR/SP and SP class, where the later shows the highest amount of GFAP positive cells. There was also a big difference detectable between the ER/LR and the LR/SP group; the latter displayed cell amounts around 6,000 cells per mm^2.

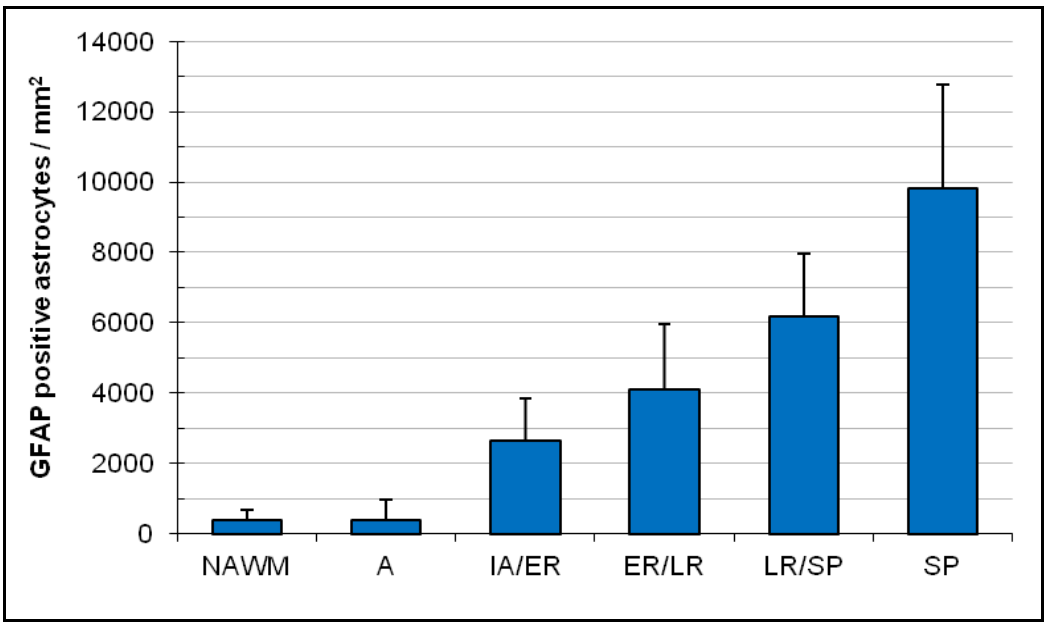

Figure 5 GFAP positive astrocytes in different lesion types. The columns represent the mean values of the counted GFAP positive astrocytes according to the particular lesion types: normal appearing white matter (NAWM), active lesion (A), inactive lesion to early remyelination (IA/ER), early remyelination to late remyelination (ER/LR), late remyelination to shadow plaque (LR/SP) and shadow plaque (SP). The bars on each column represent the standard deviation of each lesion type. Whereas NAWM and A columns show approximately the same cell amount, there is a significant increase of GFAP positive cells from column IA/ER to SP. Statistically significance was proven via t-test. This test yielded a p-value of 4.4E-48 in comparison of NAWM and SP. The statistical significance between SP and A is represented by a p-value of 1.6E-47. Comparison of SP and IA/ER resulted in a p-value of 2.2E-40 and the significance test between IA/ER and NAWM produced 1,9E-27 as a p-value. The t-test of the ER/LR and LR/SP group showed a p-value at 1.6E-12. Thus all compared groups showed a statistically significance.

In figure 6 the PLP mRNA expression of the different lesion types is displayed. There is a significant difference in PLP mRNA expression between NAWM and active lesions detectable, with hardly any PLP mRNA in the active state. Most PLP mRNA expression could be determined in the IA/ER class with around 1,600 PLP mRNA expressing cells per mm^2. With advancing remyelination, PLP mRNA expression decreases, ending with the fully remyelinated shadow plaque, showing only slightly more cells expressing PLP mRNA than the

NAWM. There are about 700 cells per mm^2 in the NAWM and also around 700 cells per mm^2 in the SP expressing PLP mRNA detectable. Statistical significance was proven via t-test. The given p-values in table 2 show the statistically significance between the tested groups.

