A Novel Calpain Inhibitor for the Treatment of Experimental Autoimmune Encephalomyelitis (EAE)

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List of Abbreviations

ACS	Average clinical score
ADEM	Acute disseminated encephalomyelitis
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APC	Antigen presenting cells
APP	Amyloid precursor protein
ATM	Acute transverse myelitis
BBB	Blood-Brain barrier
CFA	Complete Freund`s adjuvant,
CYLA	Cysteic- leucyl-argininal
CNS	Central nervous system
CSF	Cerebrospinal fluid
Dpi	Days post immunization
D-PBS	Dulbecco`s phosphate buffer saline
EAE	Experimental autoimmune encephalomyelitis
FDA	Food and Drug Administration
GA	Glatiramer acetate
GBS	Guillain-Barre syndrome
H&E	Hematoxylin and eosin
IACUC	Institutional animals care and use committees
ICAM	Intercellular adhesion molecule
IFA	Incomplete Freund`s adjuvant

IFN	Interferon
IIDDs	Idiopathic inflammatory demyelinating diseases
IL	Interleukin
i.p.	Intraperetonially
IVIG	Intravenous immunoglobulin
LFA	Leukocyte function associated antigen
LFB	Luxol fast blue
LPS	Lipopolysaccharide
MAG	Myelin associated glycoprotein
MAP	Mitogen-activated protein
MBP	Myelin basic protein
MHC	Major Histocompatibility complex
MMP	Matrix metalloproteases
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NAA	N-acetyl aspartate
NFP	Neurofilament protein
NMO	Neuromyelitis optica
NOS	Nitric oxide syntheses
RFU	Relative fluorescent unit
PE	Plasma exchange
PLC	Proteinlipase C

PLP	Proteolipid protein
PML	Progressive multifocal encephalomyelitis
PPMS	Primary progressive MS
PRMS	Progressive-relapsing MS
RRMS	Relapsing-remitting MS
SPMS	Secondary-progressive MS
SSEP	Subacute sclerosing encephalopathy
SFV	Semliki`s forest virus
TCR	T cell receptor
TLR	Toll-like receptor
TMEV	Theiler`s murine encephalomyelitis virus
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

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Acknowledgments

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I. Abstract

Aberrant activation of calpain plays a key role in the pathophysiology of neurodegenerative disorders including multiple sclerosis (MS). Calpain is increasingly expressed in inflammatory cells in experimental autoimmune encephalomyelitis (EAE) and is significantly elevated in the white matter of patients with multiple sclerosis, thus calpain inhibition could be a target for therapeutic intervention.

A novel drug, cysteic-leucyl-argininal (CYLA), was designed by attaching a cysteic acid to the protease inhibiting part of leupeptin. Cysteic acid has similar functional group as in taurine, an amino acid that is being transported across the blood-brain barrier (BBB) using two taurine transporters.

The experiments of this thesis addressed the effect of CYLA, targeted to the central nervous system (CNS), in a myelin oligodendrocyte glycoprotein (MOG)-induced EAE in C57Bl/6 mice. Daily intraperitoneal application of 0.5 to 2mg of CYLA or oral administration of 2mg of the prodrug was found to significantly reduce the clinical signs of EAE, demyelination, and inflammatory infiltration in a dose-and time-dependant manner. Measurement of calpain content by a fluorogenic method and calpain activity by Western blot analysis of substrate (alpha-fodrin) degradation revealed that this inhibitor could cross the blood-brain barrier and inhibit calpain.

In addition, the effect of CYLA in suppressing axonal injury was examined in chronic EAE. Markers for axonal injury were barely detectable in the CYLA treated mice groups. Both the free aldehyde form of CYLA, which was administered by injection, and the diacetal prodrug which was given orally, were able to ameliorate the disease course

and its progression. We concluded that CYLA has a potential for the treatment of MS and other neurodegenerative disease in which elevated calpain activity is suspected.

II. Specific Aims

Specific aim one:

To test whether or not CYLA suppresses the onset of EAE in vivo by inhibiting CNS Calpain.

We hypothesize that CYLA will either prevent the onset of EAE or at least ameliorate signs of EAE because it will cross the blood-brain barrier and inhibit calpain within the central nervous system. Based on this hypothesis, a treatment regimen was designed to examine the effect of CYLA in a dose-and time-dependent manner in a MOG-induced EAE.

Specific aim two:

To test whether or not CYLA is effective in preventing injury to the axons in chronic EAE.

In addition to myelin degeneration, axonal injury has been recognized as a major component of MS and EAE. The severity of the disease seems to correspond more to the axonal injury than to myelin degeneration. Calpain has been implicated in both inflammatory and neurodegenerative components of MS.

The second hypothesis was formed based on several reports by different investigators that neuronal calpain contributes to axonal injury, especially in chronic stages of the disease. My aim is to determine if CYLA crosses the BBB and prevents or reduces axonal injury.

III. Introduction

1. General background

MS is a chronic, autoimmune demyelinating disease of the CNS. It is the second most common disabling neurological disease of young individuals between the ages of 20-50, traumatic brain injury being the most common (Martin and McFarland, 1995; Bauer et al., 2001; Hafler et al., 2005). The general population prevalence of MS varies between 60-200/100,000 in Northern Europe and North America, and 6-20/100,000 in lower risk areas (Hernan et al., 1999; Sospedra and Martin, 2005).

Despite numerous studies, the etiology of MS remains unknown. To date there is neither a clearly identified causal agent, nor a fully established pathogenesis for MS, although genetic predisposition, viral infections and environmental factors are considered to be contributing factors in the etiology of this disabling disease (Compston, 2004; Hafler, 2004). Histological pictures of MS lesions, genetic data, results from animal model studies and response to immunomodulatory drugs support the concept that MS is probably an autoimmune disease in a susceptible individuals after exposure to not yet clearly defined environmental factors (Keegan and Noseworthy, 2002; Hafler, 2004; Lutton et al., 2004). In addition, vitamin D deficiency has also been assocated with the onset and exacerbation of MS (Hayes, 2000; Brown, 2006; Mark and Carson, 2006).

1.1. Genetics

Strong evidence for the genetic involvement comes from a series of studies on families with twins. The genetic risk is indicated by the recurrent risk in family members of the affected individual (Robertson et al., 1996). The hereditary tendency of MS is indicated by the increased relative risk of siblings compared with the general population and by the increased concordance rate of monozygotic twins compared with dizygotic twins. Twin studies from different population indicate that a monozygotic twin of a patient who has MS is at higher risk (25%-30% concordance) in developing MS than a dizygotic twin (2%-5%) (Mumford et al., 1994; Willer et al., 2003). The relative low concordance rate of identical twins indicates a contribution of the nongenetic factors to MS etiology (Dyment et al., 1997). Results from studies on half-siblings (Sadovnick et al., 1996) and adoptee (Ebers et al., 1995) support the assumption that genetics rather than the family environment plays the key factor for familial aggregation of the disease.

Giving further evidence for the genetic involvement in MS, some immunological markers are also associated with the susceptibility in developing MS. Linkage analysis studies and intensive whole genome screening conducted in several affected families that have MS yielded that there is an association of MS with the HLA Class II DR2 alleles, but failed to find other loci in MS (Ebers, 1996; Haines et al., 1996; Sawcer et al., 1996).

Gender has a strong impact on diseases that are considered autoimmune. The preferential involvement of women in autoimmune disorder is not fully understood, but their hormones, intrinsic stronger immune response and genetic factors likely play a role. Sex influences certain genes linked to MS. The MHC Class II HLA-DR2 DQ6 halotype, a susceptible gene, is more common in female than male patients (Celius et al., 2000). As

in the case of other autoimmune disorders MS afflicts preferentially women (1.5-2:1), which make up 75% of all patients with MS (Sanna et al., 2003).

Women are generally more affected than men in early life where as men are more affected later in life (Kahana, 2000; Compston and Coles, 2002). Age of onset suggests a link to hormonal factors. Risk of MS begins at puberty. Less than 1% of cases appear before the age of 10 while 90% of patients are between 15 and 50 years of age. Other studies point to the roles of hormones in MS. In a recent study, 24% of men who had MS had low testosterone level compared with matched control patients (Pelfrey et al., 2002; Pelfrey et al., 2005). In another study Men who had MS and sexual dysfunction were found to have very low testosterone level and the dysfunction improves with testosterone therapy (Wei and Lightman, 1997). Pregnancy is another state where hormonal influence plays a role. Pregnancy does not increase risk of MS, but onset of the disease is less likely to occur during pregnancy. The major effect of pregnancy on MS is a decrease in disease activity, which presumably resulted from an immunosuppressive state during pregnancy produced by several factors, such as changes in hormones and a shift towards a Th2 response (Elenkov et al., 2001).

1.2. Environmental factors and infectious agents

Among possible factors, geographic location, infectious agents and lifestyle influences have been proposed to induce or contribute to disease expansion (Coo and Aronson, 2004). MS occurs with the highest prevalence in North Europe and North America. People in the northern hemisphere, especially northern European origins, tend to be statistically more susceptible to MS than people in the southern hemisphere. It has been estimated that roughly 1 in 100 persons of susceptible northern European origin who live in temperate climates are more likely to develop MS (Lutton et al., 2004).

Environmental contribution to MS etiology is supported by different factors such as north to south gradient, a decrease in MS prevalence when people move from high risk are to low risk area before the age of 15-16 and MS distribution can not be explained by population genetics alone (Sospedra and Martin, 2005).

The geographic distribution of MS with increasing incidence in the northern hemisphere and rare cases in equatorial regions suggests a striking correlation between environmental conditions and MS. One of the many factors that are associated with the environment variation in MS incidence is the exposure to sunlight (Hernan et al., 1999; Freedman et al., 2000). MS is prevalent in areas reporting fewer than 2000 hours of sunshine annually. It also shows seasonal variability, with highest disease activity in the spring and lowest in the fall (Auer et al., 2000). A large cohort study in the US showed that 3.5 times more women residing in the northern states were diagnosed with MS, compared with women in the southern states (Hernan et al., 1999).

Vitamin D is synthesized by the conversion of 7-dehydrocholesterol to vitamin D in the skin. Exposures to ultraviolet (UV) B light are required and it is strongly associated with the level of vitamin D within the body. Vitamin D is involved in regulation of the immune system (Mark and Carson, 2006). There is a possible link between MS and decreased vitamin D synthesis. A Finnish study found that, compared with patients without MS, patients with MS have low serum vitamin D levels during the summer (Soilu-Hanninen et al., 2005). Treatment with vitamin D supplements decreased the incidence of relapses and severity of the disease both in MS and EAE (Cantorna et al., 1996; Cantorna et al., 1998). Decreased exposure to sunlight may cause insufficient production of vitamin D, resulting in an impaired immune regulation and the development of autoimmune diseases such as MS (Holick, 1987; Holick et al., 1987; Mark and Carson, 2006). Vitamin D deficiency is commonly seen in patients with MS.

Experimental evidence for the role of vitamin D came from animal studies. Mice treated with dietary vitamin D prior to the induction of EAE did not develop signs of EAE, compared with 100% incidence in the control group. EAE mice treated with vitamin D injections followed by dietary supplementation halted disease progression. The effect was reversible since the disease progressed after discontinuation of the supplement (Cantorna et al., 1996).

MS patients also show polymorphism in vitamin D receptors, suggesting a link between vitamin D deficiency and pathogenesis of MS (Baas et al., 2000; Meehan and DeLuca, 2002). Vitamin D receptors are present in most body cells including astrocytes, neurons and oligodendrocytes. Activation of vitamin D receptors plays a role in

modulation of immunity by influencing the production of cytokines (VanAmerongen et al., 2004). Decreased production of proinflammatory cytokines such as IL-2, TNG-α and IL-12 was observed by vitamin D receptor activation. Likewise, vitamin D led to the increased production of anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) and IL-4 (Cantorna et al., 2000; Mahon et al., 2003).

Viral and bacterial infections are possible candidates as environmental triggers of MS. It has been observed that demyelinating CNS and peripheral nervous system (PNS) disorders occur following a viral illness such as progressive multifocal leukoencephalopathy (PML), subacute sclerosing encephalopathy (SSEP), HIV encephalopathy and guillan-barrie syndrome (GBS) (Soldan and Jacobson, 2001). MS like illness is also observed after immunization with live attenuated viral vaccinations, especially rabies vaccination. Prospective studies have shown that MS relapses often follow viral infections (Sibley et al., 1985).

Results from animal experiments using different types of virus such as Theiler`s murine encephalomyelitis virus (TMEV), Semliki forest virus (SFV) and canine distemper virus to induce MS-like illness also support the possible involvement of viruses in demyelinating disease such as MS (Mokhtarian and Swoveland, 1987; Johnson, 1994; Soldan and Jacobson, 2001).

Two main possible mechanisms have been proposed to explain how infectious agents can cause the MS-like illness. These phenomena are known as molecular mimicry and bystander activation (Fig. 1).

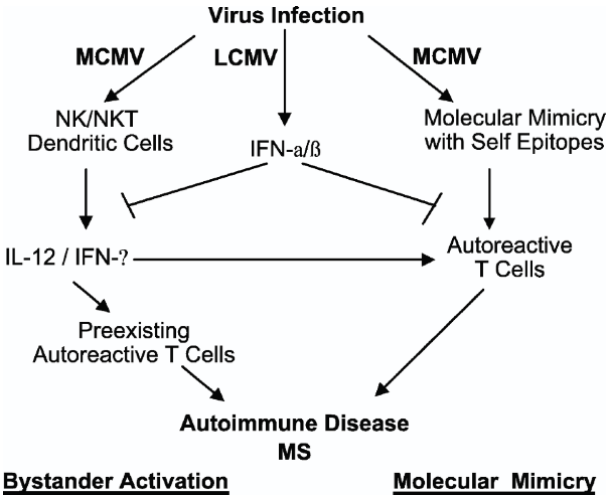


Figure 1 Two mechanisms induced by virus infection can trigger autoimmune diseases including MS. Infection with virus that has molecular mimicry with myelin antigen can prime autoimmune T cells (right). Infection with a virus, including murine cytomegalovirus (MCMV), can cause the activation of NK, NKT and dendritic cells, resulting in interleukin (IL)-12 and interferon (IFN)-γ production (left). These cytokines can cause the bystander activation of autoreactive T cells, leading to autoimmune diseases, such as MS. On the other hand, infection with a virus such as lymphocytic choriomeningits virus (LCMV) can suppress these pathways by triggering the production of IFN-α/β (center). MCMV infection may also result in molecular mimicry with self antigens leading to the activation of autoreactive T cells and autoimmune disease through TCR engagement with the induction of IL-12 driving T cell development (McCoy et al., 2006).

Molecular mimicry involves reactivity of T and B cells with either peptides or antigenic determinants shared by infectious and self-antigens such as the one after SFV-infection (Mokhtarian et al., 1999). Thymic deletion of autoreactive T cells has been considered as a major preventing mechanism of autoimmunity (Kyewski and Derbinski, 2004). However, it has been shown that these autoreactive T cells are present both in healthy individuals and MS patients (Ota et al., 1990; Pette et al., 1990). These T cells are capable of recognizing autoantigens expressed in thymus such as MBP and S100β (Wekerle et al., 1994). It is generally believed that similarity between viral peptide and components of the nervous system leads to the activation of the preexisting autoreactive

T cells, which eventually result in activation of an autoimmune cascade (Mokhtarian et al., 1999; Wekerle and Hohlfeld, 2003).

Bystander activation mechanisms can be classified into two categories. The first category refers to the fact that T cell activation occurs by means of inflammatory cytokines, superantigens and molecular pattern recognition independent of T cell receptor (TCR). The second mechanism involves the unveiling of host antigens and the adjuvant effect of infectious agents on antigen-presenting cells (APCs) in a TCR dependent way.
In the unveiling of host antigens as a consequence of cell damage that are infected with viruses, activated virus-specific T cells will recognize viral epitopes and kill infected cells which results in the destruction of self-tissue and release of autoantigens. The presentation of autoantigen in the presence of infectious agent components as an adjuvant can result in de novo activation of autoreactive T cells and later to epitope spreading. This process occurs in TMEV model of MS (Miller et al., 1997;McMahon et al., 2005).

In the latter mechanism of activation, lipopolysaccharide (LPS) from infectious agents will bind to proinflammatory factors such as Toll-like receptors (TLRs), particulamyelin oligodendrocyte glycoproteinrly TLR4 that is expressed on microglia but not on astroglia or oligodendrocytes in the CNS, and will lead to activation of these cells leading to increased expression of inflammatory cytokines and reactive oxygen species. LPS-TLR interaction may also occur in MS during an infection with bacteria (Sospedra and Martin, 2005).

1.3. Pathology

The pathologic hallmark of MS lesion since its first detailed description by Charcot in 1868 until now remains almost the same consisting of focal demyelination associated with inflammation and reactive gliosis. This leads to the scarring of the tissue and varying degrees of axonal injury (Bitsch et al., 2000; Bruck, 2005). The lesions are scattered through out the CNS with predilection for the periventricular white matter, optic nerve, spinal cord, brainstem and cerebellum(Ludwin, 2006).

The primary mechanism of injury is inflammation associated with myelin damage. Myelin a very complex substance that wraps and surrounds the nerve fibers and it is essential for the nerves to transmit electrical signals (Fig. 2).

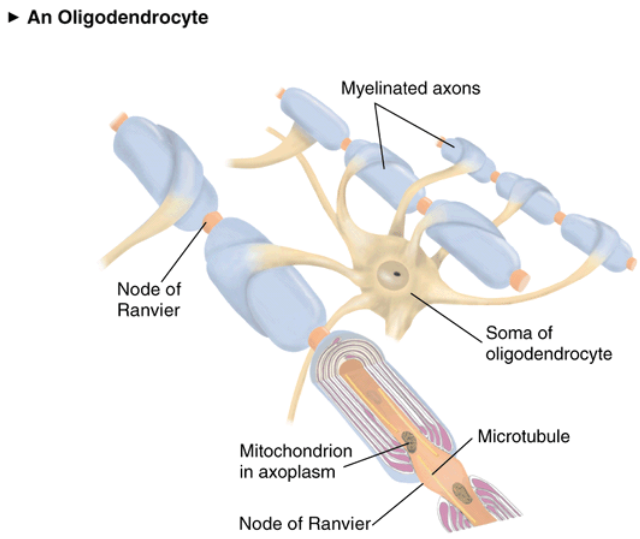


Figure 2 Cartoon showing the structure of myelinated nerve fiber within the CNS. One oligodendrocyte wraps around several axons within the CNS.

The clinical signs and symptoms in MS appear due to impaired or loss of conduction of electrical signals as a result of myelin damage (Stys et al., 1992; Rosenberg et al., 1999; Lo et al., 2002; Craner et al., 2003; Waxman et al., 2004). At the initiation of disease, a great number of patients experience acute flare-ups and subsequent improvement of the clinical symptoms. In most cases the symptoms occur frequently. This phenomenon is known as a relapse. A minority of patients experience a steady worsening of the disease from the onset. Two proposed mechanisms lead to progressive impairment of permanent disability over time.

One mechanism is stepwise worsening as a result of accumulated deficits from residua of exacerbations; the other mechanism is the gradual, inexorable progression, independent of the exacerbations (Lublin et al., 2003). Some lesions (mostly detected accidentally) don't produce symptoms and remain clinically silent when the affected area of the brain that is not associated with function or when the demyelination is minor and conduction can be maintained through the underlying axon. In these cases the lesion of MS are more often inflammatory than necrotic in the earlier stages of the disease (Smith and McDonald, 1999; Lachapelle et al., 2005).

The remissions are believed to be due to apoptosis of inflammatory cells, NO produced by macrophages and microglia, limited remyelination (Lassmann et al., 1997; Bruck et al., 2003; Lachapelle et al., 2005) and increases expression of Na^+ channels to restore electrical conduction (Waxman, 1998; Craner et al., 2003; Waxman et al., 2004).

The relapses are believed to be due to epitope spreading (Vanderlugt and Miller, 2002). Epitope spreading is a phenomenon whereby an endogenous antigen that is distinct and none cross reactive with the antigen used to induce the disease, leads to

priming of autoreactive T-cells (Fig. 3 and 4). The activated T-cells cross the BBB and initiate inflammatory cascade within the brain. This inflammatory reaction results in tissue injury. The tissue debris will be processed and presented to APC leading to activation of diffent T-cells distinct from the primary T-cells. These T-cells will then cross the BBB and cause additional tissue destruction resulting in the subsequent relapse.

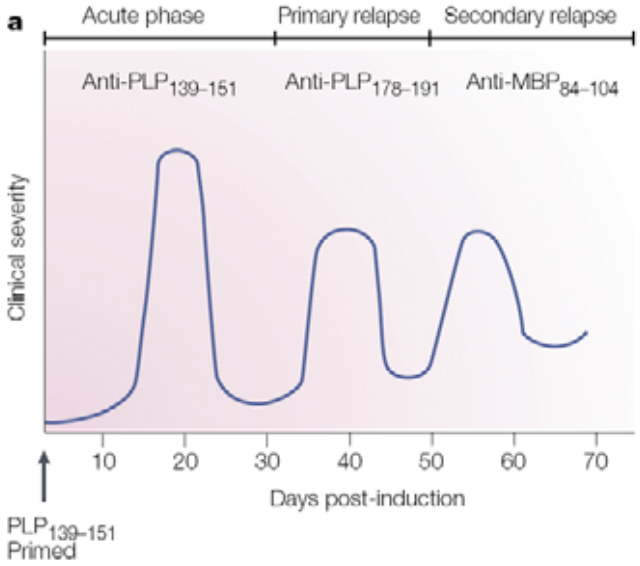


Figure 3 Hierarchical pattern of intramolecular and intermolecular epitope spreading in PLP139–151-induced relapsing EAE and Theiler's virus-induced demyelinating disease (TMEV-IDD). The relapsing–remitting clinical course and pattern of epitope spreading in relapsing-EAE in the SJL mouse induced by priming with the immunodominant PLP139–151 epitope. Acute disease is mediated by PLP139–151-specific TH1 cells, which are then downregulated, resulting in disease remission. Myelin destruction during the acute clinical episode leads to the priming of T cells to a secondary epitope, PLP178–191 (intramolecular epitope spreading), which mediates the primary relapse. After regulation of these cells, a third wave of TH1 cells is induced to the lesser immunodominant MBP84–104 epitope (intermolecular epitope spreading), which mediates the secondary relapse (Vanderlugt and Miller, 2002).

Epitope spreading initiated as a result of tissue damage it thought to play an active role in ongoing disease pathology (Vanderlugt and Miller, 2002).

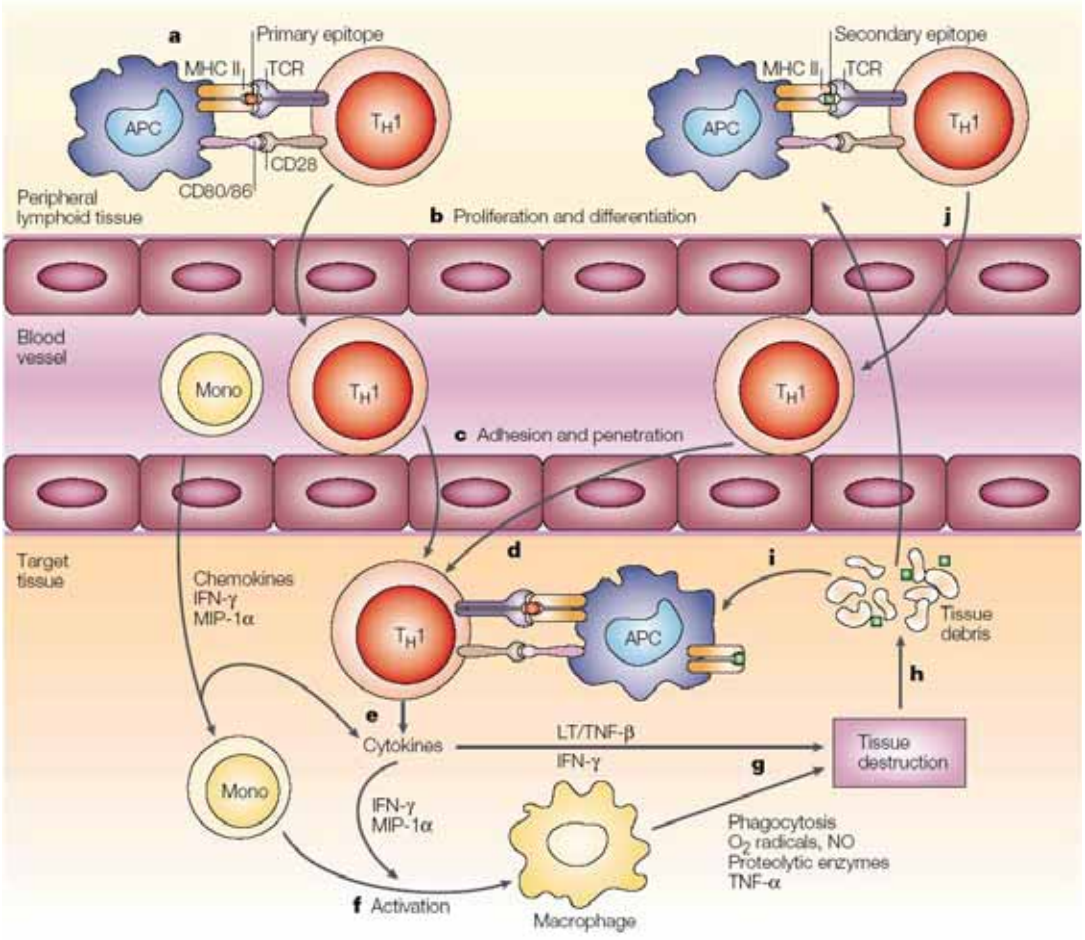


Figure 4 Mechanism of Epitope spreading. Presentation of the primary epitope (the immunodominant self or viral epitope) occurs in peripheral lymphoid tissue (**a**), resulting in activation and differentiation of autoreactive TH1 cells (**b**). The activated TH1 cells migrate (**c**) into the target tissue, where they encounter antigen presented by resident APCs. (**d**). After antigen restimulation, the pathologic TH1 cells release a cascade of chemokines and cytokines (**e**), leading to recruitment of additional mononuclear phagocytes from the peripheral blood, which are activated along with resident APCs (**f**).Activated mononuclear cells then lead to bystander tissue destruction (**g**) via phagocytic mechanisms and release of TNF-α, proteolytic enzymes, NO and O2 radicals. The tissue debris (**h**) is processed and presented on resident and peripheral APCs (**i**), leading to the activation and differentiation of a second wave of TH1 cells (**j**), which can re-enter the tissue and cause additional tissue destruction. APC, antigen presenting cell; IFN-γ, interferon-γ; LT/TNF-β, lymphotoxin/tumour necrosis factor β; MIP-1α, macrophage inflammatory protein 1α; MHC II, major histocompatibility complex class II; Mono, monocyte; TCR, T-cell receptor;TH1, T helper cells type 1 (Vanderlugt and Miller, 2002).

