Biomarkers for Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis: Possible mechanisms mediating neurodegeneration

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To my loving parents

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Chapter 1

Introduction

1.1 Objective and rationale

The main goal of the thesis is the identification of protein markers of human multiple sclerosis (MS), an auto-immune disorder of the central nervous system (CNS), in order to gain a better understanding of its molecular pathogenesis.

MS is a chronic inflammatory, demyelinating disorder of CNS white matter believed to originate from an autoimmune response directed against one or several antigens of cerebral white matter. The main clinical symptoms are neurological disabilities such as paralysis, sensory disturbances, lack of coordination and visual impairment. The disease has a largely heterogeneous pathology among patients, which contributes to the complexity of MS (Lucchinetti et al., 2000). MS is characterized pathologically by disseminated white matter lesions with perivascular inflammation and demyelination.

Extensive studies have been carried out on the pathogenesis of MS using animal models, which have led to an increased understanding of the pathogenic mechanism. The most widely used MS autoimmunity model, experimental autoimmune encephalomyelitis (EAE), recapitulates many features of human MS. EAE is induced in animals (rodents and primates) by immunization with myelin antigens or epitopes. Different EAE models facilitate investigation of various pathogenic aspects of autoimmune neuroinflammation, which may have relevance for its human counterpart (Hohlfeld, 1997; Steinman, 1999).

MS is largely inferred to and treated as a neuro-inflammatory disease (Bielekova and Martin, 2004; Kieseier et al., 2005; Killestein and Polman, 2005). The pathogenesis of the disease is believed to originate from the activation of autoreactive T-cells, directed against myelin antigens, which enter the CNS (Stein-

man, 2001a; Hohlfeld, 1997). The causes that trigger the activation of T-cells remain elusive. Studies showing axon loss (Trapp et al., 1998) and neuronal atrophy (Waxman, 2000b) indicate the neurodegenerative phase of the disease. The pathological process that correlates highly with the permanent physical disability is axonal and neuronal loss, which already commences in the very early stages of the disease (Trapp et al., 1999; Kornek et al., 2000). Therefore high emphasis and attention have also to be given to the neurodegenerative mechanisms taking place in MS and the molecules that mediate these processes need to be discovered and addressed. MS has to be understood and studied as a complex disease with contributions from multiple disease processes.

Despite the profound understanding of the different aspects of the pathogenesis of MS, effective therapeutic intervention and early diagnostic measures are still very inadequate (Murray, 2006). Moreover, the majority of therapeutic agents used in clinical trials are directed against the immunological features of the disease, leaving the process that leads to irreversible CNS dysfunction in the patients untreated (Bielekova and Martin, 2004). This shortcoming can be partially ascribed to the oversimplification of MS as an inflammatory, demyelinating disease and employment of conventional concept-driven approaches, which deal with the characteristics of one or several molecular components at a time (Robinson et al., 2003b). Hence, in order to decipher the composite molecular mechanisms that contribute to development and progression of complex diseases like MS, unbiased approaches that allow large scale monitoring of molecular level changes of gene and gene products need to be adapted. In other words, the simplified views of disease pathways should be revised and global approaches to understand the system, rather than just components, have to be implemented (Robinson et al., 2003b; Ibrahim and Gold, 2005).

Several studies using different strategies have reported large-scale transcript analyses of human MS lesions (Lock et al., 2002; Dutta et al., 2006) and the mouse model EAE (Ibrahim et al., 2001; Mix et al., 2002; Nicot et al., 2003), enabling the identification of a number of transcripts unique to MS or EAE. Protein microarrays have also been developed for the large-scale identification of autoantibodies in MS and EAE (Robinson et al., 2003a). All large scale analysis studies reflect the polygenicity and complexity of the pathological response in MS (Ibrahim and Gold, 2005). They have unfolded the involvement of unexpected candidates and pathways, (unlikely to have a role in autoimmunity), in the pathogenesis of MS, such as the role of a protein called osteopontin, lipid metabolism, allergy-associated genes etc (Chabas et al., 2001; Steinman et al., 2002; Steinman and Zamvil, 2003).

However, a purely gene-based expression pattern is not sufficient for the dis-

section of a disease phenotype. It is evident that there is no tight temporal correlation between mRNA and protein expression levels (Gygi et al., 1999). Moreover, the functional properties of many proteins are regulated by post-translational modifications (PTMs) and alternative splicing, which cannot be detected by transcript analysis. The analysis of PTMs is of high significance in studies aimed at the discovery of biomarkers relevant in the pathogenesis of diseases.

Proteome is the complete set of gene products expressed in a given organism at a given time point. Current proteomic techniques allow the detection and comprehensive analysis of several hundred to thousand proteins expressed in a system at a physiological state, and are capable of differential analysis of a normal versus altered (diseased) state. Furthermore, proteomic methods are suitable for the detection and identification of PTMs. Proteomic analysis can, therefore, supplement the above-mentioned large scale approaches in identifying and characterizing molecules relevant to the pathogenesis of MS. To this date, only a few studies have reported proteomic analysis of EAE (Duzhak et al., 2003; Alt et al., 2005; Ibrahim and Gold, 2005) and MS samples (Hammack et al., 2004; Dumont et al., 2004; Noben et al., 2006).

Our approach was to use mouse EAE brain and spinal cord tissue as the primary source of specimen in the disease marker discovery phase of MS. We examined the proteomes of the different models of EAE in early, middle and full-blown disease stages. Proteomic screening was extended to human post-mortem MS brain tissue (post-mortem).

For the proteomic analysis we applied classical two dimensional gel electrophoresis (2DE) for protein separation and mass spectrometry for the identification of protein expression differences. 2DE is ideally suited to resolve complex protein mixtures for the comparative analysis of two or more sample groups (Liebler, 2002). Moreover, one of the greatest strengths of 2DE analysis is the isolation and detection of different isoforms and PTMs of the same protein. Acknowledging the role of phosphorylation in determining the activity and molecular interaction of many proteins we looked specifically at this PTM. Hence we compared both, the whole proteome, as well as the phosphoproteome, of control vs. diseased CNS.

Animal models provide the best source of biological material in the biomarker discovery phase due to their homogeneous background in contrast to human samples. Hence, animal models are used for the thorough and reproducible proteome analysis of the different forms and stages of the disease. The subsequent extension to human samples enhances confidence in the results.

Tissue level analysis as the first step gives access to the primary mediators of

a given physiological state. The goal is to discover protein regulation or modification unique to EAE and /or MS, and then to understand the pathobiology and disease process specificity of these gene products. The functional and biological roles of the protein markers are studied by extensive database and literature searches in order to select a set of markers that can elucidate molecular mechanisms that contribute to the deleterious clinical outcome of MS, and to establish interactions between those that bring about the disease process. Functionally important markers are validated by immunoblotting. Clinical translation and evaluation of the identified biomarkers in different patient populations will enable the identification of these markers as a signature of the disease process in subsets of patients. Uncovering the wide range of protein alterations in disease states holds much promise for elucidating the disease pathogenesis, and for developing novel diagnostics and therapeutics. The new molecular markers can also serve as surrogate endpoints for monitoring therapeutic efficacy.

1.2 Multiple Sclerosis

Multiple sclerosis is understood as a chronic inflammatory, demyelinating disorder of the CNS white matter. MS is believed to originate from an autoimmune response directed against neural antigens. Main pathological features of the disease are disseminated white matter lesions with perivascular inflammation and demyelination in the brain and spinal cord. Prominent clinical manifestations include functional disabilities such as sensory disturbances, lack of coordination and visual impairment and paralysis.

MS is classified into different types according to its clinical course. The disease is often characterized (almost 80% of the patients) by relapsing episodes of neurological impairment followed by remissions (relapse remitting), which lead to a secondary chronic stage of the disease, where remissions eventually are non-existent (secondary progressive). Another type of the disease seen in 10% to 20% of the patients begins with a slow progression of neurological deterioration worsening over a period of time (primary progressive).

1.2.1 Pathogenesis of MS

Immune-mediated pathogenesis

MS is broadly regarded as a Th1-cell mediated disease characterized by an inflammatory and an eventual degenerative phase (reviewed in Wekerle, 1998;

Steinman, 2001a; Steinman et al., 2002). The pathogenesis of the disease is understood to originate from the activation of autoreactive T-cells in the systemic compartment, which access the CNS. The inflammatory response subsequent to the T-cell activation, leads to the release of cytokines, adhesion molecules and MHC class II molecules, enabling the entry of lymphocytes into the CNS. In the CNS the immune response is targeted to the myelin components. Activated T-cells that have entered the CNS get presented with neural antigen by antigen presenting cells (APCs). This leads to the recruitment of naive T-cells in an antigen independent manner at the site of inflammation, accounting for the vast majority of infiltrating cells. These cells are activated *in situ* upon recognition of self antigens released during the initial tissue damage. This might result in epitope spreading and perpetuation of inflammatory processes (Elson et al., 1995; Miller et al., 2001; Lehmann et al., 1992). Activated macrophages, microglia and astrocytes express inflammatory mediators like nitric oxide leading to the phagocytosis and destruction of the myelin sheath. However, it is evident from various studies that a purely T-cell dominated brain inflammation does not lead to major demyelination, (Lassmann, 1998). Additional demyelination augmentation factors are required to propagate myelin loss (Lucchinetti et al., 2000). It is known from various animal models that auto-reactive T-cells specific for CNS autoantigens initiate the inflammatory response and open the blood brain barrier (BBB), where a B-cell response with autoantibodies against these antigens is required to induce demyelination (Hohlfeld, 1997). Interestingly, animal studies show that although autoantibodies to myelin antigens can contribute substantially to the extent of tissue injury they cannot initiate the disease (Aboul-Enein et al., 2004). Disease relevant antibodies have been found in sub-types of MS lesions and also in MS cerebrospinal cord (CSF), (Storch et al., 1998; Reindl et al., 1999; Lennon et al., 2004). Antibody bound specifically via the Fab region to neural cells can interact via the Fc region to Fc bearing cells, namely microglia/macrophages, and direct the potential injury mediators produced by microglia toward a specific target.

Several lines of evidence from EAE and MS support the view of MS as a Th1-mediated autoimmune disease, initiated by MHC class II restricted CD4+ T-cells, which produce proinflammatory cytokines such as interferon gamma (IFNγ), interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α) and lymphotoxin. However, the cellular composition in MS lesions suggest that CD8+ T-cells outnumber the CD4+ T-cells in all stages of lesion development, and clonal expansion within MS plaques is more prominent in the CD8 population (Babbe et al., 2000; Steinman, 2001b; McCallum et al., 1987; Lassmann and Ransohoff, 2004). Moreover, the number of CD8+ cells correlates better with the extent of acute axonal injury than the number of CD4+ cells (Bitsch et al., 1998). The

feature that neurons express MHC class I molecules, (and not MHC class II), under inflammatory and stress conditions denotes the role of CD8+ T-cells in the injury process (Zang et al., 2004). Moreover, the involvement of Th2-mediated cell response has also been implicated in the pathogenesis of MS (Lafaille et al., 1997; Lassmann and Ransohoff, 2004; Pedotti et al., 2003, for review).

Immunopathological characterization of MS reveals heterogeneity in the pathophysiological mechanisms in patients with MS suggesting diverse causes and molecular mechanisms that lead to demyelination in patient subpopulations. The heterogeneous patterns of demyelination are not distinctly related to a specific clinical presentation in MS patients. The factors that amplify demyelination can be either an antibody-mediated destruction of surface components of myelin or macrophage toxins, or an impaired metabolism of myelin-supporting oligodendrocytes. (Lucchinetti et al., 2000; Kornek and Lassmann, 2003).

Cause

The trigger of the immune response directed against myelin components is being investigated and considered. The most assumed concept is that initial immune sensitization in MS results from exposure to exogenous agents through a phenomenon called molecular mimicry (Zhou and Hemmer, 2004; Wucherpfennig et al., 1997; Wucherpfennig, 2001). Peptide library-based studies indicate that myelin reactivity in many cases actually reflects cross-reactivity with exogenous peptides. Many microbial protein sequences share homologies with structures found on the myelin sheath and can lead to an attack on myelin via molecular mimicry. Indeed, relapses in MS are often triggered by common viral infections. Many viruses have genes encoding sequences that mimic those found in the major structural proteins of myelin (Steinman, 2001a). Increased frequency of CD4+ myelin reactive cells in the peripheral blood of MS patients compared to healthy donors seems to be derived from memory T-cells, implicating prior sensitization with disease relevant antigens (Wulff et al., 2003), possibly during a viral infection.

Neural-mediated disease hypothesis

Although it is commonly accepted that an autoimmune response initiated in the periphery is the central event of MS that results in demyelination and neurodegeneration (immune-initiated disease hypothesis), a possibility that the neuroinflammatory reactions are in response to primary events impacting on the neural cells, (neural-mediated disease hypothesis), is being emphasized lately (Prat and

Antel, 2005; Trapp, 2004). An acquired acute or persistent infection of neural cells can lead to the release of neural antigens, which can provoke a disease relevant autoimmune response. One of the mechanisms by which these acquired infections could impact on MS is molecular mimicry responses. It has also been observed that changes in oligodendrocytes, like apoptosis, can be the initial event in the formation of an acute MS lesion, serving to recruit an initial innate (microglia) and subsequently adaptive (T-cell) immune response, (Barnett and Prineas, 2004). The basis of apoptosis could be primary injury due to a viral infection, or insults such as trauma and ischemia.

Axonal loss

Classically, functional deficits in MS and EAE were regarded as the consequence of demyelination and the resulting impairment in salutatory conduction (Raine, 1997; McFarlin and McFarland, 1982). However, increasing evidences have proved little or no correlation between demyelination and symptom severity, suggesting the involvement of more complex factors and mechanisms that contribute to the irreversible functional deficit in MS and EAE (Antel, 1999). Supporting this, studies reporting axonal damage and dysfunction (Ferguson et al., 1997; Trapp et al., 1998, 1999; DeStefano et al., 1999; Bitsch et al., 2000; Bjartmar and Trapp, 2001; Bjartmar et al., 2001), and neuronal loss or apoptosis (Smith et al., 2000; Peterson et al., 2001), indicate that MS and EAE are not merely demyelinating diseases but neuronal disorders. (Waxman, 2000a,b; Filippi, 2001). Recent works have provided mounting evidence of axonal loss and atrophy in MS lesions accounting for the permanent loss of function (see above). Correlation between axonal loss and neurological disability has also been established in EAE (Wujek et al., 2002). Owing to their late entry into the focus of investigation, the molecular events underlying neuronal impairment that contribute to neurological deficits and permanent disability in EAE and MS remain obscure, in contrast to the well-defined histopathology. A report by Kornek et al (Kornek et al., 2000), highlights the course and advancement of axonal injury in MS and EAE in different stages of the disease, and in different kinds of lesions. Acute axonal injury occurs at a very high incidence during the active demyelination phase, at a very early time point of lesion formation, the extent of which is variable between individuals. Axonal injury in MS is not restricted to demyelinated lesions, rather it appears dispersed in the periplaque (more prominent) area, as well as in the normal appearing white matter (NAWM) areas distant from the established demyelinated plaques. These changes taking place around the plaques are likely to be secondary to the ongoing active demyelination. A direct effect of inflammation taking place throughout the CNS independently of the plaques

cannot be excluded. The presence of low burning axonal damage when no active lesion can be detected, might represent the clinical progression of the disease in the absence of MRI-activity. The mechanism of axonal injury in the absence of active demyelination can be attributed either to the inflammatory process or to the lack of trophic support provided by myelin and oligodendrocytes. This persistent axonal destruction may explain the observed profound reduction of axonal density in established MS plaques (these points are inferred from the above-mentioned publication from Kornet et al.).

1.2.2 Clinical diagnosis of MS

Multiple Sclerosis is an extremely variable disease with a range and variety of symptoms. There is no general pattern for the course of MS among patients. The symptoms vary and change in severity and duration over a period of time. The symptoms are usually combinations of various sensory and motor disturbances, as well as cognitive dysfunctions due to the impairment of different functional systems in the CNS. The high diversity in the clinical manifestation of MS contributes to the difficulty in narrowing down a diagnosis of MS from other possibilities. The guidelines for diagnosis of MS have evolved over the years to include new testing techniques, such as the MRI, for earlier and more accurate diagnosis.

The first set of diagnostic criteria for MS were the Schumacher criteria, developed in 1965. The Schumacher criteria relied largely on a neurological examination by the doctor and symptom history. If a sufficient number of abnormalities in the CNS were detected, and these abnormalities and symptoms were recurring or progressing over a period of time, and also indicated that more than one area of the CNS was involved, then a diagnosis of MS could be put forward (Schumacher et al., 1965). Since the early 1980s the Poser criteria were used to diagnose and classify MS. This relied on evidence of at least two relapses typical of MS, and the evidence of involvement of white matter in more than one site in the CNS, the concept of ”lesions disseminated in time and space” (Poser et al., 1983).

A new system of classification called the McDonald criteria was established in 2001 (McDonald et al., 2001). This new system gave the MRI scan a much larger role in MS detection, supplemented with visual evoked potentials (VEP) testing and CSF testing via lumbar puncture. CSF abnormality is defined as the presence of oligoclonal IgG bands different from any such bands in serum, and/or the presence of an elevated IgG index (Link and Tibbling, 1977; Andersson et al., 1994). Abnormal IgG bands are detected using isoelectric focussing.

CSF analysis provides supportive evidence of the immune and inflammatory nature of a lesion in cases where the imaging could not specifically detect MS-like lesions. Abnormal VEP, typical of MS, with a delayed but full wave form (Halliday, 1993) provide objective evidence of an additional lesion that affects the visual pathways given that the only clinically expressed lesion did not show involvement of visual areas. Thus, the McDonlad criteria incorporate clinical and laboratory elements in the diagnosis process, allowing an earlier confirmation of the diagnosis and earlier decisions about commencing disease-modifying therapies. The McDonald criteria also incorporate MRI to demonstrate the multiple areas of involvement and the appearance of new enhancing lesions over a period of time. It emphasizes the role of additional evidence from VEP and CSF analysis in supplementing the determination of MS diagnosis.

It is evident from the above criteria that the diagnosis of MS cannot be determined directly after the onset of the first symptoms because of the nature of its clinical presentation. The McDonald criteria allow a relatively early determination of MS, at least in patients with a relapse remitting course of the disease. However, when patients develop the first symptoms the disease has been present for a long time, and they may have evidence of old lesions on MRI and some atrophy of the brain. The diagnosis is especially difficult in patients with an insidious beginning, which later on takes a progressive form of the disease. Evidence from several sources suggest that the disease is present long before the onset of the first symptom (Murray, 2006). This is one of the major hindrances in treating the early factors that account for the progression of the disease resulting in neurological deficit. Therefore, there is a compelling need to develop very early diagnostic measures to detect MS preferably in the body fluids of patients.

1.2.3 Treatment of MS

Studies aimed at the development of therapeutic strategies for MS have been dominated by a focus on the immune phase of the disease. This is reflected in the treatments that are currently in clinical practice and trial. A vast majority of the available drugs are targeted against the inflammatory component of the disease (Killestein and Polman, 2005). Treatment is for acute attacks, prevention of relapses and progression, management of symptoms, and rehabilitation. In recent years, advances have been made in all four areas (Murray, 2006). In the case of acute attacks patients are given corticosteroids (eg. methylprednisolone) to moderate the inflammatory reaction (Morrow et al., 2004). Commonly used immunomodulatory drugs include interferon beta-1b (Betaseron); interferon beta-1a (Avonex) (Giovannoni and Goodman, 2005), and glatiramer acetate (Copax-

one) (Wolinsky, 2006; Neuhaus et al., 2000; Zeimssen et al., 2002). Symptomatic treatments include steroid treatments, antispastic agents, therapies to treat bladder and bowel dysfunctions, fatigue etc.

Recent understanding of the pathogenesis of MS using biased and unbiased methods have led to the identification of a number of other therapeutic targets, (reviewed in Kieseier et al., 2005; Zamvil and Steinman, 2003). There are around 70 drugs that are in phase I and phase II trials of drug development. The most exciting of these are Antergen and Statins. Antergen (Natalizumab) inhibits adhesion molecules that enable T-cell migration across the BBB (Miller et al., 2003; von Andrian and Engelhardt, 2003). Statin is an anti-cholesterol drug that inhibits HMG CoA reductase that is evolving as a promising treatment for MS (Stuve et al., 2003; Nath et al., 2004). One of the hypotheses put forward to explain the anti-inflammatory and neuroprotective effects of statins is the reduction of the expression of major histocompatibility (MHC) Class II molecules that present neural antigens to T-cells (Youssef et al., 2002; Wekerle, 2002). Minocycline is a compound that deactivates microglial cells, thereby reducing free radical production and attenuating neuronal apoptosis (Metz et al., 2004). It had a positive effect in many neurological diseases and is currently being tested for MS (Yong et al., 2004). Other promising therapeutic agents from animal studies are Neuregulin (glial growth factor 2) and remyelinating antibodies aimed at initiating the repair process. Immunosuppressive therapy using vaccines is another approach under investigation. The new treatments have shown promise in the short term recovery but how much or if any long term disability is prevented is not clear. However, increasing efforts are being undertaken to promote remyelination, axonal regeneration, to preserve axonal and neuronal integrity and to achieve repair of tissue damage and regain of function. Advances in therapeutic intervention, aimed at preventing or recovering from long term disability, depends largely on the thorough understanding of the molecular pathogenesis of MS.

1.3 Experimental autoimmune encephalomyelitis : Animal model for MS

The development of animal models of demyelinating diseases was attempted and accomplished in the early 1930s (Rivers and Schwentker, 1935; Rivers, 1933). The basis for the development of those models was the observation that rabies immunization, using attenuated rabies virus grown in brain tissue, leads to clinical paralysis. The injection of brain material with complete freund's adjuvant

(CFA) (an emulsion made from killed mycobacteria tuberculosis that can initiate a vigorous immune response) permitted the development of highly reproducible models of acute paralysis and white matter inflammation in monkeys (Rivers, 1933). This study established animal models of nervous system autoimmunity termed experimental allergic encephalomyelitis. Later the name was interchanged with experimental autoimmune encephalomyelitis. Thereafter, EAE was adapted in rodent species and its clinical and histopathological similarity to MS was demonstrated (Tsunoda and Fujinami, 1996; Alvord, 1984; Swanborg, 1995).

The current protocols initiate EAE in susceptible animals by systemic immunization with neural autoantigens or by the transfer of neural antigen-sensitized T-cells. Several antigens, myelin-specific and non-myelin specific, are used to induce EAE in animals, which include myelin basic protein (MBP), myelin associated glycoprotein (MAG), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and S100. Most commonly EAE is induced in rats and mice. The various EAE models show marked heterogeneity with regard to the topography and the extent of demyelination and axonal disruption, depending mainly on the immunizing agents and the genetic characteristics of the animals. These variations facilitate the investigations of the various pathogenic aspects of autoimmune neuroinflammation, which may have relevance for its human counterpart (Hohlfeld, 1997; Lassmann and Ransohoff, 2004; Steinman, 1999). The disease can be monophasic, involving an acute paralytic episode that is followed by complete recovery, relapsing-remitting, which involves multiple cycles of attack interspersed by full or partial recovery, or chronic, where disease symptoms of the initial attack either stabilize at peak levels or gradually worsen over a period of time. The dominant early clinical symptom of acute autoimmune encephalomyelitis is progressive ascending muscle weakness (Wekerle et al., 1994). The clinical symptoms of EAE are assessed by a disease score scale ranging from 0-5, starting from tail impairment and advancing to complete paralysis and death.

EAE recapitulates many features of MS with respect to the clinical presentation, genetics and pathology. EAE is regarded as a Th1 MHC class II restricted CD4+ T-cell mediated disease of the CNS (Behi et al., 2005). Both MS and EAE have a similar composition of inflammatory lesions, mainly T lymphocytes and activated macrophages, and a similar pattern of chemokine expression consistent with Th1-driven inflammatory process (Lassmann, 1983; Huang, 2000). The migration of the activated T-cells through the BBB is the origin of the inflammation in the brain in EAE and MS (according to the immune-mediated hypothesis) (Wekerle, 1998). EAE reproduces Pattern 1 and 2 of MS pathogenesis in which

macrophage-released toxins such as TNF-α and reactive oxygen species (pattern 1), or antibodies and complement (pattern ll), specifically mediate demyelination (Kornek and Lassmann, 2003).

Myelin oligodendrocyte glycoprotein-induced EAE

MOG is a CNS-specific myelin transmembrane protein expressed on the outermost surface of the myelin sheath with a single extracellular Ig-like domain (Johns et al., 1995). Compared to other myelin proteins such as PLP, MOG is a minor component of myelin (Brunner et al., 1989). MOG is found only in mammals and is highly conserved across species. It is expressed exclusively in the CNS by myelin-forming oligodendrocytes. Its location at the outermost surface of the myelin sheath (Brunner et al., 1989) makes MOG an ideal target for autoantibodies in the extracellular milieu (Breithaupt et al., 2003). MOG was first identified as a target autoantigen for antibody-mediated demyelination in a guinea pig model of EAE (Lebar et al., 1986). Consequently, there was a shift in the focus from the role of T-cell and inflammatory mediators, as a cause of demyelination, towards the role of antibody response. This was followed by the development of MOG-induced models of EAE (MOG-EAE) where demyelination is mediated by MOG-specific autoantibodies (Johns et al., 1995; Adelmann et al., 1995; Bernard et al., 1997).

There are two distinct effector mechanisms that mediate demyelination in MOG-EAE with counterparts in MS pathogenesis (Lucchinetti et al., 2000; Kornek and Lassmann, 2003). The choice of the effector mechanism is species-dependent. While demyelination is strictly antibody-mediated in rats (Adelmann et al., 1995) both, demyelinating autoantibody response and encephalitogenic T-cell response, with TNF activation contribute to demyelination in mouse models. Intriguingly, MOG is the only antigen that can induce both a pathogenic demyelinating autoantibody response, and an encephalitogenic T-cell response in experimental animals (Iglesias et al., 2001).

The pathogenesis of MOG-induced EAE can be best explained as an antibody-mediated, but T-cell dependent process (Iglesias et al., 2001). The T-cell response plays a critical role in the initiation of the disease. Genetic studies have demonstrated that the T-cell response is largely controlled by genes within the MHC (Weissert et al., 1998; Stefferl et al., 1999). The induction of an MHC class II restricted, CD4+ T-cell response against MOG commences CNS inflammation and damages the BBB. This permits the MOG immunoglobulin-like domain (Igd) -specific antibodies to access the CNS. Besides this, the T-cell response recruits and activates phagocytes (macrophages and microglia) within the

CNS, promoting immune-mediated demyelination (Vass and Lassmann, 1990). The demyelinating activity of MOG-specific mAbs is related to their ability to activate complement (Piddlesden et al., 1993). The formation of membrane attack complex (MAC) on the myelin/oligodendrocyte surface may lead directly to the lysis of oligodendrocytes and the disruption of myelin. Moreover, the disruption in calcium homeostasis, due to increased calcium influx, activates myelin-associated proteases and destabilizes myelin (Scolding et al., 1989; Menon et al., 1997). Complement activation also generates pro-inflammatory factors that further enhance the local inflammatory response, delay BBB repair and activate phagocytes within the lesions (Linington et al., 1989; Piddlesden et al., 1993).

The TNF-dependent effector mechanism in the absence of an antibody response is observed in murine models of MOG EAE and is ineffective in the rat and marmoset. (Hjelmstrom et al., 1998; Iglesias et al., 2001; Eugster et al., 1998). N-terminal Ig domain (MOG Igd)- induced EAE in SL/J mice is characterized by large cellular infiltrates reaching deep into the parenchyma. These areas are associated with large demyelination and extensive axonal damage. Passive MOG EAE, (induced by adoptive transfer of MOG epitope specific T-cells) (Iglesias et al., 2001), is associated with extensive demyelination and axonal loss.

In MOG-induced EAE this combination of immune effector mechanisms reproduces the demyelinating pathology seen in the majority of patients with MS. The clinical relevance of autoimmune responses to MOG in MS is supported by the reports showing that MS is associated with enhanced MOG specific T and B cell responses, and by the identification of MOG-specific antibodies associated with myelin debris in actively demyelinating MS lesions (Genain et al., 1999). Moreover, MOG is encoded within the distal region of MHC that is orthologous to the HLA gene loci associated with MS (Lambracht et al., 1995). Similar to MS, susceptibility to EAE in mice is linked to MHC loci (Steinman, 1999).

Proteolipid protein-induced EAE

PLP, one of the abundant protein constituents of myelin, has been recognized as a major CNS-specific encephalitogen. Immunization with discrete peptide determinants of PLP induce CD4+ T-cell mediated development of acute, chronic relapsing, and chronic progressive EAE. Relevance of PLP mediated autoimmune response in MS was demonstrated by the accumulation of activated PLP-specific T-cells in the CNS of MS patients. Moreover it has been shown that T-cell reactivity to multiple PLP determinants occurs in patients with MS (Tuohy, 1994; Stinissen et al., 1997; McRae et al., 1995).

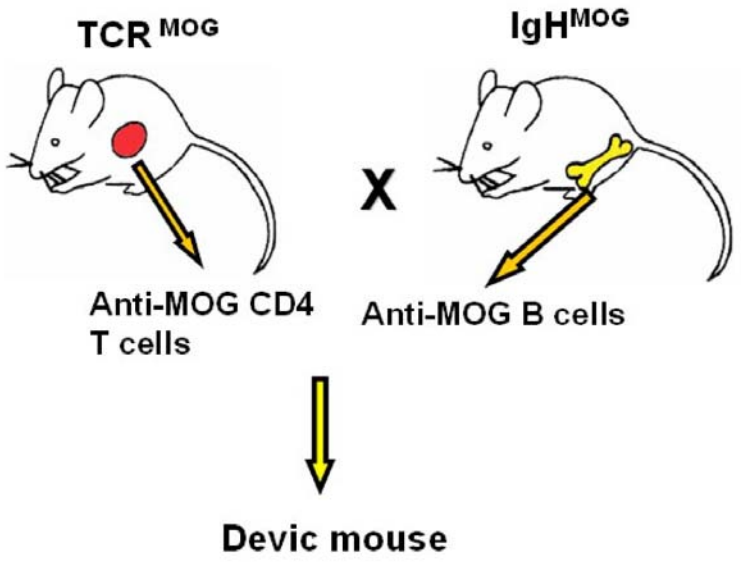

Figure 1.1: A double transgenic animal model (Devic's mice) of CNS autoimmunity, containing MOG specific T-cells and B-cells

Devic's EAE - Transgenic model of EAE

F1 double transgenic mice, generated from interbreeding T-cell receptor (TCR) transgenic mice containing MOG 35-55 specific CD4+ T-cells and IgH^{MOG} mice which have MOG specific B-cells, develop a spontaneous EAE-like disease (Krishnamoorthy et al., 2006), (Figure 1.1). The double transgenic animals (IgH^{MOG} X TCR^{MOG}) contain both MOG specific CD4+ T and B-cells. According to the authors more than 50% of the double transgenic mice develop spontaneous EAE while all the single transgenic littermates remain disease free.

What triggers the initiation of spontaneous EAE is not well understood. The (re-) activation of lymphocytes in sick double transgenic mice mainly occurs in the CNS without evidence of priming in the peripheral lymphoid organs. The presence of both MOG specific T and B cells in a MOG competent background is essential for spontaneous EAE.

The CNS lesions in sick mice resemble the human MS variant, human opticospinal MS or Devic's neuromyelitis optica. Thus the IgH^{MOG} X TCR^{MOG} are called Devic's mice. The pathological lesions in these mice are exclusively present in the optic nerves and spinal cord whereas the brain regions are completely spared from any inflammation or tissue damage, a pattern similar to Devic's neuromyelitis optica. The CNS lesions of sick Devic's mice contain mas-

sive numbers of the inflammatory cells, composed of macrophages and CD4+ T-cells. Profound demyelination and axonal damage coincide with the infiltration of inflammatory cells. Occasionally a few B-cells or CD8+ T-cells are present in the lesions. In some cases, significant numbers of eosinophils are also present in the actively demyelinating lesions. The healthy Devic's mice, on the other hand, do not develop any appreciable CNS lesions except for sparse inflammatory infiltrates around perivascular areas. Devic's mice have several salient features to study the pathogenic mechanisms of CNS autoimmunity.

1.4 Proteomics

The terms proteome and proteomics were coined in 1990 by Marc Wilkins and colleagues (Wilkins et al., 1996b).

Proteome: Total protein complement of the genome.

Proteomics: The comprehensive analysis of the protein complement of the genome of an organism or the complete set of proteins that is expressed functionally at a given time or physiological state.

Proteomics deals with the identification of proteins, their cellular localization and PTMs. It gives information on protein structure, protein-protein interactions, activities and functions of proteins, as well as protein dynamics and quantification. It is a study of a multi-protein system, where interplay of multiple distinct proteins, in their role as part of a network, is the focus rather than a single component (Liebler, 2002). So do genomics and transcriptomics, so why do we need proteomics?

There are many reasons and justifications why protein level studies are important (Wilkins et al., 1996a; Pandey and Mann, 2000).

1. Proteins define the functional state of an organism and what is happening at a cell at a given state.

2. Proteins, not genes, determine the complexity of an organism. PTMs of proteins greatly increase protein complexity and dynamics, coordinating the intricate regulation of biological events.

3. mRNA expression levels do not necessarily correlate with the levels of proteins in a cell. mRNA stability and translation efficiency varies (Gygi et al., 1999).

4. Proteins are the targets of most drug therapies.

5. Proteomics-based profiling uniquely allows delineation of global changes in protein expression patterns resulting from transcriptional and post-transcriptional control, PTMs and shifts in proteins between different cellular compartments.

Proteomics as a tool

The proteome is highly complex and dynamic in nature. Naturally, characterizing the whole proteome is intricate and challenging . Proteins are expressed in a dynamic range of $10 - 10^{10}$, which poses a major challenge while analyzing the proteome. The lack of amplification possibility is yet another drawback in the protein world. Despite these restrictions, this technology has made major advances in the last decade. This tremendous progress can be attributed mainly to the development and integration of important tools like the completed genome sequences, advanced mass spectrometry, improved bioinformatics tools for developing databases of protein sequences and spectra analysis software, and improved analytical methods in protein separation. Proteomics as a technology is welcome in many fields of biological and medical sciences for a number of different applications.

The use of proteomics is currently most appreciated in its application in disease marker and drug discovery (Hanash, 2003; Wang and Hanash, 2002; Turck et al., 2005; Fountoulakis and Kossida, 2006). Gene product alterations in diseases may occur in many different ways such as PTMs, regulatory mechanisms, protease activity, chemical modifications (e.g. oxidation) by environmental factors, etc, that are not predictable from genomic analysis. Moreover, the majority of the drugs target proteins for therapeutic action, which makes proteomics the method of choice for target selection, as well as for measuring and monitoring the response and efficacy of drugs. The overwhelming technical advancement that has taken place in the last few years offers a repertoire of methods and tools for carrying out highly sensitive, precise and high throughput proteomic investigations to answer specific questions (Steel et al., 2005).

Proteomic workflow

All proteomic methods aim at the same goal: to achieve ways to accurately detect large numbers (1000s) of distinct molecular species, most of which exist in a very low abundance and in multiple modified forms. Most biological samples are a complex mixture of proteins with varying physical properties such as molecular weight (MW), charge, solubility, etc. The first step is to extract as

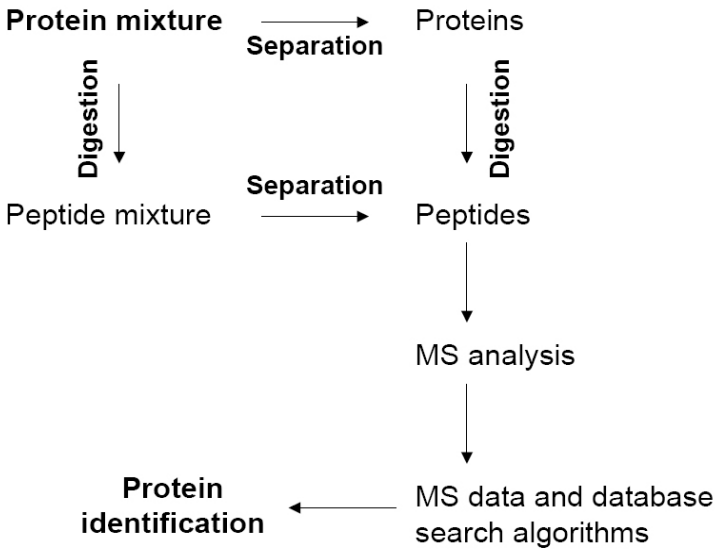

Figure 1.2: Proteomics workflow.

many proteins as possible from the biological material with limited, or no contamination of other bio-molecules, like nucleic acids or lipids. The next step is to separate the complex mixture into individual proteins or groups of proteins, making use of their diverse physical properties to reduce the complexity of the samples. The type and the number of separation methods are chosen based on the starting material and the purpose of the study. Proteins need to be digested to peptides for effective measurement by mass spectrometers and for their precise identification. Separation processes are carried out either prior to, or after the enzymatic digestion of proteins into peptides. After the separation and digestion, the peptides are applied to the mass spectrometers and identified. A schematic representation of the general proteomic workflow is given in Figure 1.2. Based on the mode of protein separation employed, there are two main proteomic processes: 1. Gel-free proteomics, 2. Gel-based proteomics. The following section describes the different procedures in detail.

Gel free proteomics

Gel-free proteomics refers to the workflow where liquid chromatography is used for protein separation. Proteins in a complex mixture are digested to peptides in the first step. The resulting peptides are separated with high performance liquid chromatography (HPLC) by exploiting the different physical properties of the peptides such as hydrophobicity, charge, etc. The diversity of the stationary

phases and the separation methods give HPLC a high resolving power. After the chromatographic separation the peptides are applied, either on-line or off-line, to a mass spectrometer for the identification of the proteins. This method is called LC-MS. The recent developments in the LC-MS process link a series of chromatographic separation modes (multidimensional chromatography) before the introduction of the peptides to the mass spectrometer (Washburn et al., 2001). This method, called the multidimensional protein identification technology (MUDPIT), has substantially increased the sensitivity and resolving power of the LC-MS process. MUDPIT spreads out peptides into more fractions and elutes more peptides to the mass spectrometer. This method also helps to reduce the suppression of the low abundant peptides.

Gel-based proteomics

Gel based proteomics is the classical proteomics method where proteins in a complex mixture are separated by 2DE, and is still in practice as one of the main techniques for comparative proteomics. For all our experiments we used 2DE for proteome separation. Principles and specification of 2DE are explained in the next section.

1.4.1 Two dimensional polyacrylamide gel eletrophoresis

2DE was developed in 1975 by O'Farrell (O'Farrell, 1975) and Klose (Klose, 1975) for resolving highly complex protein mixtures. The procedure involves resolution of proteins by two orthogonal gel electrophoresis separations. In the first dimension, called isoelectric focussing (IEF), proteins are separated according to their isoelectric point (pI). Subsequent to IEF, the focussed proteins are transferred to SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and separated according to their MW. Individual proteins are resolved as spots on the 2DE gels. The protein spots are visualized by staining the SDS gel with protein binding dyes. The protein spots are excised out for mass spectrometry-based identification. The number of individual protein spots separated on the gel is variable and largely depends on the type of sample, sample preparation, the amount of protein loaded and the staining procedure. Routinely, 2DE allows the resolution of up to a 1000 protein spots, although separation of up to 10000 protein spots has been reported (Klose, 1999).

Isoelectric focussing

Proteins are amphoteric in nature as they carry both acidic and basic groups. Isoelectric point is the pH at which a molecule (protein) has no net charge and is incapable of moving in an electric field. At a pH below their pI proteins have a net positive charge and, at a pH above their pI, they carry a net negative charge. Proteins move to the anode or cathode in an electric field depending on the pH. Consequently, when proteins are subject to an electric field in a pH gradient they can be focussed at their pI, (Figure 1.3). The development of immobilized ph gradients (IPG), using carrier ampholytes, has been a major advancement of the 2DE technique (Bjellqvist et al., 1982; Gorg et al., 1988, 2000). Carrier ampholytes are zwitterionic, low molecular weight molecules. When electric fields are applied to polyacrylamide containing a mixture of ampholyte molecule, the ampholytes migrate to their respective pI and form a stable and stationary pH gradient, without ampholyte drift at a high electric field as they are supported by the polymerized acrylamide. Length and the pH range of the strips vary and are chosen according to the purpose of the analysis. Very narrow range IPG strips are available for the separation of proteins with very similar pI with high resolution. Preparation of samples for IEF is the most important process in 2DE and often demanding as a number of factors can impede good focussing of protein, and lead to a distorted protein pattern. IPGs allow the production of a reproducible proteome pattern on 2DE gels by facilitating stable and consistent pH gradients.

SDS PAGE

After IEF, the IPG strip is transferred and joined to an SDS gel. SDS is an anionic detergent that denatures secondary and tertiary structures of proteins, and binds to proteins in proportion to their size or length. The binding of SDS imparts a net negative charge to proteins irrespective of their original charge. A polyacrylamide gel serves as a molecular sieve with pores that can fit the protein molecules. Upon application of an electric field the protein-SDS complex can migrate through the polyacrylamide matrix towards the anode. The property of this movement is proportional to the size (mass) of the protein molecules, the viscosity of the medium and the applied voltage. Since SDS imparts an approximately uniform mass:charge ratio for most proteins the distance of migration through the gel can be assumed to be directly related to the size of the protein, (Figure 1.4). Prior to the SDS PAGE, the proteins focussed on IPG strips are equilibrated with SDS and DTT to completely denature and reduce them so that the migration in SDS PAGE is not influenced by protein folding and depends only on their primary structure. Re-oxidation of the reduced cysteine groups is

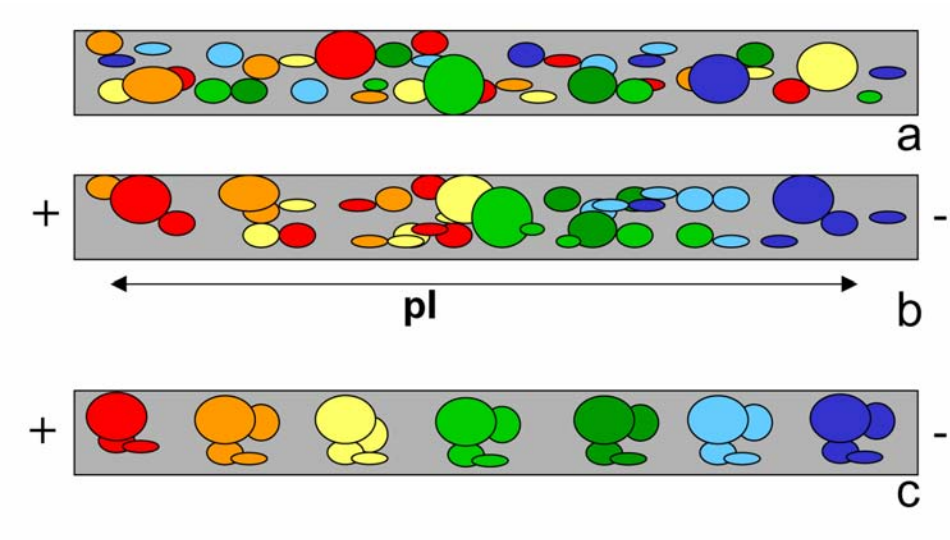

Figure 1.3: First dimension - isoelectric focussing a) A complex protein mixture added on an immobilized pH gradient (IPG) strip. b) Movement of proteins along the pH gradient in an electric field. c) Proteins focussed at their pI at high voltage.

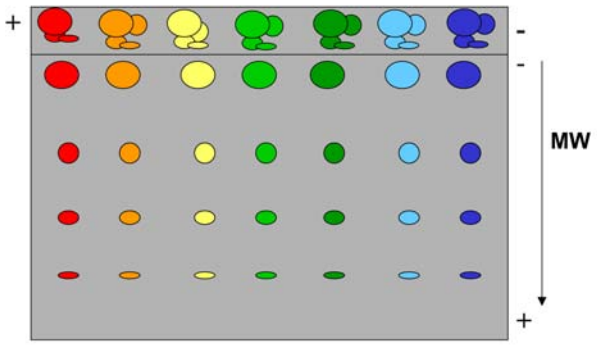

Figure 1.4: Second dimension - SDSPAGE: proteins focussed on an IPG strip are resolved according to their molecular weight in an SDS gel.

prevented by alkylating the free thiols on the cysteine groups.

Protein staining

Many staining techniques are available for visualizing the proteins separated as spots on 2DE gels. The two main deciding features of the staining methods are their sensitivity and their compatibility with mass spectrometry. Silver staining detects proteins with the highest sensitivity. However, the classical silver staining

procedure interferes with mass spectrometric identification. The most common staining method for proteomics is using Coomassie Brilliant Blue that binds to proteins in proportion to their length. Modification of the Coomassie staining procedure has improved the sensitivity of staining substantially. The protein spots are dissected out of the gels for tryptic digestion and mass spectrometry.

Phosphoproteome analysis

As mentioned earlier, the analysis of phosphorylated proteins is of great significance in proteomic studies aimed at the discovery of protein markers associated with the pathogenesis of diseases. Due to their very low abundance the modified (phosphorylated) proteins are usually "missed" during the separation procedures. Moreover, the charge state of phosphorylated peptides resists ionization and, hence, their mass spectrometric characterization. Consequently, increasing attention has been given to the development of methods to isolate and characterize them (Reinders and Sickmann, 2005).

One of the main strengths of 2DE is the ability to visualize different isoforms of the same protein. The recent commercial availability of fluorescent stains has facilitated the specific detection of PTMs such as glycosylation (ProQ Emerald, Molecular Probes), and phosphorylation (ProQ Diamond, Molecular Probes), on 2DE gels. ProQ Diamond stain binds specifically to proteins with phosphate groups on serine, threonine and tyrosine residues (Steinberg et al., 2003). Due to its fluorescent nature the ProQ Diamond stain can detect phosphorylated proteins present in as low as 4ng per spot. The staining intensity correlates with the number of phosphate groups present on the respective proteins (Steinberg et al., 2003). This high sensitivity is highly desirable in the case of phosphorylated proteins because of their very low abundance (Schlessinger, 1993). Proteins separated on 2DE gels for proteomic analysis can be stained first by the ProQ Diamond stain, and subsequently by the colloidal Coomassie stain or any other protein stain. ProQ Diamond staining allows the comparative expression profiling of a subproteome, namely phosphoproteome, both in a quantitative and a qualitative manner. Moreover, its high sensitivity increases the proteome coverage. As the dye binds non-covalently to the phosphate groups, it is compatible with mass spectrometry analysis.