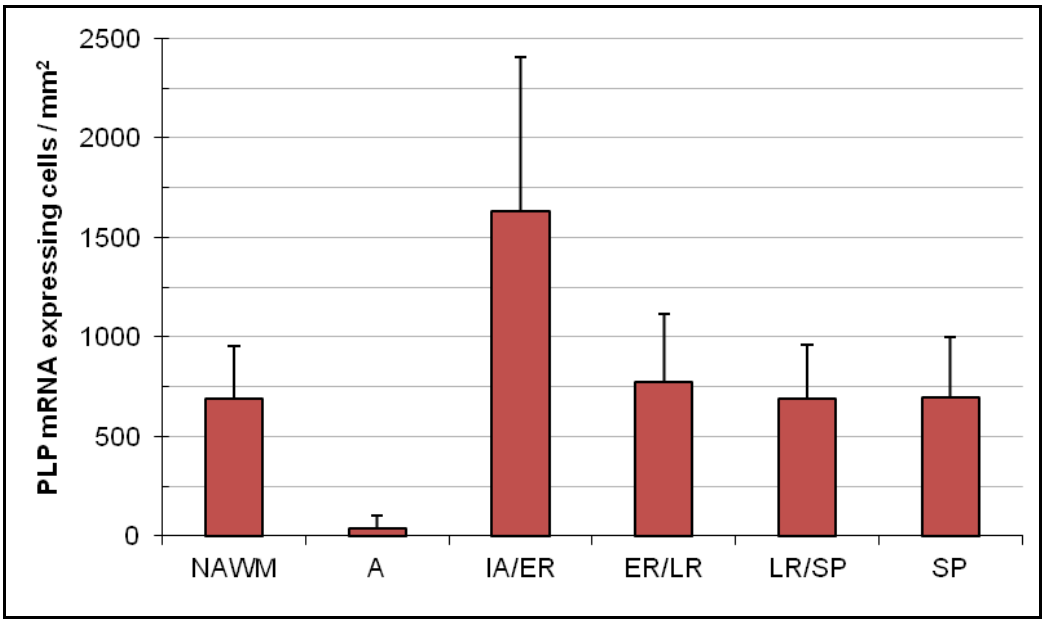

Figure 6 PLP mRNA in different lesion types. The columns represent the mean values of the counted cells expressing PLP mRNA according to the particular lesion types: normal appearing white matter (NAWM), active lesion (A), inactive lesion to early remyelination (IA/ER), early remyelination to late remyelination (ER/LR), late remyelination to shadow plaque (LR/SP) and shadow plaque (SP). The bars on each column represent the standard deviation of each lesion type. Note that the y-axis in this diagram differs from the y-axis in the GFAP diagram (figure 5) because in general there is less PLP expression than GFAP positive cells detectable. The cell amount expressing PLP mRNA rapidly decreases from NAWM to A. IA/ER shows the highest cell amount expressing PLP mRNA and expression decreases again to SP whereupon the NAWM, LR/SP and the SP show a similar cell amount. Statistical significance was reassessed by a t-test between different groups. With a p-value of 9.9E-11, the comparison of NAWM and A showed a statistically significant result. The comparison of A and IA/ER showed not surprisingly a significant p-value of 5.7E-29. T-test between NAWM and IA/ER resulted in a p-value of 3.0E-14 and the comparison of SP and IA/ER yielded a p-value of 2.4E-16. The testing of ER/LR and LR/SP resulted in a p-value of 8.5E-02.