1.4. Axonal Injury

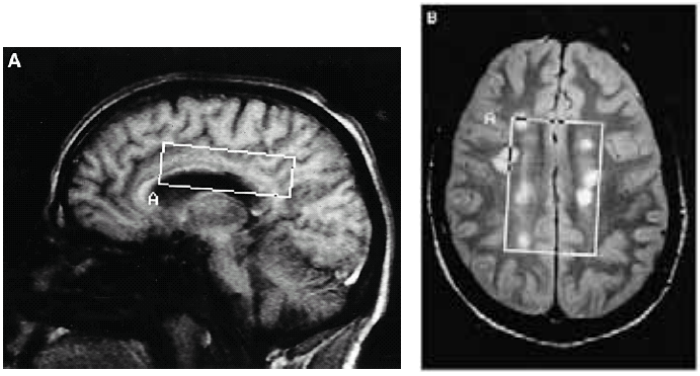
Contrary to traditional beliefs MS symptoms are not entirely a result of demyelination. Increasing evidence shows the involvement of axonal injury. In the initial phase of the disease a varying degree of axonal injury is present and this becomes more critical in the chronic phase (Ferguson et al., 1997; Trapp et al., 1998).

Convincing evidence is accumulating regarding axonal injury and their roles in the pathophysiology of MS. Axonal injury has become a hallmark of MS (Trapp et al., 1999; Bjartmar and Trapp, 2001). Axons are injured within MS lesions and the extent of axonal damage increases with the magnitude of inflammation, disease progression and duration. The magnitude of axonal loss also correlates with the degree of disability (Matthews et al., 1998; Bjartmar et al., 1999; Trapp et al., 1999; Bjartmar et al., 2000; Wujek et al., 2002; Bjartmar et al., 2003; Ghosh et al., 2004; Petzold et al., 2005).

However, the mechanism responsible for the damage to the axons that finally leads to complete transection is not known. It appears that injury to the axons is a result of the activation of a final common pathway that can be triggered in a number of different ways, the relative importance that may vary among lesions and within lesions at different stages of their development (Stys, 2005).

Axonal injury in MS was first described by Charcot in 1868. Although damage to the axons has been described as a key factor for disability in MS, much less is known about the pathogenesis of the axonal damage (Kornek et al., 2000). These have been recently studied and shown by brain imaging; histopathological and axonal transport studies (Rao et al., 1981; Guy et al., 1989; Trapp et al., 1998; Trapp et al., 1999; Zhu et al., 1999; Bitsch et al., 2000; Mancardi et al., 2001; Wujek et al., 2002). There is

accumulating evidence that axonal damage correlates with the accumulation of some neuronal markers like NAA, amyloid precursor protein (APP) and dephosphorylated neurofilament (Matthews et al., 1998; Pike et al., 1999; Ferguson et al., 1997; Trapp et al., 1998). There was little correlation between the extent of demyelination between results of T2-weighted MRI lesions volumes and clinical disability (Khoury et al., 1994; Filippi et al., 1995). In order to investigate the extent of axonal damage and its impact on permanent disability in vivo a group of MS patients with relapsing-remitting course have been followed by measuring qualitatively the concentration of NAA using magnetic resonance spectroscopy (Fu et al., 1998; Matthews et al., 1998). This study is non-invasive and helps in assessing the neuronal and axonal integrity of the CNS. NAA is a well-known axonal marker that is decreased during axonal injury. It is exclusively localized in neurons and neuronal processes in the mature brain (Matthews et al., 1998). Both irreversible and reversible axonal injuries contribute the decrease in NAA level in MS (Khan et al., 2005). Results of a lontigudinal study on patients with RR-MS and SP-MS showed that there was no significant change in the conventional T2-weighted MRI of the patients whereas there was significant decrease in NAA level of these patients and it correlated with the deterioration of their neurological function (Matthews et al., 1998). NAA has been shown to be a valuable index for assessment of axonal injury or dysfunction in vivo (Fig. 5).



(**A**) Scout MRI scan of a patient with secondary progressive multiple sclerosis in sagittal orientation, illustrating the volume of interest used for spectroscopy. (**B)** Axial view of the same volume of interest (De Stefano et al., 1998).

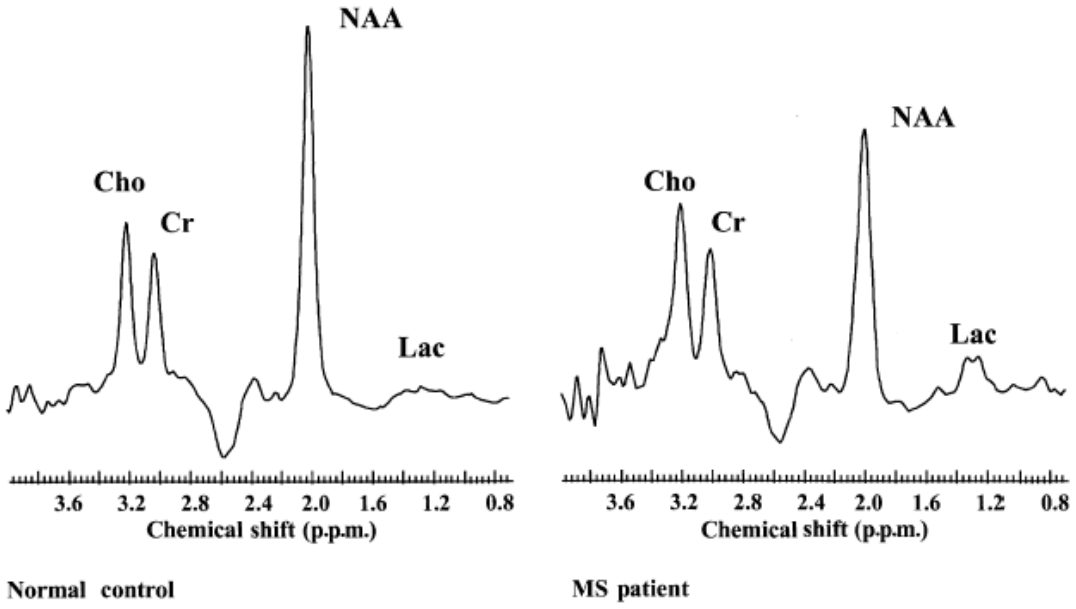


Figure 5 Comparison of the level of NAA in patients with SP-MS and normal individuals using MRS. Single-voxel proton MR spectrum from the volume of interest illustrated in Fig. 38 (right) and a spectrum from a homologous volume of interest relative to a normal control (left). Note the significant decrease in the NAA: Cr ratio in the patient's spectrum. The prominent lactate (Lac) resonance at 1.2 p.p.m. arises from lactate in the CSF, a common finding associated with ventriculomegaly.Cho = choline (De Stefano et al., 1998).

In the brains of patients with MS a diffuse global injury of the so called “normal-appearing white matter (NAWM)” is found besides areas of focal demyelination. This phenomenon has been shown by different imaging studies. One technique that has been used to investigate this process is the magnetic resonance spectroscopy (MRS) that analyzes the extent of global injury by measuring the neuronal specific marker N-acetylaspartate (NAA) level and the ratio of NAA to creatine (Dehmeshki et al., 2001). In addition, the level of other metabolites has been determined. MRS reveals that there is a decreased NAA level in the secondary-progressive MS when compared with relapsing-remitting MS and healthy individuals (Suhy et al., 2000; Dehmeshki et al., 2001). This indicates that another pathological process is going on other that demyelination. Injury to the axons is believed to be the underlying pathologic process.

Axonal injury is the major correlate for the permanent neurological deficit rather than inflammation or demyelination (Trapp et al., 1999; Bjartmar et al., 2000; Lassmann, 2001). The need for a therapeutic means that targets the suppression or the reduction of axonal injury is of crucial importance in the future.

It is not known what mechanisms lead to axonal injury, but it is believed that calpain is a major contributor to the axonal damage. Myelin damage leads to the impairment or loss of signal transmission within the damaged nerve and loss of axonal protection. Over time the nerve cells increase the expression of Na^+-channel in order to compensate the impairment of loss of electrical signal transmission (Fig.6). This leads to the increased influx of Na^+-ion. The increased Na^+-ion concentration is the drive for another process that exchanges Na^+ for Ca^{2+} leading to increased Ca^{2+}- influx and

concentration in the axon (Stys et al., 1992; Lo et al., 2002; Craner et al., 2003; Waxman et al., 2004; Stys, 2005).

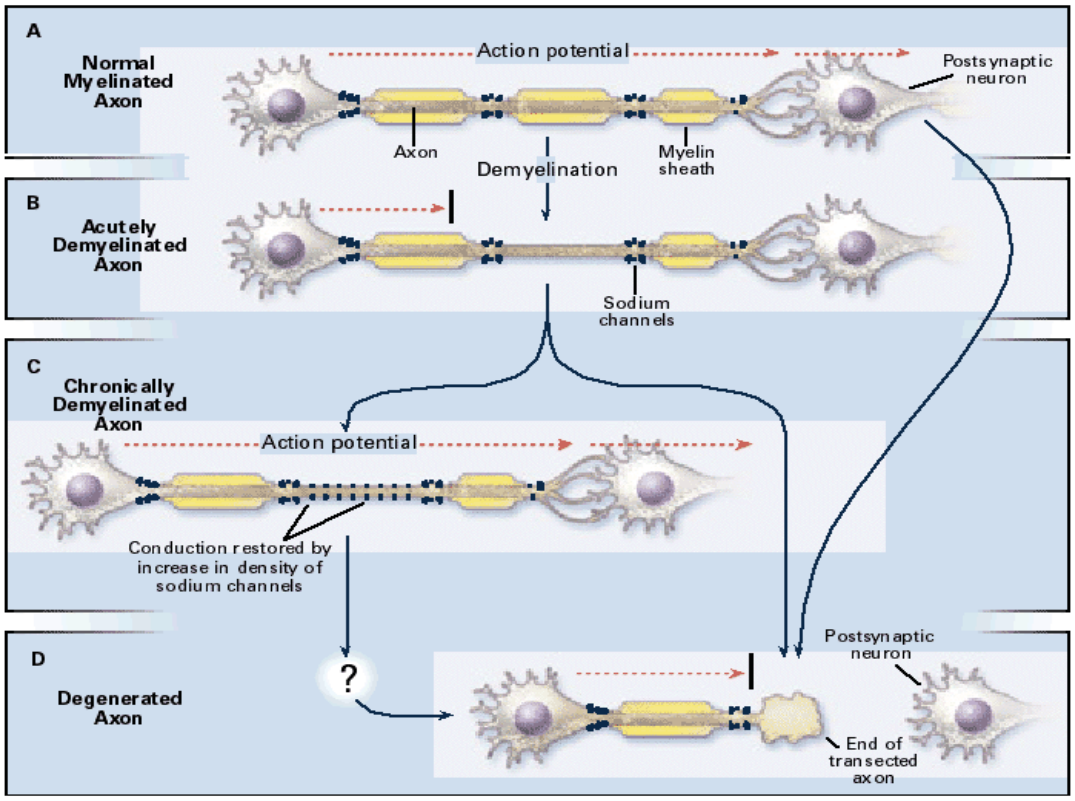


Figure 6 Schematic illustration of the demyelination associated with increased expression of Na-channels over the course of the disease. Neurons increased the density of Na-channels in demyelinated areas in order to restore impaired or lost conduction. Ultimately it comes to complete transaction of the axon.

The increased Ca^{2+}-ion concentration could lead to activation of a number of enzymatic cascades including calpain activation (Fig. 6 and 7). Calpain degrades a number of axoskeletal proteins such as neurofilament and spectrin (Lo et al., 2002; Waxman et al., 2004; Stys, 2005).

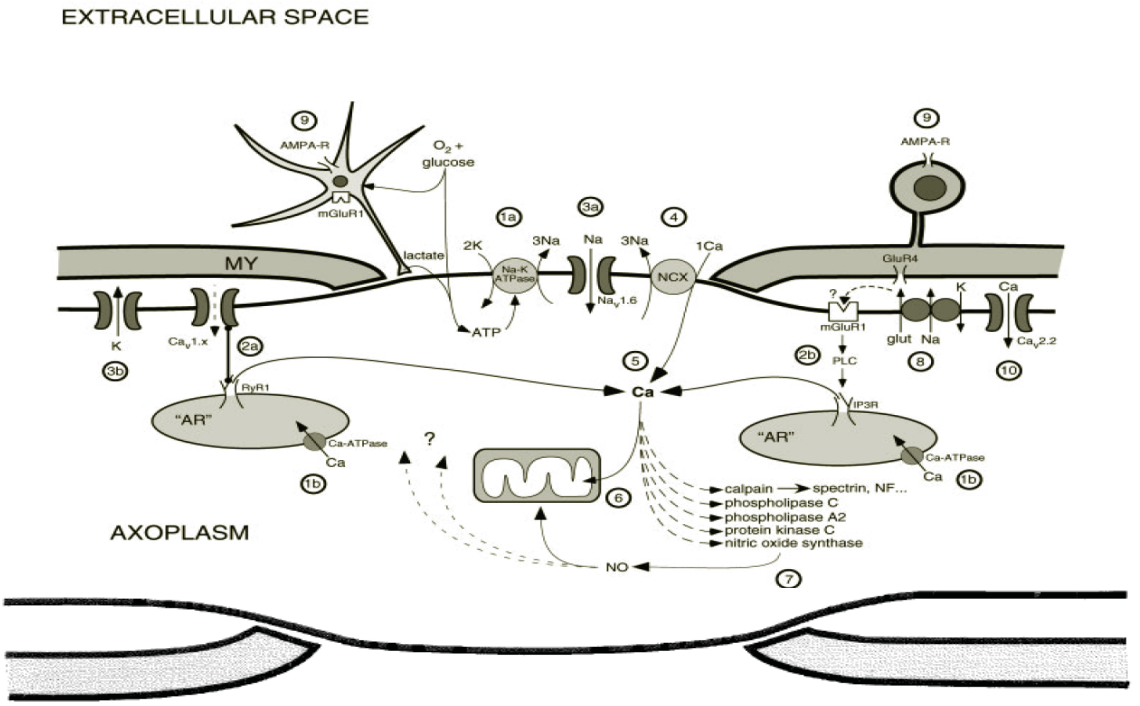


Figure 7 Proposed mechanisms for axonal injury. Summary of interdependent events in injured myelinated axons leading to axonal degeneration. The increased Na-influx as a result of increased Na-channel density at areas of demyelination leads to the activation of more Na-Ca^{2+}-exchanger. This process leads to abnormally high Ca^{2+}-influx in to the axon causing activation of a number of enymatic cascades including calpain activation. Ultimately, calpain degrades cytoskelton elements such as spectrin leading to axonal damage (Stys, 2004).

APP, among other markers of axonal injury, is a sensitive marker that accumulates within the axon to a detectable level. APP is a membrane–spanning glycoprotein that is metabolized in the neurons and transported from the neurons to the axons bulbs by fast anterograde transport (Koo et al., 1990; Hendriks et al., 2005). This transport is mediated by the direct binding of APP to the kinesin light chain, a microtubule motor protein (Koo et al., 1990; Sisodia et al., 1993). When axonal damage

occurs, APP accumulates in the axon to the degree that can be detected by immunhistochemical methods (Gilgun-Sherki et al., 2003b).

1.5. Grey matter injury

MS is generally considered a disease of the white matter of the CNS, however involvement of the grey matter and the peripheral nerves has recently been reported (Peterson et al., 2001; Cifelli et al., 2002). Regions of demyelination have been found in the deep cerebral nuclei (Cifelli et al., 2002) and the cerebral cortex. The cortical lesions may appear as a subcortical white matter lesion that extends into the adjacent cortex or as an independent perivenous cortical lesion. Some of the cortical lesions are band-like demyelinated areas without any topographic relation to cerebral veins. These lesions appear in the upper three to four cortical layers. The cortical lesions are different from the white matter lesions in that they are associated with less inflammation with a dominant effector cell population of ramified microglia and not a macrophage phenotype (Peterson et al., 2001). The global tissue texture in the cortex lesions is better preserved than the white matter lesions, suggesting a less global tissue injury. This makes it difficult to appreciate the extent of cortical demyelination on autopsy and MRI investigation of the lesions (Kidd et al., 1999). The presence of apoptotic neurons has been reported to be increased within the demyelinated cortex (Peterson et al., 2001). Cortical demyelination might have an impact on neuronal, dendritic and axonal function, viability and survival. This may be relevant to the pathogenesis of neurological and cognitive disability in MS (Cader et al., 2005; Christodoulou et al., 2005; Lazeron et al., 2005; Lynch et al., 2005; Morgen et al., 2005; Wachowius et al., 2005).

1.6. Variation of clinical courses

Clinically, MS is a heterogeneous disorder which presents with a spectrum of different courses and phenotypes (Lucchinetti et al., 1996; Lassmann et al., 2001; Lucchinetti et al., 2004). Heterogenity is also observed at the level of genetic predisposition, histological characteristics of the lesions, MRI pattern and response to therapy. This heterogeneity is also present in all animal models of MS. Depending upon the type of antigen and animal strain used, different types of clinical courses are observed (Fuller et al., 2004; Rao and Segal, 2004).

The clinical course of MS is variable and nearly impossible to predict for each individual patient. MS is divided into 4 different clinical subtypes according to the clinical courses.

The definitions derived from an international survey of people who work in the field of MS (Lublin and Reingold, 1996; McDonald et al., 2001). The results from the survey are widely accepted terms used for the clinical courses of MS (Fig. 8).

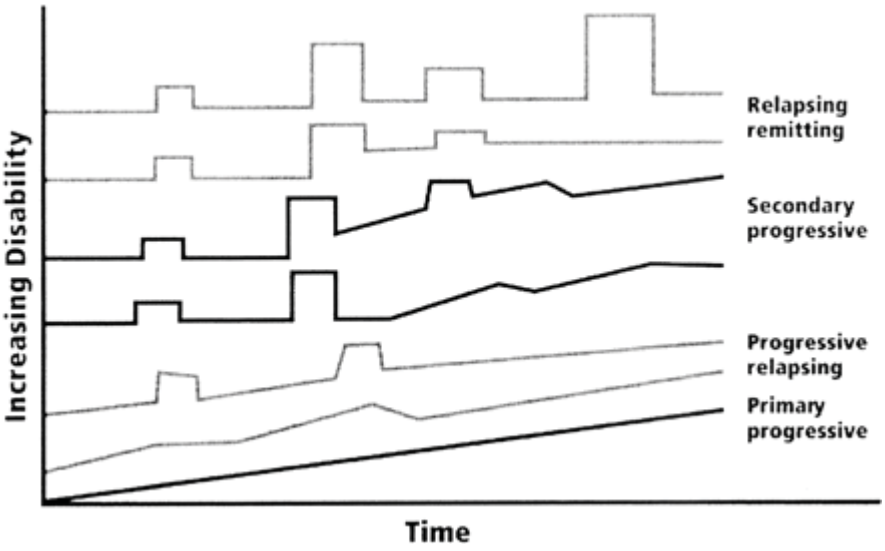


Figure 8 Graph showing the different clinical courses of multiple sclerosis over time. The increasing disability is plotted over time for the different forms of MS (Kieseier et al., 2003).

1. Primary Progressive multiple sclerosis (PPMS): In this disease course patient experience a slow but nearly continuous worsening of the disease beginning from the onset. PPMS represent about 10% of MS cases (Kieseier and Hartung, 2003).

2. Relapsing-remitting multiple sclerosis (RRMS): In this subtype patients experience clearly defined flare ups, which represent acute worsening of the neurological function followed by partial or complete remission. Periods between the relapses are characterized by the lack of disease progression. This is the most common presenting form of MS, represents about 85% of the MS cases. A relapse is defined as new symptom should be present at least for 24 hours and the symptoms should not be associated with fever, since fever can unmask subclinical lesions (Kieseier and Hartung, 2003).

3. Progressive Relapsing MS (PRMS). In PRMS patients experience an initial period of RRMS course followed by a continuous worsening of the disease with or without

relapses. The period between the relapses are characterized by continuous progression of the disease (Kieseier and Hartung, 2003).

4. Secondary progressive MS (SPMS). In SPMS patients experience an initial period of RR course followed by gradual worsening of the disease with or without relapses. The period between the relapses are characterized by continuous progression of the disease. Progression means the continuous worsening of the neurological impairment over at least 6 or 12 months. Untreated 50% of RRMS will end up in developing SPMS in about 10 years (Kieseier and Hartung, 2003; Lutton et al., 2004). Patients who have SPMS tend to have greater disability than patients who have RRMS. SPMS is considered as the sequel of the RRMS (Fig. 9) when compensatory mechanisms fail and when disability develops from incomplete recovery from the frequent relapses and from gradual disease progression (Bruck, 2005).

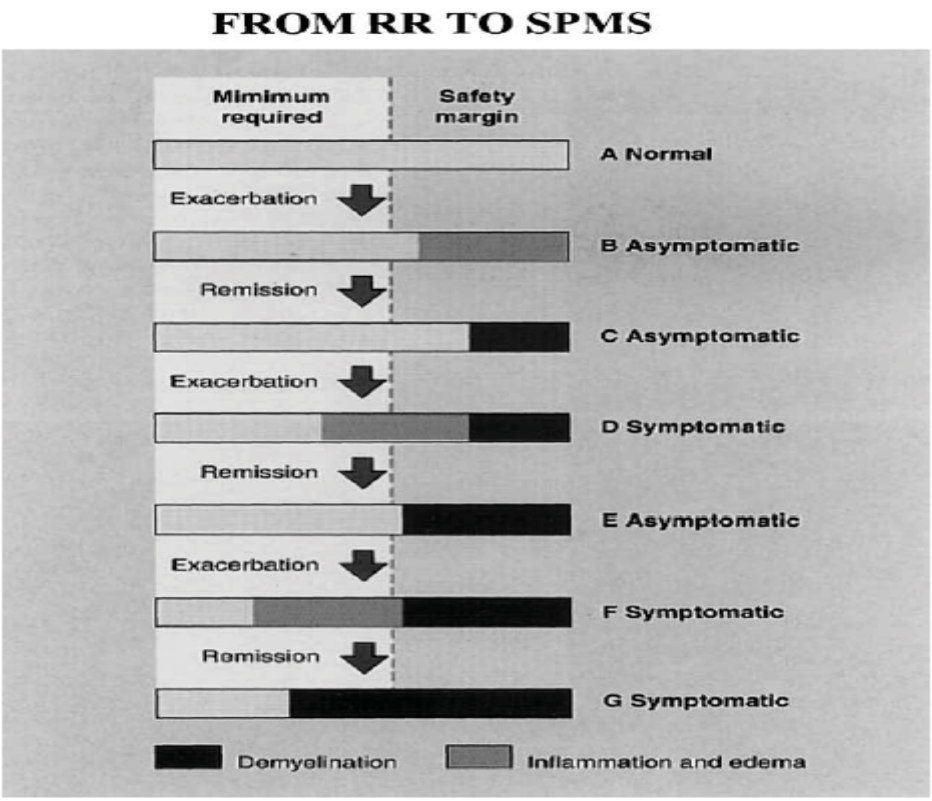


Figure 9 Stepwise worsening of relapsing-remitting course to secondary-progressive MS from repeated relapses (Poser and Brinar, 2004).

In addition, the forms of MS can be characterized by the severity, namely benign and malignant. Benign MS allows the patient to remain fully functional in all neurologic symptoms 15 years after disease onset, while malignant MS leads rapidly and progressively to significant disability in most aspects of the neurological system or death within a short period of time after the onset of the disease (Runmarker and Andersen, 1993).

1.7. Pathogenesis

All forms of MS are believed to be immune-mediated. It remains open how the individual components interact in a coordinated and sequential manner, but the general consensus on the pathophysiology of MS is that activated preexisting myelin reactive T-cells cross the blood-brain barrier (BBB) and initiate the inflammatory cascade that results in demyelination and injury to the axons (Hohlfeld and Wekerle, 2004; Sospedra and Martin, 2005).

Autoreactive T-cells are normally eliminated in the thymus by clonal selection (Kyewski and Derbinski, 2004). For unknown reasons, autoreactive T cells mange to escape the thymic clonal selection process and enter the circulation. It has been proposed that under normal condition these autoreactive cells are held in check by regulatory T-cells (Treg) in healthy individuals (Sakaguchi, 2000; Kuchroo et al., 2002). In disease state it comes to an imbalance between the Treg and autoreactive T-cells by frequent and extensive activation of the auto reactive T-cells leading to the initiation of different kinds of autoimmune disorders (Baecher-Allan et al., 2004; Viglietta et al., 2004).

Myelin reactive T-cells are present in both MS patients and healthy individuals (Ota et al., 1990; Pette et al., 1990) indicating that their presence by itself is not sufficient to initiate disease. Rather, the frequency and extent of autoreactive T-cells activation is what ultimately causes an imbalance between the protective and destructive arms of the immune system.

Activation of myelin reactive T-cells occur in the periphery by superantigens, bacterial lipopolysaccarides, molecular mimicry and other causes (Steinman et al., 2002; Sospedra and Martin, 2005, , 2006). Activated Th1 type lymphocytes enter the circulation

(Karin et al., 1993; Brocke et al., 1996). Activated helper T-cells subsets have a distinct cytokine profile that regulates immune response (Karin et al., 1993; Sospedra and Martin, 2005). T-cell subsets are divided into Th1, Th2 and Th3 (Romagnani, 1997). Th1 lymphocytes produce, in general, proinflammatory cytokines, such as interleukin 2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α). Th2 and Th3 Lymphocytes, on the other hand secrete anti-inflammatory cytokines, such as IL-4, IL-5, IL-6, IL-10 and IL-13 (Cannella et al., 1990, , 1991; Cannella and Raine, 1995; Navikas and Link, 1996).

Upon activation, these T-cells produce different types of proinflammatory cytokines, cytokine receptors (CCRs) and express adhesion molecules [very late antigen 4 (VLA-40 and leukocyte function associated antigen 1 (LFA-1)] on their surface (Karin et al., 1993; Butcher et al., 1999). Some of the cytokines produced by the activated T-cells induce or upregulate the expression of endothelial adhesion molecules [E-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1)] of the BBB (Fig. 10). These two different types of adhesion molecules allow the rolling and adherence of the T-cells and facilitate the diapedsis through the BBB (Cannella et al., 1990, , 1991; Brosnan et al., 1995; Cannella and Raine, 1995; Noseworthy et al., 2000).