Image analysis

Biological variations in the protein profile, (induced by a disease) manifest as a difference in the protein spot pattern on the 2DE gels. Image analysis software

is equipped with features that allow the comparison and relative quantification of protein spots on different gels. However, to date no software has been able to offer a protocol that can perform the differential analysis of 2DE gels without any manual input. Indeed in reality, manual interpretation of the results is a prerequisite. The initial part of my work focused on the evaluation of different commercially available 2DE softwares and the optimization of a standard procedure for the use of the PDQuest software (Bio-Rad Laboratories). Chapter 2 outlines the main features of PDQuest (Bio-Rad). A detailed protocol for the use of the software is attached in the appendix.

Strengths of 2DE

- 2DE is ideally suited for resolving proteins in a complex mixture.
- Detection of isoforms of proteins such as splice variants, PTMs and other chemical modifications.
- Standardized staining techniques and analysis software enable relative quantification and the comparison of two or more different conditions.

Limitations of 2DE

- A low dynamic range: sensitivity of 2DE depends on the dye used to visualize the proteins.
- A lack of automation: as most of the 2DE steps are performed manually, technical variation reduces the reproducibility of the proteome pattern.
- IEF is not suitable for the separation of membrane proteins, basic proteins and proteins with a high molecular weight.
- The sample preparation is complex: many factors such as nucleic acids, lipids, salt, etc interfere with IEF, leading to a distorted 2DE pattern. Sample preparation has to be optimized for every sample type.
- The efficiency of protein digestion and yield of peptides is variable during in-gel digestion. This minimizes the chances of identification of very low abundant proteins.
- The procedure is labour intensive.

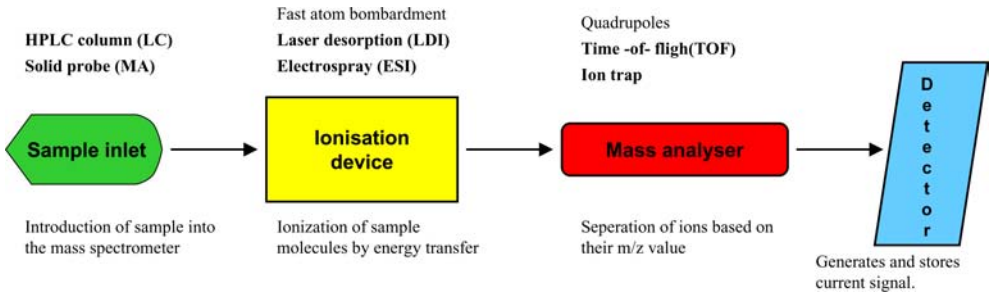

Figure 1.5: Basic scheme of mass spectrometers and the different methods.

1.4.2 Mass spectrometry

A mass spectrometer determines the molecular weight of compounds by separating molecular ions according to their mass to charge ratio (m/z). The basic principle behind mass spectrometry is that ions differ in their movement properties in an electric and /or magnetic field depending on their m/z value. A mass spectrometer ionizes molecules and subjects them to movement in an electric or magnetic field. The differing movement property, such as velocity, is measured to determine the molecular mass.

All mass spectrometers consist of 4 main components: 1. sample inlet, 2. ionization source, 3. mass analyzer, and 4. mass detector. After sample introduction into the mass spectrometer the molecules are ionized in the ionization device. The ions are then propelled into the mass analyzer where they are resolved according to their m/z value. The resolved ions are detected and converted to a signal by the detector. Technological innovations in the last decades have led to the invention of various methods of sample introduction, ionization, mass analysis and detection, all of which have contributed immensely to the potential of the instruments. There are different mass spectrometers varying in the technology and features of the different components. Figure 1.5 outlines the main parts of a mass spectrometer and their respective functions, and the different technologies that exist.

The most commonly used sample introduction and ionization techniques for proteomics are liquid chromatography/electrospray ionization (LC-ESI) and matrix assisted laser desorption and ionization (MALDI). While MALDI ionization technology is mostly combined with TOF mass analyzers, ESI sources are coupled to quadrupole and ion trap mass analyzers. In our experiments we used two different mass spectrometers namely, the ESI-ion trap (LCQ-Deca XP, Thermo-Electron) and MALDI-TOF-TOF (Ultraflex, Bruker Daltonics) for LC-MS-MS and MALDI-MS-MS measurements, respectively. These two systems will be

discussed briefly below.

ESI-ion trap

Electrospray Ionization (ESI): electrospray ionization uses an electric field to produce a spray of fine droplets containing the charged analyte molecules, thereby generating gaseous ions from a liquid solution. Samples, dissolved in a liquid phase are passed to a thin capillary needle maintained at a very high voltage. The high potential and small radius of curvature at the end of the capillary tube create a strong electric field, which causes the emerging liquid to leave the end of the capillary as a mist of fine droplets mixed with vapor called the ”taylor cone”. The sample ions are directed to the mass analyzer by applying a voltage (positive or negative) at the electrostatic lenses. Before the droplets enter the mass spectrometer either heat or dry gas (nitrogen) is applied to the droplets to desolvate the sample molecules. As the solvent molecules evaporate the droplets decrease in size and increase in their electric field density. The high electric repulsion of like charges causes the sample ions to desorb (columbic explosion) from the droplet and to be drawn into the mass analyzer inlet by electrostatic attraction, (Figure 1.6).

The main advantage of ESI is the ability to analyze samples present in liquid phase. Since molecules exist multiply charged in liquid phase, the gaseous ions entering the mass analyzer are also multiply charged. The feature of producing multiply charged ions enables the analysis of high molecular weight molecules, even with a mass analyzer with a small mass range. This has the additional benefit of accurate mass determination by averaging the masses calculated from different mass-to-charge values of the same molecule observed in the spectrum. The disadvantage is that the resulting spectrum is complex.

Advantages:

- The analysis of samples in solution.
- It can be coupled to HPLC.
- Multiply charged ions increase the range of the mass analyzer.
- The soft ionization process prevents fragmentation of the precursor ion.

Ion trap mass analyzer: A 3D ion trap mass analyzer consists of three hyperbolic electrodes: a ring electrode around the middle, and two endcap electrodes at the entrance and the exit of the ions. The amplitudes of RF (Radio Frequency

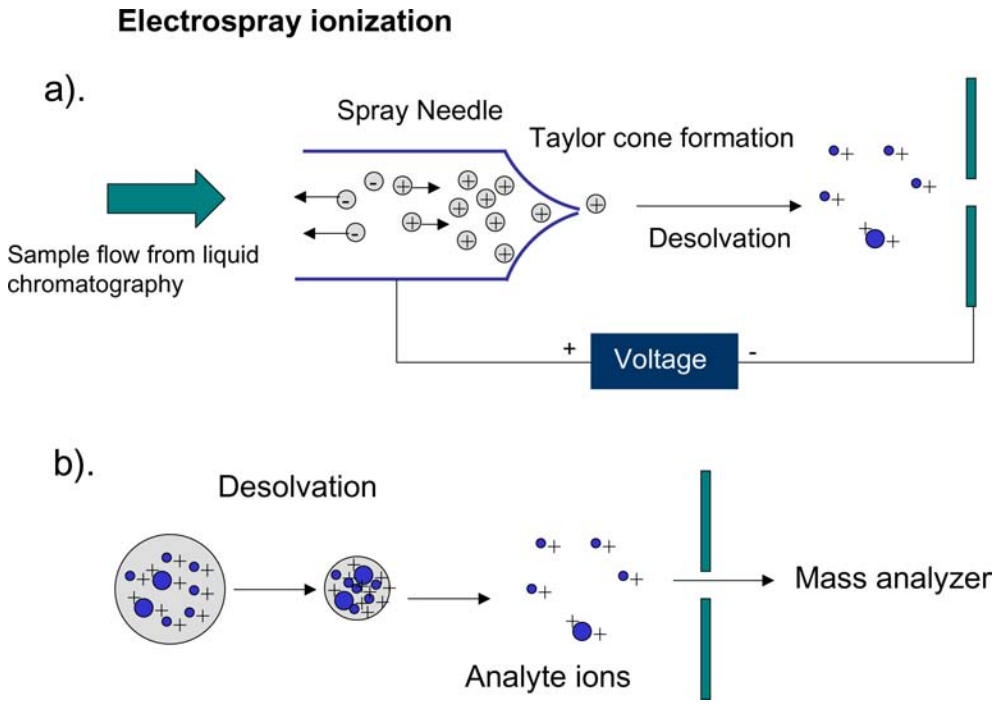

Figure 1.6: a) Electrospray ionization. b) Close up of desolvation process

- oscillating voltage), and DC voltages applied to the ring electrode, are ramped at a constant RF/DC ratio to allow stability and, hence, storage of a single m/z value in the ion trap.

The ions from the ESI source enter into the trap through the inlet in a pulsed fashion. The variable amplitude of the ring electrode RF potential produces a 3D quadrupolar potential field within the trap, and maintains the trapped ions in a stable oscillating trajectory. The exact motion properties of the ions depend on the individual mass-to-charge (m/z) ratios of the ions and the amplitude of the voltages applied. For the detection of the ions, the potentials are altered to destabilize the ion motions resulting in the ejection of the ions. This is done by scanning the amplitude of the fundamental RF voltage to make ion trajectories become unstable sequentially. Ions are ejected through holes in the exit endcap electrode and focused towards the detector to produce the mass spectrum signal, (Figure 1.7).

Full scan mode - precursor ion detection: in the full scan mode, where the precursor masses are measured, the RF is scanned to sequentially eject ions on the basis of their m/z, giving the mass spectrum of all the peptide ions in the trap at a given time or ionization period.

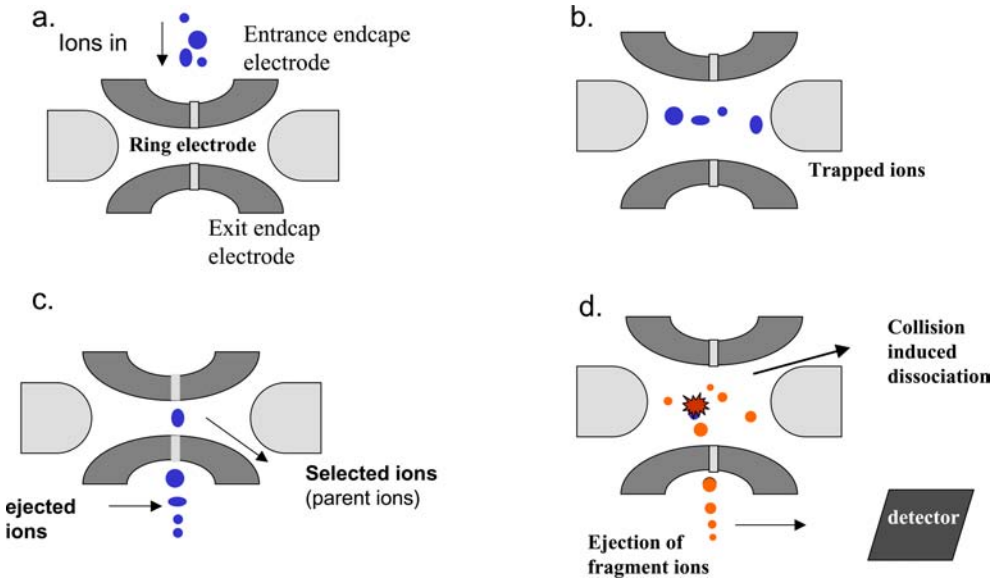

Figure 1.7: Ion trap mass analysis: a) Parts of an ion trap b) Trapped ions c) Selection of one ion d) Fragmentation by collision induced dissociation

Collision induced dissociation - MS/MS analysis: for MS/MS analysis the trap is filled with ions from the source and a particular ion, (the most intense), is selected. The voltage and energy on the trap is increased to promote high energy collision of the ions with helium gas in the trap, inducing fragmentation called collision induced dissociation (CID). The fragments are caught in the trap, scanned out in the order of their m/z value and detected.

MALDI - Matrix assisted laser desorption ionization

Analyte molecules are embedded in a small non-volatile organic compound (matrix) that can absorb laser energy. As the sample-matrix-mixture dries the matrix molecules crystallize to form a lattice into which the peptide molecules are integrated. When laser energy is applied matrix molecules absorb energy, get excited and vaporized. The matrix imparts the absorbed energy to the peptide molecules, thereby ionizing and vaporizing the peptide ions. Most ions are singly charged in MALDI ionization, and they are measured in the positive mode for peptide and protein identification by selecting the positive ions during the extraction, (Figure 1.8).

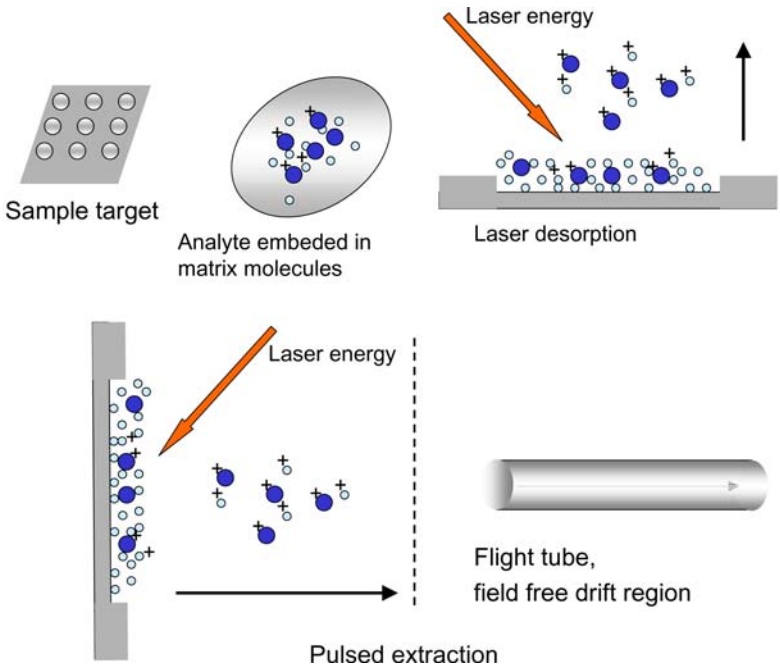

Figure 1.8: Illustration of the ionization of peptides embedded in the matrix molecules by laser energy (top), and pulsed extraction of ions (bottom)

Time of Flight (TOF) mass analyzer

TOF mass analyzer works on a very simple principle. It uses the differences in the transit time through a drift region (flight tube) to separate ions of different masses. Ions in an analyte are accelerated to the detector by imparting equal kinetic energy to all the ions. After the ions are generated, an electric field accelerates all ions into a field-free drift region with a kinetic energy (E_{kin}) of zE, where z is the ion charge and E is the applied voltage. Since the ion E_{kin} is 1/2mv^2, where m is the mass of the ion and v is the velocity it acquires in the electric field, lighter ions have a higher velocity than heavier ions and they reach the detector situated at the end of the drift region sooner. The derivation of the relationship between time of flight and m/z is as follows.

$$E_{kin} = zE = \frac{1}{2}mv^2 \tag{1.1}$$

$$v = \sqrt{\frac{2zE}{m}} \tag{1.2}$$

$$v = \frac{d}{t} \tag{1.3}$$

$$\frac{d}{t} = \sqrt{\frac{2zE}{m}} \tag{1.4}$$

$$t = \sqrt{\frac{dm}{2zE}} \tag{1.5}$$

since d, and E are constant

$$t \propto \sqrt{\frac{m}{z}} \tag{1.6}$$

Thus, the time of flight in a TOF analyzer is proportional to m/z values of individual ions and the ions reach the detector in the order of their m/z value. However, this simplified mode of generating spectra, called the linear mode measurement has a very low mass resolution due to the uneven distribution of energy to the ions during ionization and delayed ion formation.

New and improved mass spectrometers are equipped with tools that are capable of correcting these technical and experimental limitations. Reflectron mode, and pulsed ionization and delayed extraction are two features that permit high resolution in a MALDI-TOF instrument. In the reflectron mode, ions with the same or similar time of flight are first focused to a reflectron, and then deflected to the detector to increase the resolution. The reflectron is an ion mirror which makes use of an electrostatic field to reflect ions through a small angle towards a second detector. Ions with the same mass-to-charge ratio, but higher kinetic energy, penetrate deeper into the reflector, delaying their time of arrival at the reflectron detector relative to the slower low-energy ions, (Figure 1.9).

Pulsed ionization applies laser energy in a pulsed (not continuous) fashion to the peptides embedded in the matrix. Peptide extraction is delayed so that all peptides with the same or similar m/z can start their flight at the same time point.

The main advantages of MALDI-TOF:

- Spectra over a large mass range can be obtained in microseconds.
- Very high sensitivity due to the high ion transmission.
- There is no theoretical mass limit.
- The operation is simple and straightforward.
- Robust instrumentation.

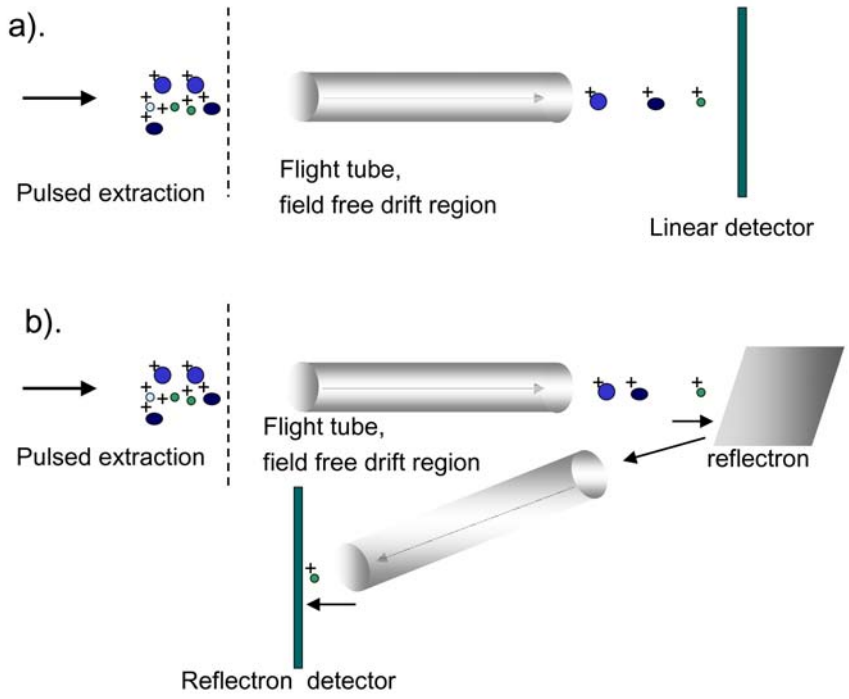

Figure 1.9: TOF mass analyzer: a) mass analysis in linear mode b) mass analysis in reflectron mode

MALDI-TOF-TOF

It is also possible to generate MS/MS spectra using a TOF analyzer. Fragmentation of precursor ions is achieved, either by post source decay (PSD), or by CID.

Post source decay: The term post-source decay refers to the fragmentation of molecular ions during their flight time in the drift tube away from the ionization source. During the lengthy flight time the precursor ions fragment resulting in smaller product ions. During fragmentation equal velocity is imparted to all the product ions but they differ in their kinetic energy (E_{kin}). Since E_{kin} is directly proportional to the mass of the ions, (for equation see above), the smaller product ions have less E_{kin} than their precursors. In the reflectron mode the fragment ions are distinguished based on the difference in their E_{kin}. This method of generating MS/MS spectra is time-consuming as the reflectron has to be voltage-tuned in steps in order to be able to detect all the fragments. To overcome this disadvantage the LIFT technology was developed (Bruker, Daltonics).

For tandem MS and protein identification, peptides of interest (most intense peaks) are selected for fragmentation after peptide mass fingerprinting (PMF).

The selection of precursor ions in a MALDI-TOF instrument is carried out by the application of an ”ion gate” that can be tuned to select an ion of a specific m/z ratio. After laser-induced dissociation or PSD the parent mass, along with the fragment ions, are accelerated and the fragment masses are measured according to their time of flight - LIFT spectra.

1.4.3 Protein identification by mass spectrometry

The key component in the identification of proteins by mass spectrometry is the availability of databases with sequence information of all expressed and hypothetical proteins in a species with completed genome sequences. The masses obtained from a mass spectrometer are searched against such a database using specified search algorithms for the identification. The analytical protein identification by mass spectrometry relies on the fact that a peptide sequence containing 6 or more amino acids is unique to a single protein.

Proteins are identified with the help of two different kinds of peptide mass information.

1. Peptide mass fingerprinting: identification of proteins by matching measured masses of proteolytic peptides to theoretical peptide masses of proteins in a database.

2. Tandem mass spectrometry (MS/MS): identification of proteins based on matching proteolytic peptide masses along with additional sequence information, (Yates et al., 1995a).

   There are specialized mass spectrometers for performing one or both of the above mass measurements. Sensitivity, resolution and mass accuracy are the three most desirable qualities of mass spectrometers that make them appropriate for mass analysis and identification of proteins.

Peptide mass fingerprinting (PMF)

Isolated proteins are cleaved into peptides using chemicals and/or proteolytic enzymes like trypsin that fragment proteins at specific amino acid residues. For example, trypsin cleaves proteins specifically after the lysine and arginine residues. Depending on the number of lysine and arginine residues, and the length of the protein, tryptic digestion generates multiple peptide sequences. After the generation of mass spectra, specialized search algorithms are used to compare

the measured mass spectra against databases containing protein sequences of a given species. The enzyme used for proteolytic cleavage is specified to minimize the complexity of the search. Search engines compare and match the measured masses to *in-silico* digested theoretical peptide masses. In the simplest approach proteins, whose predicted peptide masses match the greatest number of signal (masses) in the MS data, get the highest scores. The key factors that decide the exactness of PMF-based protein identification are the accuracy of the mass measurement and the mass or sequence coverage of the protein. Therefore, mass spectrometers that are capable of measuring mass signals with minimum error, (less than 50ppm), are most suited for PMF-based protein identification. The features of the search algorithms also play a major role in the determination of protein identity with high certainty. Improved scoring algorithms have features to correct for scoring bias due to protein size and the tendency of erroneous assignment of small peptide masses to many proteins. Some of the latest software, such as MASCOT (http://www.matrixscience.com), has incorporated statistical properties to affirm true identities (Gras et al., 1999; Fenyo, 2000).

PMF is the method of choice for high throughput analytical proteomics because of its time effectiveness, and it is most suited for the identification of proteins that are isolated, or for mixtures that do not have more than 2 or 3 component proteins. However, this method has its limitations. Unless a protein is identified with peptides of mass accuracy of +/- 50ppm, and with high sequence coverage the identity of the protein is always questionable. Furthermore, distinguishing very closely related protein species is uncertain with PMF. Since PMF is exclusively a method for identification it is not suitable for obtaining sequence or amino acid modification information.

Tandem mass spectrometry (MS/MS)

In tandem mass spectrometry, after the mass signal of the individual peptides has been obtained, the precursor ions or parent peptides are cleaved along the different bonds to generate the fragment ions. The masses of the fragment ions are analyzed and combined with the parent mass. Upon collision in the collision cell of a mass spectrometer the peptides absorb energy that induces breakage of the bonds at different sites. The types of fragment ions generated in an MS/MS spectrum depend on many factors including the instrument type, the primary peptide sequence, the amount of internal energy, how the energy was introduced, charge state, etc. The cleaved fragments are named based on the bond that is cleaved, (Figure 1.10). The accepted nomenclature for fragment ions was proposed by Roepstorff and Fohlman (Roepstorff and Fohlman, 1984).

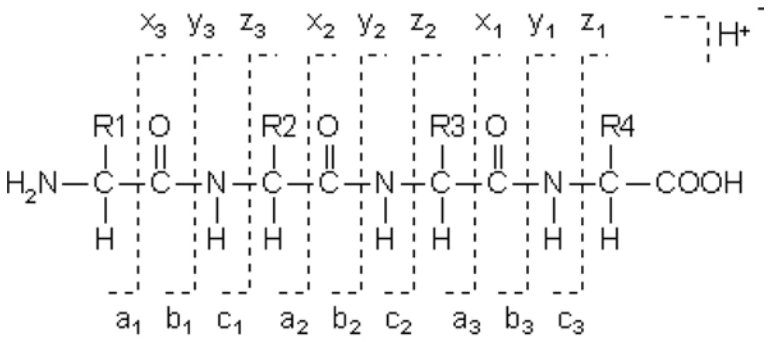

Figure 1.10: Possible fragment ions. This figure is taken from the homepage of Matrix Sciences.

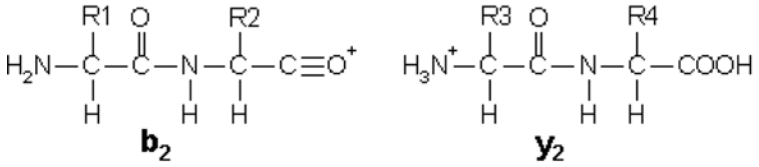

Figure 1.11: b and y ions are formed when a peptide is cleaved along its peptide backbone. This figure is taken from the home-page of Matrix Sciences.

After fragmentation, the ions carrying a proton at the N-terminal end are called a, b, or c ions whereas, when the charge is carried at the c-terminal end, the ions are called x, y or z ions. However, low energy collision induced fragmentation takes place predominantly along the peptide back bone generating b and y ions. Therefore, protein identification is generally based on b and y fragment ions, (Figure 1.11). Take together, tandem mass spectrometry generates peptide mass spectra (MS scan) along with the fragment ion spectra (MS/MS scan) of the individual precursor masses.

The identification of proteins based on tandem mass spectrometry data is facilitated by software tools (eg. SEQUEST, MASCOT) that can assign MS/MS data to peptide sequences in databases. (Yates et al., 1995b,a). The MS/MS data analysis is based on the following steps.

1. The precursor m/z value of each MS/MS scan is used to select peptides from the database with the same mass within a given mass tolerance range. The enzyme used for peptide cleavage and precursor mass charge state is specified in the settings.

2. A theoretical MS/MS spectrum is generated from each of the selected peptides.

3. The measured MS/MS spectrum is compared to the theoretical MS-MS spectra of the selected ions.

4. According to the correlation between the theoretical MS/MS spectra and the measured MS/MS scan a correlation score is given to the peptide sequence and the corresponding protein.

It is evident that the significance (score) of the identity depends on the quality of the mass spectra, and the completeness of the databases and the search algorithm itself. However, the search algorithms do not judge the quality of the spectra. At this point manual inspection of the quality of the spectra and the matching of theoretical and measured fragment ions are very important. The most reliable protein identifications are those in which several different sequences within the identified protein provide high quality matches to the MS/MS spectra in the data file.

In contrast to PMF, tandem mass spectrometry gives sequence information and, thereby, increases the confidence of the identity of a protein. High mass accuracy of the precursor peptide is not highly determinative in tandem mass spectrometry. The sequence information is also valuable in determining PTMs, or chemically induced modifications. Additionally, it also gives information about the sites of the modifications in a peptide.

Because of the high certainty in the protein identity we always used tandem mass spectrometry for protein identification in our experiments.

1.5 Biomarkers and proteomics for MS

Definitions:

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention.

A surrogate endpoint is a biomarker that is intended to serve as a substitute for a clinically meaningful endpoint, (how a patient feels, functions or survives), and is expected to predict the effect of a therapeutic intervention.

Since the mid-1800s, MS has been considered as an episodic disorder, characterized by the development of discrete lesions or plaques with disruption of myelin in the white matter of the central nervous system with relative preservation of the axons. Accumulating evidence now suggests a widespread in-

volvement even in the normal appearing white matter (NAWM) and gray matter (NAGM), as well as damage to axons and neurons (Murray, 2005). Consequently, there has been a shift in attention towards axonal and neuronal damage that are important aspects of MS, and the basis of its eventual and mostly irreversible progression. The clinical outcome of MS is the effect of a combination of several pathological processes such as inflammation, demyelination, axonal damage, excitotoxicity, apoptosis, neuronal loss and neurodegeneration. In spite of the complexity, most of the available drugs target the inflammatory components of the disease, (see section 1.2.3). Therefore developing new therapies for MS that are aimed at preventing or repairing one or more of these disease processes is of great importance. This new course in MS research calls for a better understanding of the molecular mechanisms that contribute to the clinical disability and the discovery of disease process-specific biomarkers (Bielekova and Martin, 2004). The new direction promises to solve the longstanding issues in MS research such as the identification of new disease-promoting factors and mechanisms, the discovery of new therapeutic interventions, and the identification of surrogate endpoints for monitoring the long-term therapeutic efficacy (Robinson et al., 2003b; Ibrahim and Gold, 2005).

The advent of global screening technologies like genomics and proteomics offers an opening to accelerate the search for new pathological pathways, and to identify new therapeutic targets and biomarkers in an unbiased manner. As proteins are the functional molecules that reflect the dynamic physiological status of tissue or cells, our view and approach is to analyze the global change in protein expression and modification (proteomics) in MS, and its animal model EAE, in order to identify protein biomarkers for EAE and MS.

Chapter 2

Materials and methods

2.1 EAE induction

2.1.1 Immunization of mice and evaluation of EAE

SJL/J mice were purchased from Charles River WIGA GmbH (Sulzfeld, Germany). Mice were housed in a specific pathogen-free, viral antibody-free animal facility at the Experimental Center of Dresden University of Technology. All breeding and experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Dresden University.

To induce active EAE, mice were injected with an emulsion of 200μg proteolipid protein (PLP) 139-154 in CFA (GIBCO, BRL) supplemented with 4 mg/ml inactivated Mycobacterium tuberculosis (H37 RA; Difco Laboratories, Inc., Detroit, MI) in the flanks on both sides and the tail base. The animals received an additional intraperitoneal injection of 200ng pertussis toxin (List Biological Labs, Inc., Campbell, CA) in 0.1 ml PBS on the day of immunization and again 48 h later.

Mice bred onto the C57Bl/6 genetic background were immunized with 100μg rMOG in the same way as described above. Animals were monitored daily for clinical symptoms and weight. For the clinical evaluation of EAE, the following scale was used: 0, no clinical disease; 1, tail weakness; 2, paraparesis (incomplete paralysis of one or two hindlimbs); 3, paraplegia (complete paralysis of one or two hindlimbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund or dead animals. For proteomics analysis, mice were sacrificed. Brain and spinal cord were removed and directly stored in liquid nitrogen until analysis.

2.1.2 Passive EAE by transfer of encephalitogenic T-cells

SJL mice were immunized in the above described way for the establishment of T cell lines. Ten days later, cells isolated from draining Lymph nodes (LNs) were cultured in the presence of 10 μg/ml antigen for 3 days. After growing the cells for 10-14 days in Interleukin-2-containing DMEM (GIBCO BRL), the cells were restimulated with antigen and irradiated (4,000 rads) syngeneic spleen cells; the specificity was assessed by [^{3}H]thymidine (Amersham International) incorporation for the last 16-18 h of a 3-day culture period. Activated T-cells were harvested on Lymphoprep (Nycomed, Oslo, Norway), washed once, and injected via the tail vein into mice. Animals were scored daily for clinical signs of EAE as described above.

2.2 Human MS specimen

Human tissue specimens were provided by the Human Brain and Spinal Fluid Resource Center, VAMC, Los Angeles, CA 90073, which is sponsored by NINDS/NIMH, National Multiple Sclerosis Society, VA Greater Los Angeles Healthcare System, 11301 Wilshire Blvd., Los Angeles, CA 90073 and Veterans Health Services and Research Administration, Department of Veterans Affairs.

Brain tissue from three MS patients and three control individuals was obtained for the experiment. Three different tissue sections, namely plaque, NAWM adjacent to the plaque, and the nearest NAGM from MS patients and two sections, NAWM and NAGM, from controls were included. The frozen brain sections were shipped on dry ice and were stored at -80° upon arrival. Clinical characterization of the patients is given in Figure 2.1

2.3 Two dimensional gel electrophoresis

2.3.1 Sample preparation of tissue samples

Materials and solutions required

1. Isoelectric focussing buffer (IEF): 7M Urea, 2M Thiourea (Sigma-Aldrich, Germany), 100mM Dithiothreitol (DTT), 4%(w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 0.05% biolytes 3-10,(Bio-Rad, Hercules CA) 0.001%

Sample	Age	Gender	Location	Comments
3010 MS -A plaqe NAWM NAGM	49	F	Frontal lobe	Marked demyelination and axonal loss consistent with **chronic MS.** Also classified as relapse remitting. Clinical diagnosis: MS.
3065 MS -C plaqe NAWM NAGM	61	M	Parietal lobe	Prominent demyelination, gliosis and oligodendrocyte loss, no inflammatory component. **Chronic MS** with chronic active plaques with minimal activity. Clinical diagnosis: MS.
3093 MS -B plaqe NAWM NAGM	68	M	Parietal lobe	Mild gliolis and decrease in oligodendrocyte density, demyelination, **chronic MS.** Clinical diagnosis: MS.
3175 normal NAWM NAGM	54	F	Frontal lobe	Pancytopenia. Neuropathology: no diagnosis. Clinical diagnosis: normal control.
2519 normal NAWM NAGM	80	M	Frontal lobe	Chronic obstructive pulmonary. Neuropathology: no diagnosis Clinical diagnosis: normal control
2526 normal NAWM NAGM	82	F	Frontal lobe	Lung cancer. Neuritic plaques with amyloid cores and neurofibrillary tangels not diagnostic of AD. Mild gliosis and cell loss in the white matter. Clinical diagnosis: normal control.

Figure 2.1: List of specimens, clinical characterization, diagnosis, age and gender of all the patient. MS patients are named A , B and C.

(v/v) Bromophenol blue (Sigma). Prepared and stored as 1 ml aliquots at -80°C.

2. Protease inhibitors: all protease inhibitors were added to the IEF buffer in 1X concentration just before use.

   - Pepstatin (Roche Diagnostics, Mannheim): 1000X stock prepared by dissolving 1 tablet in 1 ml ethanol and stored at -20°C up to 3 months.
   - Complete (Roche): 25X stock prepared by dissolving 1 tablet in 2 ml double distilled water and stored in aliquots at -20°C up to 3 months.
   - Phenylmethylsulfonyl fluoride (PMSF, Roche): 100mM stock (100X) prepared in methanol or ethanol and stored at 4°C.

3. Tissue sample grinding kit (Amersham, Piscataway, NJ).

4. BCA protein assay kit (Pierce, Rockford, USA).

5. Compat-Able protein assay preparation reagent set (Pierce) for cleaning up the samples for protein assay.

The following procedure was used for the preparation of animal and human tissue specimens.

Tissue homogenization: The frozen tissue was transferred to liquid nitrogen and ground to a powder in liquid nitrogen using mortar and pestle cooled on dry ice. The powdered tissue was aliquoted after the liquid nitrogen had evaporated completely, then weighed and frozen at -80°C until further processing.

Protein extraction and solubilization: An aliquot of each sample was solubilized using the sample grinding kit (Amersham) in 200μl of IEF buffer, (7M Urea, 2M Thiourea, 100mM DTT, 4% Chaps, 0.05% biolytes 3-10 (Bio Rad), 0.001% bromophenol blue) containing protease inhibitors (PMSF, Complete, and Pepstatin). After grinding the tissue for 1-2 minutes in IEF buffer the samples were centrifuged at 14000 rpm for 10 min and the supernatant was collected. Protein extraction was repeated in another 100μl of the buffer.

2.3.2 First and second dimension gel electrophoresis

Materials and solutions required

Isoelectric focussing

1. IPG strips of desired pH range (Bio-Rad) (4-7 pH range for mouse brain tissue and 5-8 pH range for mouse spinal cord, 3-10 pH range for human brain).

2. Filter wicks (Bio-Rad)

3. Isoelectric focussing apparatus (IEF Protean, Bio-Rad)

4. Equilibration trays (Bio-Rad)

Equilibration

1. Equilibration buffer base (EQB): 50 mM Tris pH 8.8 (Bio-Rad), 6M urea (Sigma-Aldrich), 2% sodium dodecyl sulphate (SDS), 20% (v/v) glycerol (Sigma-Aldrich). Stored at -20°C.
2. Equilibration buffer 1. : EQB containing 2% (w/v) DTT (Genaxxon, Germany). Dissolved a few hours before use at room temperature and kept in the dark.
3. Equilibration buffer 2. : EQB containing 2.5% (w/v) Iodoacetamide (Bio -Rad). Dissolved a few hours before use at room temperature and kept in the dark.

SDS PAGE

1. Tris buffers : 1.5 M Tris-HCl, pH 8.8, and 0.5 M Tris-HCl, pH 6.8.
2. 10% SDS. A ready-made stock of 20% w/v SDS was diluted 1:2 with water and stored at room temperature.
3. Thirty percent acrylamide/bis solution (37.5:1 with 2.6% C), (Genaxxon).
4. N,N,N,N'-Tetramethyl-ethylenediamine (TEMED, Bio-Rad).
5. Ammonium persulfate (APS) :10% w/v solution was prepared in water freshly before use.
6. Tris Glycine SDS (TGS) running buffer (10X) (Bio-Rad): 250 mM Tris, 1.920 M glycine, 1% (w/v) SDS pH 8.3. Stored at room temperature.
7. Water-saturated isobutanol. Equal volumes of water and Iso-butanol (Merck, Darmstadt, Germany) were mixed and allowed to separate overnight. Stored at room temperature.
8. Agarose overlay buffer: 0.5% (w/v) agarose (Bio-Rad) dissolved in TGS running buffer by boiling in a microwave. A few drops of Bromophenol blue was added to the buffer for colour. Stored at 4°C and melted in a microwave before use.
9. Pre-stained molecular weight markers (Bio-Rad).
10. Hinged spacer plates (Bio-Rad).
11. Gel casting chamber (Bio-Rad).
12. Gel combs for 2DE gels with one reference well (Bio-Rad).

Procedure

Isoelectric focussing: Fifteen mg tissue equivalent or 500μg protein extract of each sample was made up to a volume of 320μl with IEF buffer and centrifuged to get a clear supernatant. Brain tissue samples were focused on IPG strips with isoelectric gradients ranging between 4 and 7, whereas the spinal cord proteins were separated between 5 and 8 pH gradient. The strips were rehydrated actively at 50V for 12 hours with IEF buffer containing the protein sample, before focussing. After the rehydration was complete filter wicks dampened with water were inserted at both electrodes in order to absorb the salt content. Focussing was conducted at 500V first, and then ramped to 10,000V and focused until 60,000VH. The strips were frozen at -80°C immediately after focussing or processed for second dimension.

SDS-PAGE: The strips were incubated for 15 min in equilibration buffer containing 2% DTT (EQB1), followed by another 15 min in the same buffer containing 2.5% iodoacetamide (EQB2) for in-gel reduction and carbamidomethylation. After equilibration the strips were rinsed in 1x TGS running buffer (Bio-Rad), joined to 12% SDS gels and overlaid with agarose buffer. The gels were run at 50V overnight, and then at 200V until the run was complete.

2.3.3 Protein staining

Materials and solutions required

1. Fixing buffer 1 (for ProQ staining): 10% acetic acid, 50% methanol. Stored at room temperature or prepared before use.
2. ProQ Diamond stain (Molecular Probes, Eugene, OR), stored protected from light at 4°C.
3. Destain 1. : 20% Acetonitrile (ACN, Merck) and 50 mM sodium acetate pH 4.0 (For a stock solution, 1M sodium acetate pH 4.0 [using fuming hydrochloric acid (HCl)] was prepared and stored at room temperature.)
4. Staining trays compatible with methanol.
5. Fix buffer 2 (for Coomassie staining): 30% ethanol, 3% phosphoric acid.
6. Colloidal solution: 17 mM ammonium sulphate (Merck), 2% phosphoric acid and 34% v/v methanol, (Merck).
7. R-250 Brilliant Coomassie, (Sigma).

Procedure

Colloidal Coomassie staining: after the second dimension was complete the gels were fixed overnight in 200ml of fix buffer 2 (30% ethanol 3% phosphoric acid) and washed. The gels were incubated in 250ml of colloidal solution (34% methanol, 17mM ammonium sulphate and 2% phosphoric acid) for 1 hour before adding 250mg of Coomassie blue (1mg/ml) (R-250 Brilliant Coomassie, Sigma).

ProQ Diamond phophostaining: subsequent to the second dimension the gels were fixed overnight in fix buffer 1 (10% acetic acid, 50% methanol), followed by staining in ProQ Diamond (Molecular Probes) for 3 hours in the dark. Gels were de-stained three times for 30 min in destain 1 (20% ACN, 50mM sodium acetate solution), in the dark. After imaging the gels were washed and stained directly in colloidal Coomassie solution.

A protocol for the staining, image analysis and identification of phosphorylated proteins on 2D gels is published in Tools for functional proteomics, 2nd edition, *Methods in molecular Biology*, (2006) Human Press Inc. (Jacob AM and Turck CW (2006) Detection of PTMs by fluorescent staining of 2D-gels, Post translational modifications of proteins, 2nd Edition, Tools for functional proteomics, Methods in molecular Biology, Human Press Inc [In press])

Imaging and analysis: ProQ and Coomassie stained gels were scanned using FX imager (Bio-Rad) and GS-800 densitometer (Bio-Rad), respectively. All images were analyzed with the help of PDQuest (Bio-Rad), a 2DE gel analysis software.

2.4 Image analysis

This section outlines the main features and limitations of PDQuest, a 2DE image analysis software developed by Bio-Rad.

PDQuest analysis consists of 6 main processes:

1. Image handling tools
2. Spot detection
3. Matching
4. Normalization
5. Analysis

6. Spot review and report

Image handling tools

The main advantage of using software to analyze 2DE gels is that it enables quick, easy and identical handling of the images. The image handling tools include the following functions:

- Tiled view of images: any number of images can be opened and arranged as tiles in the same window automatically.
- Zoom function: the automatic zooming of the same area in all the open images. This tool aids manual inspection and comparison of specific areas of interest simultaneously in all gels.
- Crop: areas of no interest can be cropped out and eliminated from the gel. Parameters for cropping, such as dimension and borders, are stored and used for all gels involved in the same experiment.
- Transformation: the transformation function changes the contrast of the spots with respect to the background, and helps to detect very faint spots which are normally suppressed by the neighboring high intense spots.
- Multi-colour channel view: different digital colors are assigned to the gels and the pseudo-colored gels are overlaid on each other. This function aids quick detection of differences between the gels.

Spot detection

A 2DE proteome pattern is rather complex and crowded. Proteins separate as smudges on the gel with varying signal intensity and shape. The primary objective of a 2DE software is to detect and assign the signal pattern as protein spots. Based on the signal (optical density) each spot is given a relative intensity value. This requires optimal calculation of the boundaries of the individual protein spots. In principle, the software tries to detect and quantify the maximum number of spots. PDQuest uses a Gaussian function to calculate the relative spot intensity values for quantification. Spot detection parameters, such as sensitivity are defined by the user. All detected spots are marked individually on the corresponding gels. The spots are numbered according to their coordinates.

Matching

A representative gel, (a gel that has minimum distortion and the maximum number of spots), is chosen as the master gel for performing the match function. Master gel ideally contains all the spots that are detected in the match set.

For every detected spot PDQuest searches for the corresponding spot in all other images included in the match-set. Matching is done by detecting a spot signal in a position, with the same position coordinates as in the master gel. Spots that are matched to the master gel are marked green. Matching can be done in different degrees of extension. After matching corresponding spots, PDQuest creates a summary of the matching with the percentage matching, number of spots matched to all members, correlation coefficient of matching and the variance of the gels. A list of the total signal density and quantity measured from each gel, and the total number of spots detected on each gel, is also given.

Normalization

Normalization adjusts for the sample to sample variability, due to sample loading or staining error, before proceeding to the quantitative comparison. Normalization can be done, either based on the total density, or the total quantity.

Analysis

Analysis refers to the comparison of the different gels for determining the biological variation-induced alteration in the protein pattern. Images belonging to the same class, (diseased group or control group), are assigned as replicate groups.

Quantitative analysis set: determines the set of spots that are regulated with a certain fold difference in their intensity between two replicate groups. The fold difference and the replicate groups are specified. PDQuest calculates the average quantity of a given spot in one group and compares it to the average value in the second group. If the ratio of these values is above the specified threshold of fold difference between the groups the spot is added to the quantitative analysis set. All spots satisfying this criteria are listed in the set.

Matching analysis set: a set of spots that is matched to all the members of the match-set constitutes the matching analysis set.

Boolean function: The boolean function allows the combination of the above two sets, and the selection of spots matched to every member, and quantitatively different between the two replicate groups.

Spot review tool

The spot review tool reports all detected spots, with their intensity (quantity) in all the gels, as histograms. Spots belonging to specific analysis sets can be selected and highlighted in the gel automatically for reviewing. Any spot can be selected on the gel to view its quantity bar graph in the spot review tool.

Although adequate with a number of useful tools PDQuest has drawbacks due to its inaccuracy in spot detection and matching. These limitations make the

analysis rather time-consuming and tedious. The main shortcoming is a lack of a warping tool. A warping tool curves or stretches the gels so that they overlap exactly on top of each other. This corrects for the distortions during electrophoresis and for the change in the dimension of the gels during gel casting. The exactness of the overlapping depends on the features of the warping tool itself. We evaluated warping tools developed by different manufacturers and have come to the conclusion that the warping tool supplied by Non-Linear Dynamics provides the best warping features. Warping the gels with Non-Linear software prior to PDQuest matching considerably increased the spot match rate.

A detailed PDQuest user protocol is attached in the appendix.

2.5 Gel spot processing for mass spectrometry

Materials and solutions required

1. Destain 2 : 1:1 solution of 20 mM NH_4HCO_3, pH 8.00 and 100% ACN.
2. 1mM NH_4HCO_3, pH 8.0.
3. Trypsin (Sequencing grade modified trypsin, Promega, Madison WI). The trypsin was dissolved in 1 mM NH_4HCO_3 at 1 $\mu g/\mu l$ concentration and stored at -20°C.
4. 2% Trifluoroacetic acid (TFA, Merck).
5. 0.1% Formic acid (HCOOH, Merck).

Digestion: protein spots with at least 2-fold difference in intensity between the control and disease groups were excised out. The gel spots were destained two times in destain 2, by incubating at 37°C. The gel spots were dried and the proteins were digested overnight with trypsin at 37°C.

- For LC-MS/MS analysis each spot was digested in 0.3μg trypsin dissolved in 20mM NH_4HCO_3 at a basic pH. After overnight incubation at 37°C the peptides were extracted twice in 5% HCOOH by incubating at 37°. The peptides were concentrated until they were dried and then resuspended in 12μl 5% HCCOH for mass spectrometry.
- For mass measurement using a MALDI-TOF-TOF mass spectrometer, each gel spot was digested in 50ng trypsin dissolved in 5μl of 1mM NH_4HCO_3. After overnight digestion 1μl of 20% TFA was added to the digest mixture in order to extract the peptides.

2.6 Modified protocol for protein digestion and peptide extraction for mass spectrometry

This method was optimized for processing very low abundant protein spots (ProQ stained spots). The method is suitable for preparing the same spot for both MALDI-TOF and ESI -ion trap measurements. Very low abundant spots should be processed with care to achieve a maximum yield of peptides from the gel pieces, and to minimize the amount of salt and tryptic peptides in the peptide mixture.