4. Discussion

According to Sofroniew et al. 2010 in mild astrogliosis most astrocytes are GFAP positive. This correlates with the microscope photo in figure 4 d-f1 representing the active lesion. Looking at the demyelinated area detectable via LFB, there are distinct GFAP positive cells (figure 4 e1; GFAP, A) present in the same area on the immunohistochemically stained slide. In comparison to the quantitative statistics of GFAP (figure 5), there are far less GFAP positive cells per mm^2 displayed compared to the remyelination areas in the different states. This makes sense considering the easily countable amount of GFAP positive astrocytes in figure 4 e1 and h1 (GFAP staining, A and IA/ER) compared to the dense network in figure 4 n1 and q1 (GFAP staining, LR/SP and SP), where almost no individual astrocyte can be seen. In figure 4 f1 (A, PLP mRNA ISH/combined with PLP IHC) there is no PLP mRNA detectable in the lesion region. This region can be identified by the white background representing missing PLP reactivity, because in this area there is no PLP left (figure 4 f1; right corner). Looking at the quantitative results in figure 6, there are hardly any cells expressing PLP mRNA in the active state compared to the other states and the NAWM. Starting from inactive state there is a sharp rise of PLP mRNA expression representing ongoing repair with a maximum PLP mRNA expression in IA/ER. This is also reflected in the quantitative evaluation given in figure 6.

The IA/ER column in figure 6 shows the highest amount of cells expressing PLP mRNA. This can be confirmed by looking at figure 4 i1 (PLP mRNA ISH, IA/ER): there is a dense and expression of PLP mRNA detectable. This confirms the function of PLP mRNA in remyelination in our sample. The start of this process seems to induce a very high expression of PLP mRNA in the lesion region which then decreases slowly as remyelination nears its completion and finally reaching the same level in the SP as in the NAWM (figure 6).

In early and late remyelination the amount of astrocytes expressing GFAP increases (figure 5) and in figure 4 k1 (GFAP staining, ER/LR) there is a dense astrocytic network displayed with individual astrocytes only detectable by their nuclei (blue counterstaining). The PLP mRNA expression statistic (figure 6) also correlates with figure 4 l1 (PLP staining, ER/LR) and shows that there are less

cells expressing PLP mRNA in ER/LR than in the IA/ER state. The increase of the GFAP positive cells displayed by figure 5 correlates with the astrocytic network growing more intense and more branched. There is still PLP mRNA expression detectable occurring on a light pink background leading to the presumption that there is already some PLP protein rebuilt whereas PLP mRNA and therefore remyelination still is ongoing. Finally the shadow plaque shows a dense network of astrocytes in figure 4 q1 (GFAP staining, SP) with only the counterstaining detectable and the maximum cell count of GFAP positive cells in our sample. This is also reflected in the diagram shown in figure 5. However in the *in situ* hybridization staining, there is still ongoing remyelination detectable to the same extent as seen in the NAWM, most likely attributable to some kind of myelin maintenance. The idea of the glial scar acting as a barrier restricting the entry of OPCs is therefore not supported in our sample.

4.1 Summary and conclusion

In this work we took a closer look at the question, whether astroglial reaction actually does impede remyelination, as it is commonly assumed given by the hard macroscopic appearance of glial scars on human MS brain autopsy material. To address this question we used the EAE-MOG animal model in the DA rat because of the close resemblance in histology of the lesions to multiple sclerosis. To get a broad view, we gained tissue from all different lesion types. After standard histological lesion classification, as described in the material and methods section, we investigated both the remyelinating activity of each lesion (PLP mRNA) side by side to the astroglial reaction (GFAP staining) and a standard myelin staining (LFB) in consecutive serial sections.

In order to investigate astrocyte behaviour we performed immunohistochemistry with an anti-GFAP-antibody, a marker for (reactive) astrocytes. In actively demyelinating lesions, the number of GFAP positive astrocytes was on average slightly lower than those found in NAWM. With disease duration and lesion evolution towards plaque repair, the number of GFAP positive astrocytes increased and a dense network of astrocytic processes appeared (figure 4 and 5). The amount of GFAP positive astrocytes increased during lesion

evolution and was clearly highest in late remyelinating lesions and surprisingly reached the maximum in the shadow plaque, which in the latter case per definition meets the criteria of a glial scar (figure 4 and 5).