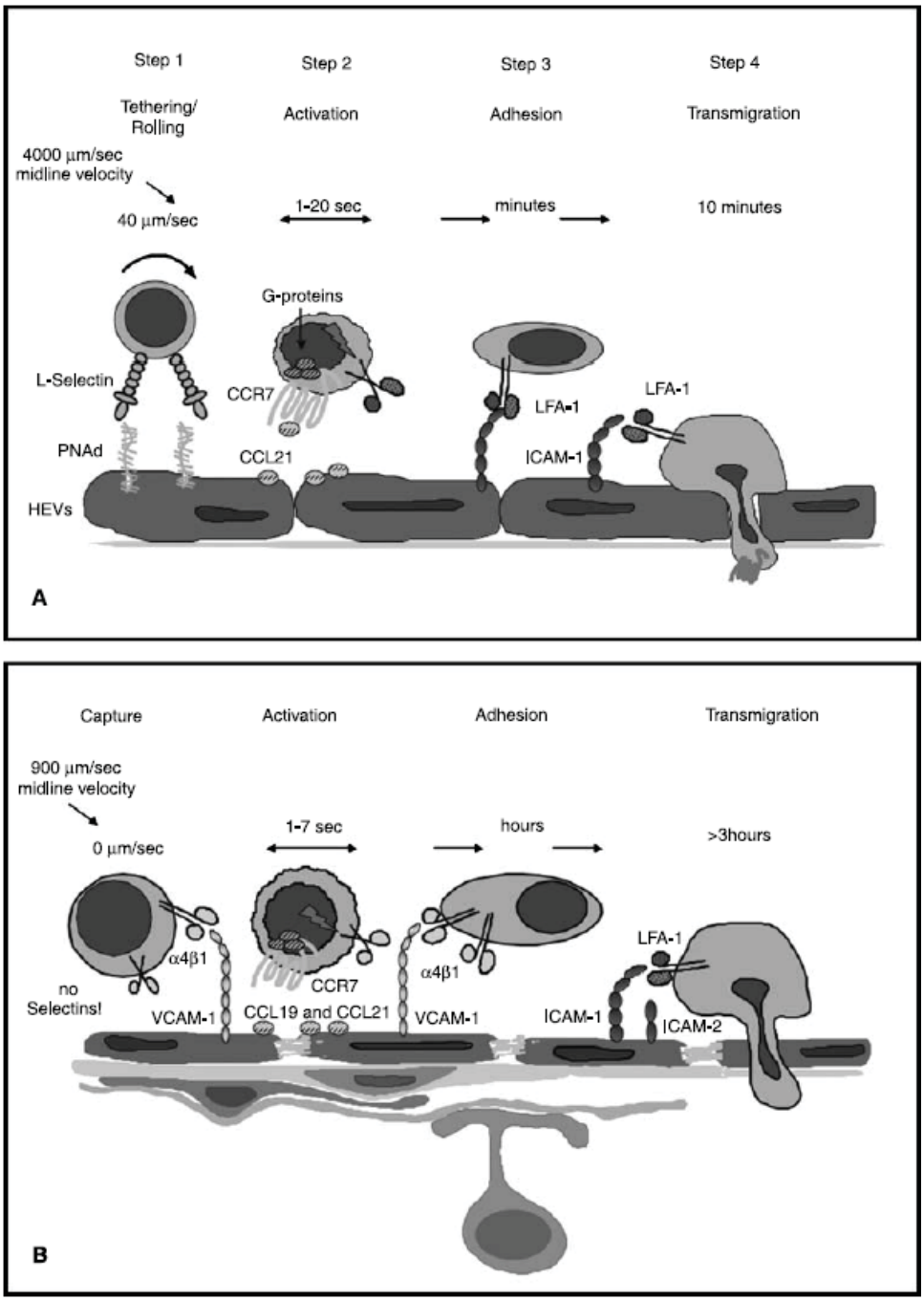


Figure 10 The multistep model of lymphocyte/endothelial recognition and recruitment from the blood. A. Molecular mechanisms appyling for the recruitment of naive lymphocytes across high endothelial venules (HEV) endothelium in peripheral lymph nodes.

B. Molecular mechanisms applying for the recruitment of T lymphoblasts across BBB endothelium in the spinal cord white matter. The velocities of free flowing lymphocytes and the time intervals observed for each individual step are derived from intravital microscopic observations (Engelhardt, 2006).

IFN-γ produced by the activated T-cells in turn activates microglia within the CNS. Upon activation microglia produce different cytokines and proteolytic enzymes. They also become competent antigen presenting cells upon activation (Benveniste, 1997). In addition, these cells up regulate and express different activation markers, such as B7-1, B7-2 and CD40 molecules on their surface (Carson et al., 1998; Mack et al., 2003).

The matrix metalloproteases (MMPs), especialy MMP9, are believed to contribute to degrading extracellular matrix molecules and enhance the disruption of the BBB (Leppert et al., 2001; Lindberg et al., 2001; Lindberg et al., 2004). Calpains, on the other hand, participate in the degradation of myelin proteins, which is one hallmark of the disease (Maeda and Sobel, 1996; Shields et al., 1998; Banik and Shields, 2000; Kouwenhoven et al., 2001; Leppert et al., 2001; Lindberg et al., 2001; Schaecher et al., 2001). The role of calpains in the pathophysiology of MS, which is the focus and major interest in this project, will be discussed in detail later.

To propagate the inflammatory cascade, T-cells, which have entered the CNS, need to be reactivated by reencountering CNS antigen. This is the most important step in the disease initiation. Without this step, disease onset does not occur. This fact has been extensively investigated and experimentally shown (Flugel et al., 2001; Tompkins et al., 2002; Kawakami et al., 2005). The reactivation of myelin reactive T-cells require the processing and presentation of CNS antigen peptides in the context of major histocompatibility complex (MHC) molecule. The trimolecular complex of antigen presentation consists of the T-cell receptor, the antigen that is processed, and the MHC molecule. In contrast non-autoreactive, ovalbumin-specifc T-cells moved through the brain and stopped only when ovalbumin was injected intracerebrally. Clinical disease

developed only when T-cells that have crossed the BBB reencounter de novo CNS processed antigen is presented to them to sufficiently reactivate them in the CNS environment (Tompkins et al., 2002; Kawakami et al., 2004; Kawakami et al., 2005). Activated microglia and macrophages express MHC antigens and are some candidates of antigen presenting cells (Mack et al., 2003; Ponomarev et al., 2005).

There are two types of MHC molecules: Class I and Class II. These molecules bind peptide antigens as part of the processing required for presentation to different T-cells (Harding et al., 1991a; Harding et al., 1991b; Peters et al., 1991). $CD4^+$ T-cells recognize antigens in the context of MHC Class II molecules, whereas $CD8^+$ T-cells recognize antigens in association with MHC Class I molecules. This activation is further enhanced by interaction of costimulatory molecules, such as CD40/CD40 ligand or B7-1 and B7-2/cytotoxic T-cell antigen-4. The full T-cell activation program is present in MS lesion. MS plaque macrophages express CD40 and B7-2, whereas lymphocytes express mainly CD40 L and B7-1 (Gerritse et al., 1996). The activated T-cells then orchestrate several cellular and humoral responses that could result in tissue damage.

2. Spectrum of demyelinating diseases

White matter disease of the CNS has a broad range of differential diagnosis (Table 1). It can be divided into vascular, metabolic, infectious and inflammatory. A variety of inflammatory demyelinating CNS disorders may produce the hallmarks of MS lesion, such as inflammation associated with demyelination and axonal injury. The different disorders vary in their clinical course, regional distribution and pathology (Fig. 11). These syndromes include acute transverse myelitis (ATM), idiopathic inflammatory demyelinating diseases (IIDDs), such as isolated optic neuritis, or brainstem demyelination, and the fulminant demyelinating disorders, such as Marburg variant of acute MS and acute disseminated encephalomyelitis (ADEM); and the recurrent disorders such as Neuromyelitis optica (NMO) also known as Devic`s disease (Dale, 2003; Poser and Brinar, 2004; Dale and Branson, 2005; Siva, 2005).

Dissemination in time	Dissemination in space		
Nonphasic[b]	Focal	Multifocal/diffuse	
		Mono-symptomatic	Poly-symptomatoc
Monophasic			
	ON	(ON)[c]	ADEM
	BSs	(BSs)	
	Myelitis	(Myelitis)	
Multiphasic	Rec. ON	(Rec. ON)	RRMS
	Rec. myelitis	(Rec. myelitis)	Devic's/OpSpMS
Progressive	Progr. ON	(Progr. ON)	Fulm. MS (Marburg)
	Progr. myelitis	(Progr. myelitis)	Progr. MS
	Progr. Cblr Syndr & Progr. OBS		

Table 1 The clinical spectrum of idiopathic neuroinflammatory demyelinating and neurodegenerative diseases of the CNSa
Abbreviations: ON, optic neuritis; BSs, brainstem syndromes; ADEM, acute disseminated encephalomyelitis; RR, relapsing-remitting; Rec., recurrent; OpSpMS, optico-spinal MS; Cbrl., cerebellar; OBS, organic brain syndrome; Progr., progressive; Fulm., fulminant. a Modified and updated from Siva et al. Frontiers in multiple sclerosis, vol. 2. London: Martin Dunitz; 1999. b Nonphasic: designates asymptomatic MS (no clinical symptoms or signs, but disease detected by MRI or postmortem).
c Monosymptomatic disease, but more widespread involvement of central nervous system as shown by MRI or other work-up (Siva, 2005).

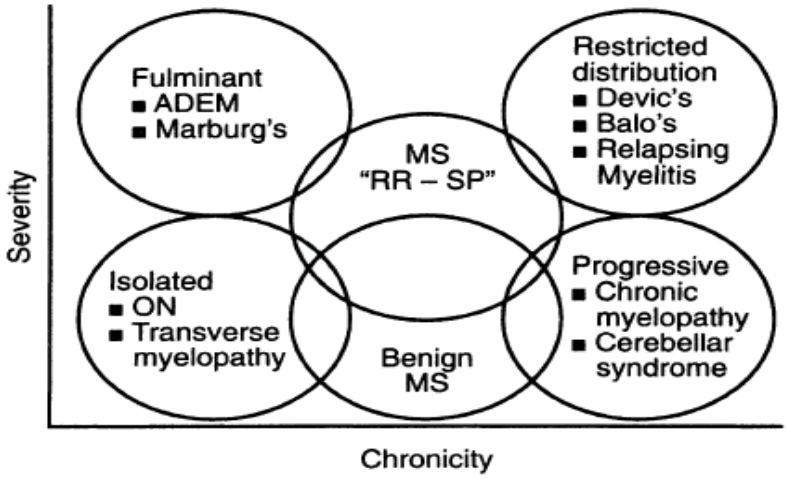


Figure 11 Different forms of demyelinating diseases grouped according to severity and chronicity (Poser and Brinar, 2004).

A central issue to understanding the pathogenesis of MS is the relationship between MS and other inflammatory demyelinating diseases. There is considerable overlap in the epidemiology, clinical, CSF, imaging and pathologic features of some of these diseases and MS. The similarities make it difficult to distinguish these diseases with reliable confidence when encountering patients after a single demyelinating event, especially ADEM and MS.

It is important to reliably distinguish these different disease entities in order to initiate appropriate long-term therapy for MS. Pathology is the gold standard, when available, to distinguish between the individual disease types. The limited extent and pattern of demyelination for example helps distinguish ADEM from MS lesions (Fig. 12 and 13; Table 2).

ADEM is an acute disorder characterized by scattered multifocal white matter involvement of the posterior fossa and cerebral hemispheres. It usually follows an

infection or vaccination. Systemic symptoms such as fever, malaise, myalgia and headache often precede the neurological symptoms (Garg, 2003). Unlike MS, ADEM is by definition an isolated monophasic event. It occurs mostly in young individuals less than 10-years old, presents with encephalopathy and a distinct pattern of MRI findings with perivascular sparing (McDonald et al., 2001; Dale, 2003; Dale and Branson, 2005).

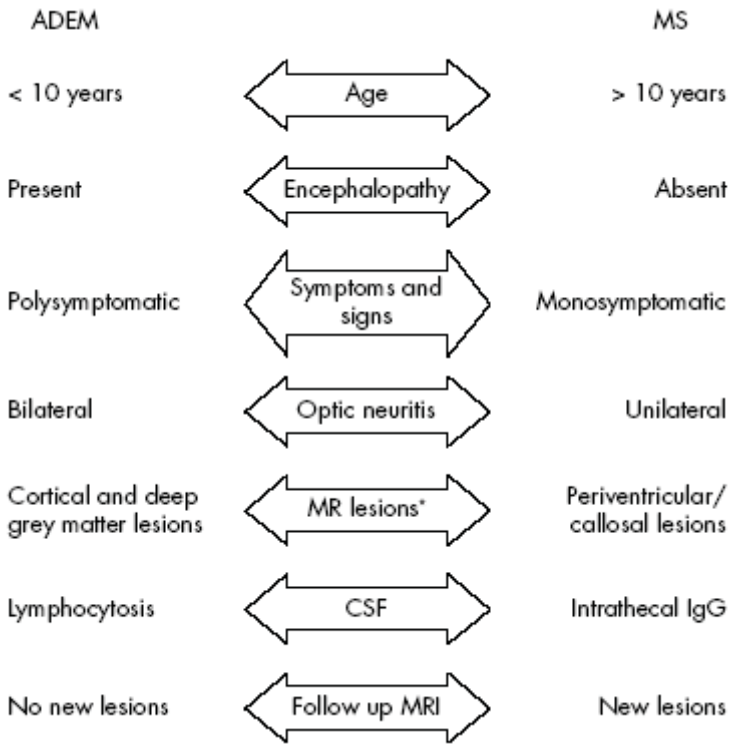


Figure 12 Clinical and investigation differences between ADEM and MS (trends only). *MR lesions other than white matter (Dale and Branson, 2005).

Feature	ADEM	Acute multiple sclerosis	Acute haemorrhagic leucoencephalitis	Neuromyelitis optica
Perivascular infiltrates				
Lymphocytes	++	++	++	++
Macrophages or monocytes	++	++	++	++
Polymorphs	–	–	++	++
Eosinophils	–	–	–	++
Perivascular haemorrhage	–	–	++	–
Necrotising venules	–	–	++	+
Perivascular demyelination	±	±	++	++
Axonal damage	±	±	++	++

Table 2 Pathological features of acute demyelinating disorders (Dale and Branson, 2005).

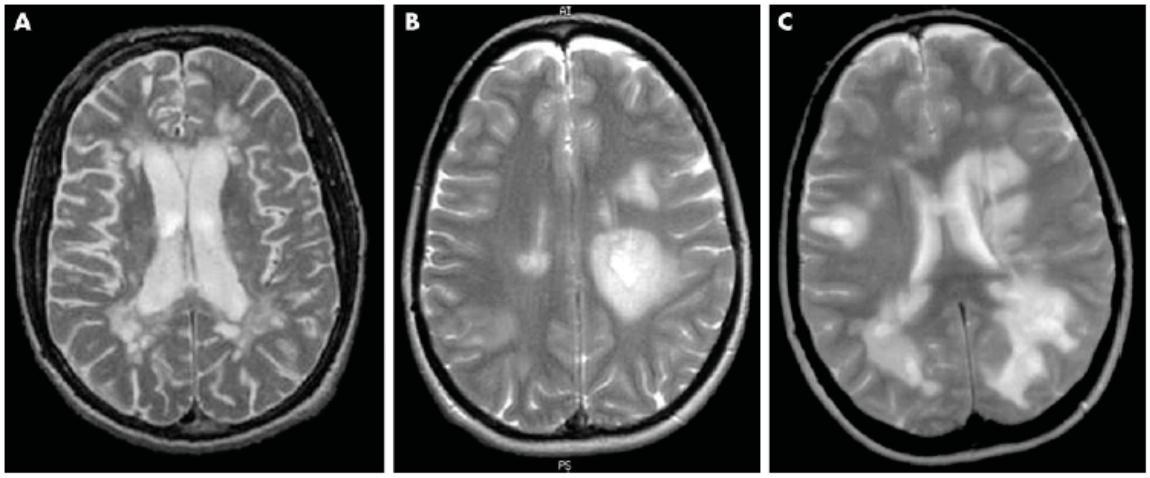


Figure 13 (A) MRI brain (T2 weighted) in MS showing well demarcated lesions in the region of the periventricular white matter. (B) MRI brain (T2 weighted) in ADEM showing a large mass-like lesion in the white matter. (C) MRI brain in ADEM showing multiple large lesions with poorly defined margins and relative periventricular sparing (Dale and Branson, 2005).

ATM is a heterogeneous inflammatory disorder affecting the spinal cord at one or several segments. The clinical symptoms evolve subacutely over hours to days. It occurs in association with viral and bacterial infections as well as in systemic autoimmune disorders. Some authors consider it as a possible first manifestation of MS (Arlazoroff et al., 1989; Dodson, 1990; Obara and Tanaka, 1991; Mills and Schoolfield, 1992; Hess and Bamborschke, 1993).

NMO also known as Devic`s syndrome is an idiopathic, severe, inflammatory disorder that preferentially affects the optic nerves and spinal cord simultaneously. Data from imaging, laboratory results, immunopathological studies and the clinical appearance suggest that it is humorally mediated disease different from MS (de Seze et al., 2002; Lucchinetti et al., 2002). The spinal cord involvement is typically continuous that extends over 3 or more vertebral segments. This finding is also confirmed by MRI studies, which shows longitudinally extensive lesions on spinal cord. NMO has a distinct serum marker for diagnostic (O'Riordan et al., 1996). It is a serum autoantibody known as NMO-IgG that has 96 % specificity and 94 % sensitivity (Lennon et al., 2004; Wingerchuk, 2006). The target antigen in this disease is the water channel aquaporin-4. It is considered as some sort of channalopathy (Nielsen et al., 1997; Agre and Kozono, 2003).

It has a severe RR disease course and the treatment of choice is high dose steroids and disease modifying agents such as interferon (IFN) and glatiramer acetate (GA) have no effect on relapses (Weinshenker, 2003; Wingerchuk, 2006).

The crucial point here is that making the correct diagnosis and the differentiation of the different disease entities is essential and has a great prognostic and therapeutic implications.

3. The role of macrophages and microglia in MS

There are two major subgroups of glial cells: the macroglia, which consists of astrocytes, oligodendrocytes and ependymal cells, and microglia.

Microglia are the principal immune cell residents in the CNS. Microglia comprise 10-20 % of all glial cells. Del Rio-Hortega first described them in 1932. He hypothesized that microglia are of mesoderm origin, have phagocytic function, and exhibit changes in morphology following insults to the CNS. However, results from the most recent studies suggest that the microglia derive from bone marrow precursors of monocytic lineage (Hickey and Kimura, 1988; Perry and Gordon, 1988; Hickey, 1991), and populate the CNS early in fetal development. Their function is similar to those of the tissue macrophages, including phagocytosis, antigen presenting, production of cytokine, eicosanoids, complement components, excitatory amino acids, proteases, oxidative radicals and NO (Banati et al., 1993; Gehrmann et al., 1993; Gehrmann et al., 1995). The production of the mediators is thought to contribute to the CNS damage through a variety of mechanisms (Fig.14).

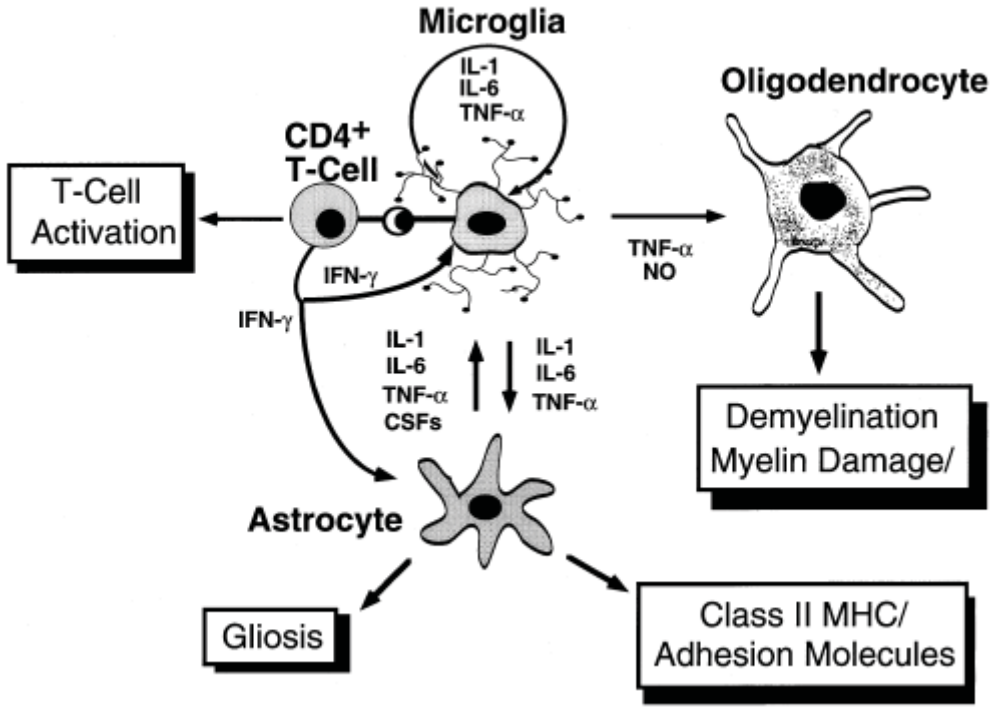


Figure 14 Potential interactions between microglia/macrophages and T-cells, astrocytes, and oligodendrocytes. Activated microglia/macrophages can present foreign antigen in conjunction with class II MHC molecules to CD4+ T-helper cells. The T-cells then become activated and secrete cytokines such as IFN-g which further contributes to the activation of microglia/macrophages. Microglia/macrophage products such as TNF-α and nitric oxide (*NO*) can damage oligodendrocytes, leading to interference with myelination and myelin gene expression. Astrocytes and microglia/macrophages also communicate via soluble mediators such as cytokines. IL-1, IL-6, and TNF-a produced by microglia/macrophages can induce astrogliosis and can regulate expression of adhesion molecules such as ICAM-1, vascular cell adhesion molecute 1, and E-selectin on astrocytes. Astrocytes in turn produce colony stimulating factors (*CSFs*) that are critical for sustaining the growth of microglia/macrophages (Benveniste, 1997).

There is accumulating evidence that intracellular and extracellular proteases from microglia contribute to various events in the CNS through proteolysis (Nakanishi, 2003). Calpain, one of the proteolytic enzymes of microglia, has been shown to play a major role in the pathophysiology of demyelinating disease such as MS by degrading myelin proteins extracelluarly (Banik et al., 1985; de Rosbo and Bernard, 1989; Banati et al.,

1993). All major myelin proteins are substrates for calpain. Furthermore matrix metaloproteases (MMP) secreted from microglia also mediate inflammation and tissue degradation through processing of pro-inflammatory cytokines and damage to the BBB (Maeda and Sobel, 1996; Kouwenhoven et al., 2001; Leppert et al., 2001; Lindberg et al., 2001). Other types of proteases from this microglia play important role in Major Histocompatibility (MHC) Class II-mediated presentation of exogenous antigen, which is also involved in the pathophysiology of MS (Benveniste, 1997; Nakanishi, 2003).

The growing knowledge about proteolytic events mediated by macroglia proteases will not only contribute to better understanding of the macroglia function in the CNS but also may help in the development of protease inhibitors as a novel neuroprotective or as a therapeutic agent in some CNS diseases.

4. The role of calpain in MS

Calpain is a calcium-activated, neutral and non-lysosomal proteolytic enzyme found mainly in the cytosol of all animal cells (Banik et al., 1985; Banik et al., 1987; Chakrabarti and Banik, 1988; Banik et al., 1992; Saido et al., 1992; Banik et al., 1994; Suzuki et al., 1995; Banik and Shields, 1999, , 2000; Huang et al., 2000; Branca, 2004). Cysteine proteases play an important role in the life and death of mammalian cells. The existence of this protease was first identified in a rat brain by Guroff in 1964. Various calpain subunits and their homologes have been identified by the technique of cDNA cloning. The tissue distribution and chromosomal localizations of various homologes of the human calpain family have been determined in the course of rapid progression since the sequencing of the human genome (Fig. 15; Table 3). Depending on the tissue distribution, calpain can be broadly classified as ubiquitous and tissue-specific (Saido et al., 1992; Ray and Banik, 2003) .

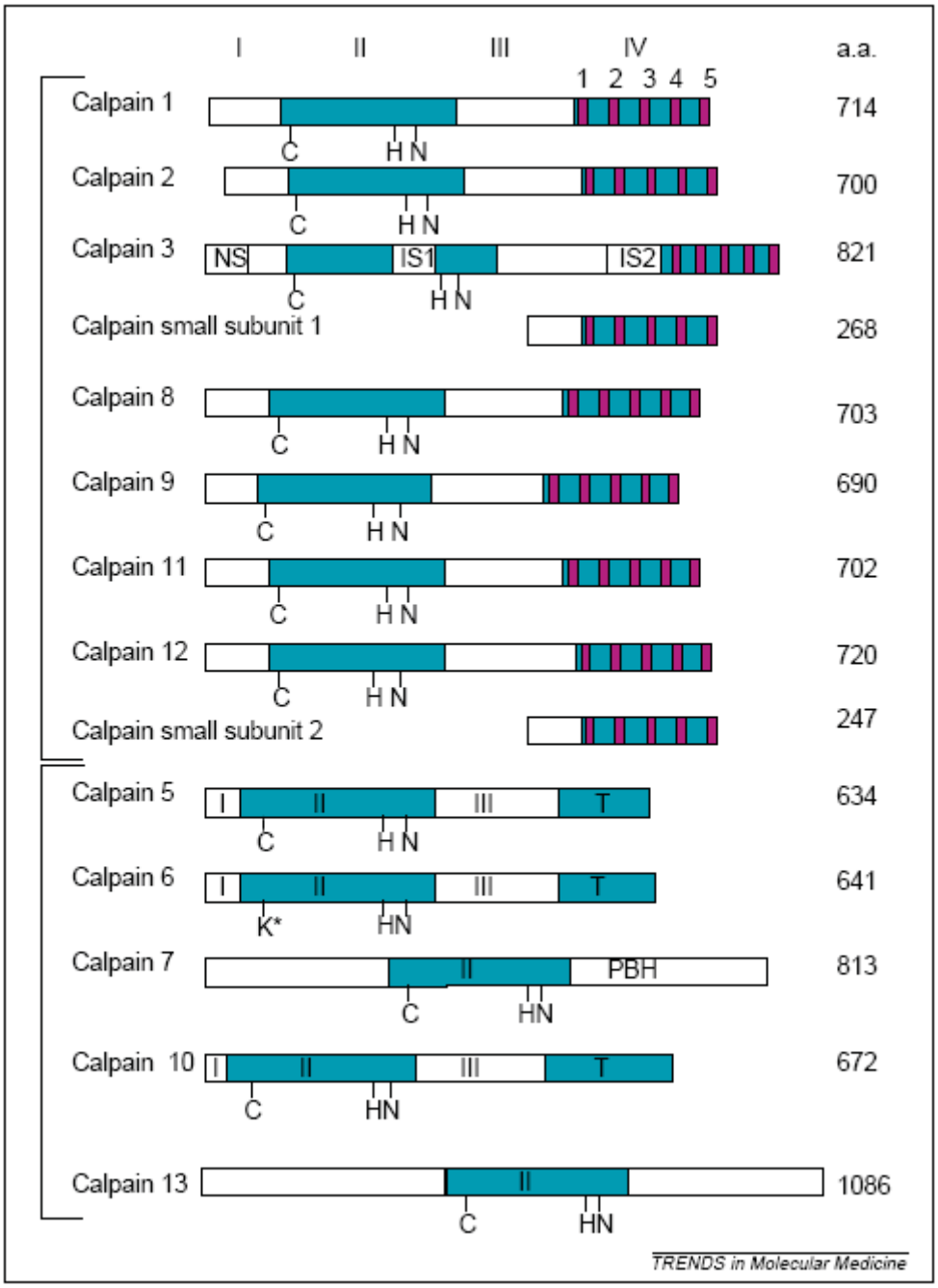


Figure 15 Mammalian calpain families.
Schematic structures of various mammalian calpains. For details on domain definition and abbreviations, see text. C, H, N represents catalytic residues Cys, His and Asn residues. K* represents the unique nonfunctional Lys residue (instead of Cys) in calpain 6. PBH is a domain homologous to a region of the protease PalB (Huang et al., 2000).