1. Prior to in-gel digestion, the dried gel spots were rehydrated at 4°C for 15 min in 5μl of 1mM NH_4HCO_3 containing up to 50ng trypsin.
2. The spots were incubated at 37°C for 5 hours for in-gel digestion.
3. After digestion, the tubes were centrifuged to collect all the liquid at the bottom.
4. 1μl of 2% TFA was added to each spot to stop the enzyme reaction.
5. For peptide extraction the tubes were incubated at 37°C for 30 min, followed by sonication for 4 minutes.
6. 3μl of the the peptide extract was transferred to another tube for MALDI mass spectrometry.
7. 10μl of 0.1%HCOOH was added to the original tubes. Vortexing and sonication were repeated.
8. The remaining peptide extract was transferred to a new tube for LC-MS/MS analysis.

2.7 Mass spectrometry and protein identification

2.7.1 MALDI-TOF-TOF analysis

MALDI target spotting - dried droplet method

Materials and solutions required: (All solvents used were of high purity to minimize contamination that would interfere with mass spectrometry.)

1. 0.1% TFA in H_2O.
2. 2:1 solution of ethanol (Merck) and acetone (Merck).
3. TA30 solution - 30% acetonitrile (ACN) and 70% 0.1% TFA.
4. α-cyano-4-hydroxycinnamic acid (HCCA, Bruker Daltonics) saturated in 30% TA. HCCA was saturated in 30% TA by sonicating for 3 min followed by 3 minutes centrifugation at top speed.
5. Anchorchip target (Bruker Daltonics).
6. Standard peptide mixture for calibration (supplied by Bruker Daltonics).

Procedure

1. $1\mu l$ (or up to $1.5\mu l$) of the sample in TFA was spotted onto an Anchorchip target ($600\mu m$ or $800\mu m$).
2. The sample drop was left to air dry until its volume was reduced to around 20% of the initial volume ($\approx$3min).
3. The saturated matrix mixture was diluted 10-fold in a 2:1 solution of ethanol and acetone
4. 1.5μl of the HCCA matrix solution was added to the sample drop and left to dry completely.
5. Each spot was washed with 2μl of 0.1% TFA to reduce the salt content.
6. For every four analyte spots, a calibration spot was prepared to enhance mass accuracy.
7. $0.5\mu l$ of the standard peptide mixture (200fmol/μl) was used as a calibrant. The calibration spots were prepared the same way as the analyte spots.

Mass spectra acquisition

The masses were measured using a MALDI Tof-Tof mass spectrometer (Ultraflex, Bruker Daltonics, Bremen). Except for rare cases the instrument was operated in an automatic mode. A spread sheet was created listing the spot positions and the method to be used for generating the spectrum. The specified method consisted of optimized parameters to be used for generating meaningful spectra from spots prepared by the dried droplet spot preparation method. These

parameters included laser energy, the raster movement of the laser shots, number of laser shots in each pulse, number of spectra to be summed up per spot, minimum peak resolution, and signal to noise ratio for the selection of precursor masses for lift spectra generation. The laser power was checked and adjusted for every measurement. All masses were detected in the reflectron mode. Masses between 800Da and 4000Da were detected.

After the PMF measurement of every spot the instrument selected the 4 most intense peaks for fragmentation. Post source decay with LIFT technology was made use of for the generation of the MS/MS spectra, or the LIFT spectra. After acquiring the PMF of all the spots, the instrument created the LIFT spectra of the selected parent masses.

Data analysis

PMF, and the LIFT spectra acquired per spot, were sorted and stored automatically into individual files. The data analysis for protein identification was carried out using the software Biotools (Bruker Daltonics). Biotools integrates the MASCOT search algorithm for the analysis. The database search was carried out, either manually for individual spots, or automatically by specifying in the spread sheet. Searches were done against the Mus musculus or Homo Sapiens databases (National Center for Biotechnology Information, Bethesda, MD, www.ncbi.nlm.nih.gov). The search parameters used were: a) Mass tolerance of 25ppm (maximum 50ppm) for parent masses and 0.7Da for fragment ions. b) Maximum of 2 missed tryptic cleavage. c) Dynamic chemical modification of 16Da for methionine oxidation, and a static chemical modification of 57Da for carbamidomethylation of cysteine residues. Routinely a true protein identity was accepted when a protein was identified with more than one peptide and one LIFT spectra. In the absence of LIFT spectra a protein identity was accepted, when more than 4 peptides with a mass error of less than 25 ppm, and 0 missed cleavage were matched to the protein, and when the protein had a score that was significant according to the MASCOT algorithm. All spectra were manually assessed for the quality.

2.7.2 LC-MS/MS analysis

A total volume of $12\mu l$ sample in 0.1% formic acid (solvent A) was injected into an HPLC prefractionation system (Dionex, Sunnyvale, CA) consisting of an auto sampler, (Famos Auto sampler), microcolum unit with a RP-C18 premicorcolumn (300μmx1cm, Dionex), micro pump (Switchos) and a detection system. Samples were applied onto a RP-C18 picofrit nano column ($75\mu m$ i.d

x 10cm, New Objective, Woburn, MA, USA). After a solvent A (0.1%HCOOH) wash for 15 minutes, the peptides were eluted by applying a linear gradient of solvent B (90% acetonitrile/0.1%formic acid/10%water) for 60 min. The eluted peptides were infused directly into the source of an ESI - ion trap mass spectrometer (LCQ DECA XP PLUS, Thermo Electron, San Jose, CA). For each pulse or ionization period, after the full scan (parent mass spectra generation) the most intense signals were fragmented by switching to MS/MS scan.

Data analysis

The resulting MS/MS raw spectra were processed and analyzed using the software Bioworks. As a first step the raw data corresponding to each gel spot were converted to DTA files, (one DTA file per MS/MS spectra), which were then searched against the non-redundant NCBI database using the SEQUEST search algorithm (J. Yates III and J. Eng, University of Washington, Seattle, WA) integrated in Bioworks. The searches were performed with a dynamic chemical modification of 16Da for methionine oxidation, and a static chemical modification of 57Da for carbamidomethylation of cysteine residues. A maximum of 2 missed cleavages during digestion was permitted. The mass tolerance was set as 3Da for the parent mass. SEQUEST sorts the protein hits according to their score criterion, X-corr and DeltaCN. Typically, a protein identified by at least two peptide hits was accepted as a true positive.

DTA files, corresponding to each spectra, were additionally searched using the online MASCOT search algorithm (www.matrix sciences.com). All DTA files belonging to the same spots were concatenated to a single file and searched with the same parameters as in the SEQUEST search. In all cases the quality of the spectra was manually assessed in order to validate the protein identification.

2.8 Membrane protein analysis

The membrane protein fraction was extracted from EAE brain and control brains using a membrane protein extraction kit (Ready Prep protein extraction kit for membrane fraction, Bio-rad). The protocol given in the kit was followed for protein extraction. The proteins were sequentially solubilized according to their hydrophobicity into two fractions: one containing the hydrophilic proteins and the other containing the hydrophobic or membrane proteins.

Twenty five mg of brain tissue was lysed and the proteins were extracted by sonication in buffer 1, supplemented with protease inhibitors, (see 2DE section for the composition of the protease inhibitors). The membrane fraction was

cleaned up using a sample clean-up kit (Bio-Rad), and prepared for one or two dimensional gel electrophoresis. For one dimensional gel electrophoresis, the membrane extract was boiled with sample loading buffer (125mM Tris buffer (pH 6.8), 9.2% SDS, 40% glycerol, 6%w/v DTT, bromophenol blue) and run on 12% SDS gels with 3% stacking gel. For 2DE the samples were made up to 320μl in IEF buffer. The mixture was left for 30 min at room temperature and centrifuged at 14000rpm for 10 min to get a clear solution for IEF on 3-10 nonlinear IPG strips. 2DE was performed, as explained in the previous section.

2.9 Western blot analysis

Materials and solutions required

1. Lysis buffer for brain tissue - RIPA buffer: 500mM Tris-HCL pH 7.4, 150mM NACl, 1mM EDTA, 1%Triton-X 100, 0.1%SDS.
2. Protease inhibitors: all protease inhibitors were added to the IEF in 1X concentration just before use.
    - Pepstatin (Roche): 1000X stock prepared by dissolving 1 tablet in 1 ml ethanol and stored at -20°C for up to 3 months.
    - Complete (Roche): 25X stock prepared by dissolving 1 tablet in 2 ml double distilled water and stored in aliquots at -20°C for up to 3 months.
    - Phenylmethylsulfonyl fluoride (PMSF), (Roche): 100mM stock (100X), prepared in methanol or ethanol, and stored at 4°C.
3. Tissue sample grinding kit (Amersham).
4. Sample loading buffer (4x): 125mM Tris buffer (pH 6.8), 9.2% SDS, 40% Glycerol, 6%w/v DTT, bromophenol blue for color.
5. Protein quantitation kit: BCA (Amersham).
6. Tris buffers: 1.5M Tris-HCl, pH 8.8, and 0.5M Tris-HCl, pH 6.8.
7. 10% SDS.
8. 30% acrylamide/bis solution (37.5:1 with 2.6% C) (Genaxxon).
9. N,N,N,N'-Tetramethyl-ethylenediamine (TEMED, Bio-Rad).

10. 10%Ammonium persulfate (APS).
11. Running buffer (10x): 250mM Tris, 1.920 M glycine, 1% (w/v) SDS pH 8.3. Stored at room temperature.
12. Pre-stained molecular weight markers.
13. Mini gel casting chamber (Bio-Rad).
14. PVDF membrane (Millipore).
15. Transfer buffer: 25mM Tris base, 0.2M Glycine, 20% Methanol (Merck).
16. Tris buffer saline (TBS) buffer: A 10x TBS solution was prepared and stored at room temperature.
17. TBST: 0.05% TBST was prepared by adding 500μl of Tween20 to 1x TBS.
18. Blocking buffer: 5% milk powder solution was prepared in 1x TBS buffer.
19. Primary antibodies: anti-Stathmin 1 antibody (Cell Signalling) anti-Stathmin 1 antibody (Calbiochem), anti-Collapsin response mediator protein 4 antibody (CRMP4, AbCam), anti-Sirtuin 2 antibody (AbCam), anti-serum amyloid P component (human and mouse) (Alpha Diognostic International Inc).
20. Secondary antibodies: HRP-linked Protein A (Amersham), HRP-linked anti-mouse antibody (Amersham).
21. ECL standard detection system and ECL plus detection system (Amersham).
22. Stripping buffer: 0.2M Glycin pH 2.2 (Bio-Rad), 0.1% SDS, 1% Tween20.

Procedure

Protein extraction and solubilization: an aliquot of each tissue sample was solubilized using the sample grinding kit (Amersham) in 200μl of RIPA buffer supplemented with protease inhibitors. After grinding the tissue for 1-2 minutes in RIPA buffer, the samples were centrifuged at 14000 rpm for 10 minutes and the supernatant was collected. Protein extraction was repeated in another 100μl of the buffer and the supernatant was combined. The protein amount was estimated using the BCA protein assay kit.

An equal amount of protein from each sample was mixed with loading buffer and boiled at 95°C for 5 min. The proteins were separated on 12% SDS gels and 5% stacking gel.

The proteins were transferred to a PVDF membrane and subsequently, the membrane was blocked for 1 hour in blocking buffer. The membranes were incubated with the respective primary antibodies overnight at 4°C. Before and after the primary antibody incubation the membranes were washed in 0.05% TBST for 1 hour with 3 changes. Secondary antibody incubation was done at room temperature for 1 hour. Antibody binding was detected using the ECL detection system. A list of the primary and secondary antibodies, and the dilutions used, are given in the following table.

Primary antibody	**Dilution**	**Secondary reagent**
Stathmin 1	1:1000	Protein A
Stathmin 1	1:20000	Protein A
CRMP4	1.10000	Protein A
Sirtuin 2	1:1000	Protein A
SAP mouse	1.000	Anti-mouse antibody
SAP human	1:500	Anti-mouse antibody

Chapter 3

Results

For the identification of biomarkers for MS we carried out a proteome analysis of brain and spinal cord tissues of EAE and MS. Protein extracts from the tissue samples were separated by 2DE and evaluated for differential protein expression. Approximately 800 to 1000 protein spots were detected on each gel. The gels were first stained with ProQ Diamond followed by colloidal Coomassie. Digital images of the gels were acquired after each staining. Colloidal Coomassie detects proteins present down to 50ng per spot. The complementary ProQ Diamond stain binds specifically to the phosphate groups of serine, threonine or tyrosine residues, and facilitates the comparative expression profiling of the phosphoproteome. ProQ Diamond can detect as little as 4ng of a phosphorylated protein. Most of the ProQ stained spots including the most intense were not detected in the Coomassie staining.

Image analysis was done in two steps, one for ProQ stained phosphorylated protein spots, and a second analysis for Coomassie stained spots. The relative quantification of the spots was based on staining intensity measured as optical density. Figure 3.1 shows an overlay of the ProQ Diamond and Coomassie stained image of the same gel. Two types of differences were examined, ”qualitative” and ”quantitative”. A quantitative difference refers to the change in expression levels of a protein observed as an intensity difference of the spot. A qualitative difference is the result of a modification of a protein detected by a change in its position on the 2DE gel, (Figure 3.2). A two-fold difference was set as the threshold for a quantitative difference. Spots of interest were identified by mass spectrometry. Protein identity was based on at least two peptide MS-MS spectra corresponding to a single protein. Spectra were manually assessed for quality. In most cases a spot represented a single protein, with a few exceptions where multiple protein hits were identified from the same spot.

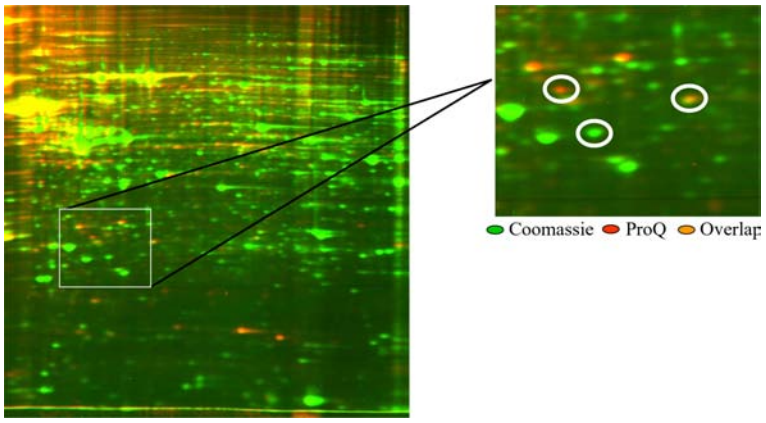

Figure 3.1: Overlap of pseudo-colored Coomassie (Green) and ProQ (red) images of the same gel. All green spots were stained only by Coomassie, red spots were stained only by ProQ stain and yellow spots by both stains.

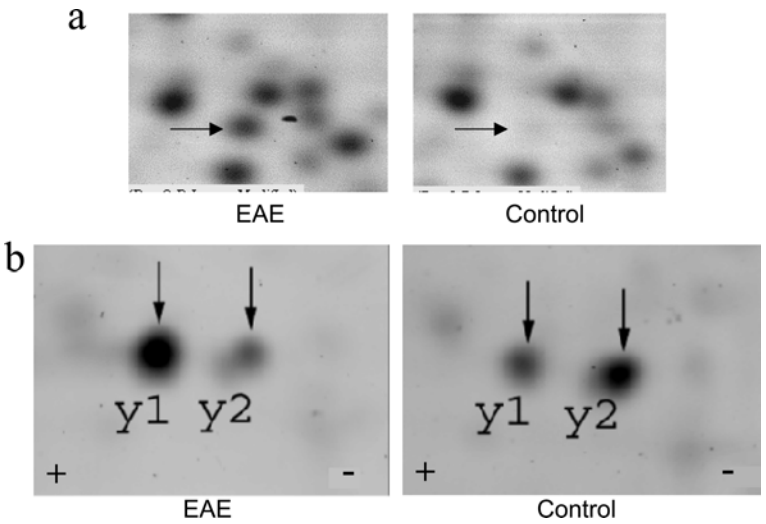

Figure 3.2: Qualitative and quantitative differences. a) A section of a 2D gel image of EAE and control spinal cord proteins. The highlighted spot is up-regulated (change in intensity) in EAE, an example of a quantitative difference in its expression. b) Stoichiometric and qualitative differences of the phosphoproteome. The two gels were stained by the phosphoprotein-specific ProQ Diamond stain. Spots y1 and y2 represent two isoforms of the same protein, which differ in the number of their phosphogroups. The hyperphosphorylated protein (the isoform carrying greater number of phosphogroups) migrates more to the acidic end of the gel. The difference in the intensity of the spots between EAE and control is due to the difference in the stoichiometry of protein phosphorylation.

3.1 Experimental autoimmune encephalomyelitis (EAE)

Several models of EAE were examined by proteomic analysis. These included MOG EAE in active and passive modes of induction, PLP EAE in active and

passive modes of induction and Devic's EAE, a spontaneous model of EAE. In some experiments all EAE animals had the same disease score, (advanced and full-blown), and in others, animals belonging to different disease scores were analyzed in the same experiment. The nature of the disease, (latency and progression), and its homogeneity among the animals, were determined by the immunogen and the mode of induction. Accordingly, the proteomic pattern and its regulation also varied among the different models of EAE. Protein extracts from both the brain and spinal cord were isolated for proteomic examination. The protein regulations were of a different nature. The following proteomic differences were found: a) up-regulation in EAE, b). down-regulation in EAE, c). proteins unique to EAE (below the detection level in the control), and d). qualitative difference due to a modification. Figure 3.3 is representative of spot differences between EAE and control proteomes on 2DE gels.

3.1.1 Protein markers in advanced EAE

Three experiments were conducted with uniform disease scores in all EAE animals. Untreated littermates or CFA and pertussis toxin-injected animals were used as controls, depending on the mode of induction. EAE induction, both in passive and active modes led to the homogeneous activation and progression of EAE, resulting in advanced stages of EAE (EAE score above "3.0"). Due to the homogeneity in the disease progression no individual variations were observed in the proteome pattern except for a few exceptions.

MOG passive EAE: passive immunization with MOG specific T-cells induced the development of an acute progressive form of EAE. Four animals reached a full-blown disease score of "4.0", 9 days after immunization. PDQuest analysis of Coomassie stained images determined several spots in the spinal cord with consistent differences in intensity compared to the controls. The brain tissue, however, gave only two proteins that were consistently different in all the animals. Only the brain samples were stained with ProQ in this experiment with a few stoichiometric and qualitative differences. Table 3.1 shows the list of all the proteins identified in passive EAE brain and spinal cord, respectively, with their nature of regulation.

PLP passive EAE: passive induction of PLP EAE gave rise to a chronic form of EAE. Animals reached a maximum disease score of "3.0". Three untreated animals were taken as a control. Coomassie staining, as well as ProQ staining revealed a number of proteins that were differentially regulated in both the brain and spinal cord. Tables 3.2, 3.3, 3.4, 3.5 list proteins identified by Coomassie and ProQ staining in the PLP passive EAE experiment.

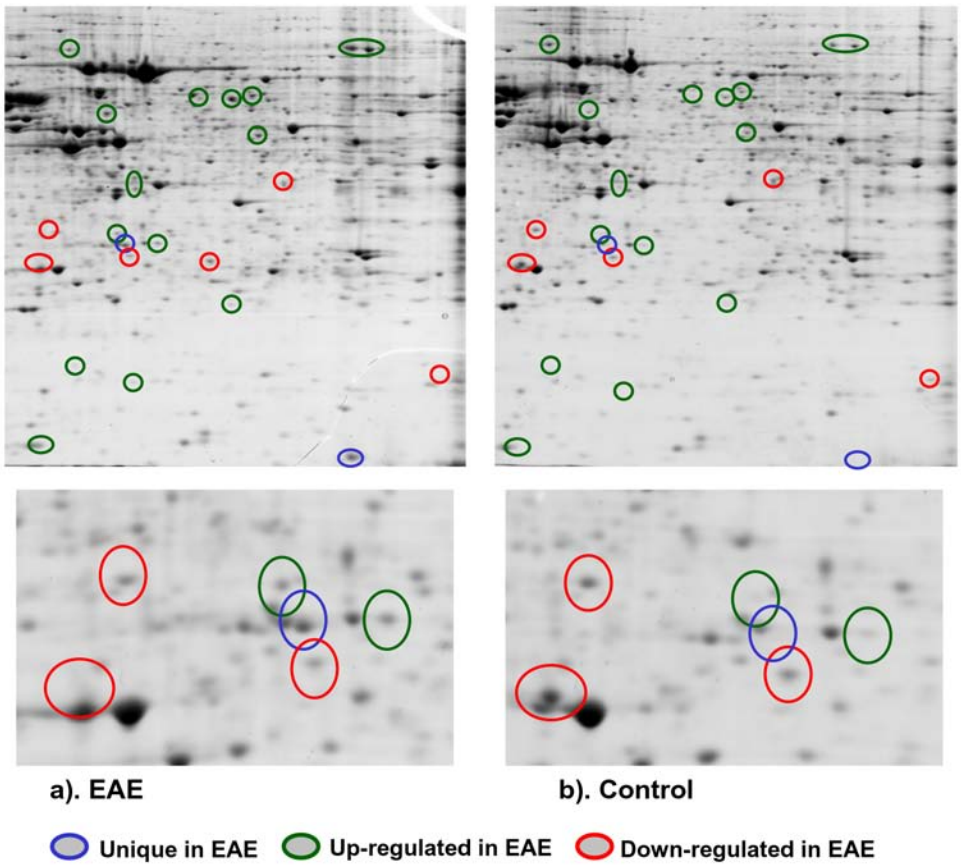

Figure 3.3: Representative 2DE gels of control and EAE spinal cord proteins. Spots circled green, red and blue stand for spots that are up-regulated, down-regulated and unique to EAE, respectively. Only the most prominent and consistent differences are marked.

MOG active EAE: although it is common that active immunization leads to variability between animals in the disease development and progression of EAE, this particular experiment gave the consistent development of EAE in 3 animals. Three CFA/PTX injected animals were taken as a control. Similar to the PLP passive experiment, Coomassie staining, as well as ProQ staining detected a number of proteins that were differentially regulated in both the brain and spinal cord. Proteins identified in this experiment are given in the supplementary material.

Accession no.	Protein name	Regulation	Sequence coverage (%)
Q9Z1Q5	Chloride intracellular protein 1	Only in EAE	19
P12246	Serum amyloid P component precursor	Only in EAE	29
P23492	Purine nucleoside phosphorylase	Only in EAE	12
Q64105	Mouse sepiapterin reductase complexed with NAD	↑ in EAE	24
P14602	Heat shock protein 27	↑ in EAE	23
Q13347	Eucaryotic translation initiation factor 3 subunit 2	↑ in EAE	7
P45886	AVCT3_BACDO actin 3	↑ in EAE	14
Q7ZUR4	Similar to F-actin capping protein alpha-1	↑ in EAE	19
P16949	Stathmin metablastin phosphoprotein	↓ in EAE	15
P02088	Hemoglobin beta	↓ in EAE	17
P07195	L-lactate dehydrogenase B chain	↓ in EAE	22
P02650	Apolipoprotein e precursor	↓ in EAE	12
Q9CR68	Ubiquinol-cytochrome c reductase	↓ in EAE	22
P31146	Coronin like protein	↓ in EAE	16
P14480	Fibrinogen beta polypeptide	↑ in EAE	9
P11413	Glucose-6-phosphate 1-dehydrogenase	↑ in EAE	17
Q9D0S8	Cysteine, serine proteinase inhibitor	↑ in EAE	26
P10107	Lipocortin, Annexin 1	Only in EAE	25
Q9JM14	Nucleosidase cytosolic	↑ in EAE	36
Q00623	Apolipoprotein 1	↑ in EAE	21
O08692	Myeloid bactenecin	↑ in EAE	31
P80314	Chaperonin subunit 2 beta*	↑ in EAE	40
Q9R1T4	Septin 6*	↑ in EAE	9
Q7SZS9	Rho, GDP dissociation inhibitor (GDI) beta	↑ in EAE	36

Table 3.1: MOG passive EAE: list of the differentially regulated proteins in the spinal cord detected by Coomassie staining. ↑ and ↓ arrows stand for an at least two-fold increase or decrease in the intensity of the spots between EAE and control proteome, respectively. Sequence coverage represents the percentage of the amino acid sequence based on which the identity was confirmed. * indicates double protein hits from a single spot. The Swiss-Prot accession numbers are given.

Accession no.	**Protein name**	**Regulation**	**Sequence coverage (%)**
Q63810	Protein phosphatase 2B regulatory subunit 1	↓ in EAE	53
P62204	Calmodulin	↓ in EAE	45
P63028	IgE-dependent histamine-releasing factor	↓ in EAE	41
I73338	Macrophage scavenger receptor II	↓ in EAE	14
O70251	Elongation factor 1-beta*	↓ in EAE	4
P28651	Carbonic anhydrase-related protein (CARP)	↓ in EAE	43
Q91V31	Laminin receptor 1	↓ in EAE	35
P11499	Heat shock protein 90 beta, hsp84	↓ in EAE	8
P07901	Heat shock protein 86	↓ in EAE	32
Q91VI7	Ribonuclease /angiogenic inhibitor	↓ in EAE	40
Q9DBJ1	Phosphoglycerate mutase 1	↓ in EAE	56
BAC25737	Septin 2	↓ in EAE	37
P62761	Visinin-like protein 1 (Neurocalcin alpha)	↑ in EAE	54
P63101	14-3-3 protein zeta/delta*	↑ in EAE	21
JC2502	Mitochondrial import stimulation factor S1 *	↑ in EAE	
O08855	Apolipoprotein A-I precursor *	↑ in EAE	47
Q9JM14	5'(3')-deoxyribonucleotidase, cytosolic *	↑ in EAE	
P20108	Thioredoxin-dependent peroxide reductase	↑ in EAE	30
P17751	Triosephosphate isomerase	↑ in EAE	
CAA27396	Actin cytoplasmic 1	↑ in EAE	26
BAB25712	Proteasome activator complex subunit 1*	↑ in EAE	23
A39682	Prohibitin*	↑ in EAE	
BAB29254	Creatine kinase	↑ in EAE	38
Q13885	Beta tubulin (Tubulin, beta 2)	↑ in EAE	45
Q8VCM7	Fibrinogen gamma chain	↑ in EAE	36
P68366	Tubulin alpha chain - mouse	↑ in EAE	29
Q8BPI0	Rho GDP dissociation inhibitor 1	only in E3	50

Table 3.2: PLP passive EAE: list of proteins and their regulation in the EAE brain. Proteins detected by Coomassie staining.* indicates double protein hits from a single spot. Swiss-Prot accession numbers are given.

Accession no.	Protein name	Regulation	No. of peptides
Q62048 [1]	PEA 15	↓ in EAE	1
IPI00110851 [3]	Mitochondrial import receptor subunit TOM22 homolog	↓ in EAE	1
IPI00331163 [3]	S-phase kinase-associated protein 1A	↓ in EAE	2
gi|14625464 [2]	Stathmin 1	↓ in EAE	2
IPI00407543 [3]	Cofilin1	↑ in EAE	3
IPI00409405 [3]	Cofilin actin depolymerizing factor	↑ in EAE	2
IPI00118384 [3]	14-3-3 protein epsilon	↓ in EAE	15
IPI00120716 [3]	Guanine nucleotide-binding protein beta 1		2
IPI00116498 [3]	14-3-3 protein zeta/delta	↑ in EAE	2
IPI00136703 [3]	Creatine kinase B-type	↑ in EAE	3
gi|2623222 [2]	ATP synthase 5b	↑ in EAE	4
IPI00404182 [3]	Rho-related GTP-binding protein RhoB	↓ in EAE	2
gi|13435747 [2]	Rho GDP dissociation inhibitor	↓ in EAE	1
gi|18606334 [3]	Calcium calmodulin dependent kinase kinase	↓ in EAE	1
IPI00129164 [3]	Sepiapterin reductase	↑ in EAE	2
IPI00399449 [3]	NSFL1 (p97) cofactor (p47)	↓ in EAE	4
IPI00607070 [3]	Actin, cytoplasmic 1	↓ in EAE	3
IPI00136107 [3]	NDRG3 protein	↓ in EAE	1
IPI00129178 [3]	Ornithine aminotransferase, mitochondrial	↓ in EAE	2

Table 3.3: PLP passive EAE: differentially regulated proteins in the brain phosphoproteome (proteins detected with ProQ staining). No. of peptides: peptides that confirmed the identity. [1], [2] and [3] represent Swiss-Prot, NCBI and EBI accession numbers, respectively.

Accession no.	Protein name	Regulation	Sequence coverage (%)
P16045	Galectin 1	↑ in EAE	44
P31725	Calgranulin B	↑ in EAE	44
O08692	Myeloid bactenecin	↑ in EAE	32
P70296	Phosphatidylethanolamine-binding protein	↑ in EAE	75
P29391	Ferritin light chain 1	↑ in EAE	37
Q8CG74	Albumin1	↑ in EAE	57
Q8BPD5	Apolipoprotein a1	↑ in EAE	49
P14602	Heat shock protein 25	↑ in EAE	49
Q9Z1Q5	Chloride intracellular channel protein 1	↑ in EAE	30
P12246	Samp	↑ in EAE	26
P97372	Proteasome activator complex subunit 2	↑ in EAE	41
Q6PEM2	Pzp protein, alpha 2 macroglobulin	↑ in EAE	10
Q921I1	Serotranferrin	↑ in EAE	37
Q61176	Arginase 1	↑ in EAE	57
Q6PFG7	Tyki protein	↑ in EAE	37
Q8VCM7	Fibrinogen gamma polypeptide	↑ in EAE	50
Q8K0E8	Fibrinogen,B beta polypeptide	↑ in EAE	27
O89053	Coronin-1A	↑ in EAE	40
Q9Z1P6	NADH-ubiquinone oxidoreductase B14.5a	↑ in EAE	35
Q9R257	Heme binding protein 1	↑ in EAE	58
P61148	Acidic fibroblast growth factor	↓ in EAE	38
P09671	Superoxide dismutase	↓ in EAE	37
Q9CPU0	Lactoylglutathione lyase	↓ in EAE	56
Q8BPI0	RHOGDI-1	↓ in EAE	47
Q9JM14	5'(3')-deoxyribonucleotidase, cytosolic	↓ in EAE	29
Q8BWR2	Thioredoxin-like protein 1	↓ in EAE	50
Q9DCF2	Hdhd2	↓ in EAE	37
P60710	Actin beta	↓ in EAE	22
Q61167	Microtubule-associated protein RP/EB 3	↓ in EAE	31
Q9DB05	Alpha-soluble NSF attachment protein	↓ in EAE	46
Q8R1G2	Hypothetical Crystallin containing protein	↓ in EAE	34
S23590	Creatine kinase	↓ in EAE	47
P35486	Pyruvate dehydrogenase E1 component a	↓ in EAE	53
BAB23362	Ndrg1	↓ in EAE	28
Q545R3	N-myc downstream regulated gene 1	↓ in EAE	31
Q8BFR5	Elongation factor Tu, mitochondrial	↓ in EAE	63
Q62188	Dihydropyrimidinase-related protein 3	↓ in EAE	17
Q8K590	EH-domain containing protein 2	↓ in EAE	20

Table 3.4: PLP passive EAE: list of proteins and their regulation in the spinal cord. Proteins detected by Coomassie staining. Swiss-Prot accession numbers are given.

Accession no.	Protein name	Regulation	Sequence coverage (%)
P54227	Stathmin	↓ in EAE	8
P54227	Stathmin	↓ in EAE	24
Q544Y7	cofilin non muscle	↓ in EAE	26
P09528	Ferritin heavy chain	↓ in EAE	67
Q8K4Z3	ApoA-I binding protein	↓ in EAE	46
AAG39256	NAD-dependent deacetylase sirtuin-2	↓ in EAE	40
P61982	14-3-3 gamma	↓ in EAE	42
P05063	aldolase 3	↑ in EAE	62
Q9DCZ1	Guanosine 5'-monophosphate oxidoreductase 1	↓ in EAE	14
O88456	Calpain A1 small subunit	↑ in EAE	
Q91XH5	Sepiapterin reductase *	↑ in EAE	43
Q9Z2L2	Truncated hsp25 *	↑ in EAE	12
AAA37430	2,3-cyclic-nulceotide 3-phosphodiesterase	↑ in EAE	6
Q62433	ndrg1	↓ in EAE	7
Q8K4Z3	ApoA-I binding protein	↑ in EAE	32

Table 3.5: PLP passive EAE: phosphoproteins (ProQ stain) regulated in the spinal cord. * indicates double protein hits from a single spot. Swiss-Prot accession numbers are given.

Animal	Disease Score
1	2.5
2	2.5
3	1.5
4	1.0

Table 3.6: MOG active EAE - animals and their disease scores.

3.1.2 Protein markers in different stages of EAE

Animals belonging to different disease stages were included in the same experiment. We used active immunization of MOG and PLP EAE for examining the protein pattern change during different stages of EAE progression. CFA and pertussis toxin-administered animals were used as controls. Animals were sacrificed at different stages of the disease. Individual variations were observed in EAE animals, most of them corresponding to the disease stages. As expected, the number of differentially regulated proteins correlated with the disease score. The higher the disease score the greater the number of protein level differences.

MOG active EAE: MOG active immunization generated 4 EAE animals with a score ranging from ”1.0” to ”2.5”, (Table 3.6).

Altered protein expression was observed in both the brain and spinal cord. Changes in the brain were not consistent in all the animals. ProQ staining gave differences only in the spinal cord but not in the brain. The number of differences observed in animal 4 with a disease score of ”1.0” was less than in the other animals. Remarkably, some of the proteins identified were regulated already at score ”1.0”, whereas others developed only when the disease had progressed to disease stage ”1.5”, (Figures 3.4 and 3.5). Noticeably, despite the disease score difference, there was no difference in the proteome pattern between animals 1, 2 and 3. In other words, while animals 3 and 4, with a minor difference in disease score, showed differences in their protein profile, animal 3, with disease score ”1.5”, did not differ from animals 1 and 2, with disease score ”2.5”. Proteins identified in this experiment are given in the supplementary data.

PLP active EAE: the ability of proteomic analysis to differentiate between disease stages inspired us to carry out an active PLP EAE experiment as a blind study. Five animals were induced to develop EAE by immunizing them actively with PLP antigen. As is typical for PLP EAE, the animals developed a chronic form of EAE. The respective disease scores ranged between ”1.5” and ”3.0”, (Table 3.7).

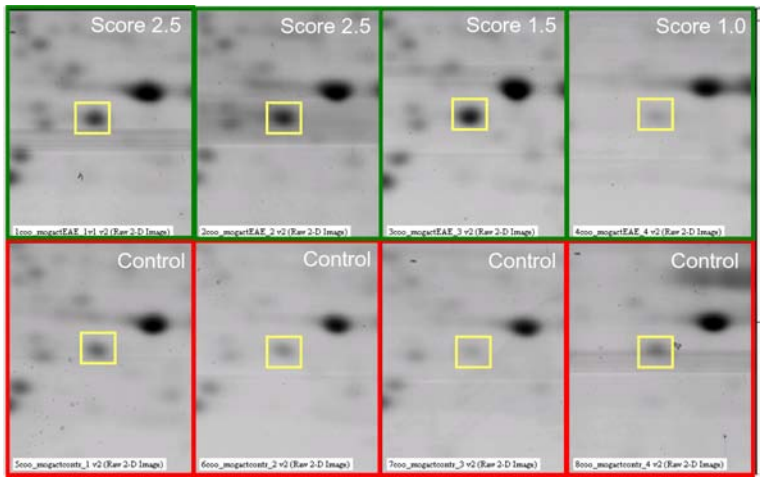

Figure 3.4: Disease score and protein regulation: up-regulation of the highlighted spot is not present in animal 4 with disease score ”1.0”

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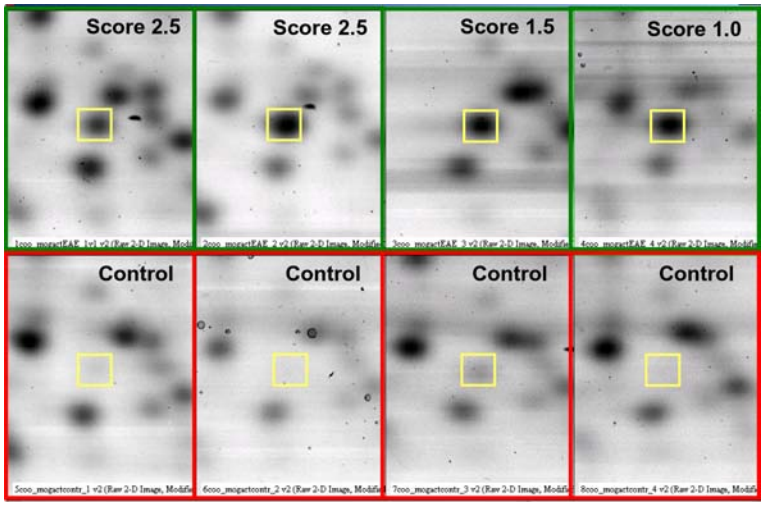

Figure 3.5: Early end point markers: consistent up-regulation of the highlighted spot in all animals, irrespective of disease score

.

After the proteome comparison the animals were grouped according to their change in protein profile compared to the control. Animals 1 and 2 showed similar changes for most spots. Therefore, they were classified as having the same disease score (group A). The proteomes of animals 3 and 4 appeared similar in most cases, but had a lot fewer spots different from the controls. Thus, animals 3 and 4 were grouped together (group B) with a disease score lower than group

Animal	Disease Score
E1	2.5 - group A
E2	2.5 - group A
E3	1.5 - group B
E4	1.5 - group B
E5	3.0 - remission

Table 3.7: PLP active EAE - animals and their disease scores.

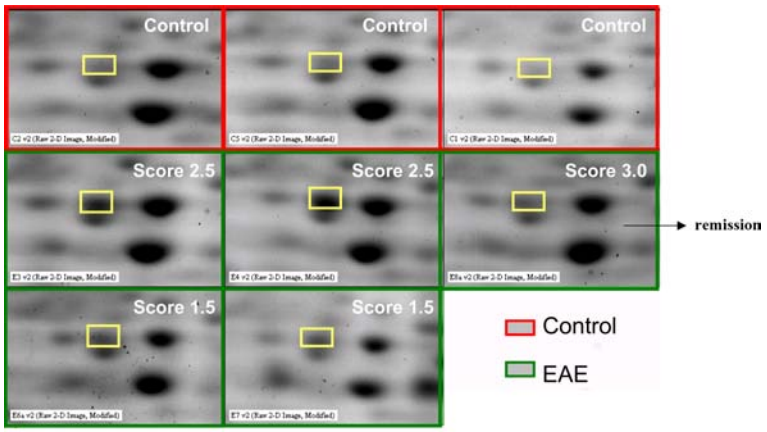

Figure 3.6: Disease score dependent variation in protein expression. The highlighted spot is up-regulated in animals with disease score "2.5" but not in animals with disease score "1.5", one of the spots that helped the classification of the animals according to their disease stage. The same spot is unaltered in the animal with disease score "3.0", (like in disease score "1.5"), because of remission.

A. However, animal 5 appeared to belong to both group A and group B. In the case of some spots it even appeared to have a disease score higher than group A, (Table3.7). Classification, based on the proteome, was then matched with the original animal experiment data. Groups A and B classification correlated well with the original disease score. Strikingly, the animal experiment data showed that animal 5 showed remission at the time of sacrifice. Figure 3.6 gives an example of a spot that helped in the classification. Variance between the animals associated with the disease score was more prominent in the brain than in the spinal cord. Both, ProQ and Coomassie staining detected differentially regulated proteins. Tables 3.8, 3.9 and 3.10 list all proteins identified in the PLP active model.

Very Early EAE: In order to determine the time of commencement of the protein level changes we performed a very early EAE study. The animals were

Accession no.	Protein name	Regulation	Sequence coverage (%)
P12460	Serum amyloid P component	only in EAE	13
P47757	F-actin capping protein B	↑ in E1, E2 E5	17
BAC34360	Albumin 1	↑ in E1, E2	11
Q8R3E6	Activator of heat shock protein 90KDa	↑ in EAE	22
P39027	Ribosomal protein S15a	↑ in E1, E2 E5	10
Q9CQM9	Thioredoxin-like protein 2	↑ in EAE	16
P39053	Dynamin 1	↑ in E1, E2	7
XP_130960 [2]	Fibrinogen B beta polypeptide	↑ in EAE	6
Q28480	Isocitrate dehydrogenase	↑ in E1, E2	9
Q62363	Tubulin beta 2 chain	↑ in E1, E2	9
P02571	Actin gamma	↑ in E1, E2	
Q9JJV2	Profilin 2	↑ in EAE	26
P67779	Prohibitin	↑ in EAE	32
Q15019	Septin 2	↑ in E2 , E5	2
P39053	Dynamin 1	↑ in E1, E2 E5	1
Q5M8R8	Acidic ribosomal phosphoprotein p0	↑ in E1, E2 E5	7
O35295	Purine rich element binding protein B	↓ in E1,E2	16
P47942	Dihydropyramidinase related protein 2	↓ in E1,E2, E5	15
NP_002794.1 [2]	Proteasome 26S Atpase subunit 2,	↑ in E1, E2 E5	8
Gi|2119272 [2]	Tubulin Alpha chain	↑ in E1, E2 E5	7
Gi|228954 [2]	T complex polypeptide 1	↓ in E1, E5	16
Gi|21594641 [2]	Dihydrolipoamide S-acetyltransferase	↑ in E1,E2 ↓ in E3, E4	6
Q61598	Rab GDP dissociation inhibitor beta-2	↑ in E1, E2 E5	18

Table 3.8: PLP active EAE: proteins and their regulation in the brain. Proteins detected by Coomassie and ProQ are separated by a double line. [2] stands for NCBI accession numbers. All the other proteins are named according to the Swiss-Prot accesion number.

induced to develop EAE by active immunization. On days 5, 7, and 9 after immunization 3 PLP-injected animals, (score="0") and 1 control animal, (adjuvant alone), were sacrificed for proteome analysis. Proteome analysis however, could not detect changes between the control animals and the EAE animals.

Accession no.	**Protein name**	**Regulation**	**Sequence coverage (%)**
P30161	ACT_COSCs	↑ in EAE	6
P50247	Adenosylhomocysteinase	↓ in EAE	4
Q8C7C7	Albumin 1	↑ in EAE	20
Q00623	Apolipoprotein A1	↑ in EAE	27
Q9DCX2	ATP synthase D chain (ATP5H)	↑ in EAE	
P37397	Calponin 3	↑ in EAE	10
Q95MF9	Chloride intracellular channel protein 1	↑ in EAE	10
AAH53398	Coronin 1a	↑ in EAE	8
Q62188	Dihydropyrimidinase related protein-3 Ulip	↑ in EAE	3
P26040	Ezrin	↑ in EAE	1
Q8K0E8	Fibrinogen beta chain	↑ in EAE	30
P50441	GATM_PIG amido transferase	only in EAE	16
Q99LB4	Capping protein (actin filament)	↑ in EAE	
Q8C2F4	Glucose regulated protein	↑ in EAE	40
Q8C2F4	Protein disulfide-isomerase A3	↓ in EAE	40
BAC25416	Glyceraldehyde 3 phosphate dehydrogenase	↓ in EAE	7
P14602	Heat shock protein 25	↑ in EAE	39
Q9CPV4	Hypothetical Glyoxalase	↑ in EAE	39
Q8C8A1	Isocitrate dehydrogenase 3 (NAD+) alpha	↑ in EAE	28
Q8VDQ8	NAD-dependent deacetylase sirtuin-2	↓ in EAE	34
Q5SVY2	Peptidylprolyl isomerase A	↑ in EAE	10
P10605	Cathepsin D	↑ in EAE	
P53810	Phosphatidylinositol transfer protein alpha	↓ in EAE	8
Q9DBJ1	Phosphoglycerate mutase 1	↓ in EAE	59
NP_031402 [2]	alpha 1 macroglobulin	↑ in EAE	3
P97371	Proteasome activator complex subunit 1	↑ in EAE	6
P49722	Proteasome subunit alpha type 2	↑ in EAE	8
Q8CHH9	Septin 8 protein	↓ in EAE	11
P12246	Serum amyloid P-component	only in EAE	21
XP_285706 [2]	Similar to carbonic anhydrase	↑ in EAE	19
Q61753	D-3 phosphoglycerate dehydrogenase	↑ in EAE	8
Q13347	Similar to eucaryotic translation initiation factor 3, subunit 2 beta	↑ in EAE	16
P99024	Tubulin beta 7 chain	↑ in EAE	4
JC4894	Ubiquitin protein ligase	↑ in EAE	13
gi|619373 [2]	Zebrin ll	↑ in EAE	21

Table 3.9: PLP active EAE: list of proteins and their regulation in the spinal cord. Proteins detected by Coomassie staining. [2] stands for NCBI accession numbers. All the other proteins are named according the Swiss-Prot accesion number.

Accession no.	Protein name	Regulation	Sequence coverage (%)
Q8K4Z3	ApoA-1 binding protein	↑ in EAE	26
O88456	Calpain small subunit 1	↑ in EAE	14
P18760	Cofilin non muscle	↓ in EAE	20
P09528	Ferritin heavy chain	↓ in EAE	12
P14602	Heat shock protein beta 1	↑ in EAE	
Q99PL6	Leucine aminopeptidase	↑ in EAE	13
Q99LC3	NADH-ubiquinone oxidoreductase 42kDa	↑ in EAE	71
P28663	N-ethylmaleimide sensitive fusion protein	↑ in EAE	9
Q6PKE6	Platelet activating factor acetlyhydrolase 1b	↑ in EAE	8
Q8C6E9	Purine rich element binding protein A	↑ in EAE	16
P52480	Pyruvate Kinase M2-type	↑ in EAE	6
Q61598	Rab GDP dissociation inhibitor beta	↑ in E2 and E5	35
P17182	Similar to Alpha enolase	↑ in EAE	12
P09411	Similar to phosphoglycerate kinase 1	↑ in EAE	5
Q8VDQ8	Sirtuin 2, SIR2L2	↓ in EAE	2
Q8VDQ8	Sirtuin like protein 2	↑ in control	26
Q9CR38	Sorcin	↑ in EAE	29

Table 3.10: PLP active EAE: list of proteins and their regulation in the spinal cord. Proteins detected by ProQ staining. All proteins are represented by their Swiss-Prot accession numbers.