We then performed in situ hybridization on adjacent slides with a PLP mRNA probe, double-stained immunohistochemically against PLP in order to detect ongoing remyelination. In all lesion types except the active lesion, PLP mRNA could be detected – the most in inactive lesions and in early remyelination. We could also detect PLP mRNA in shadow plaques, with equal levels as in the NAWM. Most interestingly, we found that shadow plaques, the hallmark of a successfully completed remyelination (and the aim of any potential treatment in existing demyelinating disease), at the same time exhibits all histological features of the dreaded glial scar, a dense network of GFAP positive astrocytes (figure 4 and 6). These results lead us to the conclusion, that at least in this animal model the appearance of a glial scar does not act as a barrier for myelinating OPC to enter the area, nor does it impede remyelination and plaque repair. In order to elucidate this finding in more detail, of course further research is required.

4.1.1 Outlook

Recent research, mostly from *in vitro* studies, has shown that astrocytes can produce neurotrophic factors such as BDNF (brain derived neurotrophic factor) and NGF (growth factor) to promote regeneration and development of neurons (Nair, 2008) and furthermore CNTF (ciliary neurotrophic factor), which acts stimulating on oligodendrocyte maturation (Stankoff, 2002). Other factors secreted by astrocytes such as FGF (fibroblast growth factor) can help OPCs to survive in an inflammatory region in order to differentiate to remyelinating oligodendrocytes (Gomez-Pinilla, 1995). At the same time, other recent studies have confirmed the remyelination inhibiting and inflammation promoting role of astrocytes in MS. For example they can express Vascular Cell Adhesion Molecule-1 (VCAM-1) (Gimenez, 2004), matrix metalloproteinases (MMP) (Pagenstecher, 1998) and a multitude of chemokines such as MCP-1 (Monocyte Chemoattractant Protein-1), CX3CL1 and CXCL12 (Ransohoff,

1993; Berman, 1996; McManus, 1998), thereby promoting the inflammation process and impeding remyelination. This might be explained by the fact that, as in all biological systems, astrocytes can take over different roles depending on their activation state and environment. This is further complicated because in human MS-disease demyelination and remyelination can coexist in close vicinity most likely explaining the seemingly contradictory findings in the literature. Still astrocytes seem to be a promising target for future therapeutic interventions.

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List of abbreviations

APAAP alkaline phosphatase anti alkaline phosphatase

BCIP 5-bromo-4-chloro-3-indolyl-phosphate

CNS central nervous system

DA Dark Agouti, a rat strain commonly used for immunological experiments

DEPC Diethylpyrocarbonate

EDTA ethylene-diamine-tetra-acetic acid

EAE experimental allergic (autoimmune) encephalomyelitis

HE Hematoxylin Eosin, a common staining method

IHC immunohistochemistry

L liter

MBP myelin basic protein

min minutes

MOG Myelin Oligodendrocyte Glycoprotein

MOG-EAE experimental autoimmune encephalomyelitis induced by myelin-oligodendrocyte glycoprotein.

MRI magnetic resonance imaging

MS Multiple Sclerosis

NAWM normal appearing white matter

NBT 4-nitro blue tetrazolium chloride

OGP oligodendrocyte progenitors

OPC oligodendrocyte precursor cells

PBS phosphate puffered saline

PFA Paraformaldehyde

PLP proteolipid protein

PLP mRNA ISH PLP mRNA *in situ* hybridisation

PLP-ISH/PLP-IHC PLP mRNA *in situ* hybridization double-stained with PLP immunohistochemically

PNS Peripheral nervous system

SDS Sodium dodecyl sulfate

TBS Tris buffered salin