Name	Typical/Atypical (C-terminal EF-hand)	Tissue Expression	Human Chromosome	Human Genebank Accession
Calpain 1	Typical	Ubiquitous	11q13	X04366
Calpain 2	Typical	Ubiquitous	1	NM_001 748
Calpain 3	Typical	Skeletal muscle, lens, retina	15q15	X85030
Calpain 4	Typical	Ubiquitous	19q13	NM_001749
Calpain 5	Atypical	Ubiquitous	11q14	NM_004055
Calpain 6	Atypical	Placenta	Xq23	NM_014289
Calpain 7	Atypical	Ubiquitous	3p24	AB028639
Calpain 8	Typical	Stomach mucosa	1 (mouse)	AA_043093
Calpain 9	Typical	Digestive tract	1	NM_006615
Calpain 10	Atypical	Ubiquitous, and tissue specific isoforms	2q37	AF089088
Calpain 11	Typical	Testis	6p12	AJ242832
Calpain 12	Typical	Ubiquitous	7 (mouse)	NM_144691
Calpain 13	Typical	Testis/lung	2p22.2	AC068689
Calpain 14	Atypical	Ubiquitous	2p22.2	AC015980
Calpain 15	Atypical	Ubiquitous	16p13.3	U85647
Calpain small subunit 2	Typical	N.D.	16	AC026802

Table 3 Human Calpain Gene Products and Expression Profiles. N.D. –not determined (Carragher, 2006).

Mammalian ubiquitous calpains exist in two major isoforms: micro-calpain (μ-calpain, a heterodimer of calpain 1 and a calpain small subunit) and milli-calpin (m-calpain a heterodimer of calpain 2 and a calpain small subunit). Calpain I requires 2-80 μM and calpain II requires 0.2-0.8 mM Ca^{2+} concentrations for half-maximum activity *in vitro*. Under physiological conditions, calpain (Fig. 16) is constitutively inhibited by its natural inhibitor calpastatin (Fig. 17).

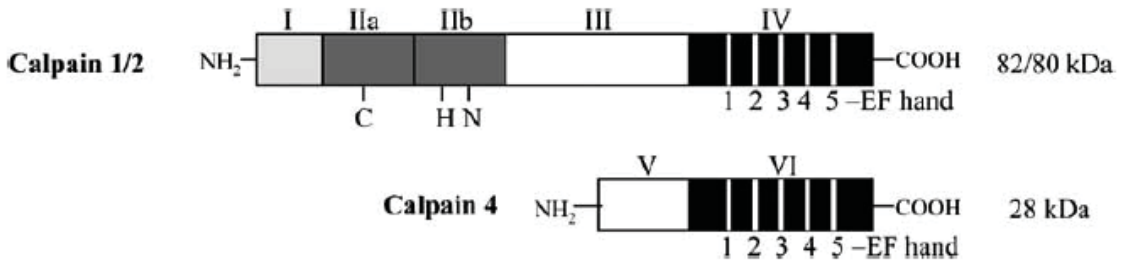


Figure 16 Schematic presentation of molecular organization of ubiquitous human calpain. A. m-calpain and m-calpain are heterodimers composed of unique catalytic subunits encoded by calpain 1 and calpain 2 respectively which combine with the small regulatory subunit encoded by calpain 4. Calpain 1 and calpain 2 share significant homology and can be subdivided into four separate domains based on amino acid sequence. Domain I is a short NH_2-terminal domain. Domain II represents a distinct cysteine protease domain containing the active site residues of cysteine (C), histidine (H) and asparagine (N). This domain is highly conserved between calpain family members. Domain III has no significant homology to other known proteins, but has been proposed to interact with phospholipids and calcium. Domain IV shares homology with calmodulin and contains five EF hand motifs. The first four EF hand motifs are thought to act as calcium binding sites while the fifth is involved in dimerization with the regulatory calpain 4 small subunit. Calpain 4 is composed of two domains, a glycine rich domain V which has no known function and domain VI which contains five EF hand motifs, the fifth being involved in binding with domain IV of the catalytic subunit (Carragher, 2006).

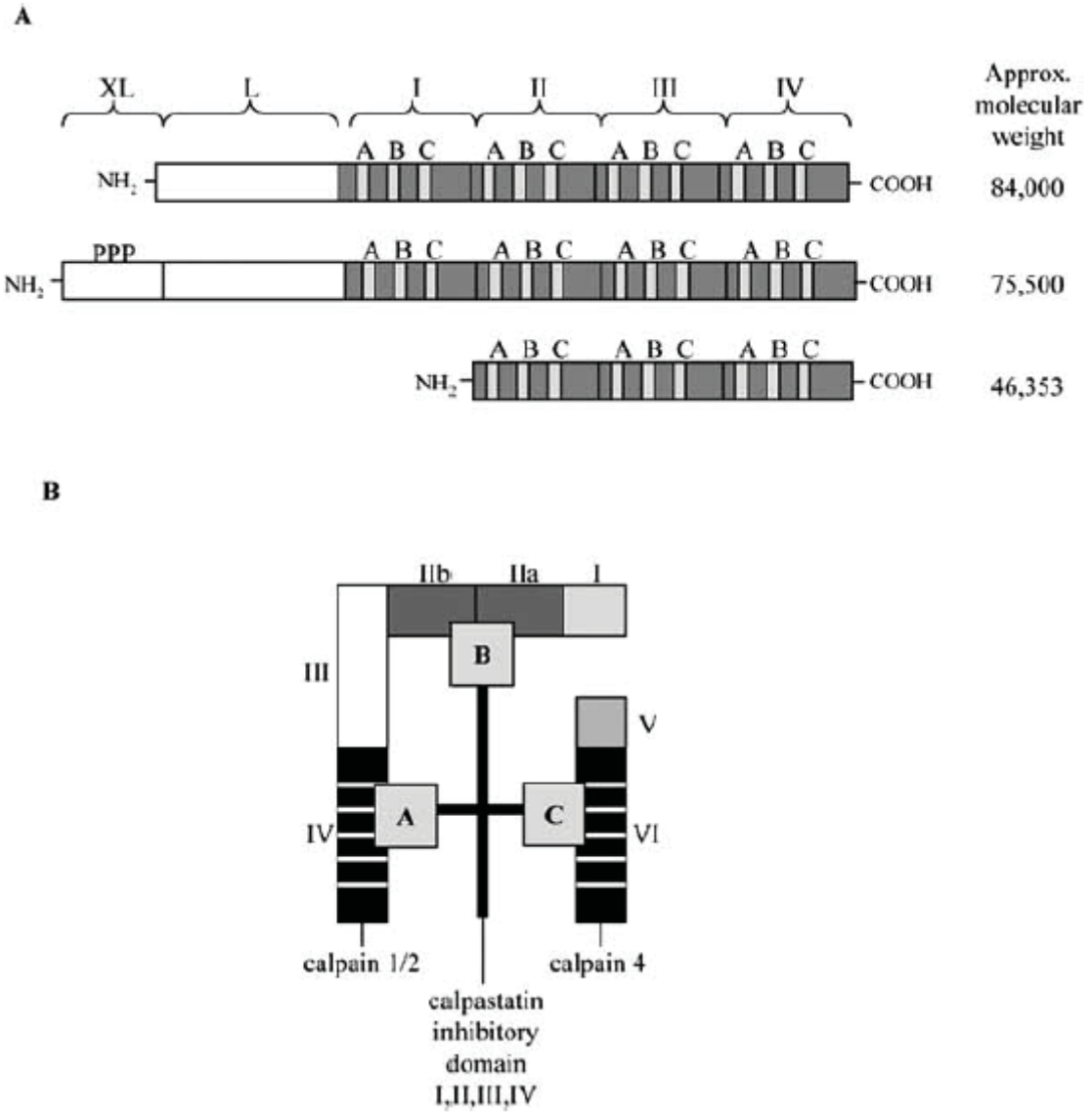


Figure 17 Domain structure of calpastatin based upon amino acid sequence. Calpastatin is composed of an NH_2-terminal domain L that has been proposed to play a role in sub-cellular localization [14]. Domain L is followed by four inhibitory domains (I-IV). Each inhibitory domain contains three sub-domains, A, B and C, which bind calpain and confer maximal inhibitory activity. Alternative splicing and distinct initiation start sites combined with proteolytic processing result in the generation of several calpastatin isoforms, including a large form containing an NH_2-terminal XL domain and a truncated form that lacks domain L and the first inhibitory domain. The XL domain contains three PKA phosphorylation sites (P) (Carragher, 2006).

The rise in cytosolic free Ca^{2+} concentration following injury or inflammation activates the proteolytic enzyme by autocleavage of its subunits and degradation of its natural inhibitor calpastatin (Zimmerman and Schlaepfer, 1984, , 1988; Cong et al., 1989; Zimmerman and Schlaepfer, 1991). The free enzyme then moves to the membrane and exerts its action (Molinari et al., 1994; Molinari and Carafoli, 1997; Pariat et al., 1997; Ray and Banik, 2003).

The cysteine protease calpain has a variety of substrates, including cytoskeletal proteins and signaling molecules, and these calpain substrates belong to different groups of proteins. Some of these groups of proteins are cytoskeletal and structural proteins [fodrin, Mitogen-activated protein2 (MAP2), myelin basic protein (MBP), neurofilament protein (NFP)], integral membrane proteins [Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid (AMPA), G-proteins], cytosolic enzymes [neuronal nitric oxide synthese (nNOS), phospholipase C (PLC), protein kinase C (PKC) etc.] and many others (Siman et al., 1984; Schlaepfer and Zimmerman, 1985; Banik et al., 1992; McGinnis et al., 1999; Ray et al., 2003a). These large groups of calapin substrates and the activation of calpain due to the increase in cytosolic free Ca^{2+} concentration suggest that calpain is involved in numerous different structural, enzymatic and transcriptional changes, depending upon the physiological or pathological functions of the cell (Ray et al., 2003b; Branca, 2004).

All major myelin proteins are calpain substrates and calpain has been implicated in myelinolysis (Yanagisawa et al., 1983; Banik et al., 1985; de Rosbo and Bernard, 1989; Ray et al., 2003b). The role of calpain in the process of demyelination was suggested from the results of a study showing inhibition of myelin degradation by

utilizing a thiol protease inhibitor E-64 to an extract of brain calpain (Yanagisawa et al., 1983).

Calpain is significantly elevated in the white matter of MS patients (Shields et al., 1999) and is increasingly expressed in activated inflammatory cells following injury to the CNS (Shields et al., 1998; Schaecher et al., 2002). The increased expression of calpain in the white matter of patient with MS (Shields et al., 1999) and in the inflamed tissue of EAE suggests its role in the pathophysiology of MS (Shields et al., 1998; Schaecher et al., 2001). Although other proteases are also involved in myelin degradation, calpain appears to play a major role in this process since neuronal protein degradation (alpha-fodrin and NFP) is significantly decreased when calpain is inhibited following CNS injury (Smith and Amaducci, 1982; Osanai and Nagai, 1984; Badalamente et al., 1987, , 1989, , 1992; Deshpande et al., 1995; Stracher, 1997; Smith et al., 1998; Stracher, 1999; Adamec et al., 2002). Thus, inhibition of calpain may help diminish the symptoms, or help treat MS that results from the demyelination of nerves.

5. Protease inhibitors

It has been shown that calpain is involved in many pathological conditions making the inhibition of calpain, if not a major, at least one essential aspect of treatment in those pathological conditions (Huang et al., 2000; Ray et al., 2003b; Branca, 2004). Several attempts have been made to identify, develop and use calpain inhibitors (Donkor, 2000; Donkor et al., 2000). The major problems in this matter have been the lack of high specificity of the inhibitor to calpain, water-solubility, cell membrane penetration and the ability to cross the BBB (Wang and Yuen, 1994; Ray et al., 2003b). Over the years some inhibitors have been developed that have a better affinity and potency, but still exhibit some of the limitations described above.

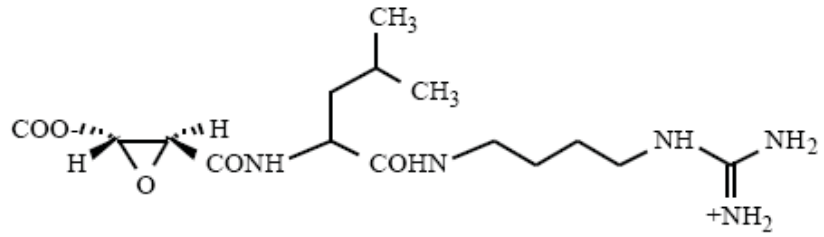
Protease inhibitors are broadly subdivided into 4 classes:

1. Peptidyl epoxides: Representative of this inhibitor group is E64, E64C and E64D (Fig. 18).

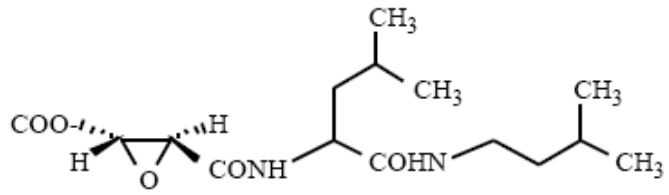
The prototypic oxirane inhibitor E-64 irreversibily inhibits calpain and other cysteine proteases by forming irreversible thioester bond with the active site cysteine (Parkes et al., 1985; Mehdi et al., 1988; Mehdi, 1991), but its charged groups prevent it from passing through the plasma membrane.

Peptidyl epoxides

Compound 1: E64



Compound 2: E64C



Compound 3: E64D

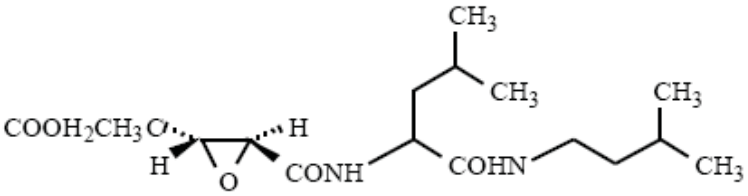


Figure 18 Representative protease inhibitors of peptidyl epoxide class (Carragher, 2006).

2. Peptidyl aldehydes: Representative of this class is leupeptin, calpeptin, MDL-28170, SJA-6017, ALLN and ALLM (Fig. 19). These groups of inhibitors exert their inhibition by forming a reversible hemiacetal with the active site thiol.

The prototypic tripeptide aldehyde inhibitor leupeptin suffers from a lack of selectivity and poor solubility, poor membrane permeability and the inability to cross the BBB (Aoyagi et al., 1984; Osanai and Nagai, 1984; Wang et al., 1999; Ray et al., 2003a). These calpain inhibitors were designed to target either the active site or an allosteric site of the enzyme, calpain, for the mediation of their inhibitory action (Wang and Yuen, 1994; Ray et al., 2003b).

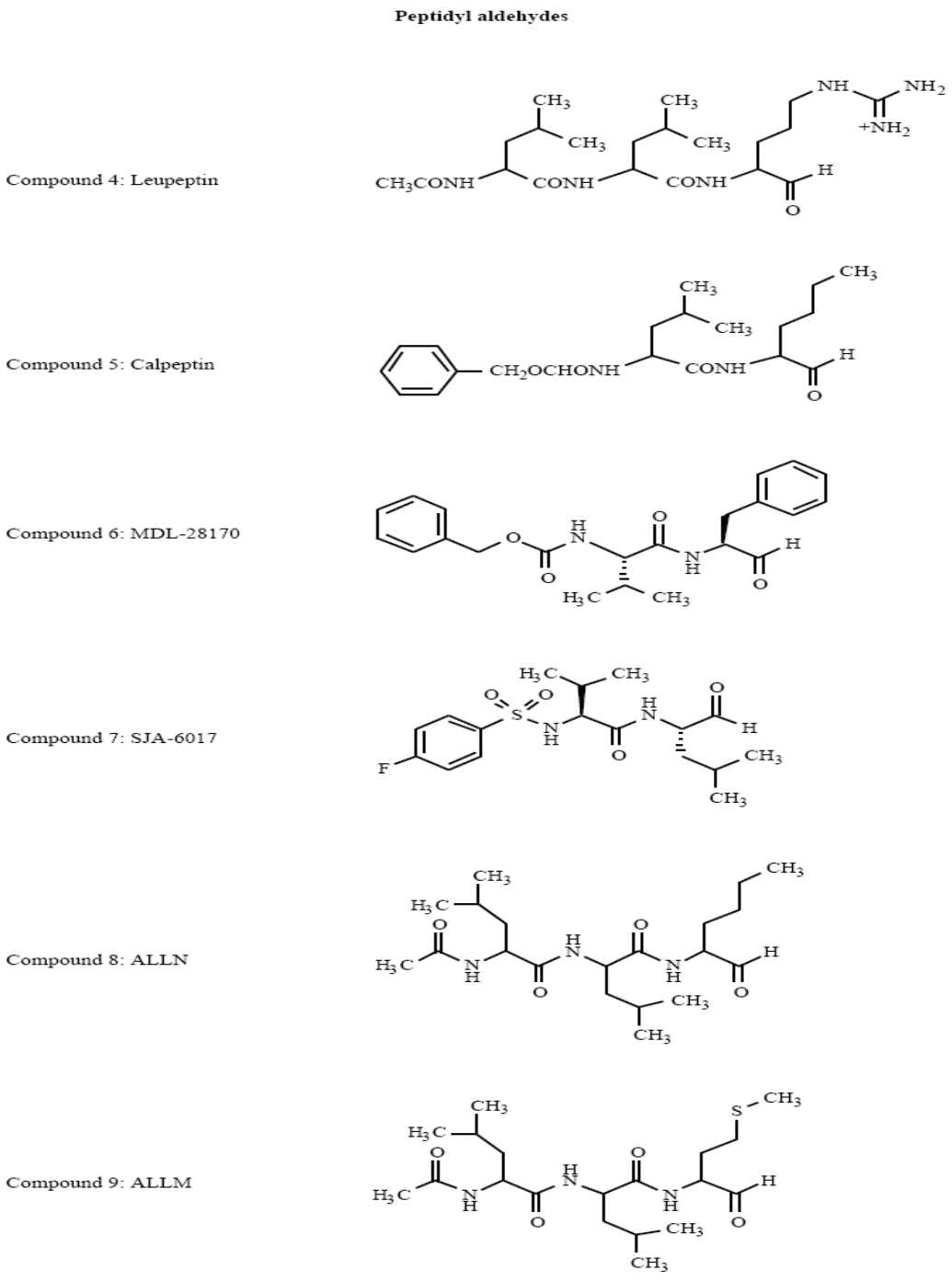


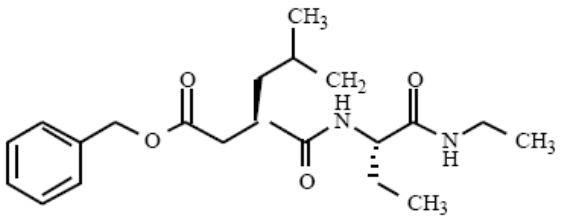
Figure 19 Representative protease inhibitors of peptidyl aldehyde class (Carragher, 2006).

3. Peptidyl α ketomides: Representatives of this class is AK275 and AK295 (Fig. 20).

These groupe of inhibitors inhibit the enzyme reversibly and show improved cell and membrane permeability than the above mentioned groups.

Peptidyl α ketoamides

Compound 10: AK275



Compound 11: AK295

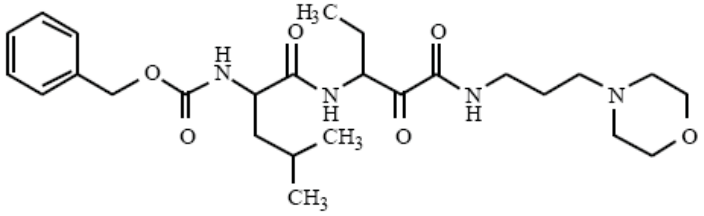


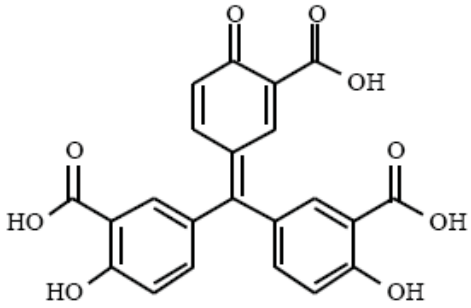
Figure 16 Representative protease inhibitors of peptidyl α ketoamide class (Carragher, 2006).

4. Non-peptide inhibitors: Representatives of these classs are ATA, quinolinecarboxamides, PD150606 and PD151746 (Fig. 21).

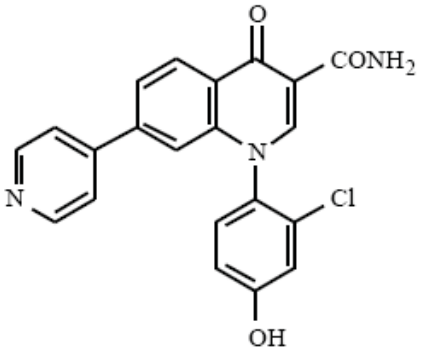
These classes of calpain inhibitors target a site other than the active site and may show better specificity to calpain than other cysteine proteases. The inhibition mode is non-competitive and reversible. The binding of the inhibitor at an allosteric site may bring an alteration in the structure of calpain leading to an impaired conformatory change and inactivation of the enzyme. One such site is for example is the calcium binding site of the catalytic domain.

Non-peptide inhibitors

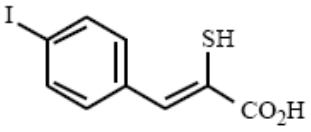
Compound 12: ATA



Compound 13: Quinolinecarboxamide



Compound 14: PD150606



Compound 15: PD151746

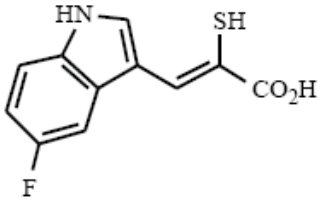


Figure 21 Representative protease inhibitors of non-peptide class (Carragher, 2006).

Ongoing efforts have been made to develop protease inhibitors with better charaterstics.

6. Cysteic-leucyl-argininal (CYLA)

In order to overcome the limitation to cross the BBB this novel calpain inhibitor (Fig. 22A) is designed from its precursor leupeptin and (Fig. 22B), a tripeptide (n-acetyl-leucyl-leucyl-argininal) that is cabable of effectively inhibiting calpain both in vitro and in vivo when applied by means of special drug delivery systems (Smith and Amaducci, 1982; Osanai and Nagai, 1984). To achieve this cysteic acid (Fig.23A and 24) was attached to the active, protease inhibitor part of leupeptin.

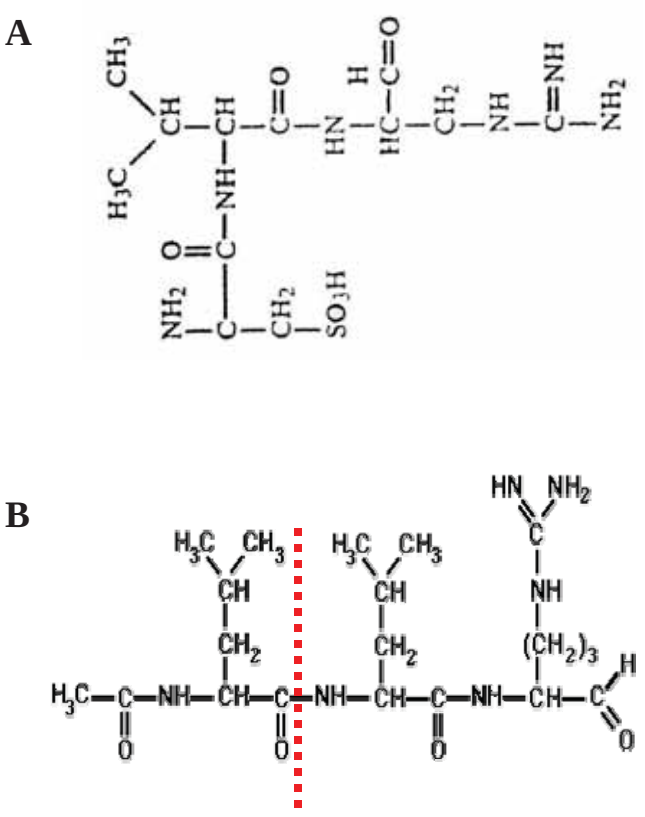


Figure 22 Structure of CYLA (A) Structure of Leupeptin (B). Red broken line=area of cleavage for CYLA synthesis.

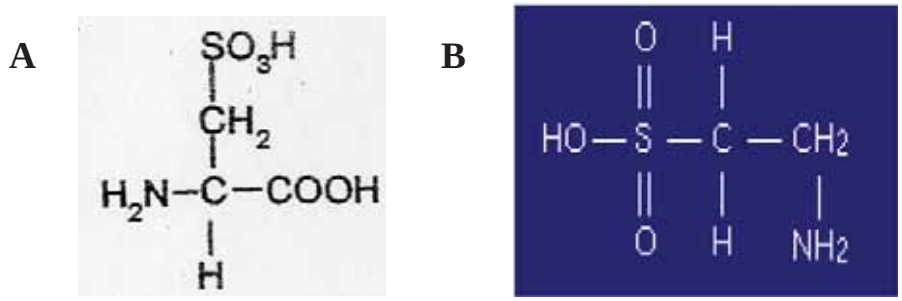


Figure 23 Structure of cysteic acid (A) and taurine (B).

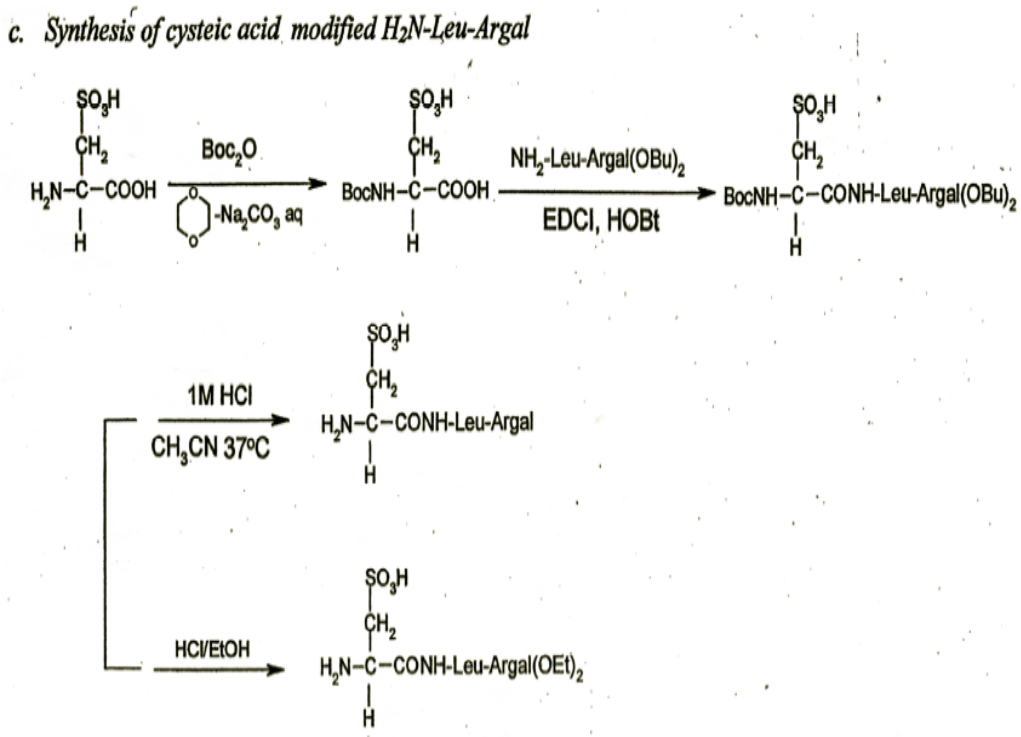


Figure 24 Multi-steps in the Synthesis of CYLA from Leupeptin. Cysteic acid is attached to the protease inhibiting part of leupeptin in a series of steps. The result of this reaction yielded CYLA.