Accesssion no.	Description	Regulation	Sequence coverage (%)
Q61171	**Type II peroxiredoxin 1**	↑ in EAE	35
Q924B0	Myo-inositol monophosphatase 1	↑ in EAE	61
P07901	**Heat shock protein HSP 90-alpha**	↑ in EAE	48
P18872	**Guanine nucleotide-binding protein G(o) A1**	↑ in EAE	49
Q7TSH1	Arp3b-pending protein	↑ in EAE	24
P63328	**Serine/threonine protein phosphatase**	↑ in EAE	19
P51863	**Vacoular ATP synthase subunit d**	↑ in EAE	51
Q9CPV4	Glyoxalase 1	↑ in control	39
P42208	**Septin 2**	↑ in control	52
Q8K2T1	RIKEN cDNA 1110025F24	↑ in control	42
Q6PDM5	Dynamin	↑ in control	46
P09528	**Ferritin heavy chain**	↑ in EAE	31
P54227	**Stathmin 1**	modification	38
P18760	**Cofilin 1**	modification	56
Q9D7V0	Prostraglandin E synthase	↑ in EAE	50
Q9D0F9	**Phosphoglycerate mutase 1**	↑ in EAE	65
Q8JZR2	CrK protein	↑ in EAE	80
Q9RDP5	Destrin	↑ in control	41
P48036	**Annexin**	↑ in EAE	70
Q15365	Poly(rC)-binding protein 1	↑ in EAE	61
P05213	**Tubulin alpha 1 chain**	↑ in control	36

Table 3.11: Devic's EAE: proteins differentially regulated in the Devic's EAE brain. Proteins detected by Coomassie and ProQ are separated by a double line. Protein markers that overlapped with the wild type EAE models are highlighted in bold letters. All proteins are represented by their Swiss-Prot accession numbers.

3.1.3 Devic's EAE

The EAE group consisted of three transgenic animals that developed spontaneous Devic's disease with EAE score "4.0" (2 animals) and "2,5", respectively. Two transgenic animals without any symptoms of EAE (score = "0") were chosen as the control. In spite of the full-blown disease stage, the protein level differences between the EAE and control animals were fewer than those observed at the same disease stage of MOG or PLP-induced EAE in wild-type animals. Both Coomassie and ProQ staining detected protein level changes. Tables 3.11 and 3.12 list the proteins identified and their mode of regulation. Altered protein levels were detected, both in the brain and spinal cord. Moreover, Devic's EAE showed a proteome pattern similar to the wild type EAE. This similarity was also obvious in the phosphorylated proteins.

Accession no.	**Description**	**Regulation**	**Sequence coverage (%)**
Q99JB2	Stomatin like protein 2	↑ in EAE	48
O89053	Coronin actin binding protein A	↑ in EAE	38
P26040	**Ezrin, cytovillin**	↑ in EAE	26
P23492	**Purine nucleoside phosphorylase**	↑ in EAE	45
Q63826	**Dihydropyrimidinase related protein 2**	↑ in EAE	33
P14602	**Heat shock protein 27kDa**	↑ in EAE	41
Q8BPI0	**Rho GDP dissociation inhibitor 1**	↑ in EAE	38
Q8K0E8	**Fibrinogen, B beta polypeptide**	↑ in EAE	40
Q545W5	Glutathione S-transferase Mu 5	↑ in EAE	47
Q9CWZ7	**Gamma-soluble NSF attachment protein**	↑ in control	45
Q8BPI0	**Rho GDP dissociation inhibitor 1***	↑ in control	47
Q9R0P9	Ubiquitin carboxyl-terminal hydrolase*	↑ in control	53
P68371	**Beta tubulin**	↑ in control	32
P45591	**Cofilin 2**	↑ in EAE	33
Q8VDQ8	**Sirtuin 2**	↑ in control	44
Q8VDT0	Glycerol- 3 phosphate dehydrogenase	↑ in EAE	25
P63104	**14-3-3 Zeta**	↑ in EAE	53
P61163	Alpha centractin	↑ in control	31
Q9DBJ1	**Phosphoglycerate mutase 1**	↑ in control	66

Table 3.12: Devic's EAE: proteins identified in the spinal cord. Proteins detected by Coomassie and ProQ are separated by a double line. * indicates double protein hits of a single spot. Protein markers that overlapped with wild type EAE models are highlighted in bold letters. All proteins are represented by their Swiss-Prot accession numbers.

Protein name	**Accession no.**	**Cellular localization**	**Regulation**	**Sequence coverage (%)**
POR1_ mouse, voltage dependent anion selective	Q60932 [1]	Mitochondrial membrane	↑ in EAE	22
POR1_ mouse, voltage dependent anion selective	Q60932 [1]	Mitochondrial membrane	↓ in EAE	16
SIR2L2	AAG39256 [1]	Cytoplasmic	↓ in EAE	12
Malate dehydrogenase	P14152 [1]	Cytoplasmic, plasma membrane	↓ in EAE	12
Guanine nucleotide binding protein beta subunit	Q08706 [1]	Plasma membrane	↓ in EAE	10
Creatine kinase-B	AAA76851 [1]	Cytoplasmic, mitochondrial	↓ in EAE	14
Glyceraldehyde-3-phosphate dehydrogenase	NP_058704 [2]	Cytoplasmic, mitochondrial	↑ in EAE	11
Glyceraldehyde-3-phosphate dehydrogenase	XP_217701 [2]	Cytoplasmic, mitochondrial	↑ in EAE	4
Neurofilament light polypeptide*	XP_127760 [2]	Cytoplasmic	↑ in EAE	10
Phospholipasae B*	NP_620253 [2]	Cytoplasmic	↑ in EAE	2

Table 3.13: Proteins differentially regulated in the membrane fraction of EAE brain. * indicates double protein hits of a single spot. [1] and [2] stand for Swiss-Prot and NCBI accession numbers, respectively.

3.1.4 Membrane protein analysis

The membrane fraction of the brain proteins from one EAE, (MOG active EAE, Score "1.5"), animal and one control animal, (adjuvant treated), was isolated by gel electrophoresis for comparison. One dimensional gel electrophoresis gave a complex picture which was not suitable for comparative analysis. Therefore, we separated the membrane protein fraction by 2DE. The proteome pattern was different from the whole proteome pattern with a considerably smaller number of proteins. Most of the isolated proteins were in the molecular range between 25 and 100kDa. We detected some differences between EAE and control brain in their membrane protein fraction, (Table 3.13). Because of the large number of steps involved in the preparation of the membrane fraction, this experiment was carried out with only one EAE and one control animal. The identification of the protein spots revealed that some of the proteins were soluble cytoplasmic proteins, which is an indication of incomplete fractionation of the hydrophilic and hydrophobic protein fractions. The low abundance of very high molecular proteins could be due to the low permeability of the IPG strips.

Description	Accession no.	MOG-P	MOG-A	PLP-A	PLP-P	Devic	Regulation
Peroxiredoxin 2	Q61171		b, sc	b	b	b	↑
Apolipoprotein A-1	Q00623	sc	b, sc	b, sc	b, sc		↑
Capping protein alpha 1 +	P47753	sc	b, sc	b			↑
Guanine nucleotide-binding protein G(o) α 1 +	P18872		b, sc				↑
Eucaryotic initiation factor elf-4A11 +	P60843	sc	b, sc	sc			↑
Fibrinogen B beta polypeptide	Q8K0E8	sc	sc	b, sc	b, sc		↑
Fibrinogen gamma chain	Q8VCM7		b, sc		b, sc		↑
Isocitrate dehydrogenase	Q28480			b, sc	sc		↑
Phosphatidylethanolamine binding protein	P70296	b			sc		↑
Serum amyloid P component	P12246	sc	b, sc	b, sc	sc		↑
Triosephosphate isomerase	P17751			sc	b		↑
Proteasome activator complex subunit 1	P97371		sc	sc	b, sc		↑
Creatine kinase	P07310		b, sc		sc	b	↓
Septin 5 /2/6/8	Q6PB74		b	sc	b		↓
Ubiquinol-cytochrome c reductase	P47985	sc	b, c				↓
5'(3')-deoxyribonucleotidase	Q9JM14		b		b, sc		both
Actin gamma	P02571		b, c				both
Actin B	P60710		b, sc		b, sc		both
Tubulin +	Q13885		sc	b, sc	b		both
Dynamin 1	P39053		sc	b		b	both
Heat shock protein 86	P07900		b, c				both
Heat shock protein 90	P08238		b and sc			b	both
Phosphoglycerate mutase 1	Q9DBJ1		b	sc	b		both
Rho GDP dissociation inhibitor 1	Q8BPI0	sc			b, sc	sc	both
CRMP2	P47942			b		sc	both
ATP synthase D	Q9DCX2			sc		b	↓

Table 3.14: Common protein markers in the brain and spinal cord: The brain and spinal cord proteome pattern always varied in the same experiment. This variation was not consistent in the different models. Every protein that was identified at least once in the brain and spinal cord, independent of the models or stage of EAE are listed. ↑ and ↓ arrows stand for an at least two-fold increase or decrease in the intensity of the spots between EAE and control proteome. "Both" refers to an inconsistent variation of a protein spot in the different experiments. + indicates different isoforms or related proteins. b - brain, sc - spinal cord, P - passive induction of EAE, and A - active induction of EAE.

3.1.5 Differential regulation in the brain and spinal cord

Although inflammation and demyelination are known to occur predominantly in the spinal cord of EAE animals, our proteomics results implicated changes in both the spinal cord and in the brain. However, in accordance with the histopathology studies, changes observed in the brain were considerably fewer than in the spinal cord. This was more evident in the ProQ Diamond staining. The number of alterations varied depending on the model and stage of the disease. Variability among EAE animals in the same experiment was more pronounced in the brain than in the spinal cord. The proteome pattern of the brain and spinal cord differed within the same experiment. However, these changes were dependent on the experiment and could not be reproduced in different experiments. A good number of proteins were identified in both, brain and spinal cord in the different experiments, (Table3.14).

3.1.6 Common protein markers in EAE

Protein lists from the different experiments were compared to each other to select all proteins that were identified in more than one experiment in spinal cord and/or brain. Despite the difference in the known histopathological characterization of the different models we identified many proteins that were commonly regulated in the EAE animals. The majority of the common protein markers were identified in both, brain and spinal cord. The following tables (Tables 3.15, 3.16 and 3.17) lists the common protein markers in EAE.

Protein name	**MOG passive**	**MOG active**	**PLP passive**	**PLP active**	**Devic**	**Accession no.**
Apolipoprotein A-1		↑	↑	↑		Q00623
Peroxiredoxin 2 +		↑	↑	↑	↑	Q61171
Serum amyloid P-component		↑		↑		P12246
Phosphoglycerate mutase 1		↑	↓		↑	Q9DBJ1
Fibrinogen B beta polypeptide +			↑	↑		XP_130960
5'(3')-deoxyribonucleotidase		↑	↑			BAB25027
Eucaryotic initiation factor elf-4A11 +	↑	↑				P60843
Beta Tubulin 1 +		↓	↑	↑		Q7Z8N3
Actin B +		↓	↑	↑		P60710
Prohibitin			↑	↑		P67779
Capping protein alpha 1 +		↑		↑		P47753
Septin 2 +		↓	↓		↑	BAC25737
Heat shock protein 86		↓			↑	P07900
Serine/Threonine protein phosphatase 2A		↑			↑	P11493

Table 3.15: Proteins commonly regulated in the brain of different models of EAE. ↑ and ↓ arrows stand for an at least two-fold increase or decrease in the intensity of the spots between EAE and control proteome, respectively. Every differentially regulated protein in the spinal cord in more than one experiment is listed. + indicates different isoforms or related proteins.

Protein name	**MOG passive**	**MOG active**	**PLP passive**	**PLP active**	**Devic**	**Accession no.**
Chloride intracellular protein 1	↑	↑	↑	↑		Q9Z1Q5
Serum amyloid P component	↑	↑	↑	↑		P12246
Purine nucleoside phosphorylase	↑	↑			↑	P23492
Myeloid bactenecin	↑		↑			O08692
Rho,GDP dissociation inhibitor	↑		↓		↑	Q7SZS9
Heat shock protein 27	↑	↑	↑	↑	↑	P14602
Eucaryotic translation initiation factor 3 subunit 2	↑	↑		↑		Q13347
F-actin capping protein alpha-1 subunit	↑	↑		↑		Q7ZUR4
Fibrinogen beta polypeptide	↑	↑	↑	↑	↑	P14480
Cysteine, serine proteinase inhibitor	↑	↑				Q9D0S8
Coronin like protein	↑	↑	↑	↑	↑	P31146
Apolipoprotein 1	↑	↑	↑	↑		NP_033822
Septin 6	↑			↓	↑	Q9R1T4
Stathmin 1	↓	↓				P16949
Ubiquinol-cytochrome c reductase	↓	↓				Q9CR68
Proteasome activator complex subunit 1		↑	↑	↑		P97371
Alpha 1 macroglobulin		↑	↑	↑		Q61838
Creatine kinase		↓	↓			P07310
Peroxiredoxin 2		↑ ↓				P35704
Superoxide dismutase		↓	↓			AAH18173
Ezrin (Cytovillin) (Villin 2)		↑			↑	P26040
Dihydropyrimidinase related protein-2			↑		↑	Q62188
Soluble NSF attachment factor +			↓		↓	Q9CWZ7
Beta Tubulin 1 +		↓	↑	↑		Q7Z8N3
Actin B +		↓	↑	↑		P60710

Table 3.16: Common set of protein markers identified in the spinal cord of different models of EAE. ↑ and ↓ arrows stand for an at least two-fold increase or decrease in the intensity of the spots between EAE and control proteome, respectively. Every differentially regulated protein in the spinal cord in more than one experiment is listed. + indicates different isoforms or related proteins.

Accession no.	Protein name	MOG passive +	MOG active	PLP passive	PLP active	Devic	Regulation
P54227	Stathmin 1		sc	b, sc		b	dephosphorylation
Q544Y7	Cofilin non muscle		b, sc	b, sc	sc	b	hyperphosphorylation
P09528	Ferritin heavy chain		b, sc	sc	sc	b, sc	↑
AAG39256	Sirtuin 2		sc	sc	sc	sc	↓
P61982	14-3-3 gamma		sc	b, sc		sc	modification
P05063	Aldolase 3		sc	sc			modification
O88456	Calpain A1 small subunit*	b	sc	sc	sc		↑
Q8K4Z3	Apo A-1 binding protein*	b	sc	sc	sc		↑
P14602	Heat shock protein 27		b, sc	sc	sc		↑
AAA37430	2,3-cyclic-nucleotide 3-phosphodiesterase		sc	sc			↑
Q62048	PEA-15 protein	b	b	b			hyperphosphorylation
Q8C6E9	Purine rich element binding protein A			b	sc		modification
NP_058580	Heterogeneous nuclear ribonucleoprotein C		b, sc				modification
Q5M9P6	Rho gdp dissociation inhibitor alpha		sc	b			modification

Table 3.17: Differences in the phosphoproteome: a common set of differentially-regulated phosphoproteins detected by ProQ staining in the EAE brain (b) and/or spinal cord (sc). The differences are caused by a qualitative or a quantitative regulation. a) A higher or lower stoichiometry in phosphorylation was detected based on the intensity of the spots. b) A hyper- or hypophosphorylation of a protein is inferred from the migration and intensity patterns of the spots. Hyperphosphorylation is based on an additional (more acidic) spot or an increase in the intensity of the more acidic form with or without the decrease in the intensity of the more basic form. Hypophosphorylation appears as an additional basic spot or the loss (in intensity) of the more acidic spot with or without the gain of the more basic spot. ↑ and ↓ arrows stand for an at least two-fold increase or decrease in the intensity of the ProQ stained spots between the EAE and control proteome. A modification is inferred when a protein was found to be up-regulated in one experiment, (in the brain or spinal cord), but down-regulated in another experiment. * indicates double protein hit from a single spot. P - passive induction of EAE, and A - active induction of EAE. + Phosphostaining was not performed with MOG passive spinal cord protein.

3.1.7 Summary: EAE results

- The proteomes of different EAE models were examined.
- The brain and spinal cord of EAE animals underwent many cellular and molecular changes that were manifested as changes in the expression and modification of proteins.
- Regulation of proteins was detected both in the brain and in the spinal cord.
- The proteome patterns of brain and spinal cord differed within the same experiment but the nature of these differences varied.
- Comparison of the different models gave a common set of proteins regulated in both brain and spinal cord.
- Disease stage dependent alterations were detected.
- ProQ Diamond stain revealed the modification of many phosphoproteins.
- ProQ diamond staining enabled the detection of many low abundant proteins and increased the overall proteome coverage.
- Many proteins were commonly regulated in different models of EAE.
- We found common protein markers in the wild type and the transgneic Devic model of EAE.

3.2 Human specimen

Three different tissue sections, plaque, NAWM, and NAGM from MS patients, and two sections, NAWM and NAGM from controls, were used for proteomic analysis. Each group consisted of three specimens. In order to understand the pathological features of the obtained brain areas, cryo-sections of the plaques were characterized by immunohistochemistry. Histopathology characterized all the plaques as inactive plaques without inflammatory or demyelinating activity. Plaques of patient A showed remyelinating activity. (Histopathology was carried out in the laboratory of Dr. Wolfgang Brueck, University of Goettingen). Each sample was run on a separate gel without pooling.

3.2.1 Comparisons and protein markers

After 2DE, different comparison were performed, as shown below.

1. All MS plaques were compared to control NAWMs
2. MS plaque vs. MS NAWM
3. All MS NAWMs were compared to control NAWMs
4. All MS NAGMs were compared to control NAGMs

1. **MS Plaque vs. Control NAWM:** all MS plaques were grouped as one replicate set and compared to all control NAWM as another replicate group. Only the changes that were strictly consistent in all MS patients were chosen and identified. However, only very few changes met this criterion, (Table 3.18). Some of the spots were isoforms of the same protein, separated at different positions.
2. **MS plaque vs. MS NAWM:** The plaque of each MS patient was compared individually to their respective NAWM. The degree of variation between plaque and its surrounding white matter differed considerably between the subjects. Patient A had the maximum number of proteins that were altered between NAWM and plaque, while Patient C showed very few changes in the protein profile between the two tissue sections. Patient B had moderate changes. All proteins identified in patients B and C were also identified in patient A, (Tables 3.19, 3.20 and 3.21).

Accession no.	Description	Regulation	Sequence coverage (%)
Q92NT0	Sirtuin 2	↓ in MS	51
P07437	Beta 5-tubulin	↓ in MS	47
Q99747	soluble NSF attachment factor gamma	↓ in MS	23
Q71U36	Tubulin alpha 3	↓ in MS	33
P02794	Ferritin heavy chain	↓ in MS	75
Q06830	Peroxiredoxin	↑ in MS	61

Table 3.18: Plaque vs. control NAWM. Proteins that were consistently regulated in all three MS patients.

Accession no.	Description	Regulation	Sequence coverage (%)
P00441	Superoxide dismutase	↑ in plaque	23
P02792	Ferritin light chain	↑ in plaque	16
P32119	Peroxiredoxin 2	↑ in plaque	50
P09211	Glutathione transferase p	↑ in plaque	61
P04792	Hsp27KDa	Only in plaque	31
P18669	Phosphoglycerate mutase 1	Only in plaque	24
Cad79979	Unnamed protein	Only in plaque	22
P00441	Superoxide dismutase	↑ in NAWM	23
AAH50637	Tubulin alpha 3	↑ in NAWM	33
P04901	Guanine nucleotide binding protein	↑ in NAWM	18
Q9H115	Beta soluble NSF attachment protein	↑ in NAWM	68
O14805	RNA-binding protein regulatory subunit	↑ in NAWM	45
P32119	Peroxiredoxin 2	↑ in NAWM	50
P62879	Guanine nucleotide binding protein 2	↑ in NAWM	18
P07951	Tropomyosin 1 alpha	↑ in NAWM	15
P09493	Tropomyosin 2 beta	↑ in NAWM	11

Table 3.19: Patient A: all proteins that showed an altered level between plaque and NAWM.

3. **MS plaque and NAWM vs. Control NAWM (individual differences):** In order to take individual variations into account we made a further comparison of all plaques and NAWMs of the MS patients with the control NAWM. Any change in MS brain, (even if only in one patient), was identified, (Table 3.22). This matching additionally considered the changes taking place in the non-lesioned NAWM areas of MS brain. Surprisingly, with few exceptions almost all the changes were common to both plaque and NAWM. Changes specific to MS NAMW or plaques were also de-

Accession no.	Description	Regulation	Sequence coverage (%)
O14805	RNA-binding protein regulatory subunit	↑ in NAWM	46
P32119	Peroxiredoxin 2	↑ in NAWM	50
P62879	Guanine nucleotide binding protein	↑ in NAWM	18
P07951	Tropomyosin 1 alpha	↑ in NAWM	18
Q86W64	Tropomyosin 2 beta	↑ in NAWM	15
O75489	NADH dehydrogenase	↑ in NAWM	47

Table 3.20: Patient B: all proteins that showed an altered level between plaque and NAWM.

Accession no.	Description	Regulation	Sequence coverage (%)
P32119	Peroxiredoxin 6	↑ in plaque	50
P31945	Peroxiredoxin 2	↑ in NAWM	28
P21266	Glutathione S transferase Mu	↑ in plaque	69

Table 3.21: Patient C: all proteins that showed an altered level between plaque and NAWM.

tected.

In accordance with the plaque to NAWM comparison, patient C was an exception with respect to the regulation of many proteins. Most of the changes were consistent in both patients A and B. Interestingly, there were examples of protein level alterations, (present in patients A and B), where the change was observed in the plaque, but not in the NAWM of patient C, (Figure 3.7). Besides this, some protein regulations specific to one of the patients, were also observed.

4. **MS NAGM vs. control NAGM:** We did not detect any protein that was consistently regulated in all MS NAGMs, compared to the control NAGM. Individual differences in the gray matter were not analyzed.

3.2.2 Summary: human specimen results

- Unlike animal experiments studies with human specimens are subject to individual variations because of the heterogeneous background.
- Only a few changes were consistently found in all three patients.

Accession no.	Description	Sequence coverage %	General regulation	Individual regulation		
				Patient A	Patient B	Patient C
P62259	14-3-3 protein epsilon	34	↓ in MS	P and NAWM	P and NAWM	
AAH10571	Brevican isoform 1	29	↓ in MS		P and NAWM	
P28161	Glutathione S-transferases Mu 2	42	Not definite	P and NAWM		
O75947	ATP synthase D chain	55	↑ in MS	NAWM	NAWM	NAWM
P02794	Ferritn heavy chain	46	↓ in MS	P and NAWM	P and NAWM	P
P06396	Gelsolin precursor	36	↓ in MS	P and NAWM	P and NAWM	P and NAWM
AAD20046	Sirtuin2	67	↓ in MS	P and NAWM	P and NAWM	P and NAWM
P04792	Heat shock protein 27	57	↑ in MS	P and NAWM		P and NAWM
P30041	Peroxiredoxin 6	43	↓ in MS	NAWM	NAWM	NAWM
P09417	Dihydropterine reductase	57	↓ in MS	P and NAWM	P and NAWM	
P02511	Alpha crystalline chain B	34	↓ in MS	P and NAWM	P and NAWM	
Q06830	Peroxiredoxin 1	61	↑ in MS	P and NAWM	P	P and NAWM
P00441	Superoxide dismutase	16	Not definite	P and NAWM		
P16949	Stathmin 1	24	↓ in MS	P and NAWM		
O14805	RNA-binding protein regulatory subunit	45	↓ in MS	P	P	
P62879	Guanine nucleotide binding protein	14	↓ in MS	P	P	

Table 3.22: All proteins that were different between patient Plaque and/or NAWM and control NAWM. Individual differences were taken into account; P - the difference was observed only in plaque; P and NAWM, same difference in both, plaque and NAWM; Not definite - the change was not in the same direction in plaque and NAWM.

- The patients varied considerably in the extent of changes between plaque and the neighboring non-lesioned white matter.
- Most protein regulations in the MS plaque were also observed in the MS NAWM.
- Most of the alterations, (MS plaque and NAWM vs. control NAWM), were

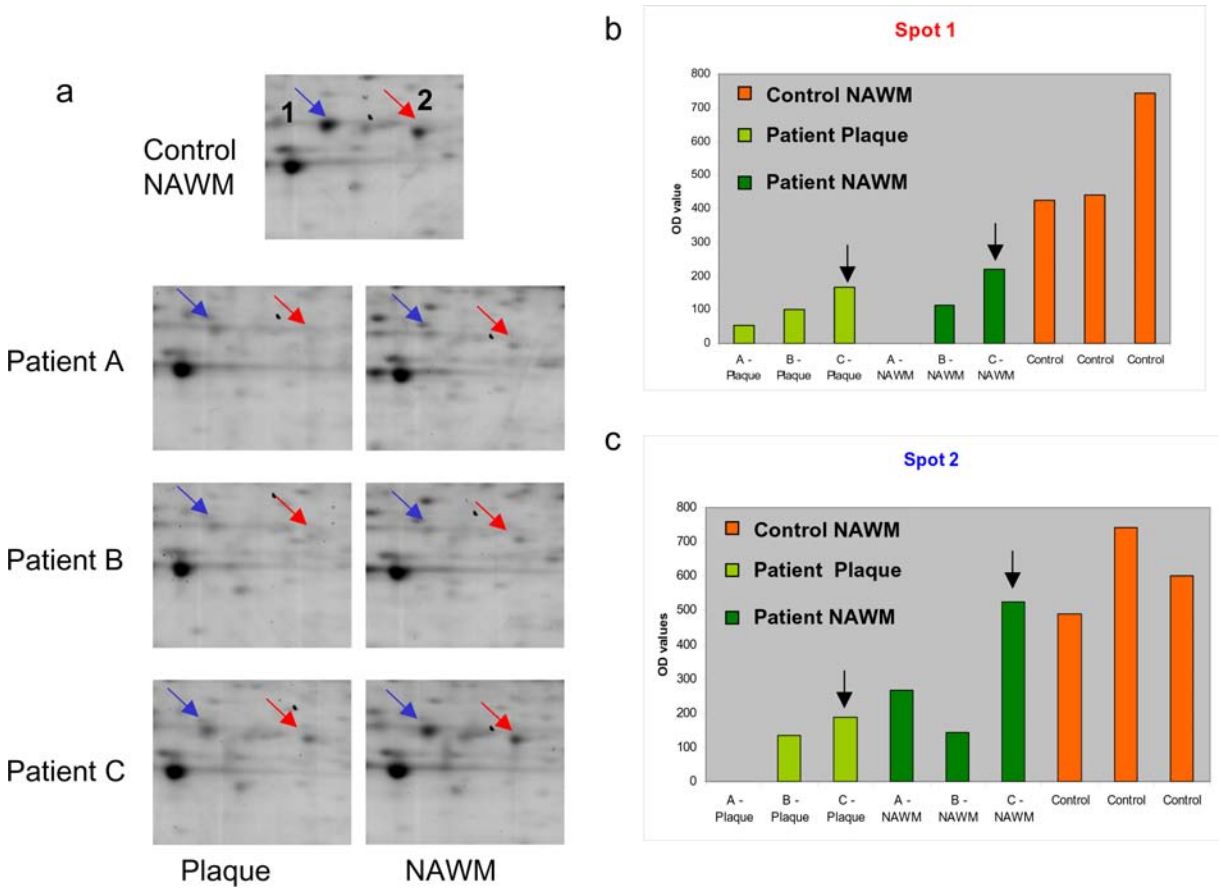

Figure 3.7: Individual variations in the regulation of proteins in MS patients. Patient C was an exception in most cases with respect to the protein pattern. a) Spot 1 is down-regulated in the plaque and NAWM of MS patients A and B. Down-regulation of spot 2 is not extended to the NAWM of patient C. b) and c) Graphs showing the intensity of spots 1 and 2.

consistent in patients A and B.

- Patient C was an exception in most cases, (Figure 3.7). Patient C had the least changes between plaque and NAWM, as well as the least variation from the control brain. In some cases the changes were observed in the plaque of patient C but not extended to the NAWM.

Description	Human	MOG passive	MOG active	PLP passive	PLP active	Devic
Sirtuin 2	↓		↓ P	↓ P	↓ P	↓ P
Beta 5-tubulin +	↓	↓	↓	↓		↓
Soluble NSF attachment factor +	↓			↓	↑ P	↓
Ferritin heavy chain	↓		↑	↑		↑
Peroxiredoxin 2 +	↑		↑			↑
Stathmin 1	↓	↓	P	P		P
heat shock protein 27	↑	↑	↑	↑	↑	↑
superoxide dismutase			↓	↓		
Glutathione S transferase	↑					↑
Phosphoglycerate mutase 1	↑		↑	↓		P
14-3-3 epsilon +	↓ P					↑ P
Guanine nucleotide binding protein +	↓					↑
ATP synthase 5 +	↑			↑p	↑	↑

Table 3.23: Common markers in EAE and MS, p - phosphorylated proteins, + - isoforms or related proteins

3.3 Protein markers common to EAE and MS

Remarkably, we were able to find a set of markers that were regulated in both EAE and MS. Proteins differentially regulated in MS and EAE are given in Table 3.23.

3.4 Selected protein markers and their validation

Serum amyloid P component

One of the most prominent protein level differences between EAE and control animals in all the experiments was the over-expression of a 25kDa protein identified as serum amyloid P component (SAP). SAP was overexpressed both in the brain and spinal cord of all the EAE animals in the different models. It was absent or expressed only at undetectable levels in all control animals. The most noticeable result is the high expression of SAP even in animals with a disease score as low as ”1.0”, (tail impairment only). This makes SAP a very early protein marker of EAE. Over-expression of SAP in EAE was confirmed by immunoblotting, (Figure 3.8b). The degree of variation in SAP levels between EAE and controls was dependent on the experiment.

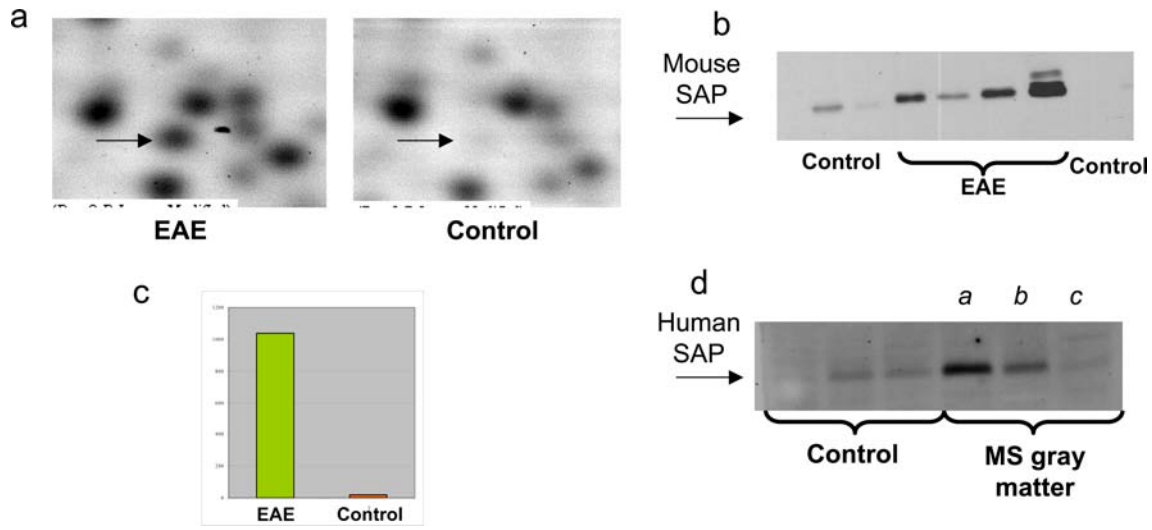

Figure 3.8: Up-regulation of SAP in the EAE spinal cord demonstrated by (a) 2DE, (b) immunoblotting. c) Graphical representation of the regulation of SAP in EAE detected by 2DE. d) Elevated SAP levels in the gray matter of 2 out of 3 MS patients. *a*, *b* and *c* represent the three individual patients.

The consistent overexpression of SAP in EAE animals motivated us to look for its regulation in MS brain using an anti-human SAP antibody. We detected up-regulation of SAP in the brain normal appearing gray matter (NAGM) of two out of three MS patient, (Figure 3.8). Intriguingly, the patient without SAP antibody reactivity was already inferred to be different from the other two MS patients based on the 2DE results. Up-regulation of SAP was restricted to gray matter and not extended to plaque or NAWM.

Sirtuin 2

In human MS brain we found a significant and consistent loss of sirtuin2 (SIRT2) protein in the diseased tissue. Changes in SIRT2, compared to the NAWM of the control brain, was observed in the plaque of all three and MS patients and in the NAWMs of patients A and B. The human SIRT2 spots were not detected in the ProQ stain. SIRT2 isoforms separated on three adjacent spots on 2DE gel with almost the same molecular weight. Two of these spots were down-regulated in MS plaques and NAWMs. Loss of SIRT2 was also observed to a minor extent in the gray matter of two patients. In the animal experiment, we consistently observed a loss of the phosphorylated form of sirtuin in the spinal cord of EAE. SIRT2 corresponded to two adjacent spots differing slightly in their molecular weight. Both spots were stained by ProQ, indicating the phosphorylation in the

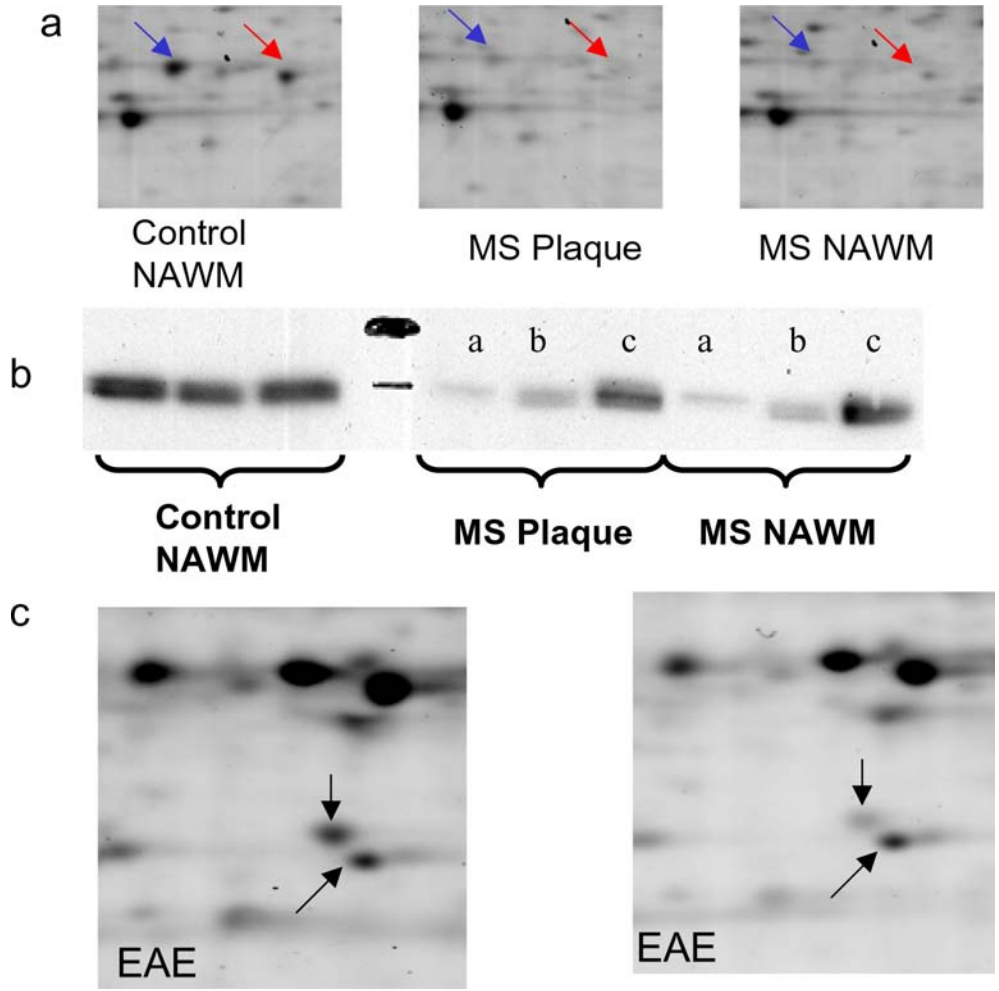

Figure 3.9: Regulation of sirtuin 2 in EAE and MS. a) Loss of two sirtuin 2 isoforms in MS plaque and NAMW. b) Loss of two sirtuin isoforms in MS demonstrated by immunoblotting, NAWM of patient C is an exception. c) Down regulation of sirtuin 2 in EAE. Two phopshorylated isoforms were detected in EAE, one was down-regulated.

mouse spinal cord. Changes in the ProQ stain were detected in both PLP and MOG models of EAE. Additionally, our immunoblotting results also replicate the loss of sirtuin isoforms in MS. Figure 3.9 summarizes these results.

Collapsin response mediator proteins

Two members of the collapsin response mediators protein (CRMP) family, namely CRMP2 (DRP2) and CRMP4 (DRP3), were identified as differentially regulated in EAE. Each protein migrated to two different positions. The two

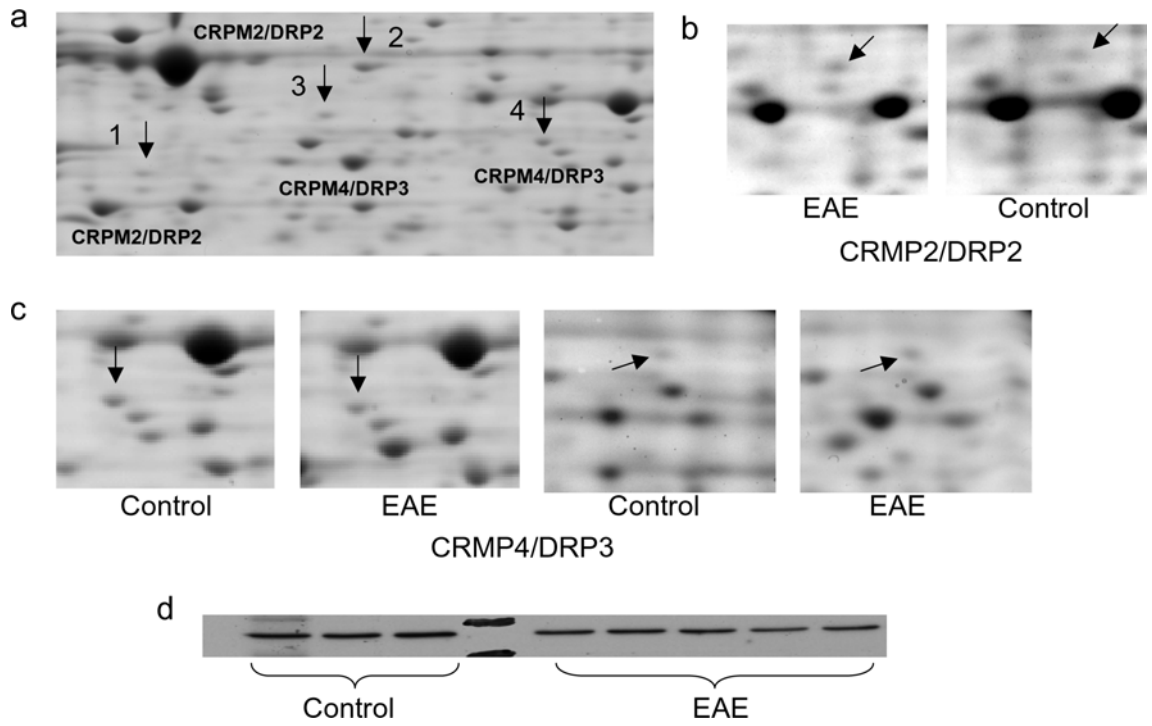

Figure 3.10: a) Collapsin response mediator proteins (CRMP) on a 2DE gel. Two different CRMPs, (CRMP2 and CRMP4), were observed to be regulated in EAE CRMP2 and CRMP4. CRMP2 and CRMP4 were identified at two different positions on the 2DE gel. b) Up-regulation of the more basic CRMP2 in EAE. c) The more basic isoform of CRMP4 was down-regulated in passive PLP EAE, whereas the more acidic isoform was up-regulated in active PLP EAE. d) Down-regulation of CRMP4 in the EAE spinal cord, detected by immunoblotting.

Experiment	Animals	Tissue and stain	CRMP/DRP	Regulation	Spot
Devic	3/3	spinal cord - C	CRMP2/DRP2	↑	1
PLP active	3/5	brain - P	CRMP2/DRP2	↓	2
PLP active	2/2	spinal cord - C	CRMP4/DRP3	↑	3
PLP Passive	3/3	spinal cord - C,P	CRMP4/DRP3	↓	4

Table 3.24: Regulation of CRMPs in EAE experiments, P and C represents ProQ and Coomassie stain, respectively.

CRMP2 spots differed in their molecualr weight. All spots were visible in the ProQ stain, indicating the phosphorylation modification of these molecules. We observed a four-fold increase of the more acidic CRMP2 spot in the Devic's model of spontaneous EAE. In contrast, in the brain of PLP active EAE animals, with disease scores "2.5" or above, the more basic DRP2 was down-regulated. Similarly, CRMP4 was also identified in two different positions on

the 2DE gel. In PLP active EAE the acidic spot representing CRMP4 was up-regulated, whereas in PLP passive EAE the more basic spot was down-regulated. Down-regulation of CRMP4 in the PLP passive experiment was seen in both the Coomassie and ProQ stain. Western blotting confirmed the slightly lower protein level of CRMP4 in the spinal cord of the EAE animals, (Figure 3.10 and Table 3.24).

Stathmin 1

We identified stathmin as a protein that was down regulated in the spinal cord of MOG passive EAE animals, (Figure 3.11a). The degree of regulation was moderate but consistent in all the animals. In the human experiment we detected a down-regulation of stathmin in the plaque and NAWM in one out of 3 MS patients in the Coomassie stain (data not shown).

In other studies changes in stathmin were detected only in the ProQ stained images. In MOG active EAE animals, (disease score ”1.0” - ”2.5”), stathmin was detected in one ProQ stained spot which was down-regulated in all EAE animals, (Figure 3.11b). The animal with a disease score of ”1.0” had the lowest degree of change in the stathmin level compared to the controls.

In passive PLP EAE, (disease score ”3.0”), stathmin migrated to 3 different positions, (Figure 3.11c). All spots were visible in the ProQ stained image but not in the Coomassie image. The staining intensity of the spots varied. One of the spots (spot 3) gave a very weak signal. In the first dimension the spots migrated as double and single spots at different positions with the same MW and a pI difference of less than 0.5 pH unit. One of the spots in the doublet migrated at a lower MW than the other spots, (Figure 3.11c).

The intensity of the stathmin spot at the most basic position on the gel was markedly reduced in the EAE animals. Of the two more acidic spots the one with the higher molecular weight was less in EAE, but the other spot, with a lower MW, was present only in EAE. The latter pattern of the two acidic spots was also observed in the brain of Devic’s EAE animals and MOG active animals with an advanced disease score.

We tested the endogenous levels of stathmin using an antibody directed against the residues flanking amino acid 38. In Western blot analysis we detected a reduced stathmin level in EAE (PLP passive animals), (Figure 3.12). Consistent with this we also observed down-regulation of another protein that bound to the stathmin antibody, (MW 60-70 kDa), in EAE animals, (data not shown). This protein is persumably a stathmin-related protein that is abundant in mouse brain.

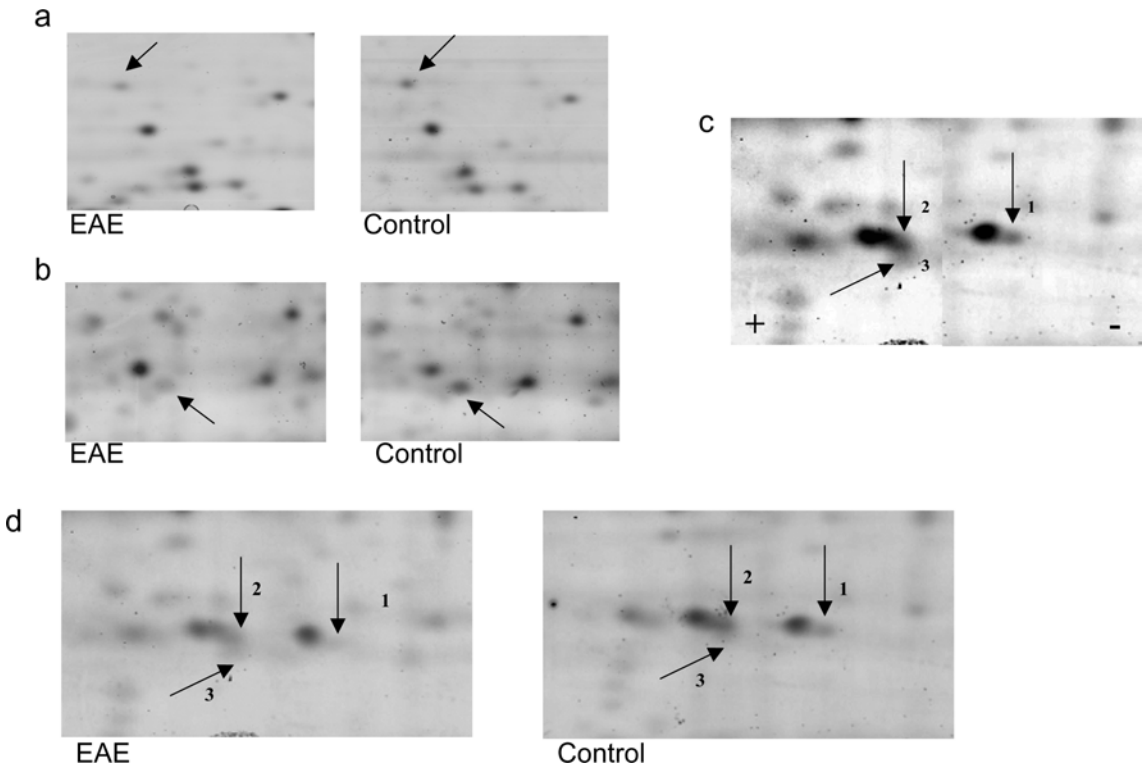

Figure 3.11: Stathmin isoforms and their regulation a) Down-regulation of stathmin 1 in the spinal cord of EAE, detected in the Coomassie stained image of MOG passive EAE experiment. b) Down-regulation of stathmin 1 in EAE, detected in the ProQ stained image of MOG active EAE experiment. c) Three stathmin isoforms were present in the mouse spinal cord. All three spots were visible only in the ProQ stained image. Spot 3 has very low intensity in the ProQ stain. Spots 1 and 2 have the same or very similar molecular weights. Spot 1 migrated to the most basic position. Spots 2 and 3 have the same pI but different molecular weights. Spot 2 is more phosphorylated than the other spots due to its high intensity in ProQ stain and its acidic position on the gel. The position and intensity of spot 1 implicates the lower degree of phosphorylation of this p-stathmin isoform. d) Difference in the levels of different stathmin isoforms in EAE. Spot 1 is down regulated in EAE. Among the doublet 2 and 3 the level of 2 is reduced, whereas spot 3 is present only in EAE. The reduction in the intensity of spot 2 (the most intense and the most acidic stahmin spot) and 1 (moderately intense) is an indication of a loss of these two phosphorylated stathmin isoforms in EAE. Spot 3 with its lower MW and very weak ProQ staining is suggestive of the presence of a hypophosphorylated isoform of stathmin in EAE. Spot 3 could also be the product of a proteolytic cleavage at the N-terminus of stathmin containing all the phosphorylation sites.