Taurine (2-aminoethanesulfonic acid) is a β-amino acid (Fig.23B) that relies upon a Na^+-dependent transport system to pass through cell membranes (Huxtable, 1996). Two distinct Na^+-dependent high affinity taurine transporters have been cloned. Taurine is the most abundant free amino acid in many tissues including the brain, where it is present in millimolar concentrations in the cerebellum and cerebral cortex (Horner and Aurousseau, 1997; Redmond et al., 1998; Pow et al., 2002; Schuller-Levis and Park, 2003). The brain synthesizes only limited amounts of taurine and thus significant amounts must be transported into those parts of the brain that require it (Kishi et al., 1988). Cysteic acid (α-amino-β-sulfo-propionic acid) shares structural similarities with taurine. It is a competitive inhibitor of taurine transport and thus utilizes the same transport mechanisms (Lahdesmaki and Oja, 1973; Koyama et al., 1990). Cysteic acid has a carboxyl group in addition to the sulfonic acid and amino groups. This provides another functional group, not required for transport, to which other compounds such as the calpain inhibitor leucyl-argininal can be attached.

The striking feature of CYLA at crossing BBB, its ability to permeate across cell membranes and the availability at the CNS are essential to enhance the uptake of this drug in nervous tissue.and for reducing calpain activity.

7. Therapeutic Strategies

The current treatment plans for MS are very complex and generally unsatisfactory. This difficulty results from the involvement of different factors in the cause and pathogenesis of the disease (Table 4). Researchers and clinicians have been testing and studying various therapeutic agents for the treatment of MS (Mokhtarian et al., 1996; Lo et al., 2002; Gilgun-Sherki et al., 2003b; Gilgun-Sherki et al., 2003a; Bechtold et al., 2004; Offen et al., 2004), but the management of the disease remains complex and unreliable.

Clinical Subform	Therapy
Acute relapse	Pulsed high-dose glucocorticosteroids *Escalation*: Plasma exchange
Clinically isolated syndromes (CIS) with high risk of of developing clinically definite MS	Interferon beta
Relapsing-remitting MS	First line: Interferon beta, glatiramer acetate Second line: IVIG, azathioprine With severe relapses and progression: Mitoxantrone
Secondary progressive MS	With relapses: Interferon beta Progressive: Mitoxantrone Second line: Cyclophosphamide
Primary progressive MS	No established therapy available
Primary-relapsing MS	No established therapy available

Table 4 Current Treatment Strategies in Multiple Sclerosis (Kieseier et al., 2003).

The therapy for MS has changed over the past several years as a result of intensive studies and drug trials on animal models. Recent findings in the immunological and pathophysiological aspects of the disease, advances in biotechnology, modern imaging and improvements in clinical trials have led to a variety of therapeutic approaches. Scientists and physicians have tried combination therapy in order to act on different aspects of the disease (Hohlfeld, 1997). This approach reduces the side effects of the individual drugs and potentiates the pharmacological efficacy of the treatment.

The therapeutic strategies vary according to the different clinical subtypes of MS. High dose steroid remains the first-line treatment for the new-onset MS, acute exacerbation or relapse. Steroids are the most potent immunosuppressive and anti-inflammatory drugs. Steroids lead to the reduction of the symptoms by inducing T cell apoptosis and diminishing the production and release of proinflammatory cytokines. In addition, they restore the integrity of the BBB and have some antiedematouse effect (Gold et al., 2001). Steroids are given intravenous for 3 days and then tapered orally. Steroid therapy can only shorten the duration of the acute exacerbation without any effect of the reduction or prevention of the relapses. Treatment of MS with steroids is very effective in the short term, but they are associated with severe side effects which limits their use. The long term use of this class of drug does not alter the course of the disease. Plasma exchange (PE) is another strategy for the treatment of acute relapse. PE is considered in persistent and severe cases of acute exacerbation that are resistant to steroid therapy (Weinshenker, 1999; Keegan et al., 2002).

Disease-modifying drugs such as interferon beta (IFN b) and glatiramer acetate (GA) (Wolinsky, 1995; Ballow and Nelson, 1997; Noseworthy, 1999) and immunsuppresive drugs (Gonsette, 1996a, 1996b) have been used extensively. Interferons [Interferon-β-1A (Avonex), Interferon-β-1B (Betaferon)] and glatiramer acetate (Copoxan) are useful for reducing the relapses and altering the progress of the disease. Both agents are the first line treatment for RRMS. Interferons are given every other day subcutaneously, while copoxan is given every day intramuscular. IFN is belived to inhibit the leukocyte proliferation, leukocyte migration across the BBB by downregulating the expression of adhesion molecules and MMP and antigene

presentation. Moreover, they modulate cytokine production towards the anti-inflammatory phenotype (Yong et al., 1998; Yong, 2002). The efficacy of IFN in the treatment of MS has been shown in several studies (Kieseier et al., 2003; Lublin, 2005).

GA is a polypepide that is arranged randomly in a specific molar ratio (L-glutamic acid, L-lysine, L-alanine, and L-tyrosine). The mechanism of action by which GA exerts its effect is believed to be immune deviation from a Th1 (proinflammatory cytokines) to a Th2 (anti-inflammatory cytokines) phenotype. In addition, GA induces antigen-specific T-suppressor cells that cross-react with putative autoantigens in the CNS and inhibit antigen presentation (Neuhaus et al., 2001; Yong, 2002). The side effects of GA are generally mild (Ziemssen et al., 2001).

Intravenous immunoglobulin (IVIG) has also been used for the treatment of MS. IVIG therapy demonstrated reduction in relapse rate, disease progression and number of gadolinium-enhanced lesions in RRMS (Fazekas et al., 1997; Achiron et al., 1998; Sorensen et al., 1998). Supportive evidence for IVIG still lags far behind that for INF beta and the optimal dosage still needs to be determined. These reasons make IVIG an alternative therapy form rather that first-line treatment option.

Immunosuppressive drugs such as azathioprine (Yudkin et al., 1991), cyclophospamide (Weiner et al., 1993; Perini et al., 2006) and Mitoxantrane are used either as a second-line drug for RRMS (Edan et al., 1997; Millefiorini et al., 1997) and/or for RRMS with severe relapses and progression (Hartung et al., 2002). Mitoxantrone is the first line drug for SPMS and PRMS (Hartung et al., 2002; Kieseier et al., 2003). These drugs have severe side effects limiting their extensive and long term use. There is no available established therapy strategy for primary progressive MS, but trial with IFN b

(Leary et al., 2003) and mitoxantrone (Pelfrey et al., 2006) were less successful (Kieseier et al., 2003). All the above mentioned drugs are immunosuppressive or immunomodulators affecting predominantly the inflammatory component of MS. These drugs do little or nothing to protect the axonal injury, which is the major correlate of permanent disability. Their efficacy is limited in the late stages of the disease where axonal injury predominates. It is crucial to develop a drug that is more effective and with a different mode of action for preventing axonal injury.

Trials with monoclonal antibodies have shown some beneficial effect in MS patients. A new drug, Tysabri (natalizumab), a monoclonal antibody against an adhesion molecule has recently been approved by food and drug administration (FDA) (Noseworthy and Kirkpatrick, 2005). Unfortunately, the drug was soon taken off the market due to a possible side effect after it was reported that two patients who were on Tysabri developed progressive multifocal leukoencephalopathy (PML). PML normally occurs in patients who are severely immunocompromised. It has been hypothesized that the inhibition of leukocyte migration by this drug may have caused an immunosupressed state in these patients.

Stem cell therapy is under intense research with some promising results (Pluchino et al., 2003). The hope from this treatment modality is that the stem cells might grow into myelin producing cells that might help the remyelination process. It appears though that stem cell therapy would be more efficacies if applied earlier in the disease process when injury to myelin is predominant. In the late stages of the disease course these therapy might not be helpful since it is the injury to the axons that leads to disability and not the myelin degradation.

Given the multiple therapeutic indications for calpain inhibition in various neurodegenerative and other disorders; an achievement of selective inhibition of these enzymes is an important pharmacological goal (Osanai and Nagai, 1984; Badalamente et al., 1987, , 1989; Bartus et al., 1994a; Bartus et al., 1994b; Saatman et al., 1996; Banik et al., 1998; James et al., 1998). Combination of drug groups, each with a different mode of action, can be used as supplemental agents to the existing MS drugs. This leads to the use of individual drugs in a minimum dose that would result in fewer side effects and in a synergistic effect of the combination of the individual drugs.

8. Animal models of MS

There are two animal model systems frequently used to study MS. These models fall into two broad groups: viral and anutoimmune non-viral (Fazakerley and Webb, 1987; Fazakerley and Buchmeier, 1993; Fazakerley and Walker, 2003; Fazakerley, 2004; Fuller et al., 2004; Rao and Segal, 2004). The non-viral animal model system for MS, experimental autoimmune encephalomyelitis (EAE), was first described in primates in the1930s and has been used extensively to study the pathophysiology and therapeutic approaches of MS (Fuller et al., 2004; Rao and Segal, 2004). The two viral models of MS are Semliki Forest Virus-induced encephalomyelitis and Theiler`s virus-induced encephalomyelitis (Mokhtarian and Swoveland, 1987; Fuller et al., 2004). Animal model systems enable the researchers to study most aspects of MS such as predisposition, clinical, physiological and histopathological features (Lassmann, 1983). Both models have led to a greater understanding of the disease process and the development of clinical therapies.

In EAE, peripheral immunization of susceptible naive experimental animals with myelin antigen(s) or transfer of presenstized lymphocytes (Fig. 25) initiates the disease (Mokhtarian et al., 1984; McRae et al., 1992). The clinical courses of the disease and the pathological features depend on the species and strain of the experimental animals used (Table 5).

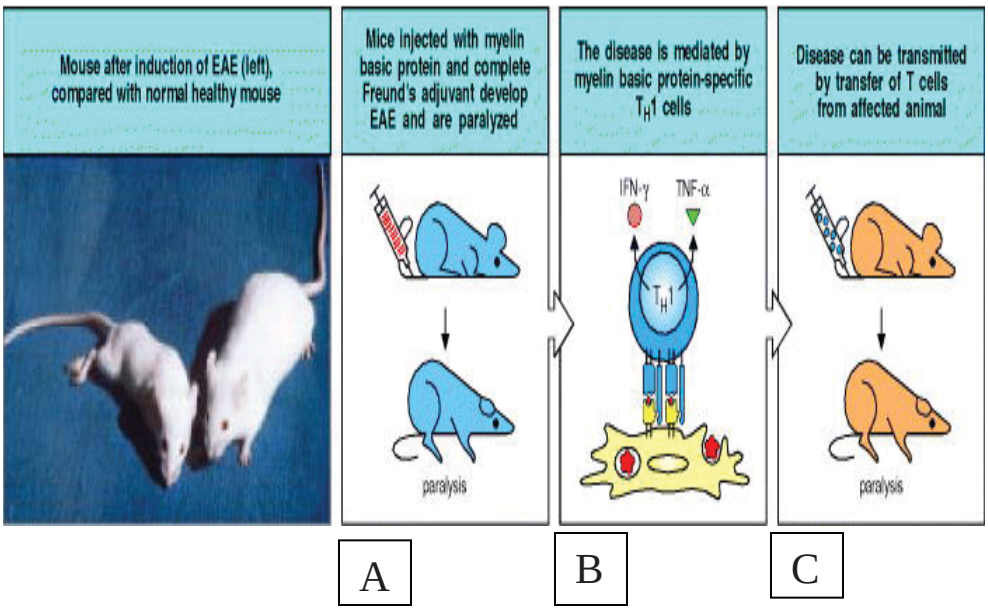


Figure 25 Modes of EAE induction. Myelin basic protein is injected into the mouse in the presence of an adjuvant to induce the disease directly (A). Senstized T-cells isolated from the diseased mice will be incubated in vitro with myelin basic protein to reactivate the cells (B). Finally, the isolated T-cells will be injected in to a naïve mouse to induce the disease indirectly (C).

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

Table 5 Commomly used rodent EAE models (Gold et al., 2006).

In addition, the disease course is also dependent upon the type of antigen used (Fuller et al., 2004). One of the disadvantages of this model is the fact that EAE fails to address the epidemiology, age-related acquisition and onset of the disease that is naturally occurring in the human population with MS (Table 6).

	Mouse	Human
General	Inbred; homozygous	Outbred; heterozygous
	Short lifespan: high fecundity	Long lifespan: low fecundity
	Fixed diet; pathogen-free	Varied diet; carriers of potential pathogens, e.g. EBV, JCV etc
	Clean environment	Open access to new infections
EAE and multiple sclerosis		
	May be monophasic	Different subtypes, usually relapsing
	Mice tested while epitopes are spreading	Epitopes must often have spread long before diagnosis
Induction	Usually with CFA	Spontaneous
Testing new therapeutics	Induction of EAE studied much more than ongoing disease	Ongoing disease
	Only a few dozen mice tested	Hundreds of multiple sclerosis patients; some side-effects are too rare to be seen in mice
Scrutiny	Less detailed	Detailed, would be missed in mice
Follow-up	Often short-term only	Several years or life-long
Molecular differences in immune response		
T-cell responses	Often stereotypical	Usually idiosyncratic, even to recurring epitope(s)
Lymphocytes in peripheral blood	75–90%	30–50%
$CD4^+$ expression	Lymphocytes	Lymphocytes, macrophages
$CD8^+$ expression	Lymphocytes, dendritic cells	Lymphocytes
IL-10 expression	T_H2	T_H1 and T_H2
IFN-α response	No preferential T_H differentiation	Promotes T_H1 response
IL-4 and IFN-γ expression by T_H	Exclusively one or the other	Sometimes both
CD28 expression	~100% of $CD4^+$ and $CD8^+$ T cells	~80% of $CD4^+$ T cells, 50% of $CD8^+$ T cells
MHC class II expression	Absent on T cells and endothelial cells	Present on T cells and endothelial cells
CD52 expression	Not found in mice	Lymphocytes
Glucocorticoid-sensitivity	High	Low and variable

Table 6 Immunological differences between mouse and human relevant for testing multiple sclerosis therapeutics (Friese et al., 2006).

EAE can be induced either by injection of whole spinal cord or brain preparation (in earlier versions of EAE models), purified components of myelin [myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG)]. Recently, it has become common practice to induce EAE in well-defined inbred animal strains (mice or rats) by targeting single major histocompatibility complex (MHC) Class II-restricted myelin epitopes such as MOG 35-55, MBP 87-116, PLP 139-151, in the presence of an adjuvant (Fuller et al., 2004; Rao and Segal, 2004). Myelin protein is a very complex heterogeneous protein containing many different components. MBP is the most abundant fraction, measuring 35% of the total protein (Campagnoni, 1988). MOG is a minor myelin component constituting of only about 0.05% of the total myelin protein and MAG is about 1% of the total myelin (Bernard et al., 1997). MOG has attracted most attention recently as an antigen for induction of EAE. It is located on the outer surface of the oligodendrocyte membrane and can be easily targeted by antibodies and T-cells (Birling et al., 1993; Amor et al., 1994; Diaz-Villoslada et al., 1999; Raine et al., 1999; Van der Goes et al., 1999). MOG activates both the cellular and antibody mediated arms of the immune response in an immunized animal. MOG-induced EAE has features typical of MS such as relapsing-remitting paralytic disease (Mendel et al., 1995; Slavin et al., 1998).

Non-myelin antigens can also induce the disease. A number of identities and similarities between protein sequences of infectious agents and known CNS autoantigens have given rise to the term "molecular mimicry". This states that infectious agents can induce autoreactivity that can lead to the onset of the disease (Mokhtarian et al., 1999).

There is strong evidence for the consideration of MS as an autoimmune disease. Most concepts of the disease initiation and progression and therapy strategies have been obtained from data that comes from the studies using EAE (Table 6). The cellular composition of the inflammation and the pattern of chemokines and chemokine receptors expression within the lesions are similar in both diseases types, EAE and MS (Huang et al., 2000). Results from the studies with animal models have shown that myelin reactive T-cells recognize myelin antigen or viral peptide presented by macrophages, microglia and dendritic cells, triggering an autoimmune cascade that leads ultimately to the destruction of the nervous tissue (Smith, 1979; Deshpande et al., 1995).

IV. Materials and Methods

1. Animals

C57BL/6J female mice (7-8-week-old) were purchased from the Jackson laboratories (Bar Harbor, MD) and housed according to Institutional Animal Care and Use Committee (IACUC) regulations in an Association for Assessment Accreditation of Laboratory Animal Care (AAALAC) certified facility.

2. Preparation of Peptide Antigen and Induction of EAE

MOG 35-55 (NH_2-MEVGWYRSPFSRVVHLYRNGK-OH) was synthesized in the Dept. of Biological Chemistry, Biosynthesis & Sequencing Facility at Johns Hopkins University School of Medicine. MOG 35-55 (300μg) was emulsified in Incomplete Freund`s Adjuvant (IFA, Sigma, St. Louis, MO) supplemented with 500μg heat inactivated mycobacterium tuberculosis (Sigma, St. Louis, MO).

To induce EAE, each mouse was injected subcutaneously (s.c.) with 100μl/flank MOG/CFA emulsion (Zhang et al., 2003) with some modification. Pertussis toxin (100ng) was given intraperitoneally (i.p.) on day 0 and on day 2 post-immunization (p.i). Animals were separated into two big groups (control and experimental). Each group has three subgroups: control group consists naïve mice (naïve control: n=4), mice immunized with only complete Freund`s adjuvant (CFA control; n=6) and naïve mice treated with only CYLA (CYLA control; n=4). The experimental group consists MOG/CFA immunized groups (EAE; n=6), EAE treated with CYLA (n=6) starting from the onset of the clinical signs (day 12pi) and EAE treated with CYLA (n=6)

starting before the onset of the clinical signs (day 7pi). EAE mice were assigned to the different subgroups randomly after induction of EAE.

3. Evaluation of neurological and body weight changes

All mice were weighed and evaluated for the extent of neurological deficit up to the termination of the experiment by two independent investigatorson a daily basis. Clinical severity was assessed according to a 0–6 scale as follows: 0, no abnormality; 1, limp tail; 2, limp tail and hind limb weakness (waddling gait), mice have difficulty righting them up when flipped on their back; 3, hind limb paralysis; 4, complete hind limb paralysis with fore limb weakness; 5, quadriplegia; and 6, moribund or death. The data were plotted as the daily mean clinical score for each group. Scores of asymptomatic mice (score = 0) and score 6 were included in the calculation of the daily average clinical score (ACS) for each group. The average body weight (ABW) of all groups is also calculated on a daily basis.

4. Preparation and application of CYLA

The prodrug, as the diethyl acetal was a gift from Ceptor Corp., Hunt Valley, MD. It shows no in vitro calpain inhibitory activity until the acetals are cleaved off. The active injected material is made by acid hydrolysis. The prodrug was incubated for 2 hours at room temperature (1mg/ml 0.1M HCl) and then dried to remove water and acid. The deacetylated CYLA is stored in the cold and prior to use, it is dissolved in Dulbecco's Phosphate-Buffered Saline (D-PBS). CYLA was administered i.p. in 200µl D-PBS per mouse, and the corresponding total daily doses were given twice a day according to the individual treatment regimen. The prodrug was administered orally in a 100µl D-PBS twice a day. CYLA was administered at two different time points after the

onset of the clinical signs (starting from day 12pi) and shortly before the onset of the clinical signs (starting from day 7pi).

5. Tissue collection

The experiment was terminated either on day 21pi for the acute phase or day 31pi for the chronic phase. Mice were anesthetized with Metofane (Schering-Plough Animal Health, Union, NJ), perfused transcardially with either D-PBS or Paraformaldehyde. For histopathological assay, spinal cord tissues were either homogenized for western blot analysis and fluorogenic assay or fixed in 4% Paraformaldehyde for 4-6h, and then transferred to sucrose gradients and subsequently embedded in Shandon M-1 embedding matrix for frozen sectioning (Thermo Electron Corporation, Pittsburgh, PA).

For the fluorogenic in vitro calpain activity assay and western blot analysis spinal cord segments were immediately taken, Spinal cord tissues were homogenized with Tris-homogenizing buffer (50mM Tris-HCL, 1mM EDTA, 0.1mM PMSF and 0.32M Sucrose) (Shields 1998). The supernatant was collected after centrifuging the homogenate for 20 min at 15000 rpm, in a pre-cooled centrifuge. Total Protein was measured using OPSYS MR Microplate reader (DYNEX Technologies, Thermo Labsystems, Franklin, MA). The supernatant was collected and kept at -80^{0}C until further use.

6. Hematoxylin and eosin (H&E) staining

Frozen sections (6-10μm) from paraformaldehyde fixed spinal cord tissues were fixed in 95% ethanol for 30 seconds (s) and then washed in distilled water for 10 s. Slides were stained with hematoxylin for 1 minute (min) and rinsed in distilled water twice for 10s each. Following this step slides were soaked in 0.1% ammonium hydroxide for 10 s until cleared. The slides were then placed in 70% ethanol for 10 s followed by two

additional steps in 95% ethanol for 10 s each. Slides were then stained in eosin for 15 s, rinsed twice in 100% ethanol, and finally dipped twice in xylene for 10 s each. Slides were then mounted and visualized with an Olympus DP11 camera. This staining method is used to identify inflammatory cells in the tissue (Smith-Norowitz et al., 2000).

7. Luxol fast blue (LFB) staining

Frozen sections (6-10μm) from paraformaldehyde fixed spinal cord tissues were rehydrated in D-PBS for 5 min. Slides were incubated with Luxol fast blue [1.0% (w/v) Solvent blue 38, Sigma, in 95% ethanol, with 5 ml 10% acetic acid] for 24h at 60°C. Slides were washed in 95% ethanol for 10 s, rinsed in distilled water for 10 s, dipped in 0.05% (w/v) Li_2CO_3 for 5 s, washed in 70% ethanol three times for 1 min each, and washed in distilled water for 1 min. The last three steps were repeated several times until a sharp contrast between the blue of the white matter and the colorless gray matter was seen. Slides were counterstained with eosin for 1 min rinsed in distilled water for 10s and stained with Cresyl violet for 1 min, before they were rinsed in H_2O and dehydrated in 95% ethanol twice for 1 min. Finally, we rinsed twice in 100% ethanol, and twice in xylene for 10 s each. Slides were then mounted and visualized with an Olympus DP11 camera. This staining method is used to detect demyelination (Smith-Norowitz et al., 2000).

8. Immunofluorescence (IF) staining and confocal microscopy

Frozen sections (50μm) were prepared from paraformaldehyde fixed spinal cord tissues for fluorescent antibody staining as described with modifications (Schaecher et al., 2002). The sections were blocked for one hour with 10% goat serum and incubated for 1h with appropriate dilutions (1:100) of the following primary antibodies: anti-calpain

antibody (kindly provided by Dr. Banik, Medical University of South Carolina, Charleston, SC), anti-CD45 antibody (leukocyte common antigen, BD PharMingen, San Diego, CA), anti-CD11b (macrophage/microglia marker, BD PharMingen, San Diego, CA). Appropriate dilutions (1:200) of the following secondary antibodies were applied for 30min: Alexa586-conjugated goat anti-rat secondary antibodies for CD11b (Caltag, Burlingame, CA), Alexa647-conjugated donkey ant-rabbit antibody for calpain (Molecular probes, Eugene, Oregon), Alexa488-conjugated goat anti-mouse for CD45 (BD PharMingen, San Diego, CA). Finally, the sections were washed, mounted with Vectashield media (Vector, Burlingame, CA) and visualized with a laser scanning confocal microscope (Olympus IX-70). Pixels were quantified as total number of pixels above background using ImageJ image analysis software from NIH and the data is graphically represented as mean ± S.E.M.

9. Fluorogenic in vitro calpain activity assay

The activity of the enzyme calpain was assayed as an indirect measure for calpain content in the different tissue samples. Calpain activity was measured using a fluorescent-labeled synthetic calpain specific substrate Suc-LLVY AMC from Oncogene (San Diego, CA). Briefly, 50µl of each sample were incubated with 100µl of activation buffer containing Ca^{2+} and the reducing agent Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and 100µl of inhibition buffer as described in the protocol. The reaction mixture was incubated for 30min at room temperature after adding the fluorescent substrate. The free AMC released by proteolysis is read with POLARstar OPTIMA plate reader (BMG Labtechnologies, North Carolina). The fluorescence determined with inhibition buffer is subtracted from the fluorescent value with activation

buffer. The net activity of calpain can then be displayed as the amount of free AMC released per unit time per amount of protein. Purified human erythrocyte calpain was used as a positive control in each assay.

10. Bielshowsky`s silver staining method

Frozen sections (6-10μm) from paraformaldehyde fixed spinal cord tissues were placed the slides in pre-warmed (40 ºC) 10% silver nitrate solution and stain for 15 minutes and sections become light brown color. Slides were then immersed the in distilled water and wash for 3 times followed by a step in which we added a concentrated ammonium hydroxide drop by drop to the silver nitrate solution, until the precipitate formed is just clear. The slides were then placed back in this ammonium silver solution and stain in 40 ºC oven for 30 min or until sections become dark brown. The slides were then placed directly back in developer working solution (8 drops of developer stock solution, 8 drops of concentrated ammonium hydroxide and 50 ml of distilled water) for 1 min. Then slides were then dipped for 1 min in 1% ammonium hydroxide solution to stop the silver reaction. After washing the slides with distilled water 3 times we placed them in 5% sodium thiosulfate solution for 5 min. Slides were washed three times with distilled water and went through the dehydration and clearing process by putting the slides in 95% ethyl alcohol, 100% alcohol and xylene. Finally, slides were mounted and visualized with an Olympus DP11 camera.

11. Western blot analysis

To evaluate abnormal calpain activity as measured indirectly by substrate degradation and increased sodium channel expression, we used western blot analysis using 50μg total protein as described with minor modifications (Shields et al., 1998). The

protein was detected using appropriate dilutions (1:1000dilutions) of anti-alpha fodrin or anti-Na^+ 1.6 primary antibodies. We used 1:2000 dilutions of the alkaline phospahtase-conjugated goat anti-rabbit secondary antibody (Sigma, Saint Louis, MO). Bands were visualized using 5-bromo-4-cholo-3-indoxyl-phophate/nitroblue tetrazolium Benzamidine (BCIP, Kirkegaard & Perry Labs, Gaithersburg, MD). Proteins were quantified using Image-pro plus 4.5.1 software (Media Cybernetics, Inc., Silver Spring, MD) and the results were expressed in optical density unit ± S.E.M.