We also tested the level of stathmin using another antibody directed against the carboxy-terminus of the protein. The stathmin antibody detected two bands, (under 20 kDa). Detectable levels of the lower band (Band 1) were present only in a few lanes. Moreover, the lower band was much weaker than the upper one,

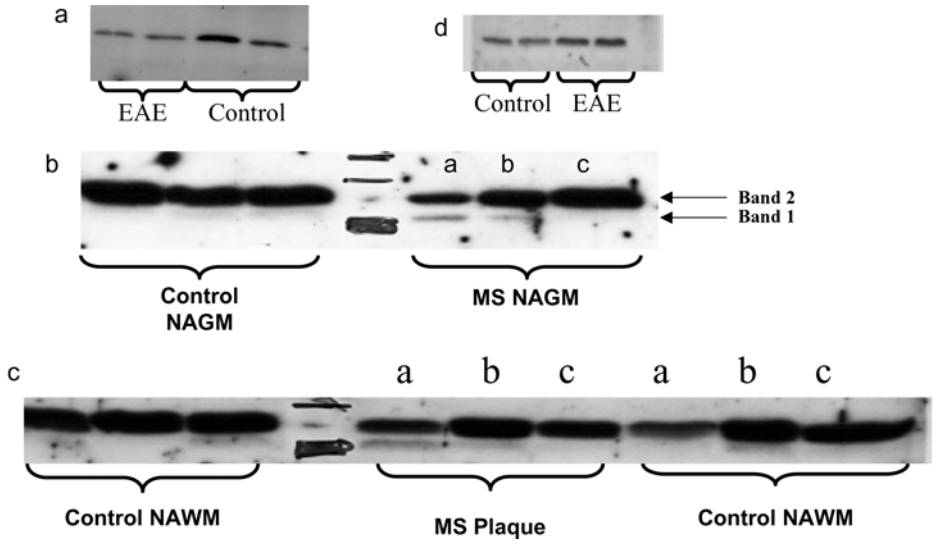

Figure 3.12: Stathmin regulation in EAE and MS, validated by Western blotting. a) Immunoblotting of stathmin using an antibody directed against the N-terminus of stathmin showed its down regulation in EAE. b) and c) Stathmin detection in MS brain using an antibody directed against its carboxy-terminus. The antibody bound to two bands. b) band 1 is up-regulated in the gray matter of two MS patients. Band 2, on the other hand, is down-regulated in the gray matter of patient A. c) Band 1 is up-regulated in the plaque of patient A and band 2 is down regulated in plaque and NAWM of patient A. d) Detection of stathmin in EAE using the antibody against its carboxy terminus. The antibody bound only to one band, which was up-regulated in the EAE spinal cord.

Figure 3.12b. The upper band (Band 2), on the other hand, gave a very intense signal in all controls and MS patients. Band 1 was up-regulated in the gray matter of two (Patients A and B) out of three MS patients, (Figure 3.12b). This band was present only at undetectable levels in all the other lanes. In contrast, band 2 was down-regulated in patient A. In plaque a distinct elevation of band 1 was observed only in patient A. a very minor increase of band 1 was also seen in patient B. A different pattern was observed in the NAWM. Band 1 was up-regulated in the NAWM of patient B but not in the other patients. Band 2 was down-regulated in the plaque and NAWM of patient A, (Figure 3.12c).

Immunoblotting of mouse spinal cord with the same antibody detected only one band. In contrast to the N-terminal directed antibody we observed an increased stathmin level in EAE, (Figure 3.12).

3.4.1 Summary: selected protein markers

- SAP was highly up-regulated in the brain and spinal cord of EAE animals.

- SAP was also up-regulated in the NAGM of 2 MS patients.
- One of the two phosphorylated isoforms of sirtuin 2 was down-regulated in the EAE spinal cord.
- Two sirtuin 2 isoforms were down-regulated in the plaque of all MS patients and in the NAWM of patients A and B.
- Migration and intensity patterns of CRMP2 and CRMP4 in the different experiments indicate a hyperphosphoylation of the two proteins.
- Immunoblotting suggests a minor down-regulation of CRMP4 in the EAE spinal cord.
- A loss of phospho-stathmin isoforms and the presence of an un/hypophosphorylated stathmin spot in EAE was observed.
- Immunoblotting using an antibody directed against the N-terminus of stathmin detected its down-regulation in EAE and in patient A.
- An antibody specific to the carboxy terminus of stathmin showed its up-regulation in EAE.
- In MS patients the same antibody revealed the up-regulation of a stathmin isoform.
- Up-regulation of the stathmin isoform was more pronounced in the NAGM of patients A and B.
- A second stathmin isoform was down-regulated in the plaque, NAWM and NAGM of patient A.

3.5 Functional classification and pathway analysis

All identified proteins were searched in the public domain, (Swiss Prot (www.expasy.org) and Human Protein Resource Database (HPRD) (www.hprd.org)), for their molecular properties and functional classification. Gene Ontology and HPRD databases categorized the protein markers as proteins involved in biological processes such as cell growth and maintenance, energy metabolism, protein regulation and metabolism, signal transduction, nucleoside regulation and immune response. The interaction between the different biomarkers and their involvement in different molecular pathways were further examined

using the literature-based pathway analysis software, Pathway Studio (Ariadne Genomics). Many of the identified markers interact directly or indirectly in cellular pathways that converge to cytoskeletal proteins such as, actin and tubulin. The most consistent and functionally relevant proteins were further studied in detail by literature mining. Closer inspection affirmed the possible involvement of some markers in diverse disease processes such as, immune response, demyelination or remyelination inhibition, protease activity, mitochondrial and energy dysfunctions, neurotoxicity, axonal damage, and neurodegeneration, (Table 3.25).

Description	**Accession no.**
Immune response	
Astrocytic phosphoprotein	PEA-15
Proteasome activator 28-subunit alpha	PA28 alpha
Ferritin heavy chain	FTH1
De/remyelination	
2',3'-cyclic-nucleotide 3'-phosphodiesterase	CN37
Stathmin 1	STMN1
Ferritin heavy chain	FTH1
Protease activity	
Cathepsin B precursor	CTSB
Calpain small subunit one	CPNS1
Ubiquitin carboxyl-terminal hydrolase 15	USP15
Ubiquitin carboxyl-terminal hydrolase isozyme L3	UCHL3
Proteasome activator 28-subunit beta	PSME2
Protease inhibitors	
Myeloid Bactenecin	NPG
Phosphatidylethanolamine-binding protein 1	PBP
Serine (or cysteine) proteinase inhibitor, clade B, member 1a	SERPINB1a
Calcium binding	
Calcium/calmodulin-dependent protein kinase kinase 2	CAMKK2
Spectrin alpha chain, brain	SPTAN1
Protein phosphatase 2B regulatory subunit 1	PP3R1
F- actin capping protein alpha 2	CAPZA2
Calpain small subunit one	CPNS1
Oxidative stress	
Superoxide dismutase 2	SOD2
Peroxiredoxin	PRDX
3-mercaptopyruvate sulfurtransferase	MPST
Glutathione s transferase Mu isoform	GST Mu
Ferritin Heavy Chain	FTH1

Mitochondria and energy damage	
Creatine Kinase B	CKB
Ubiquinol-cytochrome-c reductase complex core protein I	UQCRC1
Ubiquinol-cytochrome c reductase iron-sulfur subunit 1	UQCRFS1
Lactate dehydrogenase B Chain	LDHB
Neurotoxcity	
Serum amyloid P component	SAP
Chloride intracellular Channel protein1	CLIC1
Ferritin Heavy Chain	FTH1
Axonal integrity and transport	
Stathimin 1	STMN1
Sirtuin 2	SIRT2
Collapsin Response Mediator Protein	CRMPs
N-ethylmaleimide sensitive fusion attachment proteins	SNAPs
NSFL1 cofactor p47	NSFL1
Cofilin 1	CFL1
Axonal retraction	
Collapsin Response Mediator Protein 2	CRMP2
Cofilin1	CFL1
Rho GDP dissociation inhibitor alpha	RHO-GDI
Regeneration inhibition	
Collapsin Response Mediator Protein 2	CRMP2
Collapsin Response Mediator Protein 4	CRMP4
Stathmin 1	STMN1
Sirtuin 2	SIRT2
Ferritin heavy chain	FTH1

Table 3.25: Disease process specificity of selected biomarkers. Accession numbers follow the Swiss-Prot protein nomenclature.

Chapter 4

Discussion

4.1 General Discussion

The clinical outcome of MS is the result of a combination of several disease processes such as inflammation, demyelination, axonal damage, apoptosis, neuronal loss and neurodegeneration. In order to reduce or prevent the long-term disability that accumulates in MS, the diverse processes that contribute to the dysfunctions, need to be treated. Our aim was to identify disease process-specific protein markers that can give insight into the exact mediators of these mechanisms. These markers can serve as therapeutic targets, diagnostic markers, or as molecules that can help monitor the effect of drugs. We used the target organs, brain and spinal cord tissue in murine EAE as the primary source of specimen in the disease marker discovery phase of MS.

We examined the proteomes of three murine models of EAE such as MOG (SJL background), PLP (SJL background) and transgenic Devic's EAE (C57BL/6, background) in eight individual experiments. Animals belonging to different disease stages such as early, middle and full-blown disease, were included in the experiments. To demonstrate the significance and association of the proteins identified in the animal model for human MS, we extended the protein screening to human MS brain specimens. The brain proteomes of 3 MS patients were compared to 3 control brain specimens, diagnosed as not having MS or any other neurological disorder.

Although a few studies have already reported on a large scale transcript and protein analysis in EAE, this is the first time a comparison of different models of EAE has been carried out. Additionally, we looked at the regulation of proteins at the phosphorylation level. Repeated analysis in different animal models in-

creased the proteome coverage and helped us to replicate the results. All protein hits, reproduced in more than one experiment were accepted as definite protein markers.

Cellular protein pools that are separated on the 2DE gels mainly consist of cytosolic proteins. Classification of the proteins, that we identified based on their cellular localization, confirmed the enrichment of cytosolic proteins. We also identified minor sets of membrane and nuclear proteins. Disease marker discovery, using 2DE analysis, is partially incomplete as the method is not well-suited for the separation of many functionally important molecules like membrane proteins. However, due to the lack of a standardized method for the extraction and separation of membrane proteins or nuclear proteins for comparative analysis, we concentrated mostly on the cytosolic fraction. Moreover, limited specimen availability is another impeding factor in carrying out prefractionation of cellular compartments. We increased the proteome coverage by introducing an additional phosphoprotein analysis. More than half of the proteins that we detected by ProQ staining are known phosphorylated proteins. As most of these proteins are otherwise below the detection level, ProQ staining with its fluorescent property has enhanced the proteome coverage substantially, enabling us to compare the expression level of very low abundant proteins.

MALDI-TOF-TOF and ESI-ion trap measurements were complementary to each other in identifying the very low intense proteins. While MALDI gave a quick protein identity, ESI-ion-trap measurement with a better fragmentation processturned out to be more suitable for the identification of very low abundant proteins, (ProQ stained spots). Combining different search algorithms like MASCOT and SEQUEST helped us to gain great confidence in our protein identification without missing useful spectra information due to the bias of specific search algorithms. Manual inspection of the spectra was also helpful in avoiding false positives.

Because the brain and spinal cord are the target organs in EAE, we used the complete protein pool of the brain and spinal cord for protein analysis. The exact cell types that contribute to the identified protein markers remain uncertain. Altered regulation of the proteins can be the result of disease-specific changes taking place in neurons or glial cells. Especially in the case of cytoskeleton-organizing molecules careful examination of the cell type responsible for these modulations should follow. Some of the markers that we found were also associated with the phenotype of reactive astrocytes. Astrocytes are highly abundant in the central nervous system and their response to CNS injury can promote or impede disease progression or regeneration after injury (Egnaczyk et al., 2003). Infiltrating cells can also account for the changes observed. However, since our

analysis was not restricted to lesions alone we have diminished the effect of the infiltrating cells.

Proteome analysis of multiple murine EAE models and MS has led to the identification of many gene products that underwent change in expression or post-translational modification due to the disease process. Despite the difference in the EAE models we identified many proteins that were commonly regulated in the brain and spinal cord of EAE animals. Animals with a uniform disease score within the same experiment had a similar or identical proteome pattern. Moreover, we found a disease stage-dependent variation in the degree and number of protein level changes. In the human study we detected protein regulation consistent in all patients, as well as individual variations. Remarkably, despite the variability, we were able to show the regulation of a set of proteins common in EAE and MS.

In contrast to the EAE experiments, studies with human specimens are subject to individual variations because of the heterogeneous genetic background. The diversity is also contributed to by the well-known heterogeneous disease patterns in MS that vary in patient subtypes. Clinical classification of the tissue specimens based on the histopathological characteristics, as well as age and gender matching are very important. Moreover, variation in the post-mortem interval can lead to variable degrees of protease activities, which can result in an altered proteome pattern. Validation in a vast number of patients is a prerequisite before assigning molecular signatures to patient subpopulations.

An interesting finding was that most of the protein level alterations occurring in the plaque were also observed in the NAWM. Changes taking place in plaque and NAWM emphasize the contribution of brain areas that are proximal or distal to the plaque formation to the progression and, even to the cause of the disease (Mastronardi and Moscarello, 2005).

Patient C was an exception as was evident from both 2DE and Western blot analyses. The relatively few changes in patient C are either a sign of remission at the time of death, or disease processes that are different in this patient. Protein expression changes similar to the other patients, (present both, in plaque and NAMW), in the plaque of patient C, but not in the NAWM, can be interpreted as an indication of some NAWM changes taking place as an extension of the plaque activity.

As already highlighted, MS has a highly complex and heterogeneous pathogenesis where multiple related or independent disease processes contribute to the progress and worsening of the disease. Our aim was to identify disease process specific protein markers that can give insight into the exact mediators of these mechanisms.

All regulated proteins were classified according to their known molecular functions and the biological processes that they take part in. Proteins that were prominently and consistently regulated in most of the experiments were further investigated by extensive literature searches. Thorough literature mining helped us to associate some of these proteins to pathways that possibly contribute to the pathogenesis of MS. Additionally, we were also able to establish the interactions between some of these proteins, which enabled us to put forward some molecular mechanisms that might be responsible for disease progression in MS.

A majority of the selected proteins that we identified are involved in maintaining microtubule and actin filament organization. These proteins have important roles in regulating axonal integrity, as microtubules and microfilaments are major components of the axonal cytoskeleton. Regulation of expression and phosphorylation of these developmentally important molecules are suggestive of rearrangements of cytoskeletal elements taking place in EAE, and possibly also in MS.

Proteins that can contribute to neuronal death, either directly or indirectly, constitute another set of markers. MS is being recognized as a neuronal disease with neuronal damage and loss. Identification of the molecular mediators of neuronal death enables a focused and contained blockade of these molecules in order to limit, or repair neuronal death, without affecting other mechanisms.

Another group of proteins reflected of oxidative stress taking place in MS, and the induction of protective mechanisms and molecules to cope with this process. The most consistent difference between the plaque and NAWM of all MS patients was the expression and modification of antioxidative proteins like peroxiredoxin. Oxidative stress is a well-accepted process in MS (Gilgun-Sherki et al., 2004). The high lipid content, high rate of oxidative metabolism, and high iron content make the brain the organ most susceptible to oxidative stress.

The inflammatory component of EAE or MS was not represented much in the list of markers. Some of the predominantly regulated inflammation-related proteins include annexin 1 (an immunosuppressant), myeloid bactenecin, coronin like protein and proteasome activator complex subunit 1 (PA28 alpha).

In EAE we found the activation of PA28 alpha, a protein which has an essential function in MHC class 1 restricted antigen processing. PA28 alpha was consistently overexpressed in the brain and spinal cord of most of the EAE animals. Moreover, we also observed proteins specific for astrocyte and microglial activation. Astrocyte phosphoprotein (PEA-15) showed hyper-phosphorylation. The phosporylation of PEA-15 has an essential function in protecting astrocytes from TNF-induced apoptosis.

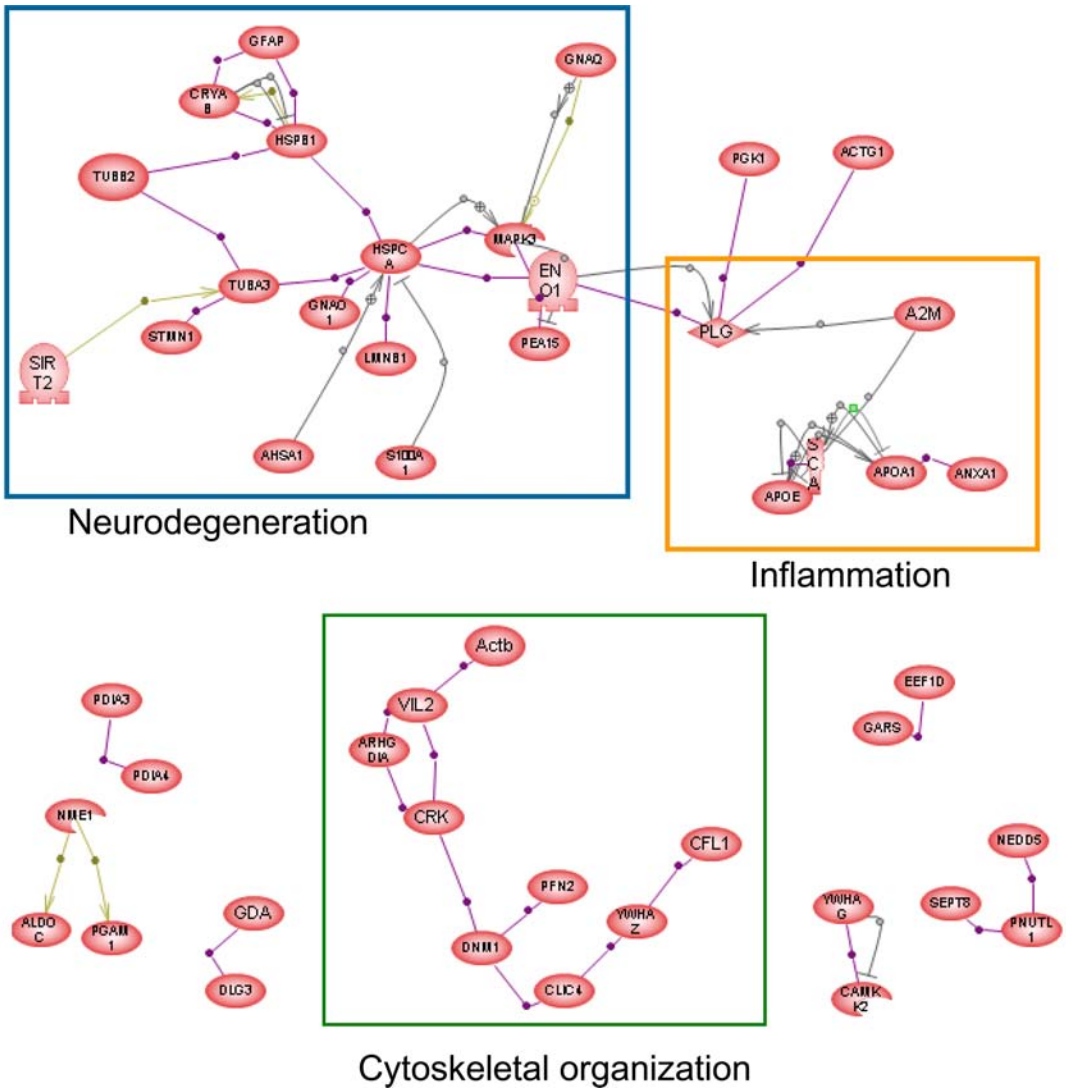

Figure 4.1: Interaction between protein markers based on literature mining tools. Proteins identified in all the different experiments were included in the pathway analysis. Proteins are named according to the Swiss-Prot nomenclature (gene name). In order to reduce the complexity of the interactions only the direct interactions between the proteins are shown in the figure. Many of the identified protein markers interact directly in cellular pathways that converge to cytoskeletal proteins. The figure is suggestive of a cross talk between the inflammatory (yellow box) and neurodegenerative processes (blue box). Most of the proteins in the blue box have been associated with neurodegenerative diseases. This scheme was created using the software Pathway Studio. Descriptions of the protein identity abbreviations used in this figure are given in Appendix B, (Table B.2).

Upon stimulation by TNF, astrocytes produce TNF and express TNF recep-

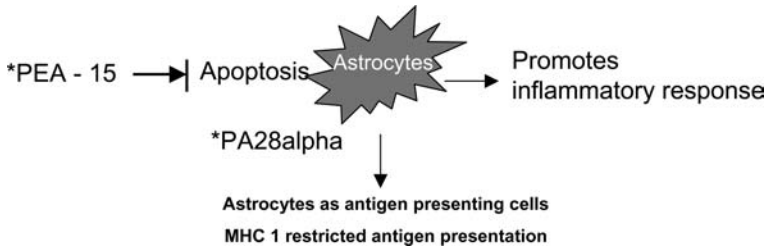

Figure 4.2: Inflammation specific protein markers

tors triggering several responses such as cell proliferation, up-regulation of TNF mRNAs, production of interleukin 8, macrophage-granulocyte colony stimulating factors (Pierre et al., 1996). However, TNF can also trigger apoptosis in astrocytes. Expression and phosphorylation of PEA-15 prevents TNF-induced apoptosis in astrocytes (Kitsberg et al., 1999). Astrocytes have both beneficial and deleterious functions in CNS inflammation. In MS and EAE they are recognized as APCs (Cornet et al., 2000). Generally, astrocytes are considered poor APCs since they do not constitutively express MHC class 2 molecules, and express very low levels of co-stimulatory molecules. Enhanced expression of MHC class I and II molecules on astrocytes have been observed in MS and EAE. MHC class I positive astrocytes can activate and present antigen to CD8+ T-cells. In MS, CD8+ cells predominate over other T-cell subsets in active lesions and are also detected in the NAWM (Hauser et al., 1986). Therefore, the activation of secondarily recruited CD8+ T-cells by MHC class 1 positive astrocytes within, or even at a distance from the inflammatory lesions, could contribute to the perpetuation of the disease process. Our data support the notion of the activation of an MHC class 1 restricted immune response in EAE.

We also observed the activation (up-regulation) of many calcium-regulated cytoskeletal-associated proteins, possibly emphasizing the role of increased calcium in the pathogenesis of MS or EAE. Activation of proteases and protease inhibitors is suggestive of protease activities also mediated by Ca2+, prevalent in EAE and possibly also in MS.

A group of proteins are involved in mitochondrial function and energy transport. Moreover, differentially regulated proteins, identified in the membrane fraction were dominated by mitochondria-related proteins. Mitochondrial dysfunction, as a major contributor for axonal degeneration in EAE, has been proposed by many studies (Dutta et al., 2006). Our results provide additional molecules that might take part in disease mechanisms mediated by mitochondrial dysfunction.

Another set of proteins that is worth mentioning is the N-ethylmaleimide sen-

sitive fusion (NSF) attachment proteins (SNAPs). Three SNAP proteins showed altered levels in MS and or EAE. Down-regulation of SNAPs was supported by the additional down regulation of NSFL1 cofactor (p47). SNAPs play an essential role in vesicular transport and release of neurotransmitters and hormones (Nishiki et al., 2001). SNAP under-expression is associated with a reduction of synaptogenesis in Down's syndrome and Alzheimer's Disease (AD), (Yoo et al., 2001). The reduced levels of SNAPs in MS and EAE can be inferred as processes leading to axonal transport dysfunction.

The following section takes a closer look at some selected proteins and elaborates on their functional implication in MS and EAE.

4.2 Selected protein markers

This part highlights some proteins and reviews the diverse functions based on literature knowledge, with possible implications in the pathogenesis of EAE and MS.

4.2.1 Neurotoxicity

Serum amyloid P component

Serum amyloid P component (SAP) was overexpressed consistently in the brain and spinal cord of EAE animals in all models of wild-type EAE. SAP was identified as an "early end point marker" due to its sustained over-expression starting from score 1 up to score 4. Consistent with the animal study we detected up-regulation of SAP in the brain NAGM of two out of three MS patient. Up-regulation of SAP was restricted to gray matter and not extended to plaque or NAWM.

SAP is a plasma glycoprotein belonging to the pentraxin family of proteins. C-reactive protein (CRP) and SAP are acute phase proteins humans and mice, respectively. They are also called short pentraxins because of their low molecular weight of around 25kDa. CRP and SAP are synthesized in the liver and secreted into the blood, and their blood concentration increases during many illnesses including CNS disorders Garlanda et al. (2005). Neuron-specific pentraxins, also called long pentraxins ($\approx$45kDa), are NP1, NP2, NP3 and PTX3. The substantial homology between the pentraxin orthologs in different mammalian species indicates the conserved functional role of the pentraxins (reviewed in Garlanda et al., 2005). SAP is important for many physiological functions. SAP protects

cells against chromatin-induced autoimmunity, and sap-/- mice develop spontaneous nuclear autoimmunity (Bickerstaff et al., 1999). The protein is involved in the binding and clearance of host or pathogen-derived cellular debris at the sites of inflammation. SAP binds to apoptotic cells and enhances their removal by macrophages and participates in complement activation and humoral amplification of innate immunity (Garlanda et al., 2005)

SAP and brain and neurodegeneration

Many studies have reported the involvement of SAP and CRP in the pathogenesis of neurodegenerative diseases (Hawkins et al., 1990; Kalaria et al., 1991a; McGeer et al., 2001). The proposed mode of activity of SAP is, either by promoting amyloid plaque formation, or by the activation of the complement pathway in an antibody-independent manner by binding to complement 1q (c1q) (Hasegawa et al., 1995; Kalaria et al., 1991b). SAP is found in association with senile plaques and neurofibrillary tangles of (AD) (Kalaria et al., 1991a). SAP knock-out mice demonstrated that targeted deletion delays and reduces amyloid formation (Botto et al., 1997). Chronic activation of pentraxins can initiate an autodestructive process and damage the host tissue (McGeer et al., 2001).

Cerebro-cortical cell culture experiments led to the finding that SAP has a direct cytotoxic effect on neurons by inducing neuronal apoptosis (Urbanyi et al., 2003, 1994). SAP gets translocated into the nuclei where it induces apoptosis of neuronal cells. Interestingly, SAP-mediated toxicity was only observed in neurons and not in glial cells. Thus, treatment of neuronal cells with SAP leads to neuronal death due to increased β amyloid formation, as well as direct cytotoxic effects of SAP on neurons. Increased BBB permeability that occurs in the inflammatory phase of neurodegenerative diseases can lead to the entry of SAP from blood into the brain. In the brain it is taken up by neurons where it mediates its toxic effect. Indeed, high levels of SAP are present in the CSF of AD patients (McGeer et al., 2001). Moreover, Yasojima et al reported the expression of SAP and CRP in neurons (Yasojima et al., 2000).

The over-expression of SAP in EAE that we found could be the result of the inflammatory response, as the serum SAP level is known to increase significantly during inflammatory diseases. The presence of SAP in the brain and spinal cord is, either due to its migration into the brain through the blood brain barrier BBB, or its expression by neurons during the disease progression. As explained above, the expression of SAP in the CNS, or its migration into the CNS, can lead to the uptake of SAP by neurons and the subsequent induction of apoptosis.

Interestingly, neuronal pentraxin, PTX3 has been shown to have a protective role in EAE and CRP transgenic mice were protected in a model of EAE (Szalai et al., 2002). These findings, together with the known function of SAP in

protection against chromatin autoimmunity, gives rise to the idea that SAP has a protective role in EAE.

Our findings show that similar to EAE, the SAP level is elevated in the brain of at least a subset of MS patients. The role of SAP-mediated cytotoxicity in MS should be investigated further.

Chloride intracellular channel protein 1

CLlC1 was greatly up-regulated in EAE, especially in later stages of EAE. In the control spinal cord CLlC1 was expressed at a very low or undetectable amounts. A recent *in vitro* study highlighted the role of CLIC1 in mediating microglia-induced neurotoxicity (Novarino et al., 2004). Activated microglia take a reactive phenotype with the production and secretion of pro-inflammatory products leading to neuronal death. *In vitro* investigations showed that activated microglia increased the expression of CLIC1 and its localization on the plasma membrane upon treatment with amyloid beta (Aβ). Aβ activated microglial cells display an ionic conductance characteristic of CLIC1. Ionic membrane currents, especially chloride conductance, have been shown to regulate microglial proliferation (Schlichter et al., 1996; Eder, 1998). Consistent with this finding, the blockade of CLIC1 inhibited microglial cell proliferation. Furthermore, blockade of CLIC1,(with the CLIC1 blocker IAA-94), inhibits the neuronal toxicity induced by Aβ activated microglial cells and leads to the increased survival of neuronal cells. There is evidence that the production of TNF alpha and nitrite molecules was reduced after treating microglia cells with IAA-94 (Novarino et al., 2004). Inflammatory events, mediated by microglial activation, contribute to neurodegenerative processes including AD, (McGeer et al., 1993, 1994; McGeer and McGeer, 1997, 1998). Several studies have emphasized that the activation of microglial cells leads to the production of various cytokines and neurotoxins, and can eventually cause neuronal injury and death (Barger and Harmon, 1997; Egensperger et al., 1998; Styren et al., 1998; Benveniste et al., 2001; Combs et al., 2001).

Microglia-mediated exacerbation of MS symptoms is recognized for therapeutic implementation. The exact mediators of microglia-induced neurotoxicity need to be pin-pointed in order to selectively block the effect. We propose CLIC1 as a target for therapeutic prevention of microglia-induced oxidative stress and neuronal death in EAE and MS.

4.2.2 Sirtuins

In human MS brain we found a significant and consistent loss of sirtuin2 (SIRT2) protein. In the animal experiments, we observed the loss of a phosphorylated form of SIRT2 in the spinal cord of EAE animals.

Silent information regulator proteins (Sirtuins) represent a new class of evolutionary conserved, nicotinimide adenine dinucleotide (NAD) dependent deacetylases of histones and other proteins. This family of proteins was originally identified in yeast as the Sir2 gene, the over-expression of which suppresses genomic instability, leading to an increase in the life span (Kaeberlein et al., 1999). The role of sirtuins in the extension of life span in different organisms has been confirmed experimentally by many studies. Recent work on sirtuins show their emerging roles in many pathophysiological conditions such as cancer, diabetes, muscle differentiation, heart failure, neurodegeneration and aging (Porcu and Chiarugi, 2005).

There are seven molecules (SIRT 1-7) in mammals, (including rodents and humans), that share the Sir2 conserved domain (Frye, 1999, 2000). SIRT family members can be recognized by the presence of a conserved core of 203 amino acid residues. The conserved core of SIRT proteins folds into an NAD+ binding protein with intrinsic protein deacetylase activity, capable of removing the acetyl moiety from the ϵ- amino group of lysine residues in protein substrates (Chang et al., 2002).

The protein deacetylase function, presumed to be intrinsic to all SIRT proteins, may be their only functional commonality. Presumably, eukaryotic SIRT proteins all share the NAD dependent deacetylase (NDAC) activity, but differ in their cellular function due to general subcellular distribution, and specific protein-protein interactions with their acetylated protein substrates, properties unique to each SIRT ortholog, and determined by the variable N and C terminal sequences (Avalos et al., 2002). Among the SIRT proteins SIRT1, 2 and 3 are categorized as class 1 sirtuins (Frye, 1999, 2000; North et al., 2003; Shi et al., 2005).

Acetylation of the lysine residues on proteins is an important PTM that can regulate protein activity. For example, acetylation of the amino terminal tails of histones increases transcriptional activity, DNA replication, repair activity and heterochromatin formation. Deacetylation of histones leads to the silencing of gene transcription (Hisahara et al., 2005).

The human ortholog of yeast sir2, SIRT1, is the best characterized among the mammalian sirtuins. Relatively little is known about the other members. SIRT1 is located in the nucleus and cytoplasm and is involved in chromatin remodelling

and the regulation of transcription factors. The others are located within the cytoplasm and mitochondria (North et al., 2003).

Sirtuin 1

SIRT1 is an emerging central regulator of both stress response and apoptotic machinery with different effects, depending on the target protein or gene (Porcu and Chiarugi, 2005). Recently, many studies have emphasized a major role of SIRT1 in cancer and neurodegeneration mediated by its interaction with its respective targets. Consistent with these findings, sirtuin activating drugs, (STACS eg. resveratrol), and their inhibitors (eg. seritinol) have emerged as promising drugs in the treatment of many diseases including neurodegenerative disorders.

SIRT1 and the brain

SIRT1 expression is high during embryogenesis in the brain, spinal cord and heart. SIRT1 deficient mice are small at birth and die during the early postnatal period. They have multiple developmental defects of retina, heart, lung and pancreas and sometimes exhibit exencephaly. SIRT1 is expressed in the adult brain but its physiological function in neurons is unknown. Sirt1 is suggested to have a role in the mechanism of neuronal cell death based on its binding and activation of FOXO1, a transcription factor that delays neuronal death (Daitoku et al., 2004).

Recently, Araki et al. reported that increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration in the *in vitro* Wallerian degeneration model (Araki et al., 2004). Delayed axonal degeneration is a characteristic of Wallerian degeneration slow mice (wlds). Nicotinamide Mononucleotide Adenylyltransferase (Nmnat1) is an enzyme in the NAD biosynthetic pathway that generates NAD within the nucleus. The study shows that the Nmnat1 part of the Wlds protein is responsible for the axonal sparing in Wlds mice, and that NAD production of the enzyme is essential to prevent axonal degradation. SIRT1 is the major effector of the increased NAD supply that effectively prevents axonal destruction. Although SIRT1 may deacetylate proteins directly involved in axonal stability, the study gives evidence that SIRT1 regulates a genetic program that leads to axonal protection. The results indicate that the molecular mechanism of axonal protection in the Wallarian degeneration slow mice (wlds) is due to increased nuclear NAD biosynthesis, that results in increased Nmnat1 activity and subsequent activation of the protein deacetylase SIRT1.

Taken together, Sir2 proteins are the effectors of the axonal protection mediated by increased Nmnat activity. Another *in vitro* study has reported the protective effect of SIRT1 against microglia-dependent amyloid toxicity (Chen et al.,

2005). SIRT1 is also found to protect cells against amyloid-induced reactive oxidant species (ROS) production and DNA damage *in vitro* (Jang and Surh, 2003; Russo et al., 2003; Savaskan et al., 2003). The neuroprotective effect of SIRT1 in Huntington's Disease(HD) has also been highlighted as a therapeutic measure (Parker et al., 2005).

Sirtuin 2

SIRT2 is predominantly localized in the cytoplasm, and to a lesser degree in the nucleus. It may regulate rDNA and telomeric silencing indirectly from its cytoplasmic location. Three different splice variants of SIRT2 with a very similar MW are reported in the protein databases. SIRT2 co-localizes strongly with the microtubule network, where it deacetylates alpha tubulin at lysine-40, (Nogales et al., 1999), (North et al., 2003). Only SIRT2 deacetylates tubulin *in vitro*, whereas the remaining SIRT proteins contain no significant tubulin deacetylase activity. Similar to the other sirtuin members, SIRT2 tubulin deacetylase activity is NAD+ dependent.

SIRT2 deacetylates tubulin, possibly regulating microtubule stability, cell structure, intracellular transport and cell motility. Tubulin acetylation increases during axonogenesis, and acetylated tubulin is resistant to depolymerization, stressing its role in the development of stable neuronal circuits (Ferreira and Caceres, 1989; Mattson, 2003). Acetylation of tubulin enhances microtubule stability and reduction in tubulin acetylation may damage neuronal function (Morales and Fifkova, 1991). However, the exact role and mechanisms of tubulin deacetylation during development, or in adult CNS, is not understood. Sirt2 may interact with and regulate other cytoskeletal proteins, and might be able to protect neural cells through mechanisms independent of tubulin.

It has also been reported that SIRT2 can deacetylate p53 (de la Monte and Wands, 2004) like SIRT3 and SIRT1 and might have an influence in regulating the insulin level in cells (Anekonda and Reddy, 2006). Cell culture investigations have shown that human SIRT2 increases in abundance during mitosis (M phase) and that SIRT2 NDAC activity controls the mitotic exit in the cell cycle (Dryden et al., 2003). Over-expression of the protein phosphatase CDC14B results in SIRT2 dephosphorylation and a substantial loss in SIRT2 protein. The hypo-phosphorylated form becomes ubiquitinylated and turns over via the 26S proteasome pathway down stream from CDC14B. Interestingly, the SIRT2 gene and protein levels are down-regulated in human brain glioma tissue (Hiratsuka et al., 2003).

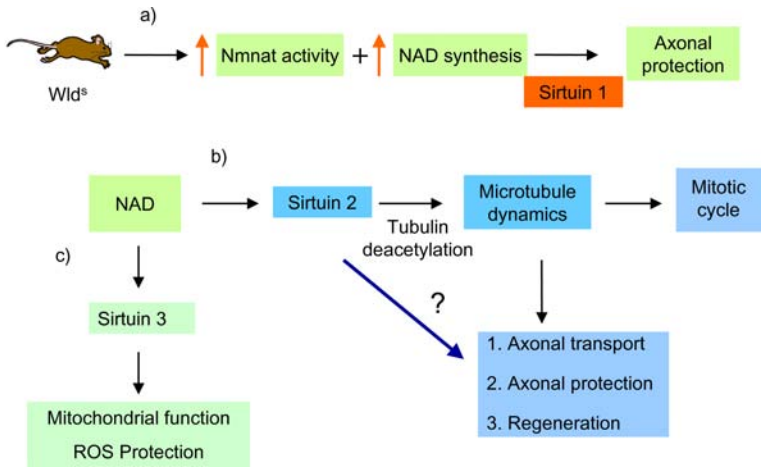

Figure 4.3: Function of sirtuin family proteins in the CNS. All sirtuins function in an NAD dependent manner : a) Sirtuin 1 is the primary mediator of delayed axonal degeneration in Wallarian degeneration slow (Wlds) mice. b) Sirtuin 2 has a tubulin deacetylation function. It might be involved in axonal protection similar to sirtuin 2, independent of its tubulin deacetylation function. c) Sirtuin 3 is important for mitochondrial function and protection against reactive oxidative species.

Sirtuin 3

SIRT3, another member of class1 human sirtuins, is localized within the inner membrane of mitochondria. SIRT3 executes NAD+ dependent protein deacetylation in mitochondria (Onyango et al., 2002; Schwer et al., 2002). It has been implicated in the reduction of ROS production and increase in respiration rates in mice, strongly indicating its role in mitochondrial function (Shi et al., 2005).

Taken together, the leading role of sirtuins has been emphasized in the context of various pathological conditions. STACs have been implicated as promising drugs in the treatment of diseases, including neurodegenerative diseases (AD, HD, axonopathy). STACs seem to work by increasing cell defense against stress and, thereby, increasing life expectancy. The sirtuin family member SIRT1 is the principle downstream mediator of these effects.

Although there is not much experimental evidence supporting the neuroprotective role of the human sirtuin SIRT2, it can be speculated that, like SIRT1, SIRT2 also has neuroprotective effects that are mediated through direct or indirect connections to its tubulin deacetylation function, or via its interaction with other cytoskeletal molecules. SIRT2 may be important for the regenerative process where the dynamics of microtubule polymerization and depolymerization are required. Another possible mechanism is the perturbation of axonal trans-

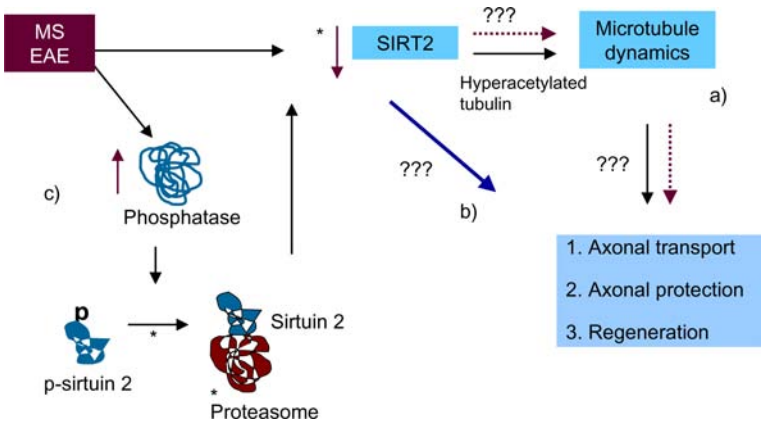

Figure 4.4: Loss of sirtuin in MS and EAE and its functional implication: a) Loss of SIRT2 can lead to accumulation of hyper-acetylated tubulin, which can hinder microtubule dynamics and impede axonal regeneration. b) SIRT2 might be involved in axonal protection similar to SIRT1. c) Reduction of phosphorylated SIRT2 in EAE might be the result of phosphatase activity, dephosphorylated SIRT2 might be degraded by proteasome. * indicates changes that we saw in EAE and/or MS.

port due to excess hyper-acetylated tubulin. An illustration of the role of sirtuin in the context of MS is presented in Figure 4.4

4.2.3 Creatine Kinase

A loss of creatine kinase brain isoform (CK-B) was detected in the brain and spinal cord of EAE animals. We also observed a marked reduction of creatine kinase in the membrane fraction of the brain protein extract of EAE animals.

The creatine kinase system consisting of a cytosolic and ubiquitous mitochondrial (uMtCK) protein, together with their substrates creatine and phosphocreatine, is the most important immediate energy buffering and transport system especially, in muscle and neural tissue. Creatine kinase catalyzes the reversible transfer of high energy phosphate from ATP to creatine, yielding phosphocreatine for energy storage and transport (Wallimann et al., 1992). There are several isoenzymes of CK with distinct tissue and cellular distributions. In the brain, the cytosolic brain-type CK (CK-B) and uMtCK comprise an energy shuttling system, which transfers energy efficiently from the site of ATP production (mitochondria) to distal parts of the cell where energy is consumed (Wallimann et al., 1998; Joubert et al., 2002), (Figure 4.5). Accumulating evidence suggests that mitochondria play a central role in neurodegeneration including the modulation of cellular energy, calcium levels, and ROS (Swerdlow et al., 2004). uMtCK has

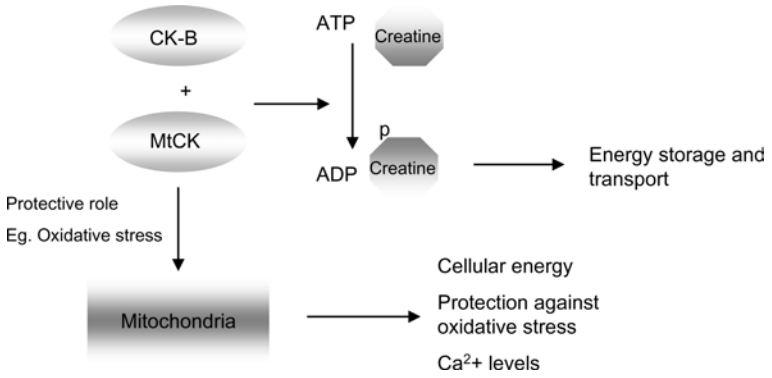

Figure 4.5: Soluble and mitochondrial forms of creatine kinase (CK-B and Mt-CK) are responsible for maintaining energy storage and transport of neural cells. Creatine kinase also protects mitochondria against insults such as oxidative stress.

been shown to be important to mitochondrial function in several ways. It has a protective role against oxidative or toxic insults to mitochondria (O'Gorman et al., 1997; Dolder et al., 2003; Hatano et al., 2004).

CKs are prime targets of oxidative damage (Dolder et al., 2001; Konorev et al., 1998). Oxidation of creatine kinase, resulting in its activity loss in the brain, has been reported in aging and brain disorders like HD and AD (Perluigi et al., 2005; Choi et al., 2004; Aksenov et al., 2000). Reduced levels of CK-B protein, but not mRNA, have also been reported in Pick's disease and Lewy Body dementia, suggesting the role of a PTM, namely oxidation, in the loss of amount and activity (Aksenov et al., 1997). Oxidation of CK leads to the disruption of the cellular energy state (Stachowiak et al., 1998). Recent reports have demonstrated the neuroprotective function of creatine therapy, delaying motor symptoms in the transgenic animal model of HD (Dedeoglu et al., 2003) and, hence, suggesting the enhancement of the cerebral energy metabolism as a protective mechanism against neurodegeneration.

Oxidative damage to proteins is of importance in explaining the metabolic dysfunctions associated with oxidative stress-mediated diseases. Production of reactive oxygen species is understood to be one of the primary mediators of neuronal toxicity in MS and EAE (Gilgun-Sherki et al., 2004; Smith and Lassmann, 2002). Our own proteomic results have also identified promoters of ROS production up-regulated in EAE animals. It can be speculated that oxidative modification of functionally important proteins like creatine kinase takes place in MS and EAE, leading to their dysfunction. Supporting this, a very old study reported reduced levels of CK-B in the serum of MS patientsVassilopoulos and Jockers-Wretou (1987). Loss and dysfunction of CK can disturb mitochondrial

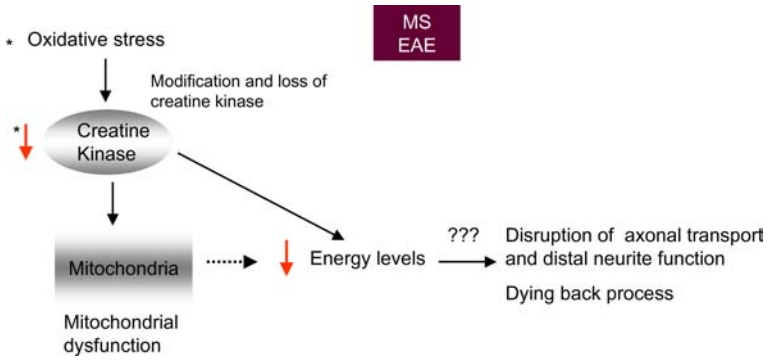

Figure 4.6: Oxidative stress modifies creatine kinase and leads to its loss of activity and reduced levels. Low creatine kinase in MS and EAE can cause insufficient energy to meet the demands, especially, of axons and thus lead to axonal dysfunction.

functions, and maintenance of energy levels in EAE. This is crucial in the case of axons where the density of mitochondrial distribution is low. We are proposing the loss of another mitochondria-associated protein, creatine kinase, in the pathogenesis of EAE.

4.2.4 Ferritin heavy chain

Ferritin is a highly conserved, ubiquitous iron binding protein. Vertebrate apoferritin consists of 24 heavy (H) and light (L) subunits. The H to L ratio varies between tissue types and is readily modified in response to physiological conditions such as inflammation, xenobiotic stress, etc (Harrison and Arosio, 1996). The critical role of ferritin in maintaining iron homeostasis is by sequestrating iron. Iron is necessary for heme functioning, and for the functioning of many enzymes executing essential cellular process. However, iron also provides electrons for the generation of reactive free radicals. Ferritin serves to capture iron and ot store it in the soluble nontoxic form, buffering the intracellular iron pool. Conversely, the level of ferritin is controlled by the level of labile iron as a protective mechanism against iron-triggered cellular toxicity (Torti and Torti, 2002). The function of ferritin in limiting the availability of iron to participate in the generation of ROS and its role as a protectant against oxidative stress, is supported by much experimental evidence (Balla et al., 1992; Cermak et al., 1993; Orino et al., 1999, 2001).

Factors, such as oxidants and inflammation, modulate the expression of ferritin and the ratio between H and L chains (reviewed in Torti and Torti, 2002). Oxidants regulate the levels of H and L ferritin chains at both the transcriptional

and post-transcriptional levels (Tsuji et al., 2000; Cairo et al., 1995). The pro-inflammatory cytokines, TNFα and ILα, increase the mRNA level of ferritin heavy chain (H-Ferritin or FHC).

The isoforms of ferritin have specific cellular distributions in the brain, depending on the cell type (Connor et al., 1994). Cellular distribution of the two subunits of ferritin in the adult brain differs depending on the cell type. In the rat brain neuronal ferritin consists of only H-subunits, whereas ferritin in microglia consists of the L-chain. Oligodendrocytes (OLCs), on the other hand, have both heavy and light chains. Astrocytes do not have either of the subunits. Differences in iron sequestration and use between neuronal and glial subtypes suggest diverse roles of each of these cells in maintaining a general brain iron homeostasis, and the relative abilities of these cells to withstand oxidative stress.

Ferritin and myelination/remyelination

Iron has a well established role in the process of myelination (Connor and Menzies, 1996). Consistent with this, FHC mRNA was detected in the myelin sheath assembly sites (Gould et al., 2000). Iron is directly involved in myelin production as a required co-factor for cholesterol and lipid biosynthesis, and indirectly because of its requirement for oxidative metabolism. During normal development, there is a switch in the predominant type of ferritin-containing cells in subcortical white matter from microglia to OLCs (Cheepsunthorn et al., 1998). This switch is also seen with iron (Connor et al., 1995). The shift in the presence of ferritin and iron is suggestive of an influence of microglia on myelination by competing with OLCs for iron (Cheepsunthorn et al., 1998). The appearance and distribution of ferritin-expressing OLCs is coincident with the pattern of subcortical white matter myelination during normal brain development (Friedman et al., 1989; Cheepsunthorn et al., 1998).

In a rat model of hypoxic ischemia (H/I) (developmental H/I model) microglial activation in the brain was coupled with ferritin expression and iron accumulation in these cells (Cheepsunthorn et al., 2001). Ferritin positive microglia accumulated in the subcortical white matter and the ratio between the H to L chain shifted more to H-ferritin. The authors claim delay and impedance of myelination due to the limited availability of iron to OLCs. Microglia compete with OLCs for iron. Activated microglia may withhold iron from the OLCs, which is a well-established mechanism observed in macrophages (Weinberg, 2000). Alternatively, activated microglia may compete more effectively than OLCs for iron being transported across the BBB. Another mechanism is the active removal of iron from OLCs through the secretion of superoxide radicals and nitric oxide during microglial activation. In the developmental H/I model remyelination was associated with an increase in H-ferritin - positive OLCs, and

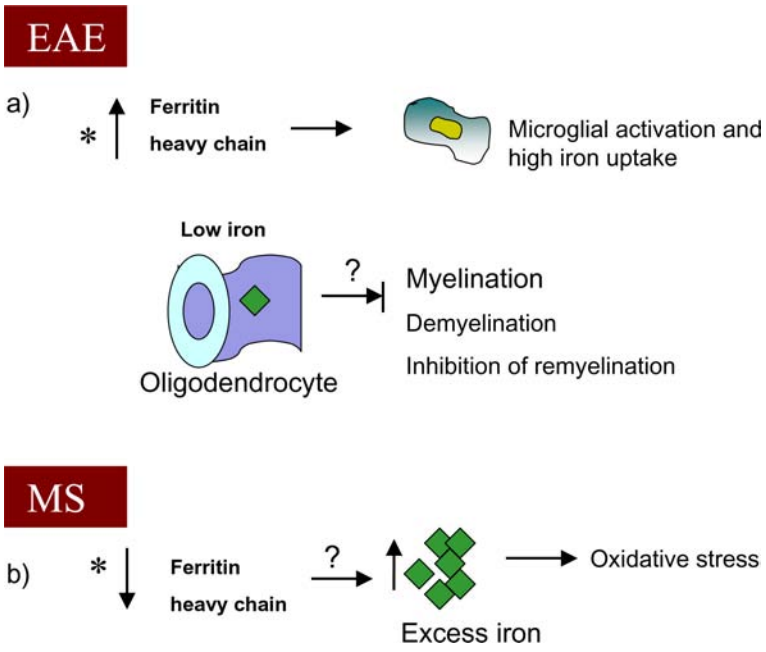

Figure 4.7: Ferritin heavy chain - functional implication. a) Microglial activation and high ferritin level deprives oligodendrocytes of iron. Lack of iron can hinder myelination or remyelination. b) Low ferritn heavy chain in MS brain can lead to iron mediated oxidative stress.

an increase in the ratio of H:L subunits.

Thus, there are two mechanisms as to how excess iron, or disruption in its sequestration can contribute to nervous system injury. 1. excess iron can promote oxidative stress and damage neurons and oligodendrocytes. 2. excess iron levels activate microglial cells and their over-expression of ferritin. Activated microglial cells compete with OLCs for iron and limit the iron availability for OLCs and prevent remyelination.

We see elevated levels of ferritin heavy chain in the spinal cord and brain of EAE animals. Up-regulation of H-ferrtin in EAE brain and spinal cord may be an indicator of microglial activation as ferritin immunoreactivity is regarded as a marker for microglia activation (Kaneko et al., 1989). A previous study has already reported increased ferritin in the spinal cord of EAE animals (Chakrabarty et al., 2003). Another explanation of the role of overexpressed ferritin is its suppression of cell-mediated immunity (Levine and Chakrabarty, 2004). Interestingly, in our experiment the H ferritin spot in the EAE brain and spinal cord was always stained by ProQ, indicating its phosphorylated state. Mass spectrometric characterization has identified phosphorylated isoforms of ferritin heavy chain and the phosphorylation sites (www.expasy.org). The influence of phos-

phorylation in regulating the function of ferritin is yet to be understood.

In contrast to EAE, the plaque and NAWM of all three MS patients had reduced levels of two H-ferritin spots. The low levels of ferritin heavy chain in the plaque and white matter could be due to an aberrant iron sequestrating function that promotes iron-mediated oxidative stress in the MS brain. The exact cell types that contribute to the altered ferritin heavy chain levels need to be further looked into. Loss of ferritin heavy chain in the white matter and plaque could also be attributed to their reduction in the OLCs, which might suggest a disrupted myelination, or remyelination and repair.

The role of iron-mediated toxic insults in MS is well investigated, and many therapeutic strategies are being put forward in this respect (Levine and Chakrabarty, 2004). Iron deposits have been detected in EAE (Forge et al., 1998) and MS brain (Craelius et al., 1982; LeVine, 1997; Valberg et al., 1989). An increased ferritin level is present in the CSF of MS patients (LeVine, 1997), suggesting the activation of a protective mechanism to limit the toxic effect of iron. A participation of ferrous iron-mediated oxidative stress in neurodegenerative diseases like Parkinsons's disease (PD) and AD, is also known (Sofic et al., 1988; Youdim et al., 1993; Kuiper et al., 1994). We found reduced H-ferritin levels in the plaque and NAWM of MS patients.

4.2.5 Proteins involved in axonal guidance

During neuronal development, neurite sprouting, growth and extension of dendrites and axons, as well as their retraction and growth cone retardation is essential for neuronal pathfinding and the establishment of the right synaptic connections. Axonal development (growth and retraction) requires regulated actin and microtubule organization (dynamics) and vesicle and protein trafficking. These composite events are mediated by various external guidance signals, and a cascade of signal transduction processes that follow after the reception of these guidance cues. Growth and retraction processes are maintained during CNS development by modulating the patterns of attractive or repellent activities.

Axonal growth and retraction are equally important in the adult CNS for neuronal plasticity and regeneration processes, following CNS injury in various neuropathological conditions. However, adult CNS is generally unable to regenerate and this has been attributed to its neuritogenic disability, and the presence of inhibitory molecules in the CNS environment that prevent neuronal growth (Tang, 2003). The lack of regeneration in the MS brain and its possible reasons, has been looked into by many researchers. For example, glial scar formation, that

takes place after CNS lesions in pathological conditions like MS, generates neuritogenic inhibitors that prevent regrowth (Fawcett and RA, 1999; Fournier and Strittmatter, 2001). Moreover, it is known that CNS myelin itself inhibits regeneration (Schwab and Caroni, 1988).

Injury-induced re-expression of repellent cues, responsible for growth cone collapse and retraction, has been considered in the inhibition of regeneration. Additionally, the retraction (axonal) process in the adult brain is implicated as a degenerative process taking place in neurodegenerative diseases (Tang, 2003).

In our EAE experiments, we found the differential regulation of a set of proteins that are important during CNS development, (axonal growth and path finding). Re-expression, or regulation and modification of these proteins in the diseased stage, can be regarded as an activation of regeneration and a repair process, or the inhibition of regeneration.

Collapsin response mediator proteins

Two members of the collapsin response mediator protein (CRMP) family, CRMP2 (DRP2) and CRMP4 (DRP3), were differentially regulated in the EAE animals. Both CRMP2 and CRMP4 migrated at two different positions, differing mostly in their pI. We observed elevated levels of the more acidic isoform of CRMP2 and CRMP4 and a down-regulation of the more basic isoforms. The two CRMP2 spots also differed in their MW, with the more acidic spot having a lower MW. All spots were visible in the ProQ stain, indicating the phosphorylation modification of these molecules. Western blotting confirmed the overall down-regulation of CRMP4. The movement and regulation pattern,with respect to the pI, is suggestive of a disease stage-dependent hyperphosphorylation of the CRMP proteins in EAE.

CRMPs, (also known as Ulip/ DRP/ Toad), are a family of cytosolic phosphoproteins involved in neuronal differentiation and axonal guidance. The CRMP family proteins are highly expressed in neurons during their peak periods of axonal growth, and thereafter strongly down-regulated (Quinn et al., 1999). Their expression and phosphorylation are spatially and temporarily regulated during development, for example in response to nerve growth factors (Minturn et al., 1995; Byk et al., 1996; Wang and Strittmatter, 1996). CRMP2 and CRMP3 are expressed throughout the spinal cord. All members have a basic region adjacent to a differentially phosphorylated region at the carboxy terminus. The basic region is responsible for the direct and indirect binding to other cellular components, which is regulated by the phosphorylation of the adjacent region.

CRMP4

CRMP4 /DRP3 protein expression in the adult CNS is restricted to regions showing neurogenesis. CRMP4 is up-regulated in neurogenesis following seizures (Parent et al., 1997; Scott et al., 1998), and in the adult sciatic motor nerves that are regrowing axons after axotomy (Minturn et al., 1995). We saw hyperphosphorylation, as well as minor loss of CRMP4 in EAE. Whether phosphorylation of CRMP4 favors or impedes the repair process needs to be looked into with respect to EAE.

CRMP2

CRMP2 mediates growth cone collapse, or axonal retraction during axonal guidance, in response to repellent molecular cues, like semaphorin (Goshima et al., 1995). Semaphorins are a family of axonal guidance molecules (Liu and LeVine, 2001). Collapsin 1 or Semaphorin 3A (Sem3A) is the best studied semaphorin as an inducer of growth cone collapse. The most prominent downstream cytoplasmic effectors of Sem3A-induced growth cone collapse signaling are the CRMPs (Goshima et al., 1995) and the Rho GTPases (Jin and Strittmatter, 1997). RHO GTPases and their modulators, such as GDP dissociation inhibitors (GDIs), lead to the activation of LIM kinase (Maekawa et al., 1999). The collapsing activity of Sem3A signaling takes place essentially through the actin depolymerizing protein cofilin (Aizawa et al., 2001). Upon activation, LIM kinase phosphorylates and inactivates cofilin (Maekawa et al., 1999), impairing its actin depolymerization function (Sumi et al., 1999). Sequential phosphorylation and dephosphorylation of cofilin are characteristic of this pathway (Aizawa et al., 2001). Phosphorylation of CRMP2 is essential for Sema3A-induced growth cone collapse. Apart from its actin-related activity CRMP2 has also been shown to be associated with the regulation of microtubule dynamics (Gu and Ihara, 2000). Association of CRMP2 with bundled microtubules implies that CRMP2 may be involved in the interaction between microtubules, or may help to link microtubules with other cellular components. Rho kinase phosphorylates CRMP2 and inactivates the ability of CRMP2 to promote microtubule assembly during growth cone collapse and axonal retraction (Arimura et al., 2005; Brown et al., 2004).

Inhibition of regeneration by Sem3A was demonstrated by studies showing an elevated expression of Sem3A after CNS nerve injury in glial scars, while it is absent or down-regulated after injury in the PNS where regeneration is uninhibited (Pasterkamp et al., 1999, 1998). *In vitro* studies provided evidence that Sem3A promotes apoptosis in neuronal cells (Shirvan et al., 1999).

Overexpression of CRMP2 in neuronal cells (neuro2a) results in blebbing of the cytoplasm and subsequent apoptosis. Blebbing of cytoplasm is believed to

be due to cytoskeletal alterations affecting the dynamics of actin, or the defective interaction between microfilaments and microtubules. In this model acetylated tubulin and CRMP2 were deposited as intra-nuclear inclusions in minor cell populations. Highly phosphorylated forms of CRMP2 were associated with neurofibrillary tangles in AD brains (Yoshida et al., 1998; Gu et al., 2000). Semaphoring lll/D and CRMP2 have been reported to mediate neuronal death in a dopamine-induced apoptotic cell model (Shirvan et al., 1999).

Cofilin

Our data suggests hyperphosphorylation of cofilin in EAE brain and spinal cord. From yeast to mammals, cofilin plays an essential morphogenetic role by promoting the depolymerizing actin filaments (Bamburg, 1999). In mammals the activity of cofilin is inhibited upon phosphorylation at a serine residue at position 3 (Agnew et al., 1995) by LIM kinase (Arber et al., 1998). Cofilin is activated by its dephosphorylation by slingshot phosphatase (Ssh) (Niwa et al., 2002).

As explained in the section on CRMPs, cofilin phosphorylation by LIM kinase is necessary for the collapsin-induced actin rearrangement during growth cone collapse (Aizawa et al., 2001). In accordance with this we found changes in the phosphorylation state of cofilin, as well as a quantitative increase in the level of phosphorylated cofilin in EAE animals.

We observed phosphorylation-specific regulation of CRMP2 and cofilin in EAE. Summing up the two findings, we are tempted to propose the role of a collapsing mediated retraction process in EAE, and possibly also in MS. Additionally, changes in the expression of RHO GDP dissociation inhibitor alpha (GDI alpha), a modulator of Rho GTPase signal transduction is more evidence that strengthens the possibility of a retraction process taking place, either as a degenerative process or as an inhibition of regeneration. Hyperphosphorylation and loss of CRMP4 , the only CRMP involved in neurogenesis, is yet another finding that emphasizes the prevalence of molecular cues (repellent cues ?) facilitating inhibition of regeneration.

4.2.6 Stathmin

Stathmin is a phylogenetically conserved cytosolic phosphoprotein belonging to the family of neuronal growth-associated proteins (GAPs). Its expression is developmental stage-specific with high expression in many immature cell types, which lowers after differentiation. Neuron-specific GAPS are SCG10, SCLIP,

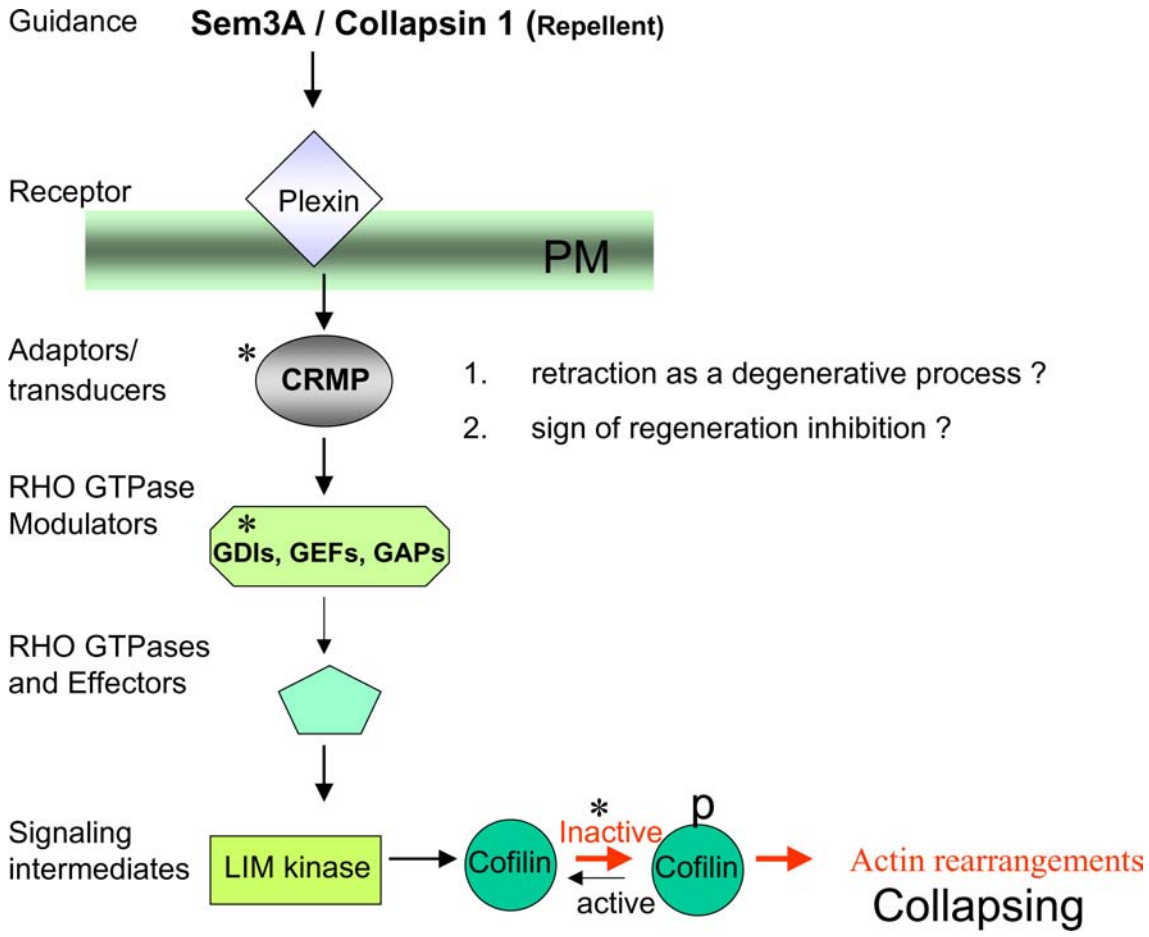

Figure 4.8: Collapsin-mediated growth cone relapse: * indicates the protein level regulation in our results.

RB3. The well-established cellular function of stathmin and SCG10-related proteins is their microtubule destabilization function. In the developing nervous system, stathmin and SCG10 are expressed in high levels during neurite outgrowth, while their levels are strongly down-regulated after synapse formation (Paolo et al., 1997). Consequently the role of stathmin and the related proteins is well appreciated in the context of neuronal development, neurite outgrowth, synaptic plasticity, degeneration and aging (Mori and Morii, 2002; Grenningloh et al., 2004).

Stathmin gets phosphorylated at its N-terminal amino acids in response to numerous extracellular stimuli that regulate proliferation and differentiation in different cell types (Sobel, 1991). Stathmin is phosphorylated at four amino acid residues. The microtubule destabilizing function of stathmin is negatively regu-

lated by its phosphorylation on one or more sites (Horwitz et al., 1997). The role of phosphorylation in modulating the function of stathmin, emphasizes stathmin as a molecule that relays extracellular stimuli to regulate cellular proliferation and differentiation.

In accordance with its function in neurite outgrowth during development, some studies have observed the up-regulation of stathmin during axonal regeneration and neurogenesis in the adult nervous system (Pellier-Monnin et al., 2001; Mason et al., 2002; Wang and Hanash, 2002; Grenningloh et al., 2004). Moreover, loss of brain stathmin is associated with age-related neurodegenerative diseases like, AD, amylotrophic lateral sclerosis (ALS) and in Down's syndrome (Strey et al., 2004).

The elevation of stathmin mRNA and protein has been demonstrated in the brain white matter of MS patients (Chabas et al., 2001; Liu et al., 2005). A detailed investigation of stathmin regulation in the MS brain by Liu et al demonstrated that stathmin levels are up-regulated in the mature OLCs in MS white matter plaque. High levels of stathmin in differentiated OLCs promote susceptibility to apoptosis. They observed two stathmin bands in the basic brain protein fraction by immunoblotting. Both bands were up-regulated in MS. The same pattern was also found in the whole protein lysate of the NAWM. Immunohistochemistry results pointed out extensive stathmin reactivity in oligodendrocytes. The authors claim a dysfunction of OLCs in remyelination and repair in MS due to the increased stathmin levels in mature OLCs.

We support the finding of Liu et al, of an up-regulation of a stathmin isoform in MS. In addition, we replicated the same pattern in the gray matter of MS patients. Moreover, we report the down-regulation of an isoform of stathmin in MS white matter and gray matter, at least in a subset of patients. One explanation is that the lower spot is the dephosphorylated form of the top isoform, which moves faster on an SDS gel, as observed in EAE. Another inference is a proteolytic cleavage of hyperphosphorylated stathmin (the upper band) at its N-terminus, thereby activating the protein.

In the mouse spinal cord we isolated 3 different p-stathmin isoforms. Migration of the stathmin isoforms at different pI is indicative of the different number of phosphorylated residues on the different residues. We observed loss of the most acidic (hyperphosphorylated) stathmin isoform, as well as the most basic p-stathmin isoform in the EAE spinal cord. In addition, we found another spot, (spot 3, see result section), with a pI similar to the most acidic spot but with a MW lower than the other isoforms. The third spot was very weakly stained by ProQ, probably indicating that it is dephosphorylated, (Figure 3.12). We infer from these results that stathmin is activated by dephosphorylation in the EAE

brain and spinal cord. Loss of the most basic p-stathmin isoform, (spot 1, fewer phosphorylated residues), in EAE indicates controlled modulation of phosphorylated stathmin, (probably phosphosite-specific), by extracellular stimuli.

Immunoblotting with an antibody directed against the carboxy-terminus of stathmin detected a single band in EAE, which was moderately up-regulated in the spinal cord of EAE animals. This probably represents the up-regulation of the hypophosphorylated stathmin isoforms. On the other hand, Western blotting, using an antibody directed against the N-terminal amino acids, clearly showed downregulation of the single stathmin band in EAE. We have no evidence of the cell type that contributes to the change in stathmin levels. Given the finding from Liu et al, we can conclude that elevated levels of dephosphorylated stathmin contribute to depolymerization of microtubules in OLCs. However, the down regulation of stathmin, that we detected even in the gray matter of one of the MS patients, prompts us to consider additional roles of stathmin, independent of OLCs in the pathogenesis of MS. Consistent with this, we also observed the down-regulation of a stathmin-related protein in EAE, as inferred from the binding of the antibody, (data not shown).

Findings from stathmin knockout mice are helpful in considering the above hypothesis (Liedtke et al., 2002). Stathmin knockout mice demonstrated that stathmin is essential for maintaining axonal integrity, suggesting a novel function possibly distinct from its microtubule destabilizing activity. Stathmin knockout mice develop, with age, a progressive axonopathy of large caliber, heavily-myelinated central and peripheral axons. The neuropathology consists of primary degenerative changes in the axoplasm. Myelin pathology appears at later stages (dys- demyelination) in association with the axonal changes. Infiltrating cells, as well as phagocytosis of degenerated axons and myelin by microglial cells and astrocytes, was observed. The pathology is ascribed to the absence of stathmin. Normal development of the knockout mice is explained as a result of a compensation of stathmin deficiency by other stathmin-related proteins. The authors assert that the neurons that give rise to the affected axons in the knockout mice normally express stathmin in their somata. The pathological lesions described were distinct from the age-related changes that may develop in normal mice, and from the pathology associated with known human diseases of the aging nervous system. Functional correlates of the structural changes are subtle neurogenic myopathy with an impaired motor nerve conduction. However, no discontinuities of microtubules or neurofilaments nor any gross alterations in axonal transport were seen in these animals. Whether a similar loss of axonal integrity is the consequence of low levels of stathmin in the neurons of MS patients needs to be investigated further.

4.3 Summary

Taken together, our proteome data demonstrate the regulation of many proteins in the CNS of EAE and MS. After extensive literature mining we are putting forward the role of some of the proteins that can directly or indirectly work as promoters of neuronal and axonal loss.

While SAP can directly induce apoptosis in neurons, CLIC1 promotes microglia-mediated neurotoxicity. Instead of complete deactivation of microglia, a selective blockade of CLIC1 in MS might turn out to be more effective in preventing microglia-mediated neuronal damage. Similarly, obscuring SAP uptake or its activity should be considered as a mode to prevent neuronal loss. Loss of ferritin heavy chain might be a causative mechanism that can promote neuronal and glial damage via oxidative stress.

Mitochondrial dysfunction as a source of axonal degeneration in EAE and MS has been demonstrated (Dutta et al., 2006). We are supplementing these findings with additional cadidate protein markers. One major change that we observed was the down- regulation of CK-B in EAE, a protein essential for energy transport in neural tissue.

Oxidative modification, and loss of activity of certain proteins and their reduced levels, have been suggested in CNS injury and diseases (Perluigi et al., 2005; Choi et al., 2004). We observed a significant loss of CK-B and lactate dehydrogenase, two proteins that undergo oxidative modification and loss. Lactate dehydrogenase is also an enzyme involved in energy metabolism.

A major set of the differentially regulated proteins is functionally associated with cytoskeletal organization. Consequently, we detected major changes (mainly loss), of actin and tubulin in MS and EAE. We observed quantitative and/or qualitative (with respect to phosphorylation) changes of some phosphoproteins in the context of cytoskeletal (dis)organization. Most of these proteins have great functional significance during development. Additional information on the phosphorylation state, provided by the phosphoproteome analysis, enabled us to correlate many proteins with possible disease mechanisms.

Our EAE results demonstrated hypophosphorylation of stathmin in EAE. Microtubule depolymerization activity of stathmin is negatively regulated by its phosphorylation on one or more of its phospho-sites. In EAE we saw a marked reduction in two hyperphosphorylated isoforms of stathmin, and an increase of one hypophosphorylated isoform. We are suggesting dephosphorylation, (possibly kinase-specific), of stathmin as a mechanism that activates a stathmin-mediated disease process in EAE. In MS we replicated the up-regulation of the

smaller isoform of stathmin that was already shown by Liu et al. In addition, we also observed a reduction in a second band of stathmin in plaque, white matter and gray matter of one of the MS patients. Liu et al have proposed the role of stathmin in OLC's. However, the effect of the stathmin level and phosphorylation on neuronal function in the context of MS and other neuronal diseases needs to be examined.

Hyperphosphorylation of cofilin is an indication of its inactivation that promotes axonal retraction. Regulation of cofilin, together with the modification of CRMP2, supports the concept of growth cone collapse as a process that promotes degeneration, or inhibits regeneration and repair. Collapsin is an inhibitory signal that is essential for axonal guidance during CNS development. It has been implicated as an inhibitor of regeneration in adult CNS after injury. Moreover, hyperphosphorylated CRMP2 was shown to be associated with neurofibrillary tangles in AD. Hyperphosphorylation and loss of CRMP4, the only CRMP protein involved in neurogenesis, is yet another clue that points to the prevalence of molecular cues (repellent cues ?) facilitating inhibition of regeneration.

Sirtuin 2 is another microtubule-associated protein that was down-regulated in MS patients. Two out of 3 SIRT2 isoforms were down-regulated in MS patients. In EAE we observed the consistent loss of one of the two phosphorylated isoforms of sirtuin. Although acetylation stabilizes microtubules, it can be argued that SIRT2 is important for regenerative processes where the dynamics of microtubule polymerization and depolymerization are required. Another possible mechanism is the disturbance of axonal transport and integrity due to hyperacetylated tubulin. The neuroprotective role of SIRT1, a sirtuin family protein, has been recognized in the context of various pathological conditions including AD, HD and axonal degeneration. There is not much experimental evidence supporting the neuroprotective activity of the human SIRT2. However, due to the immense evidence emphasizing the neuronal and axonal protective function of SIRT1, it can be speculated that SIRT2 also has a neuroprotective effect that is mediated via direct or indirect connection to its tubulin deacetylation function, or via its interaction with other molecules or histones (Anekonda and Reddy 2006).

Interestingly, in a cell culture model over-expressing CRMP2 in neuronal cells, cellular inclusions of CRMP2 and hyperphosphorylated tubulin were observed that induced apoptosis in those cells. Therefore, it is tempting to speculate a link between CRMP2 and the loss of the tubulin deacetylase, SIRT2, in modulating microtubule (axonal) instability. Additional evidence supporting disruption of axonal function is provided by the loss of the NSF attachment proteins gamma and alpha.

Axonal loss is known to correlate with the precipitation of irreversible dis-

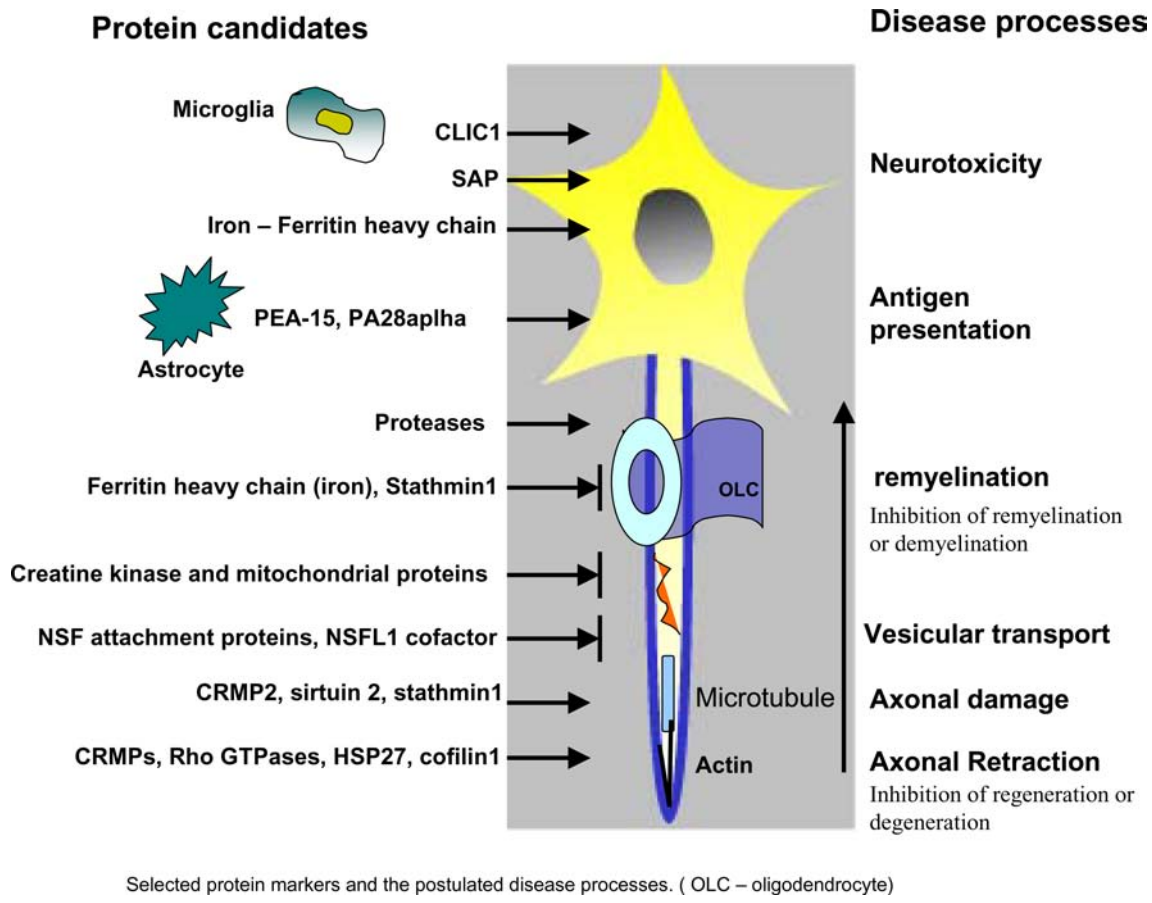

Figure 4.9: Selected protein markers and the postulated disease processes.

ability in MS and EAE (Bjartmar and Trapp, 2001; Trapp et al., 1999; Kornek et al., 2000; Wujek et al., 2002). Axonal loss is found to occur already in the early stages of EAE and MS (Kuhlmann et al., 2002). However, none of the drugs that are currently in practice or clinical trial are directed towards preventing or repairing axonal degeneration. Our results replicated the expression and post-translational modification of some proteins that have a functional correlation with the maintenance of axonal integrity and repair. The abundance of proteins involved in cytoskeletal (axonal) organization among the identified markers might be an indication of a dying-back process, where axonal damage activates neuronal degeneration (Mastronardi and Moscarello, 2005). We propose the potential of one or more of these proteins as a therapeutic target for the repair of axonal damage.

Figure 4.9 summarizes the different disease markers and the disease processes

they take part in. A closer inspection of the identified proteins points out the prevalence of protein markers that are indicative of a neurodegenerative mechanism in MS. This finding is in accordance with the recent concept of looking at MS as a neuronal disease, giving importance to the neurodegenerative disease processes (Waxman, 2000b; Prat and Antel, 2005). Markers such as CK-B, SAP, ferritin were also implicated in the pathogenesis of other neurological and neurodegenerative diseases like AD, PD, ALS etc. This finding again establishes the neurodegenerative features of MS that are similar to other diseases. All neurodegenerative disorders share the distinct commonality that a decrease in nervous system functionality takes place owing to a loss of neurons and/or glia (Mathisen, 2003). The mechanisms leading to reductions in nervous system functionality could be, either processes unique to a certain disease or some shared processes common to these diseases. While many gene targets are selected because of their specific association with the pathology of the disease, identifying similarities in the pathogenesis of neurodegenerative diseases enables certain genes to be targeted for multiple disorders. This is evident from many studies that have used proteomic profiling to identify disease markers of CNS disorders. A good majority of the proteins identified in these studies overlap. Partially, this can be ascribed to the limitations of 2DE, which detects mostly moderate to high abundant proteins. On the other hand, it is not unlikely that similar mechanisms take place in the different neurodegenerative diseases.

Intriguingly, some of the up-regulated proteins, such as HSP27 and CRMPs, are implicated as autoantigens in the serum of MS patients (Almeras et al., 2004). Up-regulation of these proteins in the CNS might be a trigger that initiates the autoimmune response against the same antigens. This inference is in accordance with the neuronal hypothesis of MS origin, where primary neurodegenerative processes stimulate a secondary immune response against CNS antigens.

One factor evident from the human study is the involvement of NAWM and NAGM in the pathogenesis of MS. Immunoblottting of SAP is an example of changes present in the gray matter that are not present in the NAWM. SAP is a protein that can induce neuronal apoptosis after its uptake into the nucleus. Factors that promote neuronal loss are more likely to be identified from the gray matter. This is consistent with the idea that MS needs to be recognized as a neuronal disease. Similarly, the regulation of stathmin in gray matter and white matter also strengthens this idea.

Additional proof of the contribution of subtle changes taking place in the CNS areas is provided by the EAE study. We found protein level variations that are common to both the brain and spinal cord. In EAE, histopathological alterations in the CNS occur in an ascending fashion and are pronounced more in the spinal

cord. The changes occurring in the brain are fewer and less in severity. Devic's model, on the other hand, is characterized by the restricted localization of lesions and inflammation in the spinal cord and optic nerve, with complete sparing of the brain. Despite the absence of lesions in the brain, we identified an almost equal number of differences at the protein level in the brain and spinal cord of EAE animals compared to the control brain and spinal cord. This is indicative of early quiescent disease processes taking place in the brain, which are not detectable as morphological changes.

In conclusion, our data has introduced many protein candidates that can possibly shed light on the molecular mechanisms of at least some of the disease processes taking place in EAE and MS. Clinical disabilities of MS are contributed to by the action of diverse disease mechanisms that have distinct roles in the progression of the disease. Functional classification of the proteins that we have identified reveals the disease process-specificity of these markers. Through extensive literature mining we are putting forward certain molecular pathways, as well as the interaction between some of the markers. Moreover, our approach to compare the phosphoproteome took us a step further in explaining the functional correlation of certain proteins in the pathogenesis of EAE and MS. The results are a reflection of the neurodegenerative process in EAE that can lead to neuronal and axonal damage. Further, there are markers suggestive of an inflammatory process, mitochondrial dysfunction, glial cell activation or damage, demyelination and oxidative stress, all of which are known processes in MS and EAE. Disease process-specific protein markers are targets for therapeutic interventions for specific and restricted blocking, or enhancement of gene products, in order to impede disease processes. Our findings justify the importance of unbiased analysis of expression and post-translational modification-specific changes of proteins in deciphering the functional aspects of complex disease mechanisms.

4.4 Future perspectives

The protein markers and mechanisms that we have introduced open a new way to the better understanding of the molecular pathogenesis of EAE and MS. Although some of these molecular markers are already associated with other neurodegenerative diseases, we have introduced some protein markers for the first time in the context of MS. We are putting forward pathways that can contribute to neuronal and axonal loss in MS. The hypotheses asserted by us need to be further investigated in animal or cell culture studies. While we have thoroughly looked at EAE, our analysis was limited to very few MS patients. The proteins

identified in EAE need to be tested in the tissue, and body fluids of a large number of MS patients in order to establish their correlation with disease activity in MS. The presence of a marker in a body fluid can serve as an (early) diagnostic marker for MS, as well as to monitor the effects of drugs. Clinical translation and evaluation of the identified biomarkers in different patient populations will enable the identification of these markers as a signature of the disease process in subsets of patients.

Our results demonstrate the similarity of the molecular mechanisms of MS to other CNS disorders. It is tempting to conclude that all neuronal diseases have similar mechanisms at the molecular level, but with a different origin and clinical manifestation, depending on the part of the CNS that is affected. The similarity of MS to other neurodegenerative diseases needs great attention. Recognizing the common molecular mediators of these diseases will promote the usage of drugs for MS which are already on trial for other diseases.

A great number of large-scale transcriptome analysis has been performed in the search for genes and gene products responsible for the MS and EAE pathogenesis. There is an urgent need to combine these diverse findings in order to determine common disease markers, or markers that interact in common disease pathways. This would help establish links between the gene products discovered in different laboratories, thereby creating a system level picture of complex disease mechanisms occurring in MS and EAE. Different pieces of the puzzle have to be joined together to solve complex diseases like MS.

Chapter 5

References

Aboul-Enein, F., Bauer, J., Klein, M., Schubart, A., Flugel, A., Ritter, T., Kawakami, N., Siedler, F., Linington, C., Wekerle, H., Lassmann, H., and Bradl, M. (2004). Selective and antigen-dependent effects of myelin degeneration on central nervous system inflammation. *J Neuropathol Exp Neurol.*, 63(12):1284 – 1296.

Adelmann, M., Wood, J., Benzel, I., Fiori, P., Lassmann, H., Matthieu, J. M., Gardinier, M. V., Dornmair, K., and Linington, C. (1995). The n-terminal domain of the myelin oligodendrocyte glycoprotein (mog) induces acute demyelinating experimental autoimmune encephalomyelitis in the lewis rat. *J Neuroimmunol*, 63:17 – 27.

Agnew, B. J., Minamide, S, L., Bamburg, and R, J. (1995). Reactivation of phosphorylated actin depolymerizing factor and identification of the regulatory site. *J. Biol. Chem.*, 270:17582 – 17587.

Aizawa, H., Wakatsuki, S., Ishii, A., Moriyama, K., Sasaki, Y., Ohashi, K., Sekine-Aizawa, Y., Sehara-Fujisawa, A., Mizuno, K., Goshima, Y., and Yahara, I. (2001). Phosphorylation of cofilin by lim-kinase is necessary for semaphorin 3a-induced growth cone collapse. *Nat Neurosci.*, 4(4):367 – 373.

Aksenov, M., Aksenova, M, Butterfield, A, D., Markesbery, and R, W. (2000). Oxidative modification of creatine kinase bb in alzheimer?s disease brain. *J. Neurochem.*, 74:2520 – 2527.

Aksenov, M. Y., Aksenova, M. V., Payne, R. M., Smith, C. D., Markesbery, W. R., and Carney, J. M. (1997). The expression of creatine kinase isoenzymes in neocortex of patients with neurodegenerative disorders: Alzheimer's and pick's disease. *Exp. Neurol.*, 146:458 – 465.

Almeras, L., Lefranc, D., Drobecq, H., de Seze, J., Dubucquoi, S., Vermersch, P., and Prin, L. (2004). New antigenic candidates in multiple sclerosis: identification by serological proteome analysis. *Proteomics.*, 4(7):2184 – 2194.

Alt, C., Duvefelt, K., Franzen, B., Yang, Y., and Engelhardt, B. (2005). Gene and protein expression profiling of the microvascular compartment in experimental autoimmune encephalomyelitis in c57bl/6 and sjl mice. *Brain Pathol.*, 15(1):1 – 16.

Alvord, E. C. (1984). The challenge: how good a model of ms is eae today? *Prog Clin Biol Res.*, 146:3 – 5.

Andersson, M., Alvarez-Cermeno, J., and Bernardi, G. (1994). Cerebrospinal fluid in the diagnosis of multiple sclerosis: a consensus report. *J Neurol Neurosurg Psychiatr*, 57:897 – 902.

Anekonda, T. S. and Reddy, P. H. (2006). Neuronal protection by sirtuins in ad. *J. Neurochem.*, 96:305 – 313.

Antel, J. (1999). Multiple sclerosis?emerging concepts of disease pathogenesis. *J Neuroimmunol*, 98:45 – 48.

Araki, T., Y, S., and Milbrandt (2004). Increased nuclear nad biosynthesis and sirt1 activation prevent axonal degeneration. *4. Araki T, Sasaki Y, Milbrandt Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. J. Science.*, 305(5686):1010 – 1013.

Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by lim-kinase. *Nature*, 393(6687):805 – 809.

Arimura, N., Menager, C., Kawano, Y., Yoshimura, T., Kawabata, S., Hattori, A., Fukata, Y., Amano, M., Goshima, Y., Inagaki, M., Morone, N., Usukura, J., and Kaibuchi, K. (2005). Phosphorylation by rho kinase regulates crmp-2 activity in growth cones. *Mol Cell Biol.*, 25(22):9973 – 9984.

Avalos, J. L., Celic, I., Muhammad, S., Cosgrove, M. S., Boeke, J. D., and Wolberger, C. (2002). Structure of a sir2 enzyme bound to an acetylated p53 peptide. *Mol. Cell*, 10:523 – 535.

Babbe, H., Roers, A., Waisman, A., Lassmann, H., Goebels, N., Hohlfeld, R., Friese, M., Schroder, R., Deckert, M., Schmidt, S., Ravid, R., and Rajewsky, K. (2000). Clonal expansions of cd8(+) t cells dominate the t cell infiltrate

in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J. Exp. Med.*, 192:393 – 404.

Balla, G., Jacob, H. S., and Balla, J. (1992). Ferritin: a cytoprotective antioxidant strategem of endothelium. *J. Biol. Chem.*, 267:18148 – 18153.

Bamburg, J. R. (1999). Proteins of the adf/cofilin family: essential regulators of actin dynamics. *Annu. Rev. Cell Dev. Biol.*, 15:185 – 230.

Barger, S. W. and Harmon, A. D. (1997). Microglial activation by alzheimer amyloid precursor protein and modulation by apolipoprotein e. *Nature*, 388:878 – 881.

Barnett, M. H. and Prineas, J. W. (2004). Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol.*, 55:458 – 468.

Behi, M. E., Dubucquoi, S., Lefranc, D., Zephir, H., Seze, J. D., Vermersch, P., and Prin, L. (2005). New insights into cell responses involved in experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol Lett.*, 96(1):11 – 26.

Benveniste, E. N., Nguyen, V. T., and O'Keefe, G. M. (2001). Immunological aspects of microglia: relevance to alzheimer's disease. *Neurochem. Int.*, 39:381 – 391.

Bernard, C. C. A., Johns, T. G., Slavin, A., Ichikawa, M., Ewing, C., Liu, J., and Bettadapura, J. (1997). Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. *J Mol Med*, 75:77 – 88.

Bickerstaff, M. C., Botto, M., Hutchinson, W. L., Herbert, J., Tennent, G. A., Bybee, A., Mitchell, D. A., Cook, H. T., Butler, P. J., Walport, M. J., and Pepys, M. B. (1999). Serum amyloid p component controls chromatin degradation and prevents antinuclear autoimmunity. *Nat Med.*, 5(6):694 – 697.

Bielekova, B. and Martin, R. (2004). Development of biomarkers in multiple sclerosis. *Brain.*, 127(7):1463 – 1478.

Bitsch, A., da Costa, C., Bunkowski, S., Weber, F., Rieckmann, P., and Bruck, W. (1998). Identification of macrophage populations expressing tumor necrosis factor-alpha mrna in acute multiple sclerosis. *Acta Neuropathol.Berl.*, 95(1998):373 – 377.

Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T., and Bruck, W. (2000). Acute axonal injury in multiple sclerosis. correlation with demyelination and inflammation. *Brain*, 123:1174 – 1183.

Bjartmar, C., Kinkel, R. P., Kidd, G., Rudick, R. A., and Trapp, B. D. (2001). Axonal loss in normal-appearing white matter in a patient with acute ms. *Neurology*, 57:1248 – 1252.

Bjartmar, C. and Trapp, B. D. (2001). Axonal damage and neuronal degeneration in multiple sclerosis: mechanisms and functional consequences. *Curr Opin Neurol*, 14:271 – 278.

Bjellqvist, B., Ek, K., Righetti, P. G., Gianazza, E., Gorg, A., Westermeier, R., and Postel, W. (1982). Isoelectric focusing in immobilized ph gradients: principle, methodology and some applications. *J Biochem Biophys Methods*, 6(4):317 – 339.

Botto, M., Hawkins, P. N., Bickerstaff, M. C., Herbert, J., Bygrave, A. E., McBride, A., Hutchinson, W. L., Tennent, G. A., Walport, M. J., and Pepys, M. B. (1997). Amyloid deposition is delayed in mice with targeted deletion of the serum amyloid p component gene. *Nat Med.*, 3(8):855 – 859.

Breithaupt, C., Schubart, A., Zander, H., Skerra, A., Huber, R., Linington., C., and Jacob, U. (2003). Structural insights into the antigenicity of myelin oligodendrocyte glycoprotein. *Proc Natl Acad Sci USA*, 100(16):9446 – 9451.