12. Statistical analysis

Statistical analysis was performed using Statistical Analysis System (SAS). The clinical scores and body weight during the day's 10-20 or 10-30 were compared for each group. The Kruskal-Wallis test followed by Wilcoxon two-sample test was used to compare the clinical scores in control groups, EAE and CYLA treated groups.

V. Results

A. Acute EAE

1. Evaluation of neurological and body weight changes

The first sign of the disease was weight loss. Starting from day 8 post immunization (pi) EAE mice showed a progressive weight loss. Early neurological signs of EAE such as floppy tail and hind limb weakness appeared in general about day 12pi. Over the course of time mice exhibited hind limb paralysis and some weakness in the fore limbs between days 14-16pi. Their neurological deficit increased progressively in all EAE mice by days 18-19pi and reached its peak by day 20pi (Fig. 26). The experiment was terminated on day 21pi.

CYLA was applied in increasing doses starting from 0.5mg/mouse/day. We chose two time points for the administration of the drug: Day 10pi shortly before the onset of the clinical signs of EAE and day 12pi after the onset of the clinical signs (neurological deficits). Treatment with the lower doses of CYLA (0.5mg/mouse/day) resulted in only a minor improvement of the ACS when applied before the onset of EAE starting from day 10pi. Treatment after the onset of the clinical signs starting on day12pi showed no improvement of the ACS (data summarized in Fig. 41).

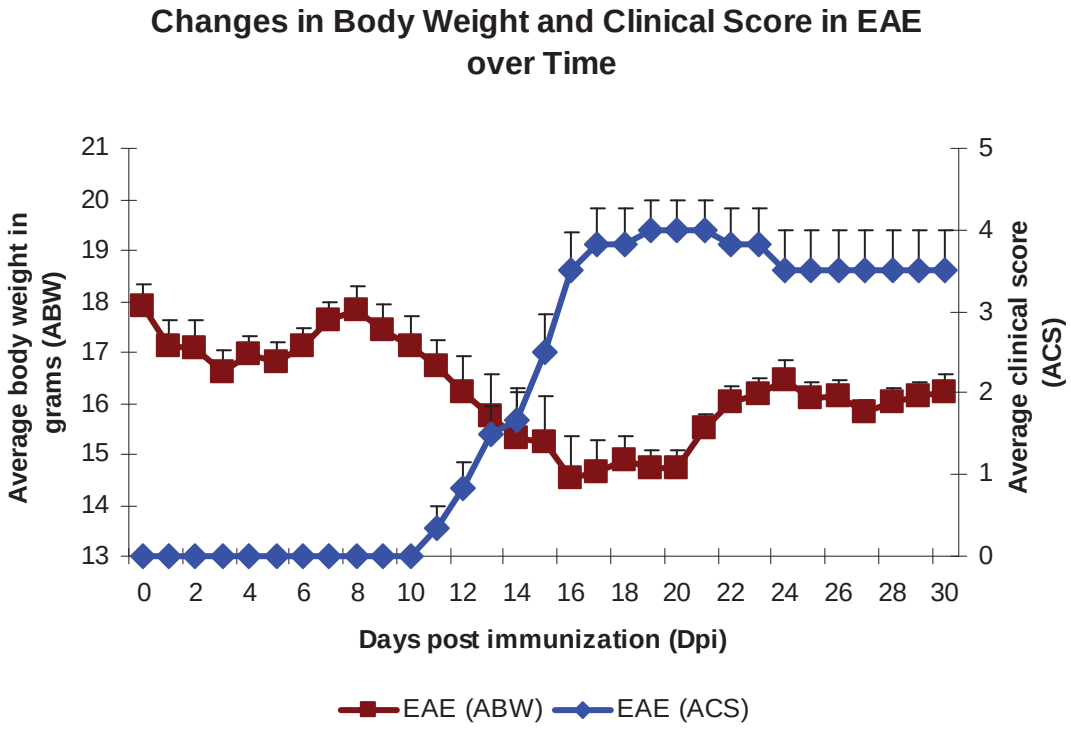


Figure 26 Representative figure showing changes in ABW and ACS of EAE group over time after induction of the disease. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. Weight loss was the first sign of the disease followed by the increasing diabilities mirroring the weight loss pattern. Starting from day 8pi EAE mice showed a progressive weight loss. Weight loss progresses until day 16pi and EAE mice maintained their body weight day 20pi. Early neurological signs such as floppy tail and hind limb weakness appeared in general about day 12pi. Over the course of time mice exhibited hind limb paralysis and some weakness in the fore limbs between days 14-16pi. Their neurological deficit increased progressively in all EAE mice and reached its peak by day 20pi.

The CYLA dose was then subsequently doubled. In addition, an earlier time point was chosen to apply the increased dose of CYLA on day 7pi, in order to examine whether or not earlier application of CYLA has a better effect on the disease onset and progression.

Animals treated with 1mg/mouse/day starting from symptom onset showed a marginal improvement of their ACS when compared to the non-treated group. The improvements of the clinical signs were indicated by the lower values in the ACS of CYLA treated versus non-treated groups. Comparision of each groups using Wilcoxon two-sample test showed no statistical significant difference in their ACS ($p<0.08$). The group of mice that received treatment with 1mg CYLA per day starting on day 7pi demonstrated a delayed onset of EAE signs (onset of the clinical signs on day 14pi) and the neurological deficits were limited to flaccid tail and mild hind limb weakness (data summarized in Fig. 41). The improvement of the disease is evidenced by the lower ACS. Comparison of the groups using Wilcoxon two-sample test showed statistically significant difference with treatment using 1mg/mouse/day starting from day 7pi ($p<0.002$). CYLA reduced the progression of the disease in this animal group. Thus, in order to produce significant reduction in clinical signs, mice had to be treated prior to the onset of the clinical signs with at least 1mg CYLA per mouse per day.

Finally the CYLA dose was increased to 2mg/mouse/day maintaining the same regiment as in the case of 1mg/mouse/day starting in one group from day 7pi and in another group from day12pi after induction of EAE. The animal group that received the 2mg/mouse/day CYLA from day 7pi showed a delayed onset of the clinical signs (onset of the clinical signs on day 16pi), minimal neurological deficit and suppression of the disease progression ($p<0.01$). Mice recieving CYLA starting from day 12pi showed improvements of the neurological deficit and suppression of the disease progression as well ($p<0.04$; Fig. 27A and B).

A

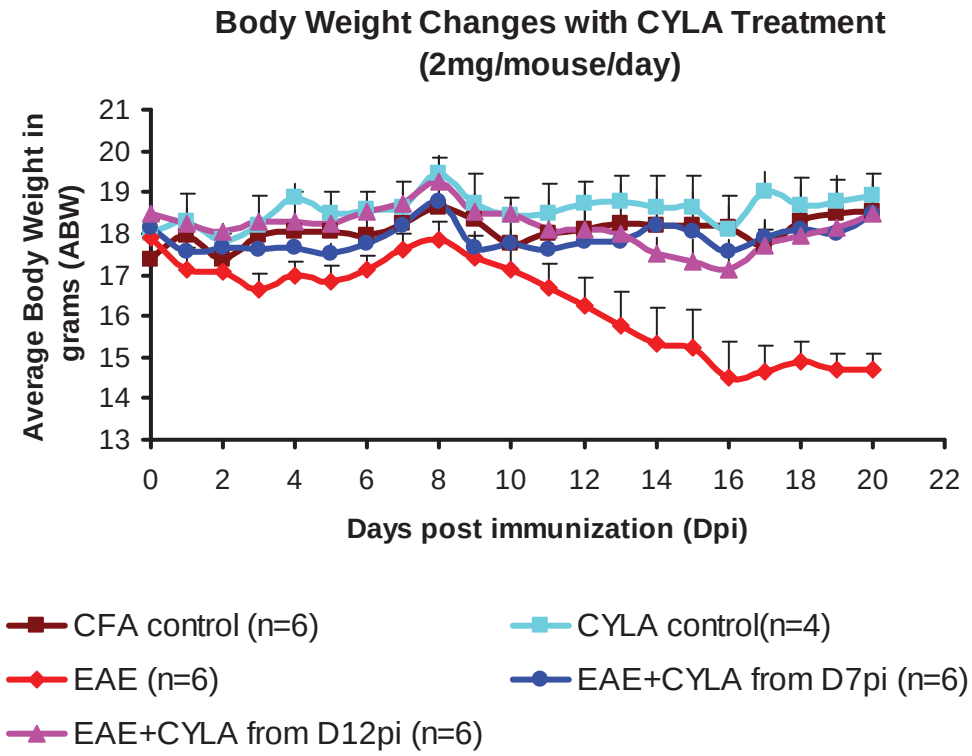


Figure 27A Changes in daily ABW of all groups over time during the course of treatment with 2mg CYLA after induction of the disease. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). EAE mice start losing weight from day 8pi. Weight loss progresses until day 16pi and EAE mice maintained their body weight day 20pi. Treatment with a total of 2mg CYLA per mouse per day showed minimal weight loss followed by quick recovery in day 12pi group. Day 7pi groups and all control groups (naïve, CFA and CYLA) showed progressive weight gain through out the observation time. Each graph presents daily ABW ± S.E.M.

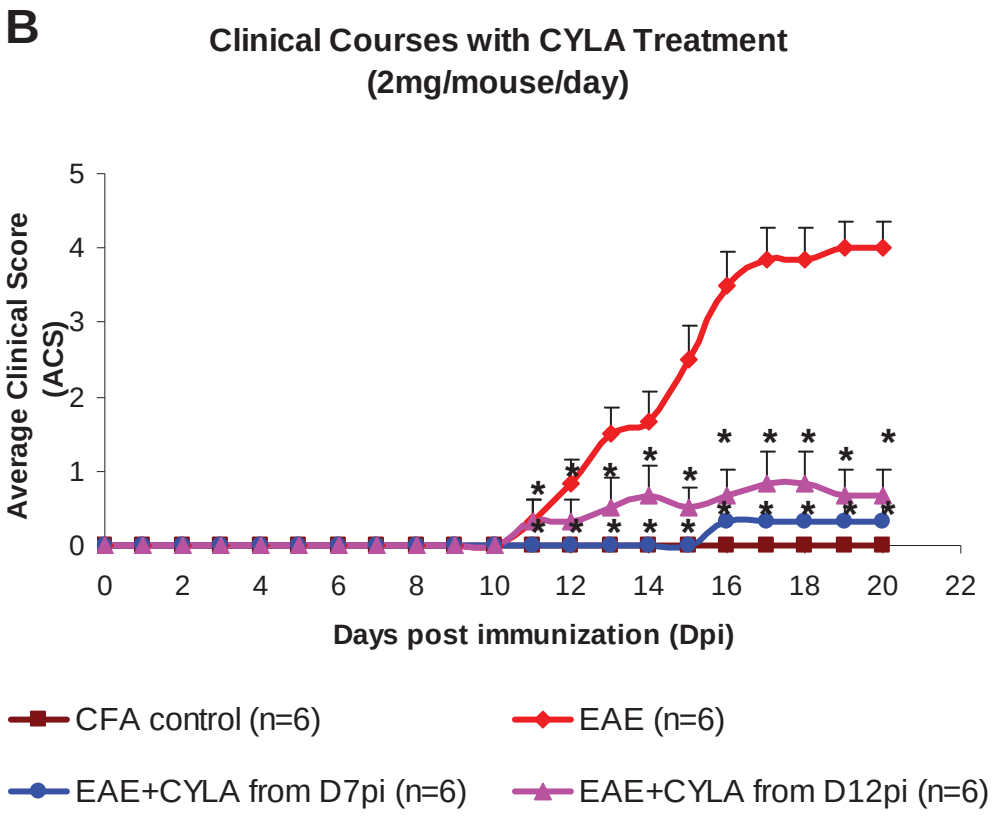


Figure 27B Changes in daily ACS of all groups over time during the course of treatment with 2mg CYLA after induction of the disease. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). Early neurological signs such as floppy tail and hind limb weakness appeared in general about day 12pi. Over the course of time mice exhibited hind limb paralysis and some weakness in the fore limbs between days 14-16pi. Their neurological deficit increased progressively in all EAE mice and reached its peak by day 20pi. Treatment with a total of 2mg CYLA per mouse per day starting from day 12pi led to a significant suppression of the clinical signs. Treatment with a total of 2mg CYLA per mouse per day starting from day 7pi showed a delayed onset and significant improvement of the clinical signs with suppression of the disease progression. All control groups (naïve, CFA and CYLA) showed no neurological deficit. The experiment was terminated on day 21pi. Each graph presents daily ACS ± S.E.M. The difference between treatments and naïve control was considered significant at $*P<0.05$.

In addition, we also administered the higher dose of the prodrug (2mg/mouse/day administered orally) and compared the results with the similar dose of the free aldehyde injected form. The prodrug was applied orally to two animal groups in the same regimen as mentioned above. One group of animals received 2mg of the prodrug via mouth starting from day 7pi. Another group of animals received 2mg of the prodrug starting from the onset of the clinical signs. The oral administration of the diacetyl prodrug was also as effective as the deacetylated form of CYLA (the ip version, $p<0.04$). The better outcome is indicated by the lower ACS when compared with non-treated EAE group (Fig. 28A and B).

In order to evaluate if CYLA by itself has toxic effect, we assessed the body weight changes of one group of mice (CYLA control). CYLA was administered to this group of naïve mice (n=4) starting from day 7pi. It was observed that naïve mice that received CYLA only showed progressive weight gain similar to that observed in the naïve control and CFA control animals.

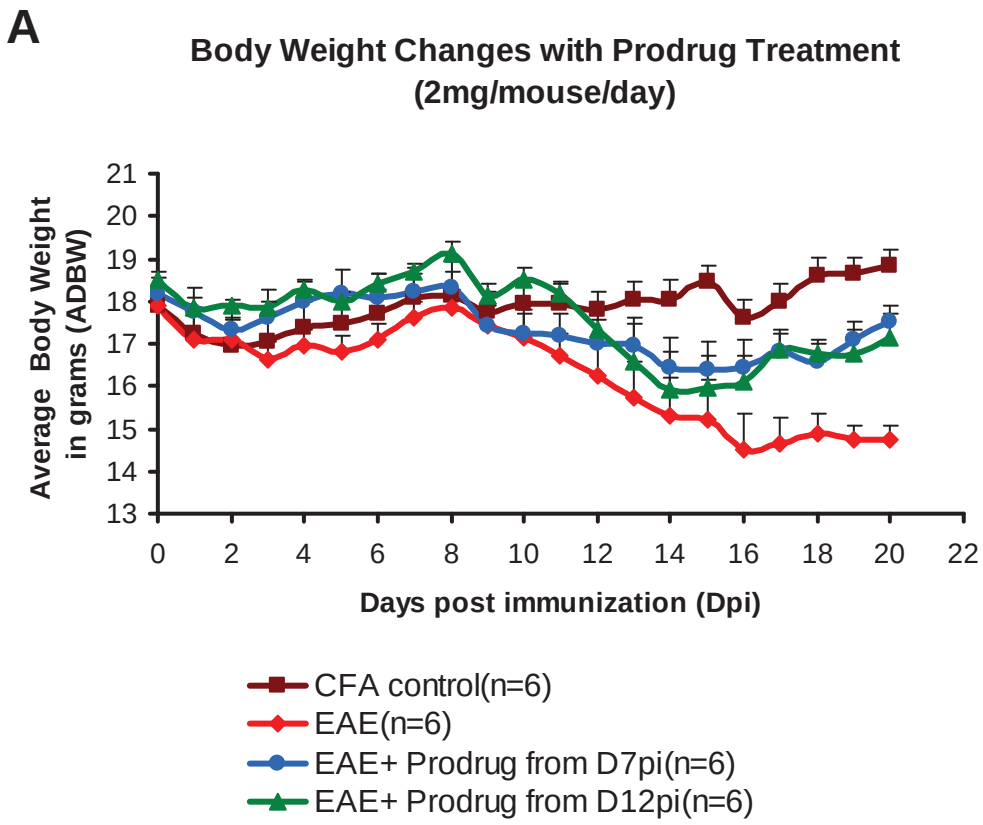


Figure 28A Changes in daily body weight of all groups over time during the course of reatment with 2mg of the prodrug after induction of the disease. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. EAE mice start losing weight from day 8pi. Weight loss progresses until day 16pi and EAE mice maintained their body weight day 20pi. The prodrug, a total of 2mg/mouse/day (1mg was given orally twice a day). EAE mice start losing weight from day 8pi. Weight loss progresses until day 16pi and improved until day 20pi. Treatment with a total of 2mg of the prodrug per mouse per day showed minimal weight loss followed by quick recovery in day 7pi group. Day 12pi groups showed similar weight loss pattern, but not to the extent observed in EAE group. All control groups (naïve, CFA) showed progressive weight gain through out the observation time. Each graph presents daily ABW ± S.E.M.

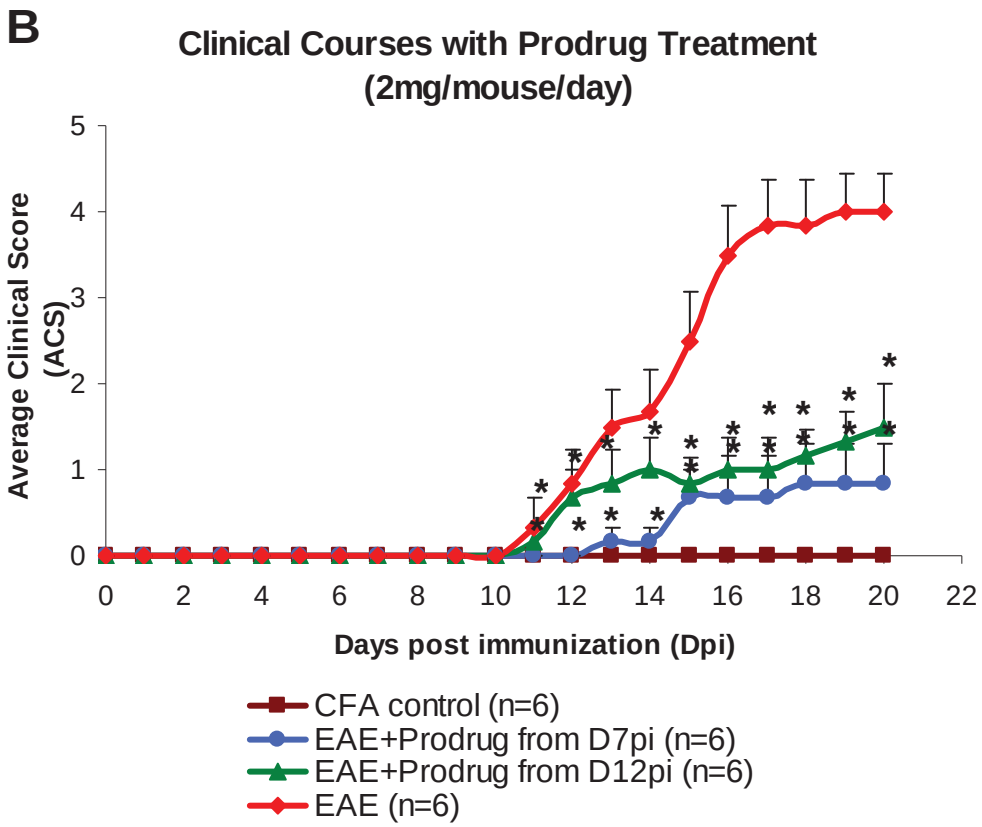


Figure 28B Changes in daily ACS of all groups over time during the course of treatment with 2mg of the prodrug after induction of the disease. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. Early neurological signs such as floppy tail and hind limb weakness appeared in general about day 12pi. Over the course of time mice exhibited hind limb paralysis and some weakness in the fore limbs between days 14-16pi. Their neurological deficit increased progressively in all EAE mice and reached its peak by day 20pi. The prodrug, a total of 2mg/mouse/day (1mg was given orally twice a day). Treatment with a total of 2mg of the prodrug per mouse per day starting from day 12pi led to suppression of the clinical signs to a significant level. Treatment with a total of 2mg of the prodrug per mouse per day starting from day 7pi showed a delayed onset and significant improvement of the clinical signs with suppression of the disease progression. All control groups (naïve, CFA) showed no neurological deficit. The outcome of the treatment with the prodrug was similar to the effect achieved using the injectable form. The experiment was terminated on day 21pi. Each graph presents daily ACS ± S.E.M. The difference between treatments and naïve control was considered significant at $*P<0.05$.

2. Hematoxylin and eosin (H&E) staining of spinal cord tissues

Hematoxylin and eosin (H&E) staining was used to show cellular infiltration of spinal cord white matter of mice with acute EAE in CYLA treated, non-treated EAE and all control groups. During the disease course inflammatory cells will migrate through the blood vessel and accumulate around it. This phenomenon is known as perivascular cuffing (white arrow; Fig. B). According to the severity of the disease the inflammatory cells invade the adjusent tissue (white arrow head; Fig. 29B). The inflammatory infiltration was distributed throughout the spinal cord white matter with a large number of cells (See arrows). The data demonstrated that there was a high amount of cellular infiltration and perivascular cuffing in the MOG/CFA mice, corresponding to the severe clinical symptoms of acute EAE. There were no inflammatory infiltrates in the naïve control group (Fig. 29A). Tissues from CYLA-treated mice showed minimal cellular infiltration localized subpial (black arrow; Fig.29C).

A. Naïve control **B. EAE** **C. CYLA treated (day 7pi)**

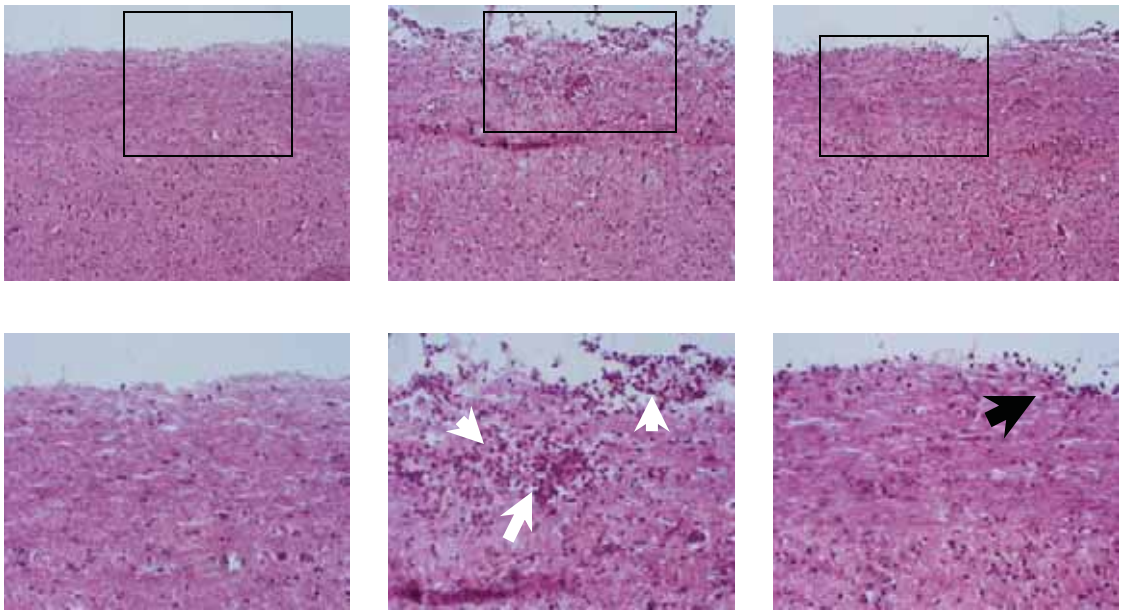


Figure 29 Representative histopathology pictures showing lumbar spinal cord sections from control, EAE and CYLA treated mice. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). H&E was performed on 6-10μm cryosections. The spinal cord sections of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] showed normal cellular content and no inflammatory infiltration of the white matter (A). EAE mice exhibited increased cellular infiltration perivascular (arrows) involving the adjacent parenchyma (Inset) and subpial (arrow head) in the white matter (B). Treatment with a total of 2mg CYLA per mouse per day reduced the inflammatory infiltration (C). Similar results were observed for both treatment groups (day 7pi and day 12pi). 10X magnification.

3. Luxol fast blue (LFB) staining of spinal cord tissues

This staining method identifies changes in myelin content of spinal cord tissue in all animal groups. Luxol fast is a basic dye that stains for myelin. Loss of myelin leads to less staining with luxol fast which appears pale. All control groups showed intense blue staining as a positive sign for myelin (Fig.30A). The staining pattern from the MOG/CFA groups was significantly paler than the previous groups and showed vacuolization

(Fig.30B), which represented a loss of myelin, one hallmark of the pathological process. The demyelination occurred in areas of cellular infiltration (black arrow). The sections from CYLA treated animal groups (Fig.30C) showed preserved myelin content as evidenced by the similar staining to the naïve control groups (Fig.30A).