Brown, M., Jacobs, T., Eickholt, B., Ferrari, G., Teo, M., Monfries, C., Qi, R. Z., Leung, T., Lim, L., and Hall, C. (2004). Alpha2-chimaerin, cyclin-dependent kinase 5/p35, and its target collapsin response mediator protein-2 are essential components in semaphorin 3a-induced growth-cone collapse. *J. Neurosci.*, 24:8994 – 9004.

Brunner, C., Lassmann, H., Waehneldt, T. V., Matthieu, J.-M., and Linington, C. (1989). Differential ultrastructural localization of myelin basic protein, myelin/oligodendrocyte glycoprotein, and 29,39-cyclic nucleotide 39-phosphodiesterase in the cns of adult rats. *J. Neurochem.*, 52:296 – 304.

Byk, T., Dobransky, T., Cifuentes-Diaz, C., and Sobel, A. (1996). Identification and molecular characterization of unc-33-like phosphoprotein (ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product. *J. Neurosci.*, 16:688 – 701.

Cairo, G., Tacchini, L., Pogliaghi, G., Anzon, E., Tomasi, A., and Bernelli-Zazzera, A. (1995). Induction of ferritin synthesis by oxidative stress: transcriptional and post-transcriptional regulation by expansion of the "free" iron pool. *J. Biol. Chem.*, 270:700 – 703.

Cermak, J., Balla, J., and Jacob, H. S. (1993). Tumor cell heme uptake induces ferritin synthesis resulting in altered oxidant sensitivity: possible role in chemotherapy efficacy. *Cancer Res.*, 53:5308 – 5313.

Chabas, D., Baranzini, S. E., Mitchell, D., Bernard, C. C., Rittling, S. R., Denhardt, D. T., Sobel, R. A., Lock, C., Karpuj, M., Pedotti, R., Heller, R., Oksenberg, J. R., and Steinman, L. (2001). The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science*, 23(294):1731 – 1735.

Chakrabarty, A., MR, E., and SM, L. (2003). Heme oxygenase-1 in sjl mice with experimental allergic encephalomyelitis. *Mult Scler.*, 4(4):372 – 381.

Chang, J. H., Kim, H. C., Hwang, K. Y., Lee, J. W., Jackson, S. P., Bell, S. D., and Cho, Y. (2002). Structural basis for the nad-dependent deacetylase mechanism of sir2. *J. Biol. Chem.*, 277(37):34489 – 34498.

Cheepsunthorn, P., Palmer, C., and Connor, J. R. (1998). Cellular distribution of ferritin subunits in postnatal rat brain. *J Comp Neurol.*, 400(1):73 – 86.

Cheepsunthorn, P., Palmer, C., Menzies, S., Roberts, R. L., and Connor, J. R. (2001). Hypoxic/ischemic insult alters ferritin expression and myelination in neonatal rat brains. *J Comp Neurol.*, 431(4):382 – 396.

Chen, J., Zhou, Y., Mueller-Steiner, S., Chen, L. F., Kwon, H., Yi, S., Mucke, L., and Gan, L. (2005). Sirt1 protects against microglia-dependent amyloid-beta toxicity through inhibiting nf-kappab signaling. *J. Biol. Chem.*, 280(48):40364 – 40374.

Choi, J., Forster, M. J., McDonald, S. R., Weintraub, S. T., Carroll, C. A., and Gracy, R. W. (2004). Proteomic identification of specific oxidized proteins in apoe-knockout mice: relevance to alzheimer's disease. *Free Radic Biol Med.*, 36(9):1155 – 1162.

Combs, C. K., Karlo, J. C., Kao, S. C., and Landreth, G. E. (2001). Amyloid stimulation of microglia and monocytes results in tnf -dependent expression of inducible nitric oxide synthase and neuronal apoptosis. *J. Neurosci.*, 21:1179 – 1188.

Connor, J. R., Boeshore, K. L., Benkovic, S. A., and Menzies, S. L. (1994). Isoforms of ferritin have a specific cellular distribution in the brain. *J. Neurosci. Res.*, 37(4):461 – 465.

Connor, J. R. and Menzies, S. L. (1996). Relationship of iron to oligodendrocytes and myelination. *Glia*, 17:83 – 89.

Connor, J. R., Pavlick, G., Karli, D., Menzies, S. L., and Palmer, C. (1995). A histochemical study of iron-positive cells in the developing brain. *J Comp Neurol*, 355:111 – 123.

Cornet, A., Bettelli, E., Oukka, M., Cambouris, C., Avellana-Adalid, V., Kosmatopoulos, K., and Liblau, R. S. (2000). Role of astrocytes in antigen presentation and naive t-cell activation. *J Neuroimmunol.*, 106(1-2):69 – 77.

Craelius, W., Migdal, M. W., Luessenhop, C. P., Sugar, A., and Mihalakis, I. (1982). Iron deposits surrounding multiple sclerosis plaques. *Arch Pathol Lab Med.*, 106(8):397 – 399.

Daitoku, H., Hatta, M., Matsuzaki, H., Aratani, S., Ohshima, T., and Miyagishi, M. (2004). Silent information regulator 2 potentiates foxo1-mediated transcription through its deacetylase activity. *Proc. Natl. Acad. Sci. USA*, 101:10042 – 10047.

de la Monte, S. M. and Wands, J. R. (2004). Alzheimer-associated neuronal thread protein mediated cell death is linked to impaired insulin signaling. *J. Alzheimers Dis.*, 6:231 – 242.

Dedeoglu, A., Kubilus, K, J., Yang, L, Ferrante, L, K., Hersch, M, S., Beal, F, M., Ferrante, and J, R. (2003). Creatine therapy provides neuroprotection after onset of clinical symptoms in huntington?s disease transgenic mice. *J. Neurochem.*, 85:1359 – 1367.

DeStefano, N., Narayanan, S., Matthews, P. M., Francis, G. S., Antel, J. P., and Arnold, D. L. (1999). In vivo evidence for axonal dysfunction remote from focal cerebral demyelination of the type seen in multiple sclerosis. *Brain*, 122:1933 – 1939.

Dolder, M., Walzel, B., Speer, O., Schlattner, U., and Wallimann, T. (2003). Inhibition of the mitochondrial permeability transition by creatine kinase substrates. requirement for microcompartmentation. *J. Biol. Chem.*, 278:17760 – 17766.

Dolder, M., Wendt, S., and Wallimann, T. (2001). Mitochondrial creatine kinase in contact sites: interaction with porin and adenine nucleotide translocase, role in permeability transition and sensitivity to oxidative damage. *Biol. Signals Recept.*, 10:93 – 111.

Dryden, S. C., Nahhas, F. A., Nowak, J. E., Goustin, A. S., and Tainsky, M. A. (2003). Role for human sirt2 nad-dependent deacetylase activity in control of mitotic exit in the cell cycle. *Mol Cell Biol.*, 23(9):3173 – 3185.

Dumont, D., Noben, J. P., Raus, J., Stinissen, P., and Robben, J. (2004). Proteomic analysis of cerebrospinal fluid from multiple sclerosis patients. *Proteomics*, 4(7):2117 – 2124.

Dutta, R., McDonough, J., Yin, X., Peterson, J., Chang, A., Torres, T., Gudz, T., Macklin, W. B., Lewis, D. A., Fox, R. J., Rudick, R., Mirnics, K., and Trapp, B. D. (2006). Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. *Ann Neurol*, 59:478 – 489.

Duzhak, T., Emerson, M. R., Chakrabarty, A., Aletrman, A. M., and Levine, S. M. (2003). Analysis of protein induction in the cns of sjl mice with experimental allergic encephalomyelitis by proteomic screening and immunohistochemistry. *Cell Mol Biol (Noisy-le-grand).*, 49(5):723 – 732.

Eder, C. (1998). Ion channels in microglia (brain macrophages). *Am J Physiol*, 275:C327 – C342.

Egensperger, R., Kosel, S., von Eitzen, U., and Graeber, M. B. (1998). Microglial activation in alzheimer disease: association with apoe genotype. *Brain Pathol*, 8(439-447):439 – 447.

Egnaczyk, G. F., Pomonis, J. D., Schmidt, J. A., Rogers, S. D., Peters, C., Ghilardi, J. R., Mantyh, P. W., and Maggio, J. E. (2003). Proteomic analysis of the reactive phenotype of astrocytes following endothelin-1 exposure. *Proteomics.*, 3(5):689 – 698.

Elson, C. J., Barker, R. N., Thompson, S. J., and Williams, N. A. (1995). Immunologically ignorant autoreactive t cells, epitope spreading and repertoire limitation. *Immunol Today.*, 16:71 – 76.

Eugster, H.-P., Frei, K., Kopf, M., Lassmann, H., and Fontana, A. (1998). Il-6- deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *Eur J Immunol*, 28:2178 – 2187.

Fawcett, J. W. and RA, A. (1999). The glial scar and central nervous system repair. *Brain Res Bull.*, 49(6):377 – 391.

Fenyo, D. (2000). Identifying the proteome: software tools. *Curr. Opin. Biotechnol.*, 11(4):391 – 395.

Ferguson, B., Matyszak, M. K., Esiri, M. M., and Perry, V. H. (1997). Axonal damage in acute multiple sclerosis lesions. *Brain*, 120:393 – 399.

Ferreira, A. and Caceres, A. (1989). The expression of acetylated microtubules during axonal and dendritic growth in cerebellar macroneurons which develop in vitro. *Brain Res Dev Brain Res.*, 49(2):205 – 213.

Filippi, M. (2001). Multiple sclerosis: a white matter disease with associated gray matter damage. *J Neurol Sci*, 185:3 – 4.

Forge, J. K., Pedchenko, T. V., and LeVine, S. M. (1998). Iron deposits in the central nervous system of sjl mice with experimental allergic encephalomyelitis. *Life Sci.*, 63(25):2271 – 2284.

Fountoulakis, M. and Kossida, S. (2006). Proteomics-driven progress in neurodegeneration research. *Electrophoresis*, 27(8):1556 – 1573.

Fournier, A. E. and Strittmatter, S. M. (2001). Repulsive factors and axon regeneration in the cns. *Curr Opin Neurobiol.*, 11(1):89 – 94.

Friedman, B., Hockfield, S., Black, J. A., Woodruff, K. A., and Waxman, S. G. (1989). In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody. *Rip. Glia*, 2:380 – 390.

Frye, R. A. (1999). Characterization of five human cdnas with homology to the yeast sir2 gene: Sir2-like proteins (sirtuins) metabolize nad and may have protein adp-ribosyltransferase activity. *Biochem. Biophys. Res. Commun.*, 260(1):273 – 279.

Frye, R. A. (2000). Phylogenetic classification of prokaryotic and eukaryotic sir2-like proteins. *Biochem. Biophys. Res. Commun.*, 273(2):793 – 798.

Garlanda, C., Bottazzi, B., Bastone, A., and Mantovani, A. (2005). Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. *Annu Rev Immunol.*, 2:337 – 366.

Genain, C. P., Cannella, B., Hauser, S. L., and Raine, C. S. (1999). Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nature Med*, 5:170 – 175.

Gilgun-Sherki, Y., Melamed, E., and Offen, D. (2004). The role of oxidative stress in the pathogenesis of multiple sclerosis: the need for effective antioxidant therapy. *J Neurol.*, 251(3):261 – 268.

Giovannoni, G. and Goodman, A. (2005). Neutralizing anti-ifn-beta antibodies: how much more evidence do we need to use them in practice? *Neurology*, 65:6 – 8.

Gorg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., and Weiss, W. (2000). The current state of two-dimensional electrophoresis with immobilized ph gradients. *Electrophoresis.*, 21(6):1037 – 1053.

Gorg, A., Postel, W., and Gunther, S. (1988). The current state of two-dimensional electrophoresis with immobilized ph gradients. *Electrophoresis.*, 9(9):531 – 546.

Goshima, Y., Nakamura, F., Strittmatter, P., and Strittmatter, S. M. (1995). Collapsin-induced growth cone collapse mediated by an intracellular protein related to unc-33. *Nature*, 376(6540):509 – 514.

Gould, R. M., Freund, C. M., Palmer, F., and Feinstein, D. L. (2000). Messenger rnas located in myelin sheath assembly sites. *J. Neurochem.*, 75(5):1834 – 1844.

Gras, R., Muller, M., Gasteiger, E., Gay, S., Binz, P. A., Bienvenut, W., Hoogland, C., Sanchez, J. C., Bairoch, A., Hochstrasser, D. F., and Appel, R. D. (1999). Improving protein identification from peptide mass fingerprinting through a parameterized multi-level scoring algorithm and an optimized peak detection. *Electrophoresis.*, 20(18):3535 – 3550.

Grenningloh, G., Soehrman, S., Bondallaz, P., Ruchti, E., and Cadas, H. (2004). Role of the microtubule destabilizing proteins scg10 and stathmin in neuronal growth. *J Neurobiol.*, 58(1):60 – 69.

Gu, Y., Hamajima, N., and Ihara, Y. (2000). Neurofibrillary tangle-associated collapsin response mediator protein-2 (crmp-2) is highly phosphorylated on thr-509, ser-518, and ser-522. *Biochemistry*, 39(15):4267 – 4275.

Gu, Y. and Ihara, Y. (2000). Evidence that collapsin response mediator protein-2 is involved in the dynamics of microtubules. *J. Biol. Chem.*, 275(24):17917 – 17920.

Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999). Correlation between protein and mrna abundance in yeast. *Mol Cell Biol.*, 19(3):1720 – 1730.

Halliday, A. M. (1993). Evoked potentials in clinical testing. *2nd ed. London: Churchill Livingstone*.

Hammack, B. N., Fung, K. Y., Hunsucker, S. W., Duncan, M. W., Burgoon, M. P., Owens, G. P., and Gilden, D. H. (2004). Proteomic analysis of multiple sclerosis cerebrospinal fluid. *Mult Scler.*, 10(3):245 – 260.

Hanash, S. (2003). Disease proteomics. *Nature*, 422(6928):226 – 232.

Harrison, P. M. and Arosio, P. (1996). The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta*, 1275:161 – 203.

Hasegawa, M., Kawashima, S., Shikano, M., Kojima, K., Yanai, T., Hassegawa, H., Tomita, M., Murakami, K., Kawamura, N., and Katsumata, H. (1995). C1q complexes with beta-2-microglobulin and amyloid p-component in sera of patients with dialysis-associated amyloidosis. *Nephron.*, 69(4):380 – 383.

Hatano, E., Tanaka, A., Kanazawa, A., A, Tsuyuki, S., Tsunekawa, S., Iwata, S., Takahashi, R., Chance, B., and Yamaoka, Y. (2004). Inhibition of tumor necrosis factor-induced apoptosis in transgenic mouse liver expressing creatine kinase. *Liver Int.*, 24:384 – 393.

Hauser, S. L., Bhan, K, A., Gilles, H, F., Kemp, M, Kerr, C, Weiner, and L, H. (1986). Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. *Ann. Neurol.*, 19:578 – 587.

Hawkins, P. N., Wootton, R., and Pepys, M. B. (1990). Metabolic studies of radioiodinated serum amyloid p component in normal subjects and patients with systemic amyloidosis. *J Clin Invest.*, 86(6):1862 – 1869.

Hiratsuka, M., Inoue, T., Toda, T., Kimura, N., Shirayoshi, Y., Kamitani, H., Watanabe, T., Ohama, E., Tahimic, C. G., Kurimasa, A., and Oshimura, M. (2003). Proteomics-based identification of differentially expressed genes in human gliomas: down-regulation of sirt2 gene. *Biochem. Biophys. Res. Commun.*, 309(3):558 – 566.

Hisahara, S., Chiba, S., Matsumoto, H., and Horio, Y. (2005). Transcriptional regulation of neuronal genes and its effect on neural functions: Nad-dependent histone deacetylase sirt1 (sir2alpha). *J Pharmacol Sci.*, 98(3):200 – 204.

Hjelmstrom, P., Juedes, E. E., Fjell, J., Ruddle, N. H., and Immunol, J. (1998). B-cell-deficient mice develop experimental allergic encephalomyelitis with demyelination after myelin oligodendrocyte glycoprotein sensitization. *Proteins*, 161:4480 – 4484.

Hohlfeld, R. (1997). Biotechnological agents for the immunotherapy of multiple sclerosis. principles, problems and perspectives. *Brain*, 120(5):865 – 916.

Horwitz, S. B., Shen, H. J., He, L., Dittmar, P., R, N., J, C., and UK, S. (1997). The microtubule-destabilizing activity of metablastin (p19) is controlled by phosphorylation. *J. Biol. Chem.*, 272(13):8129 – 8132.

Huang, D. (2000). Chemokines and chemokine receptors in inflammation of the nervous system: manifold roles and exquisite regulation. *Immunol. Rev.*, 177(2000):52 – 67.

Ibrahim, S. M. and Gold, R. (2005). Genomics, proteomics, metabolomics: what is in a word for multiple sclerosis. *Curr Opin Neurol*, 18:231–235.

Ibrahim, S. M., Mix, E., Bottcher, T., Koczan, D., Gold, R., Rolfs, A., and Thiesen, H. J. (2001). Gene expression profiling of the nervous system in murine experimental autoimmune encephalomyelitis. *Brain.*, 124(10):1927 – 1938.

Iglesias, A., Bauer, J., Litzenburger, T., Schubart, A., and Linington, C. (2001). T- and b-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. *GLIA*, 36:220 – 234.

Jang, J. H. and Surh, Y. J. (2003). Protective effect of resveratrol on betaamyloid-induced oxidative pc12 cell death. *Free Radic. Biol. Med.*, 34:1100 – 1110.

Jin, Z. and Strittmatter, S. M. (1997). Rac1 mediates collapsin-1-induced growth cone collapse. *J. Neurosci.*, 17(16):6256 – 6263.

Johns, T. G., de Rosbo, N. K., Menon, K. K., S, S. A., Gonzales, M. F., and Bernard, C. C. (1995). Myelin oligodendrocyte glycoprotein induces a demyelinating encephalomyelitis resembling multiple sclerosis. *J Immunol*, 154:5536 – 5541.

Joubert, F., Hoerter, J. A., and Mazet, J. L. (2002). Modeling the energy transfer pathways. creatine kinase activities and heterogeneous distribution of adp in the perfused heart. *Mol Biol Rep*, 29(1-2):177 – 182.

Kaeberlein, M., McVey, M., and Guarente, L. (1999). The sir2/3/4 complex and sir2 alone promote longevity in saccharomyces cerevisiae by two different mechanisms. *Genes Dev.*, 13:2570 – 2580.

Kalaria, R. N., Galloway, P. G., and Perry, G. (1991a). Widespread serum amyloid p immunoreactivity in cortical amyloid deposits and the neurofibrillary pathology of alzheimer's disease and other degenerative disorders. *Neuropathol Appl Neurobiol.*, 17(3):189 – 201.

Kalaria, R. N., Golde, T. E., Cohen, M. L., and Younkin, S. G. (1991b). Serum amyloid p in alzheimer's disease. implications for dysfunction of the blood-brain barrier. *Ann. N.Y. Acad. Sci.*, 640:145 – 148.

Kaneko, Y., Kitamoto, T., Tateishi, J., and Yamaguchi, K. (1989). Ferritin immunohistochemistry as a marker for microglia. *Acta Neuropathol (Berl).*, 79(2):129 – 136.

Kieseier, B. C., Hemmer, B., and Hartung, H.-P. (2005). Multiple sclerosis ? novel insights and new therapeutic strategies. *Current Opinion in Neurology*, 18:211 – 220.

Killestein, J. and Polman, C. H. (2005). Current trials in multiple sclerosis: established evidence and future hopes. *Curr Opin Neurol.*, 18(3)):253 – 260.

Kitsberg, D., Formstecher, E., Fauquet, M., Kubes, M., Cordier, J., Canton, B., Pan, G., Rolli, M., Glowinski, J., and Chneiweiss, H. (1999). Knock-out of the neural death effector domain protein pea-15 demonstrates that its expression protects astrocytes from tnfalpha-induced apoptosis. *J. Neurosci.*, 19(19):8244 – 8251.

Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. a novel approach to testing for induced point mutations in mammals. *Humangenetik*, 26(3):231 – 243.

Klose, J. (1999). Large-gel 2-d electrophoresis. *Methods Mol Biol.*, 112:147 – 172.

Konorev, E. A., Hogg, N., and Kalyanaraman, B. (1998). Rapid and irreversible inhibition of creatine kinase by peroxynitrite. *FEBS Lett.*, 427:171 – 174.

Kornek, B. and Lassmann, H. (2003). Neuropathology of multiple sclerosis - new concepts. *Brain research bulletin*, 61:321 – 326.

Kornek, B., Storch, M. K., Weissert, R., Wallstroem, E., Stefferl, A., Olsson, T., Linington, C., Schmidbauer, M., and Lassmann, H. (2000). Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am. J. Pathol.*, 157:267 – 276.

Krishnamoorthy, G., Lassmann, H, Wekerle, H., and Holz, A. (2006). Ose mouse: a spontaneous double-transgenic mouse model of human opticospinal multiple sclerosis and autoimmune t-b cooperation. *J Clin Invest.*, In press.

Kuhlmann, T., Lingfeld, G., Bitsch, A., Schuchardt, J., and Bruck, W. (2002). Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain.*, 125(10):2202 – 2212.

Kuiper, M. A., Mulder, C., vanKamp, G. J., Scheltens, P., and Wolters, E. C. (1994). Cerebrospinal fluid ferritin levels of patients with parkinson's disease, alzheimer's disease, and multiple system atrophy. *J Neural Transm Park Dis Dement Sect.*, 7(2):109 – 114.

Lafaille, J. J., Keere, F. V., Hsu, A. L., Baron, J. L., Haas, W., Raine, C. S., and Tonegawa, S. (1997). Myelin basic protein-specific t helper 2 (th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J Exp Med.*, 186(2):307 – 312.

Lambracht, D., Prokop, C., Hedrich, H.-J., Lindahl, K.-F., and Wonigeit, K. (1995). Mapping of h2-m homolog and mog genes in the rat mhc. *Immunogenetics*, 42:418 – 421.

Lassmann, H. (1983). Comparative neuropathology of chronic experimental allergic encephalomyelitis and multiple sclerosis. *Schriftenr. Neurol.*, 25(1983):1 – 135.

Lassmann, H. (1998). Pathology of multile sclerosis. *3rd ed. Churchill livingstone , Edinburg*, 3.

Lassmann, H. and Ransohoff, R. M. (2004). The cd4-th1 model for multiple sclerosis: a crucial re-appraisal. *Trends Immunol.*, 25(3):132 – 137.

Lebar, R., Lubetzki, C., Vincent, C., Lombrail, P., and Boutry, J. M. (1986). The m2 autoantigen of central nervous system myelin, a glycoprotein present in oligodendrocyte membrane. *Clin Exp Immunol*, 66:423 – 434.

Lehmann, P. V., Forsthuber, T., Miller, A., and Sercarz, E. E. (1992). Spreading of t-cell autoimmunity to cryptic determinants of an autoantigen. *Nature*, 358(9):155 – 157.

Lennon, V. A., Wingerchuk, D. M., Kryzer, T. J., Pittock, S. J., Lucchinetti, C. F., Fujihara, K., Nakashima, I., and Weinshenker, B. G. (2004). A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet*, 364(9451):2106 – 2112.

LeVine, S. M. (1997). Iron deposits in multiple sclerosis and alzheimer's disease brains. *Brain Res.*, 760(1-2):298 – 303.

Levine, S. M. and Chakrabarty, A. (2004). The role of iron in the pathogenesis of experimental allergic encephalomyelitis and multiple sclerosis. *Ann. N.Y. Acad. Sci.*, 1012:252 – 266.

Liebler, D. C. (2002). *Introduction to proteomics*.

Liedtke, W., Leman, E. E., Fyffe, R. E., Raine, C. S., and Schubart, U. K. (2002). Stathmin-deficient mice develop an age-dependent axonopathy of the central and peripheral nervous systems. *Am J Pathol.*, 160(2):469 – 480.

Linington, C., Morgan, B. P., Scolding, N. J., Wilkins, P., Piddlesden, S. J., and Compston, D. A. (1989). The role of complement in the pathogenesis of experimental allergic encephalomyelitis. *Brain*, 112:895 – 911.

Link, H. and Tibbling, G. (1977). Principles of albumin and igg analysis in neurological disorders. iii. valuation of igg synthesis within the central nervous system in multiple sclerosis. *Scand J Clin Lab Invest*, 37:397 – 401.

Liu, A., Stadelmann, C., Moscarello, M., Bruck, W., Sobel, A., Mastronardi, F. G., and Casaccia-Bonnefil, P. (2005). Expression of stathmin, a developmentally controlled cytoskeleton-regulating molecule, in demyelinating disorders. *J. Neurosci.*, 25(3):737 – 747.

Liu, B. P. and LeVine, S. M. (2001). Semaphorin-mediated axonal guidance via rho-related g proteins. *Curr Opin Cell Biol.*, 13(5):619 – 626.

Lock, C., Hermans, G., Pedotti, R., Brendolan, A., Schadt, E., Garren, H., Langer-Gould, A., Strober, S., Cannella, B., Allard, J., Klonowski, P., Austin, A., Lad, N., Kaminski, N., Galli, S. J., Oksenberg, J. R., Raine, C. S., Heller, R., and Steinman, L. (2002). Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med.*, 8(5):451 – 453.

Lucchinetti, C., Bruck, W., Parisi, J., Scheithauer, B., Rodriguez, M., and Lassmann, H. (2000). Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol.*, 47(6):707 – 717.

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999). Signaling from rho to the actin cytoskeleton through protein kinases rock and lim-kinase. *Science*, 285(5429):895 – 898.

Mason, M. R., Lieberman, A. R., Grenningloh, G., and Anderson, P. N. (2002). Transcriptional upregulation of scg10 and cap-23 is correlated with regeneration of the axons of peripheral and central neurons in vivo. *Mol Cell Neurosci*, 20:595 – 615.

Mastronardi, F. G. and Moscarello, M. A. (2005). Molecules affecting myelin stability: A novel hypothesis regarding the pathogenesis of multiple sclerosis. *J. Neurosci. Res.*, 80(3):301 – 308.

Mathisen, P. M. (2003). Gene discovery and validation for neurodegenerative diseases. *Drug Discov Today.*, 8(1):39 – 46.

Mattson, M. P. (2003). Methylation and acetylation in nervous system development and neurodegenerative disorders. *Ageing Res Rev.*, 2(3):329 – 342.

McCallum, K., Esiri, M. M., and Tourtellotte, W. W. (1987). T cell subsets in multiple sclerosis lesions. *Brain*, 110:1297 – 1308.

McDonald, W. I., Compston, A., Edan, G., Goodkin, D., Hartung, H. P., Lublin, F. D., McFarland, H. F., Paty, D. W., Polman, C. H., Reingold, S. C., Sandberg-Wollheim, M., Sibley, W., Thompson, A., van den, N. S., Weinshenker, B. Y., and Wolinsky, J. S. (2001). Recommended diagnostic criteria for multiple sclerosis: guidelines from the international panel on the diagnosis of multiple sclerosis. *Ann Neurol.*, 50(1):121 – 127.

McFarlin, D. E. and McFarland, H. F. (1982). Multiple sclerosis (first of two parts). *N Engl J Med*, 307:1183 – 1188.

McGeer, E. G. and McGeer, P. L. (1997). The role of the immune system in neurodegenerative disorders. *Mov Disord*, 12:855 – 858.

McGeer, E. G. and McGeer, P. L. (1998). The importance of inflammatory mechanisms in alzheimer disease. *Exp Gerontol*, 33:371 – 378.

McGeer, E. G., Yasojima, K., Schwab, C., and McGeer, P. L. (2001). The pentraxins: possible role in alzheimer's disease and other innate inflammatory diseases. *Neurobiol Aging.*, 22(6):843 – 848.

McGeer, P. L., Kawamata, T., Walker, D. G., Akiyama, H., Tooyama, I., and McGeer, E. G. (1993). Microglia in degenerative neurological disease. *Glia*, 7:84 – 92.

McGeer, P. L., Walker, D. G., Akiyama, H., Yasuhara, O., and McGeer, E. G. (1994). Involvement of microglia in alzheimer's disease. *Neuropathol Appl Neurobiol*, 20:191 – 192.

McRae, B. L., Vanderlugt, C. L., Canto, M. D., and Miller, S. D. (1995). Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *Journal of Experimental Medicine*, 182:75 – 85.

Menon, K., Piddlesden, S. J., and Bernard, C. C. A. (1997). Demyelinating antibodies to myelin oligodendrocyte glycoprotein and galactocerebroside induce degradation of myelin basic protein in isolated human myelin. *J. Neurochem.*, 69:214 – 222.

Metz, L. M., Zhang, Y., Yeung, M., Patry, D. G., Bell, R. B., Stoian, C. A., Yong, V. W., Patten, S. B., Duquette, P., Antel, J. P., and Mitchell, J. R. (2004). Minocycline reduces gadolinium-enhancing magnetic resonance imaging lesions in multiple sclerosis. *Ann Neurol.*, 55(5):756.

Miller, D. H., Khan, O. A., Sheremata, W. A., Blumhardt, L. D., Rice, G. P., Libonati, M. A., Willmer-Hulme, A. J., Dalton, C. M., Miszkiel, K. A., and O'Connor, P. W. (2003). A controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med.*, 348(1):15 – 23.

Miller, S. D., Katz-Levy, Y., Neville, K. L., and Vanderlugt, C. L. (2001). Virus-induced autoimmunity: epitope spreading to myelin autoepitopes in theiler's virus infection of the central nervous system. *Adv Virus Res.*, 56:199 – 217.

Minturn, J. E., Geschwind, D. H., Fryer, H. J. L., and Hockfield, S. (1995). Early postmitotic neurons transiently express toad-64, a neural specific protein. 355:369 – 379.

Mix, E., J, P., and SM, I. (2002). Gene-expression profiling of experimental autoimmune encephalomyelitis. *Neurochem. Res.*, 27(10):1157 – 1163.

Morales, M. and Fifkova, E. (1991). Distribution of acetylated alpha-tubulin in brain. in situ localization and biochemical characterization. *Cell Tissue Res.*, 265(3):415 – 423.

Mori, N. and Morii, H. (2002). Scg10-related neuronal growth-associated proteins in neural development, plasticity, degeneration, and aging. *J. Neurosci. Res.*, 70(3):264 – 273.

Morrow, S. A., Stoian, C. A., Dmitrovic, J., Chan, S. C., and Metz, L. M. (2004). The bioavailability of iv methylprednisolone and oral prednisone in multiple sclerosis. *Neurology*, 63:1079 – 1080.

Murray, T. J. (2005). Multiple sclerosis: the history of a disease.

Murray, T. J. (2006). Diagnosis and treatment of multiple sclerosis. *BMJ*, 332(7540):525 – 527.

Nath, N., Giri, S., Prasad, R., Singh, A. K., and Singh, I. (2004). Potential targets of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor for multiple sclerosis therapy. *J Immunol.*, 172(2):1273 – 1286.

Neuhaus, O., Farina, C., Yassouridis, A., Wiendl, H., Bergh, F. T., Dose, T., Wekerle, H., and Hohlfeld, R. (2000). Multiple sclerosis: Comparison of copolymer-1-reactive t cell lines from treated and untreated subjects reveals cytokine shift from t helper 1 to t helper 2 cells. 97(13):7452 – 7457.

Nicot, A., Ratnakar, P. V., Ron, Y., Chen, C. C., and Elkabes, S. (2003). Regulation of gene expression in experimental autoimmune encephalomyelitis indicates early neuronal dysfunction. *Brain.*, 126(2):398 – 412.

Nishiki, T., Nihonmatsu, I., Tsuhara, Y., Kawasaki, M., Sekiguchi, M., Sato, K., Mizoguchi, A., and Takahashi, M. (2001). Distribution of soluble n-ethylmaleimide fusion protein attachment proteins (snaps) in the rat nervous system. *Neuroscience.*, 107(3):363 – 371.

Niwa, R., Nagata-Ohashi, K., Takeichi, M., Mizuno, K., and Uemura, T. (2002). Control of actin reorganization by slingshot, a family of phosphatases that dephosphorylate adf/cofilin. *Cell*, 108(2):233 – 246.

Noben, J. P., Dumont, D., Kwasnikowska, N., Verhaert, P., Somers, V., Hupperts, R., Stinissen, P., and Robben, J. (2006). Lumbar cerebrospinal fluid proteome in multiple sclerosis: characterization by ultrafiltration, liquid chromatography, and mass spectrometry. *J. Proteome Res.*, 5(7):1647 – 1657.

Nogales, E, Whittaker, M, Milligan, A, R., Downing, and H, K. (1999). High-resolution model of the microtubule. *Cell*, 96:79 – 88.

North, B. J., Marshall, B. L., Borra, M. T., Denu, J. M., and Verdin, E. (2003). The human sir2 ortholog, sirt2, is an nad+-dependent tubulin deacetylase. *Mol Cell.*, 11(2):437 – 444.

Novarino, G., Fabrizi, C., Tonini, R., Denti, M. A., Malchiodi-Albedi, F., Lauro, G. M., Sacchetti, B., Paradisi, S., Ferroni, A., Curmi, P. M., Breit, S. N., and Mazzanti, M. (2004). Involvement of the intracellular ion channel clic1 in microglia-mediated beta-amyloid-induced neurotoxicity. *J. Neurosci.*, 24(23):5322 – 5330.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.*, 250(10):4007 – 4021.

O'Gorman, E., Beutner, G., Dolder, M., Koretsky, A. P., Brdiczka, D., and Wallimann, T. (1997). The role of creatine kinase in inhibition of mitochondrial permeability transition. *FEBS Lett.*, 414:253 – 257.

Onyango, P., Celic, I., McCaffery, J. M., Boeke, J. D., and Feinberg, A. P. (2002). Sirt3, a human sir2 homologue, is an nad-dependent deacetylase localized to mitochondria. *Proc. Natl. Acad. Sci. USA*, 99(21):13653 – 13658.

Orino, K., Lehman, L., Tsuji, Y., Ayaki, H., Torti, S. V., and Torti, F. M. (2001). Ferritin and the response to oxidative stress. *Biochem. J*, 357:241 – 247.

Orino, K., Tsuji, Y., Torti, F. M., and Torti, S. V. (1999). Adenovirus e1a blocks oxidant-dependent ferritin induction and sensitizes cells to pro-oxidant cytotoxicity. *FEBS Lett.*, 461:334 – 338.

Pandey, A. and Mann, M. (2000). Proteomics to study genes and genomes. *Nature*, 405(6788):837 – 846.

Paolo, G. D., Lutjens, R., Osen-Sand, A., Sobel, A., Catsicas, S., and Grenningloh, G. (1997). Differential distribution of stathmin and scg10 in developing neurons in culture. *J. Neurosci. Res.*, 50(6):1000 – 1009.

Parent, J. M., Yu, T. W., Leibowitz, R. T., Geschwind, D. H., Sloviter, R. S., and Lowenstein, D. H. (1997). Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J. Neurosci.*, 17:3727 – 3738.

Parker, J. A., Arango, M., Abderrahmane, S., Lambert, E., Tourette, C., Catoire, H., and Neri, C. (2005). Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons. *Nat Genet.*, 37(4):555.

Pasterkamp, R. J., Giger, R. J., Ruitenberg, M. J., Holtmaat, A. J., Wit, J. D., Winter, F. D., and Verhaagen, J. (1999). Expression of the gene encoding the chemorepellent semaphorin iii is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal cns. *Mol Cell Neurosci.*, 13(2):143 – 166.

Pasterkamp, R. J., Giger, R. J., and Verhaagen, J. (1998). Regulation of semaphorin iii/collapsin-1 gene expression during peripheral nerve regeneration. *Exp Neurol.*, 153(2):313 – 327.

Pedotti, R., DeVoss, J. J., Youssef, S., Mitchell, D., Wedemeyer, J., Madanat, R., Garren, H., Fontoura, P., Tsai, M., Galli, S. J., Sobel, R. A., and Steinman, L. (2003). Multiple elements of the allergic arm of the immune response modulate autoimmune demyelination. *Proc. Natl. Acad. Sci. USA*, 100(4):1867 – 1872.

Pellier-Monnin, V., Astic, L., Bichet, S., Riederer, B. M., and Grenningloh, G. (2001). Expression of scg10 and stathmin proteins in the rat olfactory system during development and axonal regeneration. *J Comp Neurol*, 433:239 – 254.

Perluigi, M., Poon, H. F., Maragos, W., Pierce, W. M., Klein, J. B., Calabrese, V., Cini, C., Marco, C. D., and Butterfield, D. A. (2005). Proteomic analysis of protein expression and oxidative modification in r6/2 transgenic mice: a model of huntington disease. *Mol Cell Proteomics.*, 4(12):1849 – 1861.

Peterson, J. W., Bö, L., Mörk, S., Chang, A., and Trapp, B. D. (2001). Transected neurites, apoptotic neurons and reduced inflammation in cortical multiple sclerosis lesions. *Ann Neurol*, 50:389 – 400.

Piddlesden, S. J., Lassmann, H., Zimprich, F., Morgan, B. P., and Linington, C. (1993). The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. *Am J Pathol*, 143:555 – 564.

Pierre, B. A. S., Merrill, J. E., and Dopp, J. M. (1996). Effects of cytokines on cns cells: glia. in: Cytokines and the cns. *Boca Raton: CRC.*, pages 151 – 168.

Porcu, M. and Chiarugi, A. (2005). The emerging therpeutic potential of sirtuin-interacting drugs: form cell death to lifespan extension. *Trends in pharmacological sciences*, 26(2):94–103.

Poser, C. M., Paty, D. W., Scheinberg, L., McDonald, W. I., Davis, F. A., Ebers, G. C., Johnson, K. P., Sibley, W. A., Silberberg, D. H., and Tourtellotte, W. W. (1983). New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol.*, 13(3):227 – 231.

Prat, A. and Antel, J. (2005). Pathogenesis of multiple sclerosis. *Curr Opin Neurol.*, 18(3):225 – 230.

Quinn, C. C., Gray, G. E., and Hockfield, S. (1999). A family of proteins implicated in axon guidance and outgrowth. *J Neurobiol.*, 41(1):158 – 164.

Raine, C. S. (1997). *Demyelinating diseases*.

Reinders, J. and Sickmann, A. (2005). State-of-the-art in phosphoproteomics. *Proteomics*, 5(16):4052 – 4061.

Reindl, M., Linington, C., Brehm, U., Egg, R., Dilitz, E., Deisenhammer, F., Poewe, W., and Berger, T. (1999). Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain*, 122(11):2047 – 2056.

Rivers, H. M. and Schwentker, F. F. (1935). Encephalomyelitis accompanied by myelin destruction experimentally produced in monkeys. 61:689 – 705.

Rivers, T. M. (1933). Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. *J exp med*, 58:39 – 53.

Robinson, W. H., Fontoura, P., Lee, B. J., de Vegvar, H. E., and et. al (2003a). Protein microarrays guide tolerizing dna vaccine treatment of autoimmune encephalomyelitis. *Nat. Biotechnol.*, 21:1033 – 1039.

Robinson, W. H., Utz, P. J., and Steinman, L. (2003b). Genomic and proteomic analysis of ms opinion. *Current opinion in immunology*, 15:660 – 667.

Roepstorff, P. and Fohlman, J. (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed Mass Spectrom*, 11(11):601.

Russo, A., Palumbo, M., Aliano, C., Lempereur, L., Scoto, G., and Renis, M. (2003). Red wine micronutrients as protective agents in alzheimerlike induced insult. *Life Sci.*, 72:2369 – 2379.

Savaskan, E., Olivieri, G., Meier, F., Seifritz, E., Wirz-Justice, A., and Muller-Spahn, F. (2003). Red wine ingredient resveratrol protects from beta-amyloid neurotoxicity. *Gerontology*, 49:380 – 383.

Schlessinger, J. (1993). Cellular signaling by receptor tyrosine kinases. *Harvey Lect.*, 89:105 – 123.

Schlichter, L. C., Sakellaropoulos, G., Ballyk, B., Pennefather, P. S., and Phipps, D. J. (1996). Properties of k+ and cl- channels and their involvement in proliferation of rat microglial cells. *Glia.*, 17(3):225 – 236.

Schumacher, F. A., Beeve, G. W., and Kibler, R. F. (1965). Problems of experimental trials of therapy in multiple sclerosis. *Ann. N.Y. Acad. Sci.*, 122:552 – 568.

Schwab, M. E. and Caroni, P. (1988). Oligodendrocytes and cns myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. *J. Neurosci.*, 8(7):2381 – 2393.

Schwer, B., North, B. J., Frye, R. A., Ott, M., and Verdin, E. (2002). The human silent information regulator (sir)2 homologue hsirt3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase. *J. Cell Biol.*, 158(4):647 – 657.

Scolding, N. J., Morgan, B. P., Houston, W. A., Linington, C., Campbell, A. K., and Compston, D. A. (1989). Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement. *Nature*, 339:620 – 622.

Scott, B. W., Wang, S., Burnham, W. M., Boni, U. D., and Wojitowicz, J. M. (1998). Kindling-induced neurogenesis in the dentate gyrus of the rat. *Neurosci Lett*, 248:73 – 76.

Shi, T., Wang, F., Stieren, E., and Tong, Q. (2005). Sirt3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. *J. Biol. Chem.*, 280(13):560 – 567.

Shirvan, A., Ziv, I., Fleminger, G., Shina, R., He, Z., Brudo, I., Melamed, E., and Barzilai, A. (1999). Semaphorins as mediators of neuronal apoptosis. *J. Neurochem.*, 73(3):961 – 971.

Smith, K. J. and Lassmann, H. (2002). The role of nitric oxide in multiple sclerosis. *Lancet Neurology*, 1:232 – 241.

Smith, T., Groom, A., Zhu, B., and Turski, L. (2000). Autoimmune encephalomyelitis ameliorated by ampa antagonists. *Nat Med*, 6:62 – 64.

Sobel, A. (1991). Stathmin: a relay phosphoprotein for multiple signal transduction? *Trends Biochem. Sci.*, 16(8):301 – 305.

Sofic, E., Riederer, P., Heinsen, H., Beckmann, H., Reynolds, G. P., Hebenstreit, G., and Youdim, M. B. (1988). Increased iron (iii) and total iron content in post mortem substantia nigra of parkinsonian brain. *J Neural Transm.*, 74(3):199 – 205.

Stachowiak, O., Schlattner, U, Dolder, M, Wallimann, and T (1998). Oligomeric state and membrane binding behaviour of creatine kinase isoenzymes: implications for cellular function and mitochondrial structure. *Mol. Cell. Biochem.*, 184:141 – 151.

Steel, L. F., Haab, B. B., and Hanash, S. M. (2005). Methods of comparative proteomic profiling for disease diagnostics. *J Chromatogr B Analyt Technol Biomed Life Sci.*, 815(1-2):275 – 284.

Stefferl, A., Brehm, U., Storch, M., Lambracht-Washington, D., Bourquin, C., Wonigeit, K., Lassmann, H., and Linington, C. (1999). Myelin oligodendrocyte glycoprotein induces experimental autoimmune encephalomyelitis in the resistant brown norway rat: disease susceptibility is determined by mhc and mhc-linked effects on the b cell response. *J Immunol*, 163:40 – 47.

Steinberg, T. H., Agnew, B. J., Gee, K. R., Leung, W. Y., Goodman, T., Schulenberg, B., Hendrickson, J., Beechem, J. M., Haugland, R. P., and Patton, W. F. (2003). Global quantitative phosphoprotein analysis using multiplexed proteomics technology. *Proteomics*, 3(7):1128 – 1144.

Steinman, L. (1999). Assessment of animal models for ms and demyelinating disease in the design of rational therapy. *Neuron*, 24(3):511 – 514.

Steinman, L. (2001a). Multiple sclerosis: a two-stage disease. *Nature Immunology*, 2(2001):762 – 764.

Steinman, L. (2001b). Myelin-specific cd8 t cells in the pathogenesis of experimental allergic encephalitis and multiple sclerosis. *J. Exp. Med.*, 194:F27 – F30.

Steinman, L., Martin, R., Bernard, C., Conlon, P., and Oksenberg, J. R. (2002). Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. *Annu Rev. Neurosci.*, 25:491 – 505.

Steinman, L. and Zamvil, S. (2003). Transcriptional analysis of targets in multiple sclerosis. *Nat Rev Immunol.*, 3(6):483 – 492.

Stinissen, P., Raus, J., and Zhang, J. (1997). Autoimmune pathogenesis of multiple sclerosis: role of autoreactive t lymphocytes and new immunotherapeutic strategies. *Crit Rev Immunol.*, 17(1):33 – 75.

Storch, M. K., Piddlesden, S., Haltia, M., Iivanainen, M., Morgan, P., and Lassmann, H. (1998). Multiple sclerosis: in situ evidence for antibody- and complement-mediated demyelination. *Ann Neurol*, 43(4):465 – 471.

Strey, C. W., Spellman, D., Stieber, A., Gonatas, J. O., Wang, X., Lambris, J. D., and Gonatas, N. K. (2004). Dysregulation of stathmin, a microtubule-destabilizing protein, and up-regulation of hsp25, hsp27, and the antioxidant peroxiredoxin 6 in a mouse model of familial amyotrophic lateral sclerosis. *Am J Pathol.*, 165(5):1701 – 1718.

Stuve, O., Youssef, S., Steinman, L., and Zamvil, S. S. (2003). Statins as potential therapeutic agents in neuroinflammatory disorders. *Curr Opin Neurol*, 16:393 – 401.

Styren, S. D., Kamboh, M. I., and DeKosky, S. T. (1998). Expression of differential immune factors in temporal cortex and cerebellum: the role of alpha-1-antichymotrypsin, apolipoprotein e, and reactive glia in the progression of alzheimer's disease. *J Comp Neurol*, 396:511 – 520.

Sumi, T., Matsumoto, K., Takai, Y., and Nakamura, T. (1999). Cofilin phosphorylation and actin cytoskeletal dynamics regulated by rho- and cdc42-activated lim-kinase 2. *J. Cell Biol.*, 147(7):1519 – 1532.

Swanborg, R. H. (1995). Experimental autoimmune encephalomyelitis in rodents as a model for human demyelinating disease. *Clin. Immunol. Immunopathol.*, 77:4 – 13.

Swerdlow, R. H., Khan, and M, S. (2004). A "mitochondrial cascade hypothesis" for sporadic alzheimer's disease. *Med. Hypotheses*, 63:8 – 20.

Szalai, A. J., Nataf, S., Hu, X. Z., and Barnum, S. R. (2002). Experimental allergic encephalomyelitis is inhibited in transgenic mice expressing human c-reactive protein. *J. Immunol.*, 168:5792 – 5797.

Tang, B. L. (2003). Inhibitors of neuronal regeneration: mediators and signaling mechanisms. *Neurochem. Int.*, 42(3):189 – 203.

Torti, F. M. and Torti, S. V. (2002). Regulation of ferritin genes and protein. *Blood*, 99(10):3505 – 3516.

Trapp, B. D. (2004). Pathogenesis of multiple sclerosis: the eyes only see what the mind is prepared to comprehend. *Ann Neurol*, 55:455 – 457.