A. Naïve control **B. EAE** **C. CYLA treated (day 7pi)**

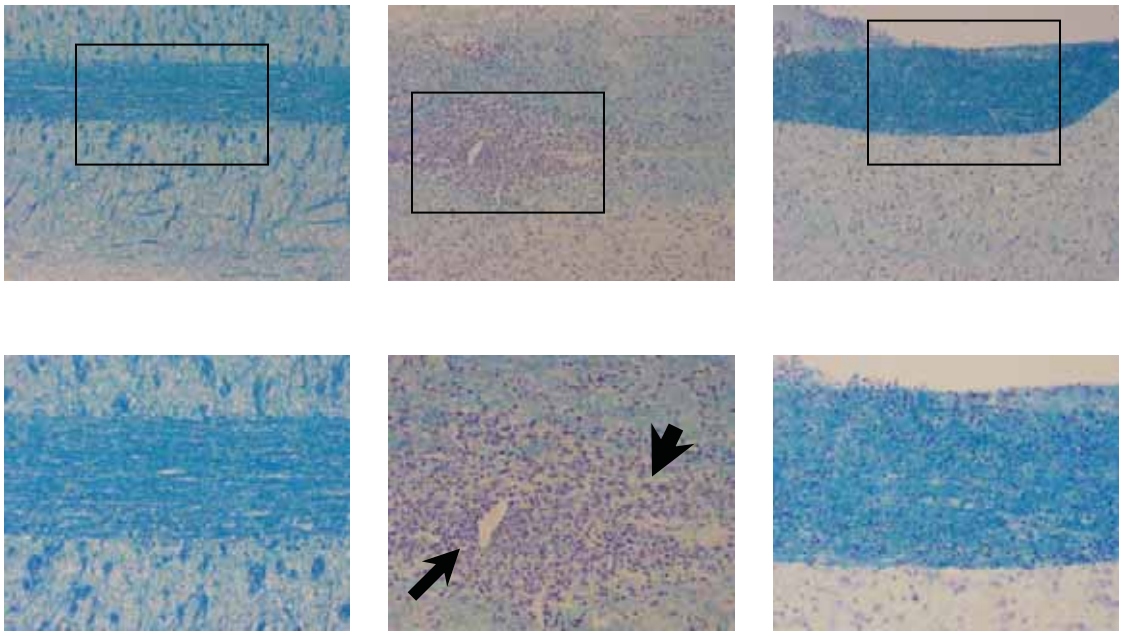
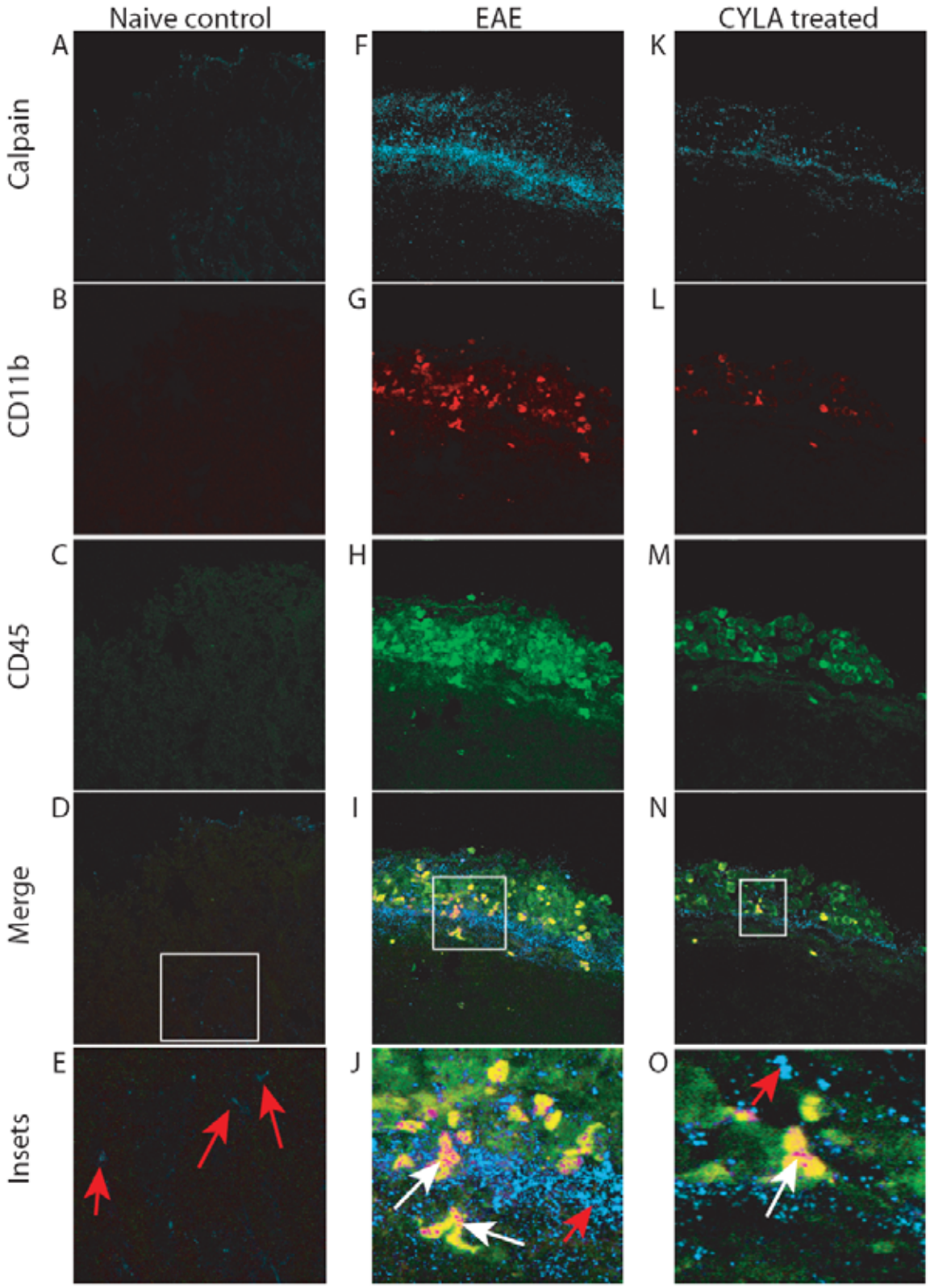


Figure 30 Representative histopathology pictures showing lumbar spinal cord sections from control, EAE and CYLA treated mice. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). LFB was performed on 6-10μm cryosections. The spinal cord sections of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] showed normal myelin content of the white matter (A). EAE mice exhibited myelin degradation in areas around cellular infiltration (B). Treatment with a total of 2mg CYLA per mouse per day reduced the myelin degradation (C). Similar results were observed for both treatment groups [day 7pi (n=6) and day 12pi (n=6)]. 10X magnification.

4. Immunofluorescent staining of spinal cord tissue

Changes in calpain content, infiltrating and resident mononuclear phagocytes of the spinal cord white matter were evaluated using IF labeling with anti-calpain antibody and cell surface markers. There was no appreciable staining for inflammatory cells and minimal staining for calpain in the naive control groups (Fig. 31A-E). By contrast, increased inflammatory infiltrates both in the subpial and adjacent white matter of the non-treated EAE mice spinal cord white matter were visible. Accumulation of calpain macrophages and microglia were seen in the inflamed white matter (Fig. 31F-J). Increased calpain was detected within the inflammed white matter and within the inflammatory cells detected (Fig. 31I-J). The increased calpain in the acute phase of the disease is accumulated within the white matter and not within the neurons (Fig. 32) as evidenced by double staining with NeuN (neuronal marker) and calpain. Treatment with of the higher doses of CYLA decreased the inflammatory infiltration into the CNS and the elevated calpain content within these cells (Fig. 31K-O), corresponding to the lower ACS. In contrast to non-treated EAE group, there was less calpain within the spinal cord white matter and within the inflammatory cells of CYLA treated groups (Fig. 31N-O). Semiquantitative analysis of pixels from the individual stainings was quantified as total number of pixels above background using NIH Imager (Fig. 31P).


Naive control
EAE
CYLA treated
Calpain
CD11b
CD45
Merge
Insets
A
F
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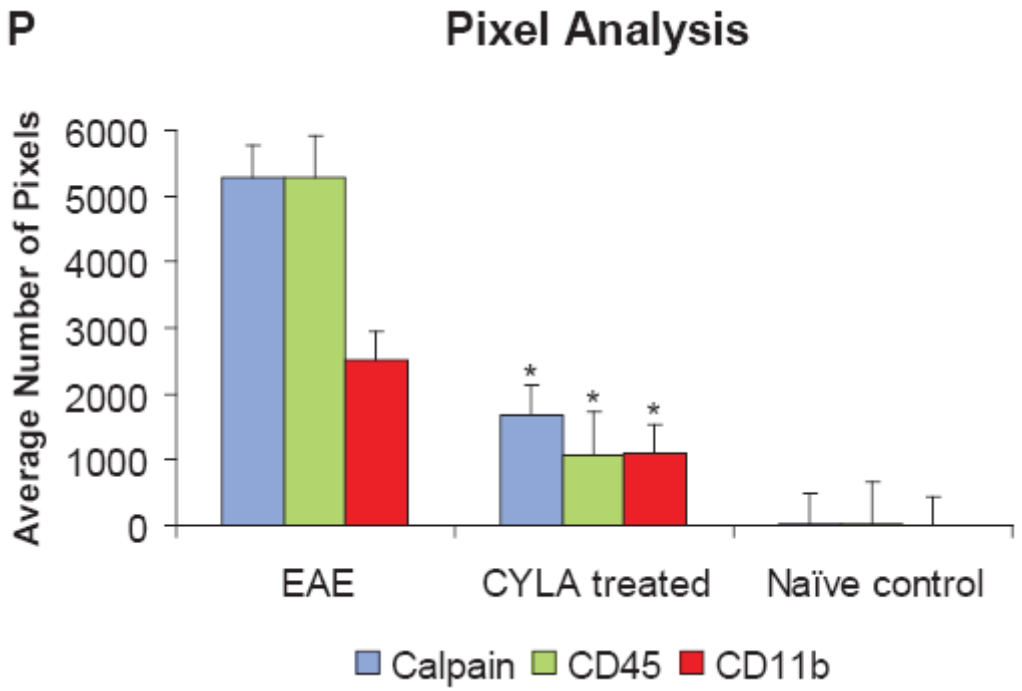


Figure 31 Changes in expression and distribution of calpain and inflammatory cells. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). Triple IF Staining of 50μm cryosections of the lumbar spinal cord were used for the IF staining. The representative images are pseudocolored to facilitate comparison. The spinal cord sections of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] showed no IF staining for inflammatory cells and normal staining for calpain (A-E). EAE mice exhibited increased calpain staining within the white matter and increased calpain positive inflammatory cell infiltration (F-J). CYLA treated mice (treatment with 2mg from day 7pi (n=6) is shown here) demonstrated decreased amount of calpain within the white matter and calpain positive inflammatory cells. Similar results were observed from day 12pi groups (n=6, K-O).
Blue represents calpain (red arrows), red represents CD11b+ cells and green represents CD45+ cells. Cells that stain positive for calpain appear pink (white arrows), and CD 45+ /CD $11b^{+}$ cells appear yellow. CD11b+/CD45+ cells (yellow, macrophage/microglia population) showed increased calpain content (pink) in EAE spinal cord section. These cells produce the increased calpain within the inflamed white matter. The quantitative data is graphically represented through pixel analysis using image analyzing software ImageJ from NIH public domain. Pixels were quantified as total number of pixels above background. Data was expressed as mean ± S.E.M. of 3 separate IF experiments (after scanning at least ≥ 9 slides each) and compared by one-way ANOVA followed by Tukey post hoc test. The difference between treatments and naïve control was considered significant at $*P<0.05$ (P). 60X magnification with oil.

A. EAE calpain **B. EAE NeuN** **C. EAE merges**

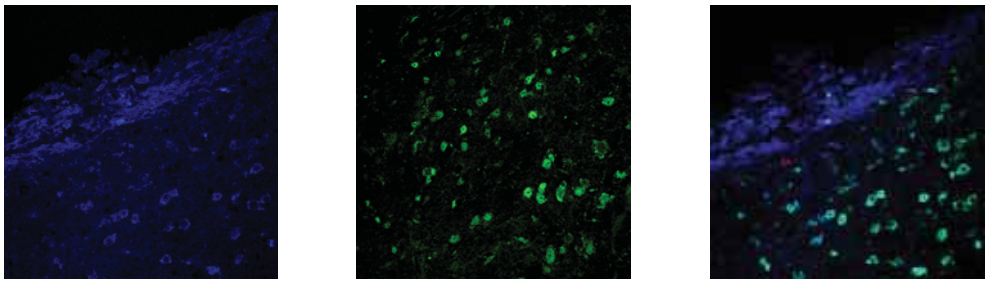


Figure 32 Double IF to localize the distribution of calpain within the spinal cord section in acute EAE. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). More calpain blue labeled (A) is accumulated within the white matter of the spinal cord section in the acute phase of the disease. The amount of calpain within the neuron green labeled with NeuN (B) is less compared with that in the inflamed white matter (merge picture, C) as showen by the distribution of calpain within the white matter and inflammatory cells (Fig. 32J) within spinal cord white matter. 60X magnification with oil.

5. Fluorogenic in vitro calpain activity

Calpain activity of spinal cord tissues was determined by a fluorogenic procedure. The calpain activity in spinal cord tissue indirectly measured the calpain content of the individual tissues. The enzyme activity in spinal cords of non-treated EAE group (n=6) was nearly twice as high as in the naive control spinal cords (n=4). Treatment with 1mg CYLA (Fig. 33A) reduced the tissue calpain content by 46% (from 203% in EAE group to 157%) in CYLA-treated group (n=6) ($P< 0.05$, Fig. not shown). Treatment with 2mg CYLA (Fig. 33B) reduced the tissue calpain content of the CYLA-treated spinal cord tissue (n=6) by 96% (from 215% to 119%) compared with non-treated EAE group (n=6) ($P<0.001$). Similar readings were observed in both groups (day 7pi and day 12pi). The results from CYLA control and CFA control were similar to that obtained in naïve control group. The net values of are given as percentages compared to naïve control. Results of at least 3 individual assays from each group were compared by one-way ANOVA followed by Tukey post hoc test. The difference between treatments and control was considered significant at $P<0.05$.

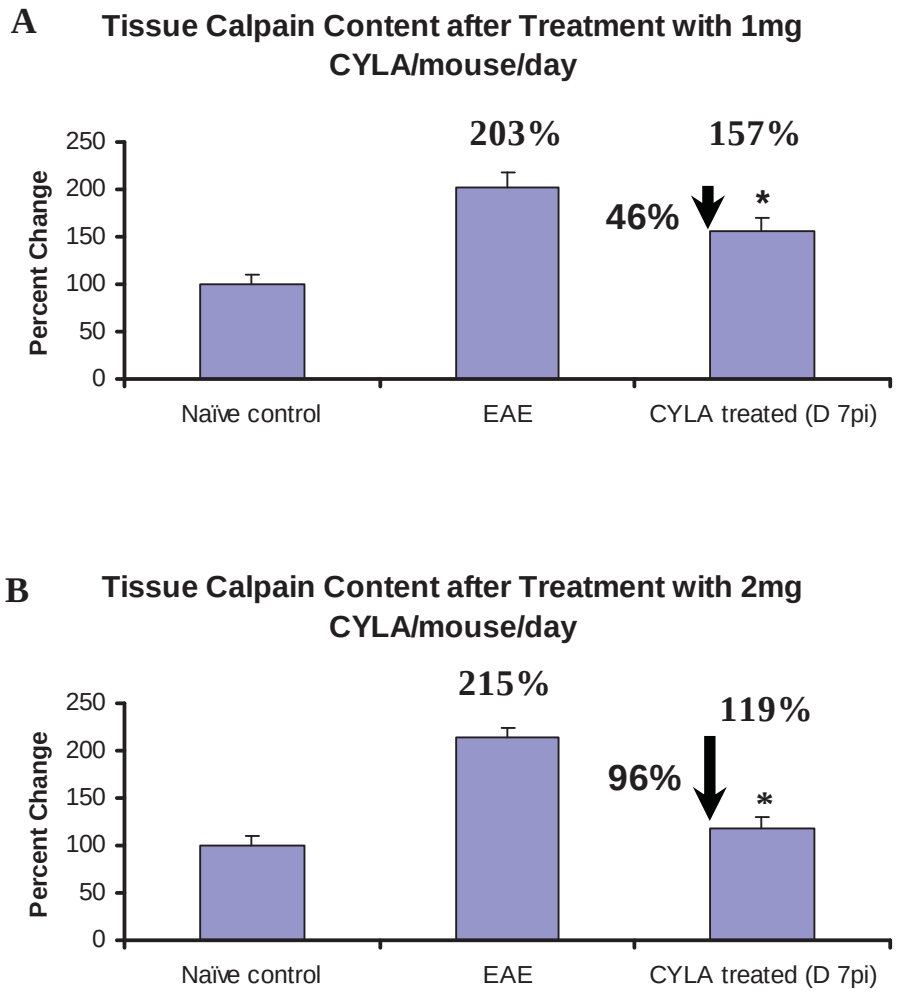


Figure 33 Calpain content as determined from the in vitro fluorogenic enzyme activity assay. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, (A) a total of 1mg/mouse/day (0.5mg per injection twice a day) or (B) a total of 2mg/mouse/day (1mg per injection twice a day) was applied i.p. Spinal cord homogenates were incubated with a calpain specific fluorescent substrate in the presence of activation and inhibition buffer separately. The net activity of calpain is obtained by subtracting the fluorescent units with the inhibitor from that of the activator. (A) The net values of are given as percentages compared to naïve control (n=4). Treatment with a total of 1mg/mouse/day (n=6) reduced the elevated total spinal cord calpain content by 46% (A). Treatment with a total of 2mg/mouse/day (n=6) reduced the elevated total spinal cord calpain content by 96% (B). The results of the other control groups [CFA (n=6) and CYLA (n=4)] were similar to that obtained in naive control groups. Similar results were observed from day 12pi groups (n=6). Data from three independent experiments was expressed as mean ± S.E.M. Differences in calpain content was compared by one-way ANOVA followed by Tukey post hoc test and was considered significant at $*P<0.05$.

B. Chronic EAE

1. Evaluation of neurological and body weight changes

We also studied the effect of the higher dose of CYLA in chronic EAE. All mice were observed for 30 days post immunization. After day 20pi, all EAE mice more or less maintained their clinical scores for the rest of the time they have been observed. Weight loss showed similar pattern, with progressive weight loss until day 20pi. EAE mice lost body weight during the first 20 days mirroring the neurological deficits and then gradually regained some weight during the last 10 days. The body weight of the control mice increases progressively and uniformly over time. Treatment CYLA starting from day 7pi and day 12pi arrested the progression of the disease. Comparison of the average weight and ACS of the groups using Wilcoxon two-sample test showed statistically significant difference with treatment using 2mg/mouse/day ($p<0.002$; Fig.34A and B).

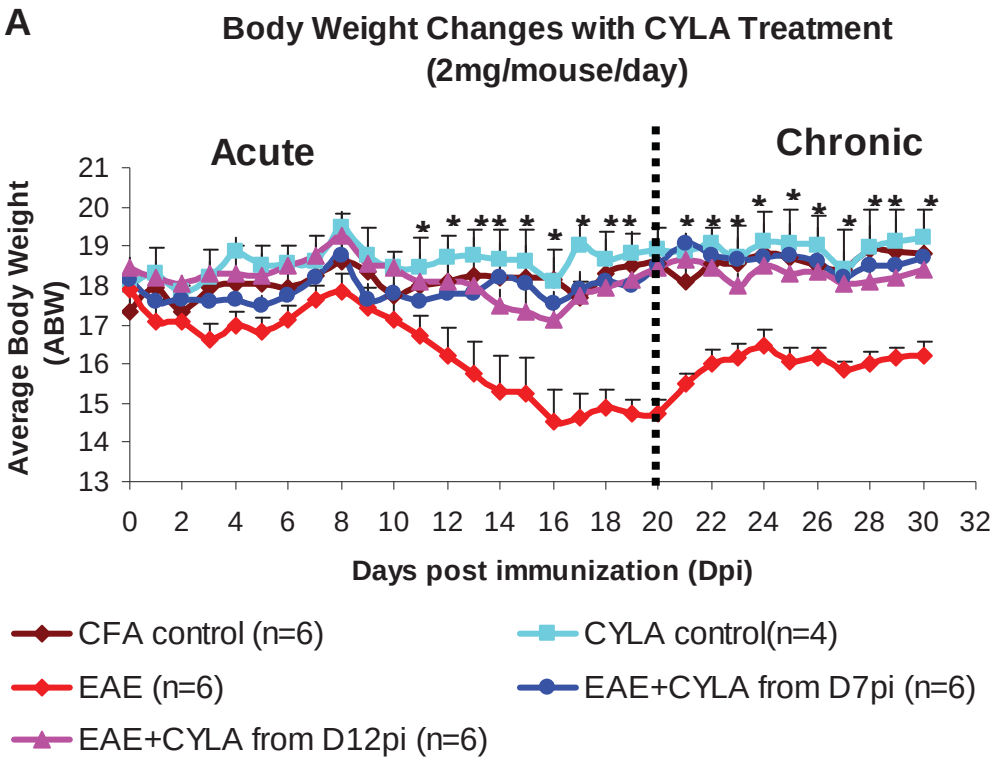


Figure 34A Changes in daily ABW of all groups over time during the course of treatment with a total of 2mg/mouse/day CYLA after induction of the disease. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. Starting from day 8pi EAE mice kept losing weight. Weight loss progressed until day 16pi and then EAE mice maintained their body weight until day 20pi. After day 20pi non-treated EAE mice gradually regained some weight until day 30pi. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). Treatment with a total of 2mg CYLA per mouse per day showed minimal weight loss followed by quick recovery in day 12pi group. Day 7pi groups and all control groups (naïve, CFA and CYLA) showed progressive weight gain through out the observation time. The experiment was terminated on day 31pi. Each graph presents daily ABW ± S.E.M. The difference between treatments and naïve control was considered significant at $*P<0.05$.

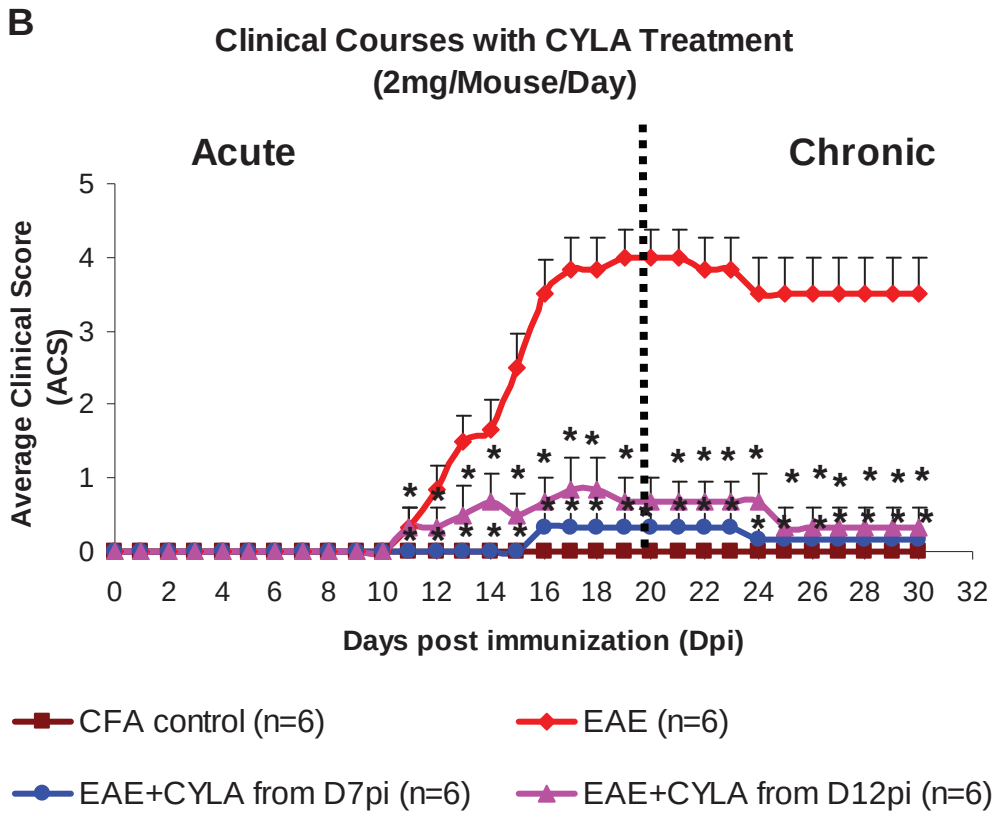


Figure 34B Changes in daily ACS of all groups over time during the course of treatment with 2mg CYLA after induction of the disease. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). Early neurological signs such as floppy tail and hind limb weakness appeared in general about day 12pi. Over the course of time mice exhibited hind limb paralysis and some weakness in the fore limbs between days 14-16pi. Their neurological deficit increased progressively in all EAE mice and reached its peak by day 20pi. After day 20pi until day 30pi EAE mice more or less maintained their ACS. Treatment with a total of 2mg CYLA per mouse per day starting from day 12pi led to suppression of the clinical signs to a significant level. Treatment with a total of 2mg CYLA per mouse per day starting from day 7pi showed a delayed onset and significant improvement of the clinical signs with suppression of the disease progression. All control groups (naïve, CFA and CYLA) showed no neurological deficit. The experiment was terminated on day 31pi. Each graph presents daily ACS ± S.E.M. The difference between treatments and naïve control was considered significant at $*P<0.05$.

2. Bielshowsky`s silver staining

Qualitative analysis for axonal injury assessed with Bielshowsky`s silver staining method. The naïve control groups showed linear staining patter with continuous axonal fibers without interruptions. In the spinal cord tissue of EAE mice the silver staining demonstrated extensive axonal injury with interruptions of the axons at areas corresponding to the lesions with extensive inflammation and demyelination (Fig.35B). The staining pattern of the CYLA treated mice exhibited preserved axonal morphology (Fig.35C), similar to that observed in the control group (Fig.35A).

A. Naïve control **B. EAE** **C. CYLA treated (day 7pi)**

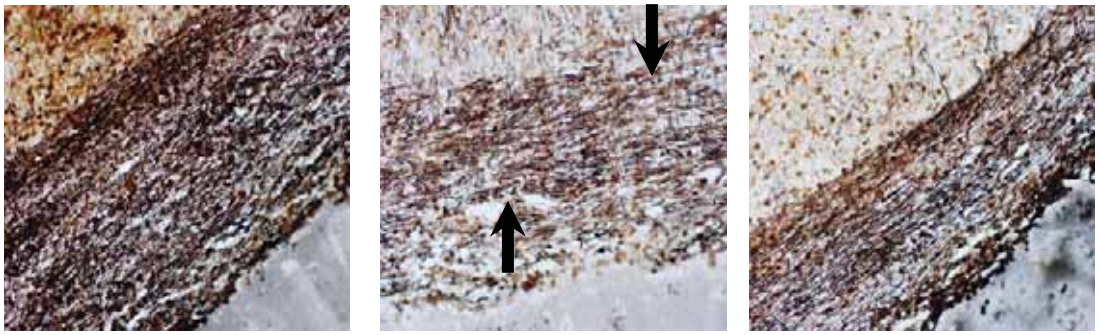


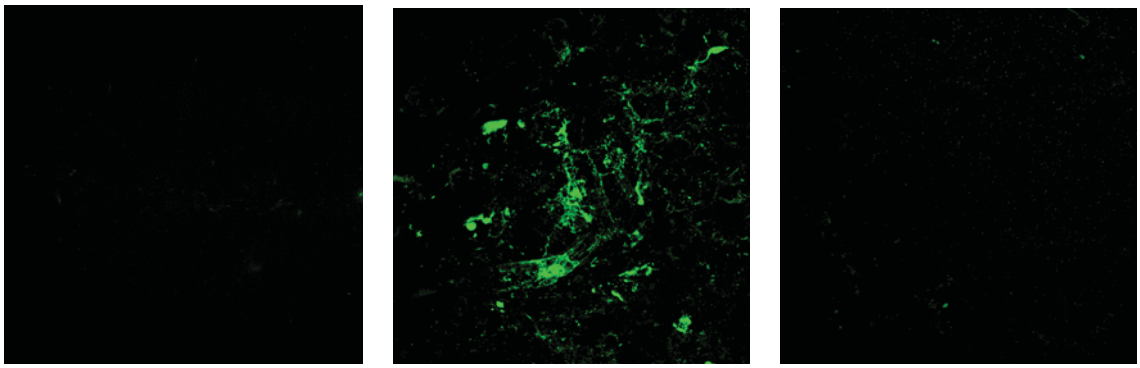
Figure 35 Qualitative assessment of axonal integrity of the lumbar spinal cord with silver impregnation method from all groups. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). Silver staining was performed on 6-10μm cryosections. The spinal cord sections of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] showed normal axonal morphology (A). Extensive axonal injuries (arrows) were visible in the spinal cords of chronic EAE mice (B). Preserved axonal morphology was observed after 2mg CYLA treatment (C). The staining for day 12pi group was similar to that observed in day 7pi groups. 20X magnification.