Trapp, B. D., Peterson, J., Ransohoff, R. M., Rudick, R., Mork, S., and Bo, L. (1998). Axonal transection in the lesions of multiple sclerosis. *N Engl J Med*, 338:278 – 285.

Trapp, B. D., Ransohoff, R., and Rudick, R. (1999). Axonal pathology in multiple sclerosis: relationship to neurologic disability. *Curr Opin Neurol.*, 12(3)):295 – 302.

Tsuji, Y., Ayaki, H., Whitman, S. P., Morrow, C. S., Torti, S. V., and Torti, F. M. (2000). Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. *Mol Cell Biol.*, 20:5818 – 5827.

Tsunoda, I. and Fujinami, R. S. (1996). Two models for multiple sclerosis: experimental allergic encephalomyelitis and theiler's murine encephalomyelitis virus. *J Neuropathol Exp Neurol.*, 55(6):673 – 686.

Tuohy, V. K. (1994). Peptide determinants of myelin proteolipid protein (plp) in autoimmune demyelinating disease: a review. *Neurochem. Res.*, 19(8):935 – 944.

Turck, C. W., Maccarrone, G., Sayan-Ayata, E., Jacob, A. M., Ditzen, C., Kronsbein, H., Birg, I., Doertbudak, C. C., Haegler, K., Lebar, M., Teplytska, L., Kolb, N., Uwaje, N., and Zollinger, R. (2005). The quest for brain disorder biomarkers. *J Med Invest.*, 52:Suppl: 231 – 235.

Urbanyi, Z., Laszlo, L., Tomasi, T. B., Toth, E., Mekes, E., Sass, M., and Pazmany, T. (2003). Serum amyloid p component induces neuronal apoptosis and beta-amyloid immunoreactivity. *Brain Res.*, 988(1-2):69 – 77.

Urbanyi, Z., V, L., and SL, E. (1994). Serum amyloid p component-induced cell death in primary cultures of rat cerebral cortex. *Eur. J. Pharmacol.*, 270(4):375 – 378.

Valberg, L. S., Flanagan, P. R., Kertesz, A., and Ebers, G. C. (1989). Abnormalities in iron metabolism in multiple sclerosis. *Can J Neurol Sci.*, 16(2):184 – 186.

Vass, K. and Lassmann, H. (1990). Intrathecal application of interferon gamma. progressive appearance of mhc antigens within the rat nervous system. *Am J Pathol.*, 137(4):789 – 800.

Vassilopoulos, D. and Jockers-Wretou, E. (1987). Serum creatine kinase b levels in diseases of the central nervous system. *Eur Neurol.*, 27(2):78 – 81.

von Andrian, U. H. and Engelhardt, B. (2003). Alpha4 integrins as therapeutic targets in autoimmune disease. *N Engl J Med.*, 348(1):68 – 72.

Wallimann, T., Dolder, M, Schlattner, U, Eder, M, Hornemann, T, Gorman, O., E, Ruck, A, Brdiczka, and D (1998). Some new aspects of creatine kinase (ck): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetics and physiology. *BioFactors*, 8:229 – 234.

Wallimann, T., Wyss, M, Brdiczka, D, Nicolay, K, Eppenberger, and M, H. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the fphosphocreatine circuit for cellular energy homeostasis. *Biochem. J*, 281(1):21 – 40.

Wang, H. and Hanash, S. M. (2002). Contributions of proteome profiling to the molecular analysis of cancer. *Technol Cancer Res Treat.*, 1(4):237 – 246.

Wang, L. H. and Strittmatter, S. M. (1996). A family of rat crmp genes is differentially expressed in the nervous system. *J. Neurosci.*, 16:6197 – 6207.

Washburn, M. P., Wolters, D., and Yates, J. R. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.*, 19(3):242 – 247.

Waxman, S. G. (2000a). Do demyelinating diseases involve more than myelin? *Nat Med*, 6:738 – 739.

Waxman, S. G. (2000b). Multiple sclerosis as a neuronal disease. *Arch Neurol*, 57:22 – 24.

Weinberg, E. D. (2000). Modulation of intramacrophage iron metabolism during microbial cell invasion. *Microbes Infect*, 2:85 – 89.

Weissert, R., Wallstrom, E., Storch, M. K., Stefferl, A., Lorentzen, J., Lassmann, H., Linington, C., and Olsson, T. (1998). Mhc haplotype-dependent regulation of mog-induced eae in rats. *J Clin Invest*, 102:1265 – 1273.

Wekerle, H. (1998). *Immunology of multiple sclerosis*, volume 3.

Wekerle, H. (2002). Tackling multiple sclerosis. *Nature*, 420(6911):39 – 40.

Wekerle, H., Kojima, K, Lannes-Vieira, J, Lassmann, H, Linington, and C (1994). Animal models. *Ann. Neurol.*, 36:S47 – S53.

Wilkins, M. R., JC, S., AA, G., RD, A., I, H.-S., DF, H., and KL, W. (1996a). Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev.*, 13:19 – 50.

Wilkins, M. R., Pasquali, C., Appel, R. D., Ou, K., Golaz, O., Sanchez, J. C., Yan, J. X., Gooley, A. A., Hughes, G., Humphery-Smith, I., Williams, K. L., and Hochstrasser, D. F. (1996b). From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. *Biotechnology (N Y)*, 14(1):61 – 65.

Wolinsky, J. S. (2006). The use of glatiramer acetate in the treatment of multiple sclerosis. adv neurol. *4. Wolinsky JS The use of glatiramer acetate in the treatment of multiple sclerosis. Adv Neurol.*, 98:273 – 292.

Wucherpfennig, K. W. (2001). Structural basis of molecular mimicry. *J Autoimmun.*, 16(3):293 – 302.

Wucherpfennig, K. W., Catz, I., Hausmann, S., Strominger, J. L., Steinman, L., and Warren, K. G. (1997). Recognition of the immunodominant myelin basic protein peptide by autoantibodies and hla-dr2-restricted t cell clones from multiple sclerosis patients. identity of key contact residues in the b-cell and t-cell epitopes. *J Clin Invest.*, 100(5):1114 – 1122.

Wujek, J. R., Bjartmar, C., Richer, E., Ransohoff, R. M., Yu, M., Tuohy, V. K., and Trapp, B. D. (2002). Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. *J Neuropathol Exp Neurol.*, 61(1):23 – 32.

Wulff, H., Calabresi, P. A., Allie, R., Yun, S., Pennington, M., Beeton, C., and Chandy, K. G. (2003). The voltage-gated kv1.3 k(+) channel in effector memory t cells as new target for ms. *J Clin Invest.*, 111(11):1703 – 1713.

Yasojima, K., Schwab, C., McGeer, E. G., and McGeer, P. L. (2000). Human neurons generate c-reactive protein and amyloid p: upregulation in alzheimer's disease. *Brain Res.*, 887(1):80 – 89.

Yates, J. R., Eng, J. K., and McCormack, A. L. (1995a). Mining genomes: correlating tandem mass spectra of modified and unmodified peptides to sequences in nucleotide databases. *Anal. Chem.*, 67(18):3202 – 3210.

Yates, J. R., Eng, J. K., McCormack, A. L., and Schieltz, D. (1995b). Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal. Chem.*, 67(8):1426 – 1436.

Yong, V. W., Wells, J., Giuliani, F., Casha, S., Power, C., and Metz, L. M. (2004). The promise of minocycline in neurology. *Lancet Neurol.*, 3(12):744 – 751.

Yoo, B. C., Cairns, N., Fountoulakis, M., and Lubec, G. (2001). Synaptosomal proteins, beta-soluble n-ethylmaleimide-sensitive factor attachment protein (beta-snap), gamma-snap and synaptotagmin i in brain of patients with down syndrome and alzheimer's disease. *Dement Geriatr Cogn Disord.*, 12(3):219 – 225.

Yoshida, H., Watanabe, A., and Ihara, Y. (1998). Collapsin response mediator protein-2 is associated with neurofibrillary tangles in alzheimer's disease. *J. Biol. Chem.*, 273(16):9761 – 9768.

Youdim, M. B., Ben-Shachar, D., and Riederer, P. (1993). The possible role of iron in the etiopathology of parkinson's disease. *Mov Disord.*, 8(1-2):255.

Youssef, S., Stuve, O., Patarroyo, J. C., Ruiz, P. J., Radosevich, J. L., Hur, E. M., Bravo, M., Mitchell, D. J., Sobel, R. A., Steinman, L., and Zamvil, S. S. (2002). The hmg-coa reductase inhibitor, atorvastatin, promotes a th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature*, 420(6911):78 – 84.

Zamvil, S. S. and Steinman, L. (2003). Diverse targets for intervention during inflammatory and neurodegenerative phases of multiple sclerosis. *Neuron*, 38(5):685 – 688.

Zang, Y. C., Li, S., Rivera, V. M., Hong, J., Robinson, R. R., Brietbach, W. T., Killan, J., and Zhang, J. Z. (2004). Increased cd8+ cytotoxic t cell responses to myelin basic protein in multiple sclerosis. *J Immunol*, 172:5120 – 5127.

Zeimssen, T., Kümpfel, T., Klinkert, W. E. F., and Hohlfeld, O. N. R. (2002). Glatiramer acetate ?specific t-helper 1-and 2-type cell lines produce bdnf: implications for ms therapy. *Brain*, 125:2381 – 2391.

Zhou, D. and Hemmer, B. (2004). Specificity and degeneracy: T cell recognition in cns autoimmunity. *Mol Immunol.*, 40(14-15):1057 – 1061.

Appendix A

Results: MOG active EAE

The following are the lists of proteins identified in the MOG active EAE experiments.

Accession no.	**Protein name**	**Regulation**	**Sequence coverage (%)**
P63028	Translationally-controlled tumor protein	↓ in EAE	41
P21107	tropomyosin 3	↓ in EAE	25
P60710	Actin B	↓ in EAE	32
Q7TNP3	Platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit.	↓ in EAE	18
Q99J99	3-mercaptopyruvate sulfurtransferase	↓ in EAE	45
Q6PB74	Sept5 protein	↓ in EAE	50
Q8BKZ9	pyruvate dehydrogenase protein X component, mitochondrial	↓ in EAE	27
P63028	Translationally-controlled tumor protein	↑ in EAE	45
Q9JKB1	ubiquitin carboxyl-terminal hydrolase isozyme L3	↑ in EAE	70
P21107	tropomyosin 3	↑ in EAE	35
O35658	(Complement component 1, q subcomponent binding protein*	↑ in EAE	35
JC1237	apolipoprotein A-I precursor*	↑ in EAE	37
BAB25027	5'(3')-deoxyribonucleotidase, cytosolic type*	↑ in EAE	
Q9QYB1	Chloride intracellular channel protein 4	↑ in EAE	52
Q6PKE6	Platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit.	↑ in EAE	18
Q9DBJ1	Phosphoglycerate mutase 1	↑ in EAE	58
Q99J99	3-mercaptopyruvate sulfurtransferase	↑ in EAE	57
P18872	Guanine nucleotide-binding protein G(o), alpha subunit 1	↑ in EAE	35
BAC33120	Pyruvate dehydrogenase protein X component, mitochondrial*	↑ in EAE	27
BAC36976	Heterogeneous nuclear ribonucleoprotein H'*	↑ in EAE	
Q61171	Peroxiredoxin 2	↑ in EAE	37
P70296	Phosphatidylethanol-amine binding protein	↑ in EAE	34
Q9D1C8	Vacuolar protein sorting 28	↑ in EAE	15
P12246	Serum amyloid P-component (SAP)	↑ in EAE	23
P47753	Capping protein alpha 1	↑ in EAE	41
P11493	Serine/ Threonine protein phosphatase 2A	↑ in EAE	20
P60843	Eucaryotic initiation factor elf-4A11	↑ in EAE	19
Q6TNR9	Phosphoglycerate mutase 1	↑ in EAE	25
Q9JKB7	Guanine deaminase	↓ in EAE	5
Q7Z8N3	-Tubulin 1	↓ in EAE	25
Q9CZ13	Ubiquinol-cytochrome c reductase core protein 1	↓ in EAE	9
P07900	Heat shock protein 86	↓ in EAE	10
P08238	Heat shock protein 90	↓ in EAE	25

Table A.1: MOG active EAE: List of proteins and their regulation in brain. Proteins detected by Coomassie staining.

Accession no.	**Protein name**	**Regulation**	**Sequence coverage (%)**
CANB1	calcineurin B subunit isoform 1, protein phosphatse 2B regulatory subunit 1	↑ in EAE	8
Q62048	PEA-15 protein - mouse	↑ in EAE	36
Q62048	PEA-15 protein - mouse	↑ in EAE	46
Q9D6E6	myosin regulatory light chain A	↑ in EAE	27
q8ved9	galectin binding protein	↑ in EAE	26
P09528	Ferritin heavy chain (Ferritin H subunit).-	↑ in EAE	48
Q9CX22	Cofilin actin depolymerizing factor	↑ in EAE	4
AAh18352	Calpain small subunit 1*	↑ in EAE	45
Q8K4Z3	Apo A-1 binding protien*	↑ in EAE	38
Q8C454	Metaxin2	↑ in EAE	19
Q64105	sepiapterin reductase*	↑ in EAE	32
AAA18336	Heat-shock protein beta-1, hsp25*	↑ in EAE	25
NP_291039	Eucaryotic translation initiation factor 4h	↑ in EAE	15
P99027	60S acidic ribosomal protein P2	↓ in EAE	40
LDHB	L-lactate dehydrogenase B chain	↓ in EAE	27
q9d6g1	heterogeneous nuclear ribonucleoprotein A/B	↓ in EAE	5

Table A.2: PLP active EAE: List of proteins and their regulation in brain. Proteins detected by ProQ staining.

Accession no.	Description	Regulation	Sequence coverage (%)
BAC27205	L-plastin, Lymphocyte cytosolic protein 1	↑ in EAE	20
P14733	Lamin B1	↑ in EAE	28
Q6NXW4	Protien disulphide Isomerase A4	↑ in EAE	28
P07901	Heat shock protein HSP 90-alpha (HSP 86)	↑ in EAE	32
P11499	Heat shock protein HSP 90-beta (HSP 84)	↑ in EAE	40
Q8K0E8	Fibrinogen, B beta polypeptide.-	↑ in EAE	52
O89053	Coronin-1A*	↑ in EAE	32
Q9D5D4	Ankyrin6*	↑ in EAE	
P24527	Leukotriene A-4 hydrolase	↑ in EAE	39
P26040	Ezrin (p81) (Cytovillin) (Villin 2)*	↑ in EAE	16
Q9EPZ3	Natural killer cell receptor Ly-49G1*	↑ in EAE	
Q5WAC6	Syntaxin binding protein 1	↑ in EAE	30
BAC39532	Serotransferrin	↑ in EAE	28
AAM22156	Plasminogen*	↑ in EAE	33
Q6TCI0	Angiostatin*	↑ in EAE	
Q9JL62	Glycolipid transfer protein (GLTP)*	↑ in EAE	50
S20990	Myosin regulatory light chain 2*	↑ in EAE	
Q64471	Glutathione S-transferase theta 1	↑ in EAE	26
C3MS	Complement C3 precursor	↑ in EAE	37
P97371	Proteasome activator complex subunit 1	↑ in EAE	32
P23492	Purine nucleoside phosphorylase	↑ in EAE	51
P97372	Proteasome activator complex subunit 2	↑ in EAE	33
P12246	Serum amyloid P-component	↑ in EAE	25
Q9Z1Q5	Chloride intracellular channel protein 1	↑ in EAE	50
P57776	Elongation factor 1-delta (EF-1-delta).	↑ in EAE	38
Q9CRE2	Mus musculus ES cells cDNA, RIKEN, clone:C330004K01	↑ in EAE	28
Q8VDZ8	Cab39 protein	↑ in EAE	36
Q6PEM2	Pzp protein,alpha-2-macroglobulin - mouse	↑ in EAE	10
P21279	Guanine nucleotide-binding protein G(q), alpha subunit	↑ in EAE	39
Q9D964	Glycine amidinotransferase, mitochondrial	↑ in EAE	32
Q76LX3	Methionine adenosyltransferase II alpha subunit.	↑ in EAE	31
Q8VCM7	Fibrinogen, gamma polypeptide.-	↑ in EAE	52
Q811A6	Spliceosome RNA helicase Bat1	↑ in EAE	19
P21614	Vitamin D-binding protein precursor*	↑ in EAE	34

Table A.3: MOG active EAE: List of proteins and their regulation in spinal cord. Proteins detected by Coomassie staining, the table is divided into three parts, this table is part 1.

Accession no.	Description	Regulation	Sequence coverage (%)
S26413	T-complex protein Tcp-10*	↑ in EAE	
Q61233	L-plastin, Lymphocyte cytosolic protein	↑ in EAE	19
BAB24239	Ubiquitin-conjugating enzyme E2 N	↓ in EAE	56
P15532	Nucleoside-diphosphate kinase	↓ in EAE	73
P17742	Peptidyl-prolyl cis-trans isomerase A	↓ in EAE	49
Q04447	Creatine kinase	↓ in EAE	25
A39608	Alpha-crystallin chain B	↓ in EAE	54
Q03265	H+ transporting two-sector ATPase alpha	↓ in EAE	22
AAH18173	Superoxide dismutase [Mn], mitochondrial	↓ in EAE	24
P20108	Thioredoxin-dependent peroxide reductase	↓ in EAE	23
PRDX6	Peroxiredoxin 6(Antioxidant protein 2)	↓ in EAE	38
AAH10785	Pyridoxine-5'-phosphate oxidase	↓ in EAE	21
CAA27396	Actin, cytoplasmic 1 (beta)	↓ in EAE	33
Q99JZ6	Tubulin, beta, 2	↓ in EAE	48
Q5XJF8	Tubulin, alpha 1.	↓ in EAE	29
BAB23362	NDRG1 protein	↓ in EAE	37
P52480	Pyruvate kinase, isozyme M2	↓ in EAE	22
Q9CQL9	Adenylosuccinate synthase	↓ in EAE	42
Q99K08	Phosphofructokinase, muscle.	↓ in EAE	10
BAB27759	Dynamin 1	↓ in EAE	40
Q8C7C7	Albumin 1	↓ in EAE	17
Q925K3	Glial fibrillary acidic protein	↓ in EAE	68
BAB27703	Spectrin alpha chain, brain	↓ in EAE	26
Q8BNF8	Valosin containing protein	↓ in EAE	30
Q8BIA1	Hypothetical ARM repeat structure containing protein	↓ in EAE	11
P17182	Alpha enolase, phosphopyruvate hydratase	↓ in EAE	27
PGK1	Phosphoglycerate kinase 1	↓ in EAE	22
Q9CR95	Adaptin ear-binding coat-associated protein 1	↓ in EAE	37
AAH13507	Cytosolic acyl coenzyme A thioester hydrolase	↓ in EAE	21
Q8K0Z0	Neurofilament, light polypeptide	↓ in EAE	12
Q91Z83	Beta myosin heavy chain	↓ in EAE	
Q04447	Creatine kinase B	↓ in EAE	30
Q7KZI7	MAP/microtubule affinity-regulating kinase 2	↑ in EAE	2

Table A.4: MOG active EAE: List of proteins and their regulation in spinal cord. Proteins detected by Coomassie staining, the table is divided into three parts, this table is part 2.

Accession no.	**Description**	**Regulation**	**Sequence coverage (%)**
Q9CZD3	Glycyl-tRNA synthase	↑ in EAE	4
P47985	Ubiquinol-cytochrome c reductase	↓ in EAE	12
P07310	Creatine kinase	↓ in EAE	11
P35704	Peroxiredoxin 2	↑ in EAE	8
P02675	fibrinogen precursor (P14480)	↑ in EAE	3
Q00623	Apolipoprotein A-1	↑ in EAE	24
O89053	Coronin 1	↑ in EAE	
Q9D0S8	Serine proteinase inhibitor clade B	↑ in EAE	29
Q8C7C7	Albumin 1	↑ in EAE	4
P47754	Capping protein Z alpha 2	↑ in EAE	8

Table A.5: MOG active EAE: List of proteins and their regulation in spinal cord. Proteins detected by Coomassie staining, the table is divided into three parts, this table is part 3.

Accession no.	Description	Regulation	Sequence coverage (%)
A53812	Cofilin, muscle	↓ in EAE	19
O08692	Myeloid bactenecin (F)	↑ in EAE	56
AAA37612	Ferritin heavy chain	↓ in EAE	47
PSD3_mouse	26s proteasome non-ATPase regulatory subunit 3	↓ in EAE	4
Q5M9P6	Rho GDP dissociation inhibitor alpha	↑ in EAE	15
Q8K4Z3	APO-A 1 binding protein*	↑ in EAE	32
AAH18352	Calpain small subunit*	↑ in EAE	
AAA18336	Heat-shock protein beta-1	↑ in EAE	40
GSTM1_mouse	Glutathione transferase 1	↓ in EAE	16
Q7TQD2	Tubulin polymerization promoting protein	↓ in EAE	7
AAH04754	Transaldolase	↑ in EAE	34
ALDOC_mouse	Fructose-biphosphate aldolase 3 +	↓ in EAE	45
Q7TNT7	Septin 3 +	↓ in EAE	6
AAK56084	2',3'-cyclic-nucleotide 3'-phosphodiesterase	↑ in EAE	32
Q63844	Microtubule-associated protein 2 kinase	↑ in EAE	16
NP_058580	Heterogeneous nuclear ribonucleoprotein C	↑ in EAE	3
Q9DBJ1	Phosphoglycerate mutase 1	↑ in EAE	10
Q8VDQ8	SIR2L2	↑ in EAE	6
Q9CS50	Protein kinase, CAMP dependent regulatory type 1 alpha	↑ in EAE	5
Q9JI46	NudT2 protein	↑ in EAE	8
P61981	14-3-3 protein gamma	↑ in EAE	6
P09528	Ferritin heavy chain	↑ in EAE	11
P54227	Stathmin 1	↓ in EAE	24
P45591	Cofilin 1	↑ in EAE	5
P17751	Triose Phosphate isomerase	↓ in EAE	11

Table A.6: MOG active EAE: List of proteins and their regulation in spinal cord. Proteins detected by ProQ staining.

Appendix B

Abbreviations

2DE	2 Dimensional Gel Electrophoresis
ACN	Acetonitrile
AD	Alzeimer's Disease
ALS	Amylotrophic Lateral Sclerosis
APC	Antigen Presenting Cells
APS	Ammonium Per Sulphate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CFA	Complete Freund's Adjuvant
CID	Collision Induced Dissociation
CK-B	Creatine Kinase brain isoform
CNS	Central Nervous System
CLIC	Chloride Intracellualr Channel Protein 1
CRMP	Collapsin Response Mediator Protein
CSF	Cerebrospinal Fluid
DTT	Dithiothreitol
EAE	Experimantal Autoimmune Encephalomyelitis
EQB	Equilibration Buffer
ESI	Electrospray ionization
FTH	Ferritin Heavy Chain
HCCA	α-cyano-4-hydroxycinnamic acid
HCOOH	Formic Acid
HD	Huntington's Disease
Ig	Immunoglobulin
IL	Interleukin
IPG	Immobilized pH Gradient
IFN	Interferon

IEF	Iso Electric Focussing
MALDI	Matrix Assisted Laser Desorption and Ionization
MOG	Myelin Oligodendrocyte Glycoprotein
MOG-A	MOG Active EAE
MOG-P	MOG Passive EAE
MS	Multiple Sclerosis
MS/MS	Tandem mass spectrometry
MW	Molecular Weight
MUDPIT	Multidimnesional Protein Identification Technology
NAWM	Normal Appearing White Matter
NAGM	Normal Appearing Gray Matter
NAD	Nicotinamide Adenine Dinucleotide
NDAC	NAD dependent Deacetylase
Nmnat	Nicotinamide mononucleotide adenylyltransferase
OLCs	Oligodendrocytes
PAGE	Polyacrylamide Gel Electrophoresis
PD	Parkinson's Disease
pI	Isoelectric point
PMF	Peptide Mass Fingerprinting
PMSF	Phenylmethylsulphonylfluoride
PLP	Proteolipid Protein
PLP-A	PLP Active EAE
PLP-P	PLP Passive EAE
ProQ	ProQ Diamond staining
PSD	Post Source Decay
PTM	Post-translational modification
ROS	Reactive Oxygen Species
RT	Room Temperature
SAP	Serum Amyloid P Component
SDS	Sodiumdodecylsulphate
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline Tween
TEMED	N,N,N,N'-Tetramethyl-ethylenediamine
TFA	Trifluroacetic Acid
Th	T-helper cells
TNF	Tumor Necrosis Factor
TOF	Time Of Flight
Wlds	Wallarien degeneration slow

Gene names and their description

This table describes the protein names, used in Figure 4.1, based on the Pathway Studio database.

A2M	Alpha-2-macroglobulin
Actb	Actin, beta
ACTG1	Actin, gamma 1
AHSA1	Activator of heat shock 90kDa protein ATPase homolog 1
ANXA1	Annexin A1
APOA1	Apolipoprotein A-I
APOE	Apolipoprotein E
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha
CAMKK	Calcium/calmodulin-dependent protein kinase kinase 2, beta
CFL1	Cofilin 1 (non-muscle)
CLIC4	Chloride intracellular channel 4
CRK	V-crk sarcoma virus CT10 oncogene homolog
CRYAB	Crystallin, alpha B
DLG3	Discs, large homolog 3 neuroendocrine-dlg
DNM1	Dynamin 1
EEF1D	Eukaryotic translation elongation factor 1 delta
ENO1	Enolase 1, (alpha)
FER	Fer (fps/fes related) tyrosine kinase
GARS	Glycyl-tRNA synthetase
GDA	Guanine deaminase
GFAP	Glial fibrillary acidic protein
GNAO1	Guanine nucleotide binding protein (G protein), alpha
GNAQ	Guanine nucleotide binding protein q polypeptide
HSPB1	Heat shock 27kD protein 1
HSPCA	Heat shock 90kDa protein 1, alpha
LMNB1	Lamin B1
MAPK3	Mitogen-activated protein kinase 3
PEA15	Phosphoprotein enriched in astrocytes 15
PFN2	Profilin 2
PGK1	Phosphoglycerate kinase 1
PLG	plasminogen
S100A1	S100 calcium binding protein A1
SCARB1	Scavenger receptor class B, member 1
SIRT2	Sirtuin 2
STMN1	Stathmin 1/oncoprotein 18
TF	Transferrin

TUBA3	Tubulin, alpha 3
TUBB2	Tubulin, beta 2
VIL2	Villin 2 (ezrin)
YWHAG	14-3-3 protein gamma
YWHAZ	14-3-3 protein, zeta

Table B.2:

Appendix C

T-cell sample preparation

The following method was optimized for the extraction and processing of T-cell proteins for 2DE.

Materials and solutions required

1. Lysis buffer: 40mM Tris base (Bio-rad), 2% NP-40 (Nonidet p-40), Sigma), 4% Chaps (Bio-rad), 200mM DTT (Bio-rad), 5mM Magnesium Chloride (MgCl2, Amersham). Stored in aliquots at -20°C. 100μl is used for up to 20x10^6 cells.

2. DNase (Sigma) 150Units/100μl lysis buffer.

3. RNase (sigma) 150 Units /100μl lysis buffer.

4. IEF buffer: (2M thiourea, 7M urea, 2% chaps, 0.2% Biolyte(3-10), 50mM DTT) Stored in aliquots at -80°C.

5. Protease inhibitors as given in the section on : All protease inhibitors are added to the lysis buffer in 1X concentration just before use.

Cell lysis and preparation for IEF

1. Frozen T-cells (10 Million cells) were thawed in 100μl lysis buffer.

2. Cells were incubated at 37°C for 45min with occasional vortexing (every 5-10 min).

3. After lysis the samples were allowed to cool to room temperature.

4. 220μl IEF buffer was added to the mixture and incubated for 30min at RT.
5. The mixture was centrifuged at top speed (14000 rpm) for 20 minutes.
6. 320μl of the clear supernatant were applied to 17cm 3-10 non linear IPG strip.

Appendix D

PDQuest image analysis

This section describes the optimized user manual for PDQuest 2DE gel analysis. The protocol was developed in collaboration with Bio-Rad Laboratories for the hands-on use of the software.

Image handling

Opening the files: Open all the images to be analyzed - (open PDQuest, file-open- (select directory, arbeits platz, c drive, programme, biorad, discovery series, sample images PDQuest and then the respective folder containing the images). You can click on the first image along with the "**shift**" key and then click on the last one (in the same column) without releasing the "**shift**" button. If the gels are not in the desired order use the "**cntrl**" key along with all the gels to be opened.)

Tile function: View all the images in the same screen - go to "**Window**" button and click on "**Tile**".

Zoom function: To zoom into any part of the image - click on the "**zoom box**", (the cursor changes to a cross) select the area to be zoomed in by drawing a box and then click on "**zoom**". To undo zooming, click back on the image. To zoom into the same area in all the gels - go to window and click on "**imitate zoom**". Using the "**grab**" button the image can be moved within the zoomed region, this is also possible with the arrow keys.

Transform function: To change the display of the image - go to "**image**" menu and click on "**transform**". A new window appears where you can adjust the contrast and brightness of the images. This has to be done individually for each gel. The changes do not affect the real images, but only the display of the

images. The "**always auto-scale**" option can be used when zoomed into an area and this shows the highest background to spot contrast. When you move from one region to another in the image the contrast is maintained. This is especially useful when defining the spot detection parameters.

Cropping the images: Cropping helps you to leave out the unwanted area of the gel. Small variation in scanning size does not affect cropping or matching, but make sure that there is no big difference in the size. Select "**advanced crop**" from the image menu and then select "**define crop area**". Draw a box around the area of interest. "**Image-advanced crop- place cross hair**", now place the cursor on a spot that is present in all the gels and click on it. This will position the box. Now go to "**image - advanced crop**" and click on "**save crop settings**". There is a pop up window, which appears and you have to give a name to the crop settings and click on "**apply**". After this is done move the cursor within to change the cursor to a scissor. Click on the image and in the pop up window select "**copy and crop**". This would mean that a new copy of the image is formed, which is now cropped. The original image is not altered. Now go to the next image and click on "**image - advanced crop - load crop settings**". Select the saved setting by clicking on it in the pop up bar. This would give a box almost within the same area that we chose for the previous gel. Now the cursor can be placed exactly on the point that was selected before. This would select the same area that was chosen for the first gel. Move the cursor around to get the scissor symbol. Clicking once on the gel would let you select copy and crop from the pop up window. Apply this for all the gels to be analyzed. Save the cropped images and close the raw ones. When the cropped images are saved they automatically get selected for spot detection.

Spot detection and matching

Go to "**spots menu**" and click on "**automated spot detection and matching**". This would give you a window where the cropped images that are opened are listed. If they are not opened pick the images from the respective folders.

Step 1. Click on one gel that looks the best to your eye (more distinct spots, minimum streaks etc) and click on the "**master**" button to select it as the master gel.

Step 2. To edit the parameters for spot detection, there is an option to select an already existing parameter set. But usually you don't do this unless you know that the gel load and the stain are the same. When you choose to "**edit new parameters**", a new window appears where you can define the parameters. First, zoom into an area in the gel containing very faint spots. Go to "**transform**" and

set always "**auto-scale**" this gives a better contrast. Select "**faint spot**" in the new window. Click on the faintest spot in the area. Now zoom out; the faint spot will be marked on the gel. If you find another spot that is fainter than the marked one just click on it, this would mark the new spot as the faintest spot.

Sometimes the software detects speckles and asks you if this should be removed after you have clicked on the edit button. In this case you can say "**ok**". If the faintest spot is not the same as the smallest spot you can activate smallest spot from the menu and then mark the smallest spot like you did with the faintest spot.Now proceed to the "**large spot cluster**" button and box the largest spot cluster that you find in the image. The next part is streak removal, mark streaks vertical and horizontal. This would remove the streaks.

You are now ready to find the spot centers; click on "**find spot centers**" the software finds and marks all the spots that match the given parameters. If you think there are spots missing you can reset the given conditions, the best way is to zoom in and transform the image by "**always auto-scale**" and then select another faint spot or try to increase the **sensitivity**" and decrease the "**minimum peak**". Increasing sensitivity alone will not help to have more spots, the minimum peak should be decreased at the same time. But the best way is to change the specified faint spot. When you are satisfied with the result, click on " **process all gels**". In the pop up bar give a name for the settings. The same set of parameters can be used for gels that are processed the same way (run, stained). Sometimes the software does not accept your faint spot as the smallest spot; in that case you may have to choose other spots.

Note: Any change in spot detection parameters should be done before going to step 3. Once the matchset is created these parameters cannot be changed. So make sure that you have best spot detection before matching. After the matching step is complete it is not possible add new images to the same matched set.

Now you are back in the automated detection and matching.

Step 3. Give a name to the match set you want to create.

Step 4 is to match gels. Here you have to set the match parameters to "**50-classic**". Click on "**edit**", there you get another window. Where the primary matching is activated. By default it is set to "**50**", now go to "**extended matching**" and select "**classic**". Now click on "**Go**".

The gels can be saved at this point by disabling matching. This is helpful when you want to use the same gel with spots detected for another analysis. The image with the spot detection completed can be stored as a separate file. This is not possible once the matching is done otherwise you loose the previous matchset including this image.

Once the matching is over you get a match summary, where all the gels are listed with the number of spots, correlation co-efficient, variance, match rate, number of spots that are matched in all gels etc. Go through the values and if not satisfactory you can do another extended matching. This can be done by going to "**re-match**". Try to change the different parameters and match again. For example try to change from 50-classic to "**20**" or "**30**" or try to change from classic to "**restricted**". See if the values in the match summary improve or not. "**Correlation Coefficient**" (Corr.Cof) should increase, "**mean CV**" should decrease, no of spots matched should increase. If the changes are not improved go back and select the old parameters, and rematch all. All spots that appear in green are matched in all gels with the primary matching, the spots in blue were matched after the extended matching and the red ones are the ones that are not matched.

Changing the Master gel: This is another way of improving the matching. Go to "**Match**" - "**Match set tool**" and click on another gel as master and save. If one gel has a lot of spots that are not there in others, all spots in this gel can be added to the master by the "**auto add spot to master function**". A new window appears where you can select the gel. Always rematch manually after these changes.

Image tools: The following helps easy handling of matchsets. When you right click on an image a list of button appears, which can be used for better image handling.

1. "**Show cross hairs**": shows centers of all detected spots with a yellow cross.
2. "**Show symbols**": shows all the detected spots in green red or blue, depending on its matched status.
3. "**Hide overlays**": hides any symbol
4. "**Show offsets**": shows the vector offset of each spot compared to the corresponding spot in the master gel.
5. "**Show quantity**": gives a value corresponding to each spot in the image. This is helpful to get a quick comparison of spot intensities.
6. "**View entire image**": to zoom out and see the whole image

Creation of replicate groups: A minimum of three gels are required per group. "**Analyze- replicate groups -create groups**". This gives you a new

window with all the gels in the match set. Select gels belonging to the same group and name it. Now you can go to match summary tool to see the match properties in the replicate groups. The "**mean CV**" should be low.

Ways to deal with red spots: Zoom in one area and "**auto-scale**", here we can see that most of the red spots are either not spots or too low in intensity. Go to "**spots**" and click on "**show quality**" and click. To screen them you can go to spots- "**show low quality**". When you now click on an image a window appears where you can enter a threshold value in the same range and cancel those spots, which are less intense than the threshold. These spots will no longer be marked with a crosshair. If there are too many spots numbered as low intense spots the threshold can be decreased. These spots can also be restored. This step has to be done for each gel. "**Rematch**" after the changes. Screening of red spots can be done in the same way using quantity threshold. But this is not a necessary step.

Note: The above step has to be performed before normalization in case you choose to do it later.

Normalization: Normalization can be done using "**quantity**" or "**density**". Arbitrary method using house keeping genes cannot be used unless you are 100% sure that they are housekeeping genes. "**Analyze - Normalize**". A new window appears. Click on "**enable normalization**" and select " **total quantity of spots**", (this method cannot be used when there is a lot of up and down regulation) or "**total density in gels**" and click on "**done**". A comparison of the density and quantity values of the images gives a rough idea of the load and stain variation between the gels. Now we can again look at the matching summary to see how the normalization has affected the matching. If not satisfied you can change from one method of normalization to another.

Analysis:

First step in analysis is to create the analysis sets. Any number of sets can be created using the "**analysis set manager**". The different types include "**quantity**", "**quality**", "**arbitrary**", "**statisctic**" and "**boolean**".

1. **Quantitative analysis set:** A quantitative analysis set consists of all spots that have difference in their intensity between two gels or two replicate groups. Select "**gels**" or "**replicate groups**". The value of quantitative difference that you expect can be specified. "**2 up**" or "**2 low**" etc. Mark "**above upper limit**" or "**below lower the limits**" to specifically define spots that are above or below the limit. This would mean that the software would pick all the spots that are 2 times more intense in group A compared to group B. Repeat this for group

B to create a set containing all the spots that are 2 times up or low in group B compared to group A. "**Analyze-analysis set manager - create - quantitative - select two groups - specify the fold increase or decrease required - name the analysis set - save - evaluate".**

2. **Matching analysis set:** A set of spots that are matched to all the members of the matchset makes the matching analysis set. Analyze-analysis set manager - create - matching - spots matched to all member.

3. **Boolean analysis:** Now you are ready to create analysis sets from the spots that are present in the existing sets. This is done by the "**Boolean analysis**", which helps you to select two analysis sets and create another set which is a union or intersection of the two selected sets. "**Analyze-analysis set manager - create- Boolean - add set- select- matching and one of the quantitative sets- intersection - name - save - evaluate**" This selects all spots matched to every member and is quantitatively different between the two replicate groups.

The analysis sets can be edited when clicked on the dashed line that appears next to the set in the set manager.

Spot review tool: You find it in the "**analysis menu**". Spot review tool gives histograms containing the relative intensity of all the spots compared between all the gels. You can choose between a display of all the spots or the spots belonging to a certain analysis sets. By default the "**all spots**" button is activated. By simply clicking on any of the histograms (it appears blue) the spot is activated in the gel, (the spot is gets zoomed into in all the gels in the matchset). Sometimes the spots may be located in the corner and therefore can be ignored. Go through each spot in the set. The spots that are relevant or significant can be selected and saved as another set by clicking on "**save selections**" and giving a new name to the set. A spot is selected by clicking on the histogram. A second click on the histogram will deselect the spot.

When the "**spot review tool**" is open, right clicking on the image would give you a menu. Choosing "**view entire image**" will help you locate the spot on the entire image.

Quantitation report: To get a quantitative data of the selected spots, "**go to report - quantity table report - mark analysis set and choose the set you want - mark replicate groups - activate group CV and ratio - select the denominator - done**". This gives a table with the spot "**ssp number**" and the ratio of the spot intensity in each group to the group which is selected as the denominator. All the spots belonging to the analysis set chosen are listed in the table. A table containing the intensity and ratio of all the spots can be obtained by marking all spots instead of spot analysis set.

Annotation: This tool helps to mark or flag a spot that you want to get back to for reference, go to"'**Analyze - Annotate**". A window appears where you can select the "**category**" that it belongs to from the list given or add a "**new category**". Activate "**show spot annotations**" and highlight the target spot. Select the spot by its SSP number and give a name or description at the "**annotation entry**" and "**apply**" the entry.

Appendix E

Curriculum Vitae

Archana Mary Jastorff

Date / place of birth:	19/08/1977, Mavelikara (India)
Citizenship:	Indian
Marital status:	Married
Maiden name:	**Jacob**

Education:

1991 - 1993	High School, Mount Carmel Girls High School, Kottayam, India
1993 - 1995	Higher Secondary, Baselius College, Kottayam, India Major subjects: English, Biology, Physics, Chemistry
1996 - 2000	Undergraduate study, Bachelor of Technology, Anna University, Chennai, India Major: Industrial biotechnology, 8 Semesters
2000 - 2002	Postgraduate study - Master of Science, Graduate School of Neural and Behavioural Sciences and International Max Planck Research School, Tuebingen, Germany Major: Neural sciences, 4 Semesters
11/2002 - 11/2006	Doctoral thesis, Dept of Proteomics and Biomarkers, Max Planck Institute of Psychiatry, Munich, Germany

Research Experiences:

06-07/ 1998	Summer project, Early sex determination in papaya and casuarinas using PCR and plant tissue culture on micropropagation in sugar cane. Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore, India
06-07/ 1999	Summer project, Effect of indigenous drugs on tumor cells in combination with Radiation therapy and Hyperthermia. Department of Radiobiology, Manipal Academy of Higher Education, Karnataka, India
01-04/ 2000	**Bachelor Thesis**, Clinical evaluation of a calorimetric diagnostic procedure for Shigella flexineri. Centre for Biotechnology, Anna University, Guindy, Chennai - 25, India
03-04/ 2001	Lab rotation I , Effect of Neurofascin on Neurite outgrowth. Department of Molecular Biology Natural and Medical Science Institute Reutlingen, Germany

09-10/ 2001	Lab Rotation II, Route planning strategies in humans. Department of Biological Cybernetics, Max Planck Institute, Tuebingen, Germany
11-12/ 2001	Lab Rotation III, Effect of context on object-based and reference- based hemi-neglect. Department of Cognitive Neurology University Clinic, Tuebingen, Germany
01-02/ 2002	Lab Rotation IV, Re-characterization of a retinal ganglion cell line. Department of Neurology University Clinic, Tuebingen, Germany
03-06/ 2002	**Master's Thesis**, Expression of GDNF and its receptor components in the retino-collicular pathway of the rat. Department of Neurology University Clinic, Tuebingen, Germany
11/2002 - 11/2006	PhD fellow, Dept of proteomics and biomarkers, Max Planck institute of psychiatry, Munich, Germany

Honors, awards:

- Poster Prize, Institute Symposium 2005, Max Planck Institute of Psychiatry, Munich, Germany.

Positions held:

- PhD student's representative, 2003 and 2004 Max Planck Institute of Psychiatry, Munich, Germany.

Invited Talks:

- Jacob AM (2004) Identification of Markers for Multiple Sclerosis using the Experimental Autoimmune Encephalomyelitis Mouse Model Dept of Neurophysiology, Institute of Physiology, Ruhr Universitt, Bochum, Germany.
- Jacob AM (2005) Identification of Disease markers using 2DE: Strengths and Limitations. Expression Proteomics Seminar, Vienna and Munich, Bio-Rad Laboratories

Lectures Given:

- Jacob AM (2005) Proteomic analysis of Murine models of EAE. International Max Planck research School (IMPRS) orientation Program Munich 2005
- Jastorff AM (2006) Identification of Disease markers using 2DE: Strengths and Limitations. International Max Planck research School (IMPRS) orientation Program Munich 2006

Publications:

- Kretz A, **Jacob AM**, Tausch S, Straten G, Isenmann S (2006). Regulation of GDNF and its receptor components GFR-alpha1, -alpha2 and Ret during development and in the mature retino-collicular pathway. Brain Res. 2006 May 23;1090(1):1-14. Epub 2006 May 2.
- Ditzen C, **Jastorff AM**, Kessler MS, Bunck M, Teplytska L, Erhardt A, Kromer SA, Varadarajulu J, Targosz BS, Sayan-Ayata EF, Holsboer F, Landgraf R, Turck CW (2006). Protein biomarkers in a mouse model of extremes in trait anxiety. Mol Cell Proteomics. 2006 Jun 30;
- Turck CW, Maccarrone G, Sayan-Ayata E, **Jacob AM**, Ditzen C, Kronsbein H, Birg I, Doertbudak CC, Haegler K, Lebar M, Teplytska L, Kolb N, Uwaje N, Zollinger R (2005): The quest for brain disorder biomarkers. J Med Invest. 2005 Nov;52 Suppl:231-5.
- **Jacob AM**, Ziemssen T, Weber F, Turck CW (2005). Proteomic profiling of Experimental Autoimmune Encephalomyelitis (EAE): Quantitative and phosphorylation specific protein expression changes in different mouse models. Molecular and Cellular Proteomics Vol 4, 8 (Suppl. 1).
- **Jacob AM** and Turck CW (2006): Detection of PTMs by fluorescent staining of 2D-gels, Post translational modifications of proteins, 2nd Edition, Tools for functional proteomics, Methods in molecular Biology, Human Press Inc [In press]

- Kronsbein H, **Jastorff AM**, Maccarrone G, Panhuysen M, Putz B, Vogt-Weisenhorn D, Stalla G, Wurst W, Holsboer F, Turck CW, Deussing J (2006): CRH receptor type 1 signaling dependent proteome and transcriptome analysis provides novel insights into molecular transmission of anxiety-relatd behavior (submitted).
- **Jastorff AM**, Haegler K, Maccarrone G, Holsboer F, Weber F, Ziemssen T, and Turck CW (2006): Biomarkers of EAE and Multiple sclerosis: Possible mechanisms mediating neurodegeneration. [In preparation].

Conference Publications:

Oral Presentations:

- Jacob AM (2004) Multiple sclerosis: a proteomic approach to identify new markers of disease. Institute symposium: Max Planck Institute of Psychiatry, Munich Germany

Poster Presentations:

- **Jacob AM**, Ziemssen T, Weber F, Turck C W (2003) Multiple sclerosis: A proteomic approach to identify disease markers. Proteomic forum 2003, Munich, Germany
- Rosenhagen M, Milfay D, Maccarrone G, Birg I, **Jacob AM**, Uhr M, Holsboer F, Turck CW (2003). Analysis of Cerebrospinal fluid of depressed patients Institute symposium: Max Planck Institute of Psychiatry, Munich, Germany
- Maccarrone G, **Jacob AM**, Birg I, Rosenhagen M, Turck CW (2004). The human CSF proteome: Quantitative and qualitative analysis. Institute symposium: Max Planck Institute of Psychiatry, Munich, Germany
- **Jacob AM**, Ziemssen T, Weber F, Turck CW (2005). Identification of biomarkers for Multiple sclerosis. 6th Meeting of the German Neuroscience Society, 30th Gttingen Neurobiology Conference 2005
- **Jacob AM**, Ziemssen T, Weber F, Turck CW (2004). Identification of biomarkers for Multiple sclerosis. Proteomic Basics Summer school 2004, Opatija, Croatia

- **Jacob AM**, Ziemssen T, Weber F, Turck CW (2005). Proteomic profiling of Experimental Autoimmune Encephalomyelitis (EAE): Quantitative and phosphorylation specific protein expression changes in different mouse models. Hupo 4th World Congress 2005
- Ditzen C, **Jacob AM**, Maccarrone G, Teplytska L, Turck CW (2005) Biomarker discovery in the CSF of depressed patients. Institute symposium: Max Planck Institute of Psychiatry, Munich Germany)

Bachelor Thesis:

- Jacob AM (2000) Clinical Evaluation of a calorimetric diagnostic procedure for Shigella flexineri, Center for Biotechnology, Anna University, Chennai, India

Masters Thesis:

- Jacob AM (2002) Expression of GDNF and its receptor components in the retino-collicular pathway of the rat