3. IF staining of spinal cord tissue

3.1 IF staining with anti-APP antibody

Qualitative assessment of axonal injury was measured using IF staining with antibody against APP. Accumulation of extensive APP was visible in non-treated mice spinal cord sections (Fig.36B), corresponding to the extensive axonal injury within the spinal cords of this group. APP was barely detectable in the CYLA treated group (Fig.36C). No APP staining was visible in all control groups (Fig.36A). Treatment with CYLA significantly reduced the accumulation of APP (Fig. 36D).

A. Naïve control **B. EAE** **C. CYLA treated (day 7pi)**



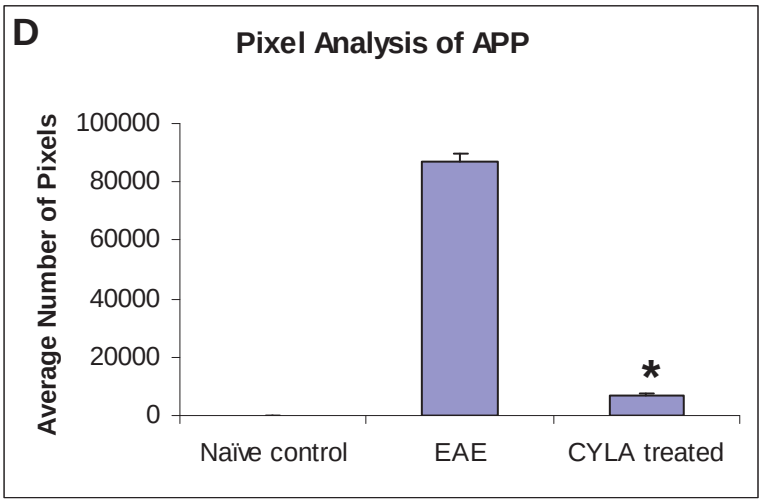
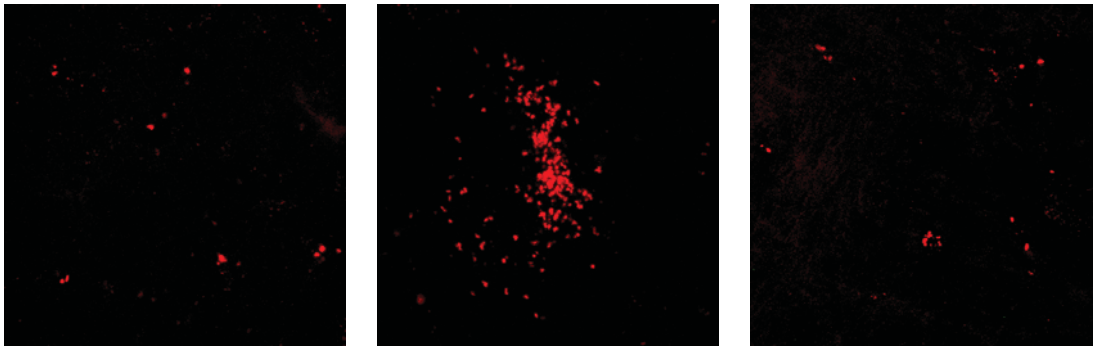


Figure 36 Evaluation of axonal injury with APP from control, EAE and CYLA treated groups. Disruption of axonal transport leads to accumulation of APP within the injured axons. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). IF was performed on 50μm cryosections. The spinal cord sections of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] showed no APP staining (A). Extensive accumulation of APP was visible in spinal cord of chronic EAE (B). APP staining in sections of CYLA treated (day 7pi group) was similar to control group (C). Similar result as in day 7pi groups was observed in day 12pi groups. The quantitative data is graphically represented through pixel analysis using image analyzing software ImageJ from NIH. Pixels were quantified as total number of pixels above background. Data was expressed as mean ± S.E.M. of 3 separate IF experiments (after scanning at least ≥9 slides each) and compared by one-way ANOVA followed by Tukey post hoc test. The difference between treatments and control was considered significant at $*P<0.05$ (D). 120X magnification with oil.

3.2. IF staining with anti-Na_v 1.6 antibody

Staining for Na channels demonstrated increased expression and abnormal distribution of Na_v1.6 channels (clustered at areas of axonal injury) in non-treated EAE mice (Fig.37B) compared to normal scattered distribution of these subtypes of Na channels in CYLA treated (Fig.37C) and control groups (Fig.37A). Concomitantly, there is increased staining for calpain within the neurons in areas of axonal injury. Treatment with CYLA significantly reduced the expression of Na_v1.6 channels (Fig. 37D).

A. Naïve control **B. EAE** **C. CYLA treated (day 7pi)**



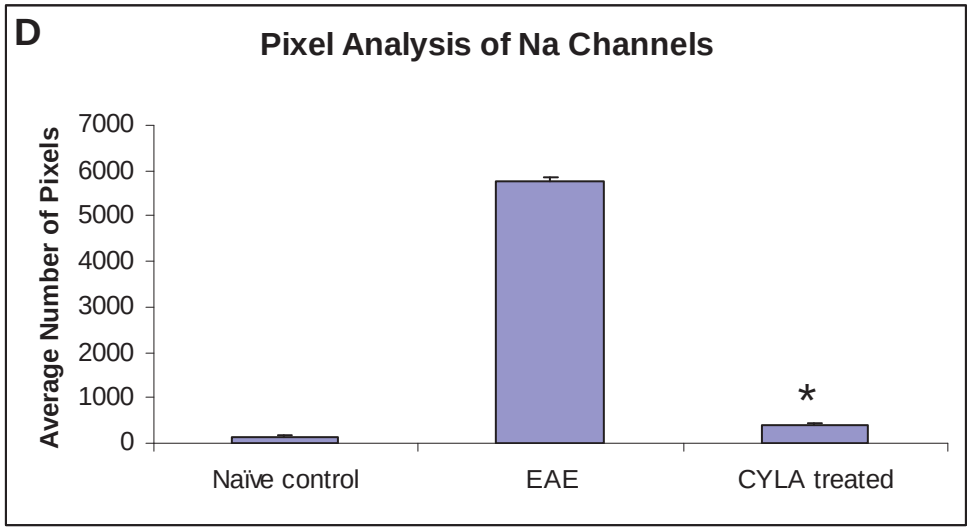
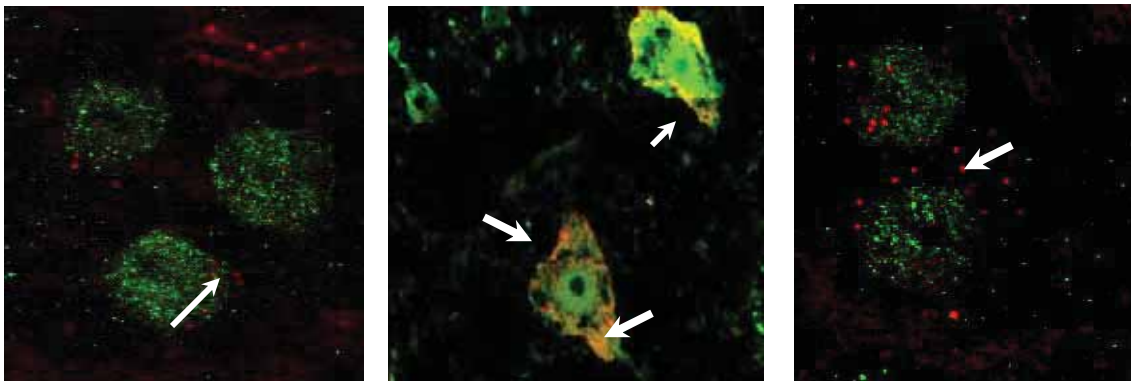


Figure 37 Changes in $Na_v1.6$ expression in the axons of lumbar spinal cord from control, EAE and CYLA treated groups. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). IF was performed on 50μm cryosections. The spinal cord sections of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] showed normal scattered distribution of $Na_v1.6$ channels corresponding to the areas of node of Ranvier (A). Increased and clustered $Na_v1.6$ channels in areas of demyelination in EAE mice spinal cord. Similar expression of $Na_v1.6$ channels in spinal cords of treated mice as in control group (D). The results from day 7pi and day 12pi groups were similar. The quantitative data is graphically represented through pixel analysis using image analyzing software ImageJ from NIH. Pixels were quantified as total number of pixels above background. Data was expressed as mean ± S.E.M. of 3 separate IF experiments (after scanning at least ≥ 9 slides each) and compared by one-way ANOVA followed by Tukey post hoc test. The difference between treatments and control was considered significant at $*P<0.05$ (D). 120X magnification with oil.

3.3. IF staining with antibodies against calpain and NeuN

Changes in calpain content, within the neurons and infiltrating and resident mononuclear phagocytes of the spinal cord sections were evaluated using IF labeling with anti-calpain antibody, NeuN (neuronal marker) and cell surface markers (Fig.38). There was no appreciable staining for inflammatory cells and any significant staining for calpain in the naive groups (Fig. 38A). By contrast, increased calpain staining within NeuN positive cells and less calpain in the inflammatory cells were observed (Fig. 38B). Treatment with CYLA significantly decreased the calpain content within NeuN positive cells (Fig. 38C-D), corresponding to the lower ACS.

A. Control **B. EAE** **C. CYLA treated**



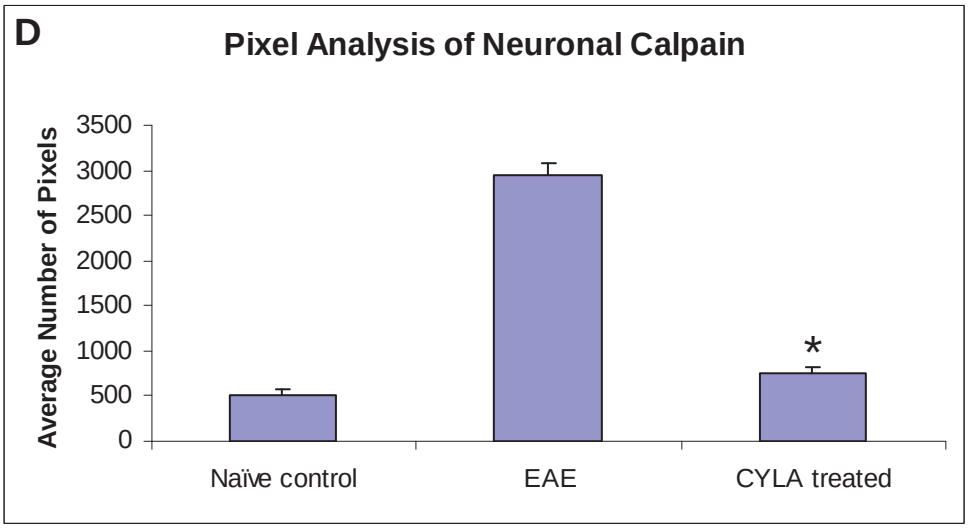


Figure 38 Double Fluorescent staining with NeuN of spinal cord sections to identify the distribution f calpain within the neurons in chronic EAE. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). IF with NeuN (neuronal marker) and calpain was performed on 50μm cryosections. Normal calpain distribution was visible as red dots within NeuN positive neuron of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] (A). Increased calpain expression was observed within NeuN positive neurons of EAE mice (B). The calpain distribution within NeuN positive spinal cord neurons of CYLA treated mice (day 7pi) was similar to control groups (C). The results of day 7pi and day 12pi groups were similar. The quantitative data is graphically represented through pixel analysis using image analyzing software ImageJ from NIH. Pixels were quantified as total number of pixels above background. Data was expressed as mean ± S.E.M. of 3 separate IF experiments (after scanning at least ≥ 9 slides each) and compared by one-way ANOVA followed by Tukey post hoc test. The difference between treatments and control was considered significant at $*P<0.05$. 120X magnification with oil.

4. Western blot analysis

4.1. Western blot analysis of Na_v 1.6 Channel Expression

The expression of $Na_v1.6$ channels were also evaluated using western blotting method. The results demonstrated that there was increased expression of this type of sodium channels in EAE mice (B, increased by 153% compared to (A) naïve control) and treatment with CYLA significantly reduced the expression of this channel in the CYLA treated group (C-D, Fig. 39).

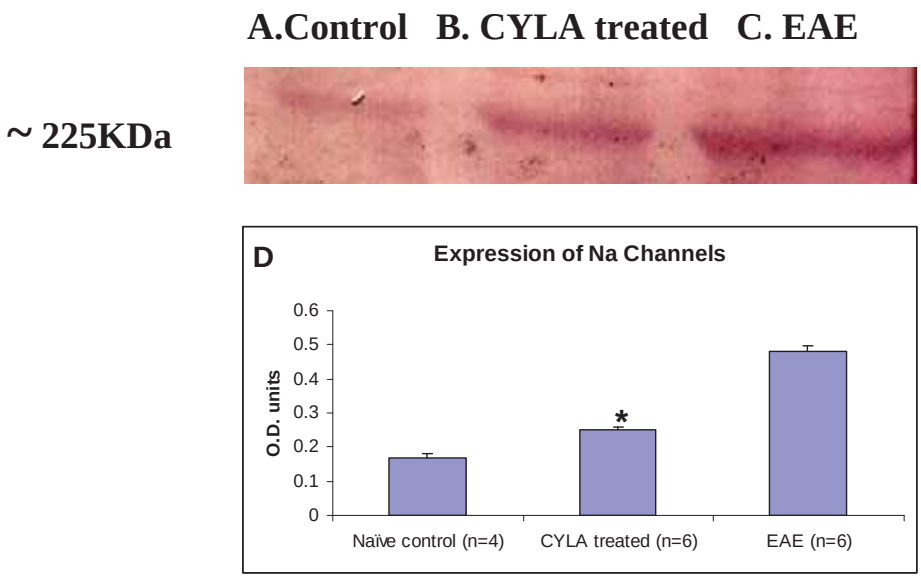


Figure 39 Determination of $Na_v1.6$ channel expression in spinal cord homogenates of naïve control (n=4), EAE (n=6) and 2mg/mouse/day CYLA treated group (day 7pi, n=6). EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). Spinal cord segments were quickly homogenized and used for this assay. Representative Immunblot showing the degree of increased Na^+ 1.6 channel expression in naïve control (A), EAE (B) and CYLA treated mice (C). The spinal cord sections of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] showed similar results. The results of day 7pi (n=6) and day 12pi (n=6) were similar as well. Densitometric analysis of the bands quantifies the degree of expression of this particular Na channel. Data from three independent assays is expressed as mean ± S.E.M. The difference between treatments and control was

compared by one-way ANOVA followed by Tukey post hoc test and was considered significant at *$P<0.05$ (D).

4.2. Western blot analysis of spectrin degradation by calpain

We observed the calpain activity by detecting products of substrate degradation using western blotting techniques. Calpain degrades the 230-kDa subunit of the cytoskeletal protein alpha-fodrin to yield a 150-kDa calpain-cleaved fodrin breakdown fragment that can be detected by the specific antibody using 50μg total protein (Fig. 40). The results of the western blot analysis showed that there was substantially increased degradation of alpha-fodrin in the non-treated EAE mice (C, n=6) [5 fold increase in EAE compared to naïve control (A, n=4)]. The treatment with CYLA (2mg/mouse/day) supressed significantly the degradation of alpha-fodrin in the spinal cords of the treated group (B, n=6) ($p<0.05$, Fig. 40). Slightly increased optical density (O.D.) was observed in the groups that were treated with 1mg CYLA (data not shown). The results obtained from the two treatment groups (day 7pi and day 12pi) were similar.

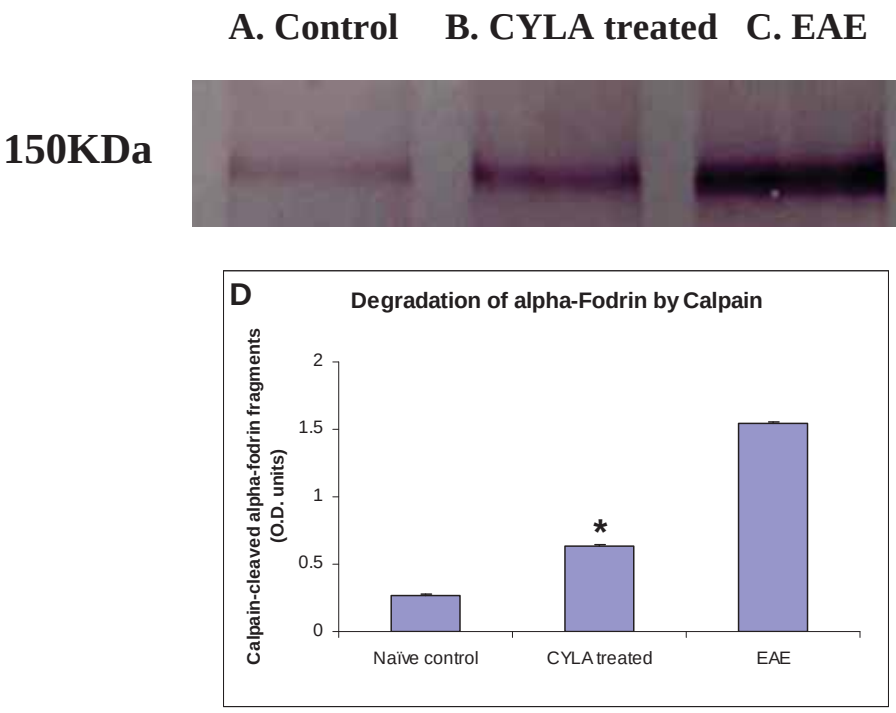


Figure 40 Determination of alpha-fodrin proteolysis as indirect measure of calpain activity in spinal cord homogenates of naïve control (n=4), EAE (n=6) and 2mg/mouse/day CYLA treated group (day 7pi, n=6). EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). Spinal cord segments were quickly homogenized and used for this assay. Representative Immunblot showing the degree of alpaha fodrin degradation in naïve control (A), EAE (C) and CYLA treated mice (B). The spinal cord sections of all control groups [naïve (n=4), CFA (n=6) and CYLA (n=4)] showed similar results. The results of day 7pi (n=6) and day 12pi (n=6) were similar as well. Densitometric analysis of the bands quantifies degradation of calpain-cleaved α-fodrin fragments. Data from three assays is expressed as mean ± S.E.M. The difference between treatments and control was compared by one-way ANOVA followed by Tukey post hoc test and was considered significant at $*P<0.05$ (D).

VI. Discussion

CYLA is a newly designed targeted calpain inhibitor. In this project, we investigated the effect of CYLA in ameliorating and/or suppressing the clinical signs and symptoms of EAE, a well-known and widely used animal model of MS. The effect of CYLA was investigated in a dose- and time-dependent manner as well in its different forms, free aldehyde (intraperitoneal) or its prodrug, diacetal form (oral). In addition, the effect of CYLA on inflammation, demyelination, CNS calpain content as well as activation of brain resident glia cells in acute and chronic forms of EAE was studied. Furthermore, we evaluated the effect of CYLA in suppressing axonal injury, the major correlate of permanent disability, in chronic EAE. The results of the experiments clearly demonstrated improvement of the clinical courses, clinical severity, improved histopathology and suppression of axonal injury.

In a series of experimental settings CYLA was applied in increasing doses starting with 0.5mg/mouse/day. The different doses of CYLA were applied to different groups at two different time point during the disease course. We chose the first time point to be shortly before the onset of the clinical signs of the disease (day 7pi) and the second time point was chosen at the onset of the clinical signs (day 12pi). These two time points were chosen for the following reasons: (1) if CYLA shows a better outcome when applies before the onset of the clinical signs, it could be used for the treatment of relapsing-remitting MS, during the remission period, in order to prevent the subsequent relapses; (2) if CYLA shows better outcome when applied after the onset of the clinical signs, it could be used for the treatment of acute disease exacerbation or it could be given with

other currently used anti-inflammatory drugs to influence the course of the acute flare-up and the subsequent disease progression.

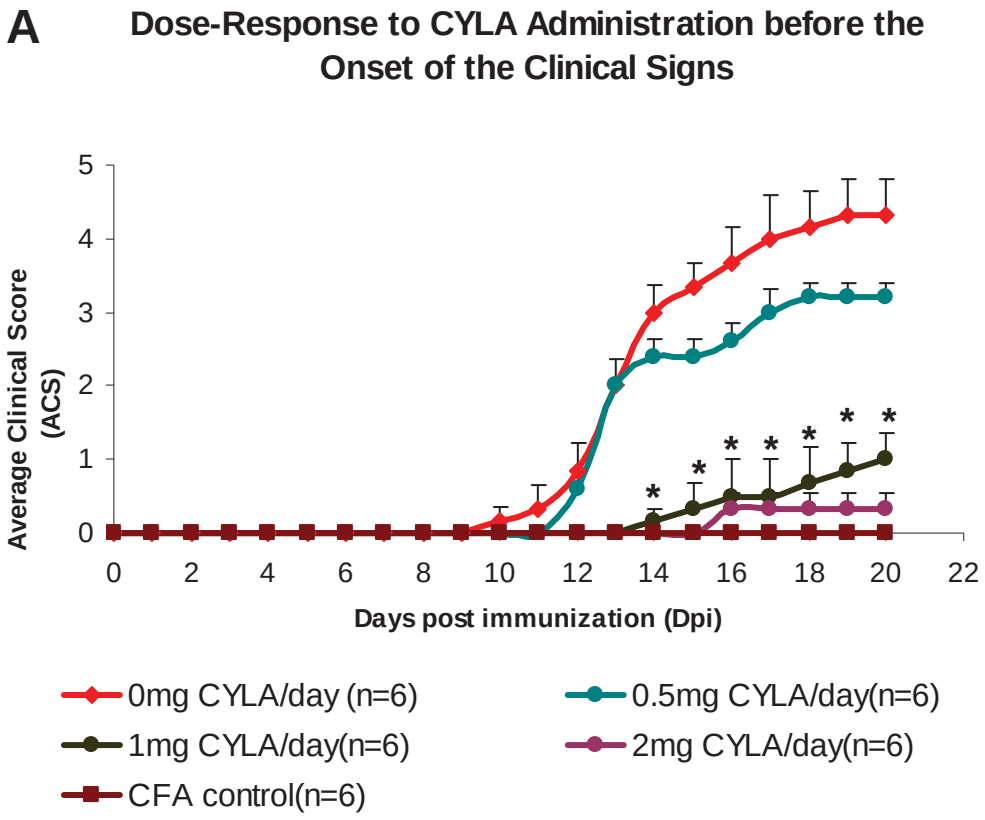
Our results from these series of experiments showed that application of 0.5mg CYLA/mouse/day did not show any improvements of the clinical signs when applied at both time points.

Increasing the dose to 1mg CYLA/mouse/day showed significant improvement of the clinical signs only when administered before the onset of the clinical signs, starting from day 7pi. Administration of CYLA starting from day 12pi after the onset of the clinical signs showed a minimal improvement of the ACS. The disease increased in severity and reached the average clinical score (ACS) of non-treated groups. Improvement of the clinical course with suppression of the disease was observed only when the 1mg/mouse/day dose was applied starting from day 7pi (before the onset of the clinical signs). This treatment regimen delayed the disease onset (disease onset on day 14pi as compared to disease onset in non-treated groups on day 12pi) and the progression of the disease as evidenced by the lower ACS (Fig. 41).

Increasing the dose to a 2mg/mouse/day showed a significant decrease in neurological deficit as evidenced by the lower ACS as well as suppression of the progression of the disease regardless of the time point of CYLA administration. The neurological deficits were very minimal and the disease progression was also suppressed in both therapy regimens (Fig. 41).

In summary, from these experiments it could be concluded that the effect of CYLA in the acute phase of EAE is both dose-and time-dependent. A higher dose of CYLA was necessary to achieve most improvement of the disease when applied at the

onset of the clinical signs. Medium dose of CYLA (1mg/mouse/day) achieved significant improvement of the clinical sign only when applied before the onset of the clinical signs (Fig. 41A and B).



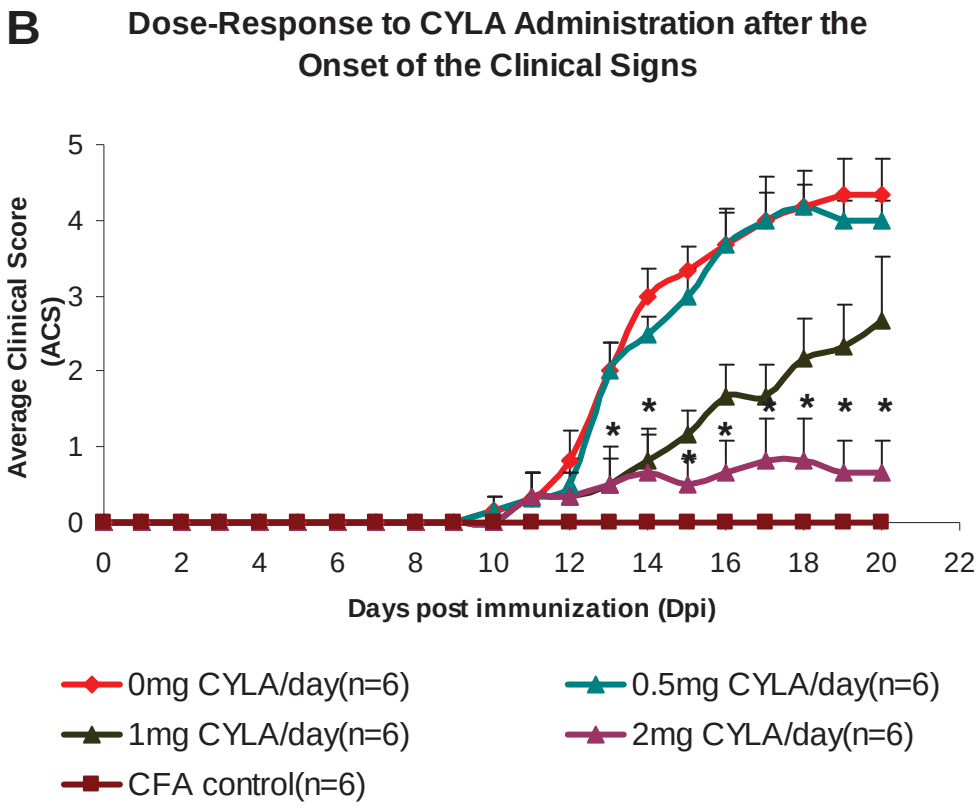


Figure 41A and B Time-and dose-dependency after intraperitoneal administration of CYLA (A) Dose-dependency of intraperitoneal CYLA treatment when applied before the onset of the clinical symptoms Treatment with CYLA starting from day 7pi before the onset of the clinical signs showed significant improvement of the clinical symptoms when CYLA is applied at a dose of 1mg a day. (B) Treatment starting from day12pi after the onset of the clinical signs showed significant improvement of the clinical symptoms only after administration of CYLA at a dose of 2mg a day. The response to the increasing CYLA doses showed a dose dependency in addition to a time dependency.

The weight changes in the disease course in EAE has been associated with the increased leptin production from the inflammatory cells (Sanna et al., 2003). Leptin is a 16KDa adipokine that is produced and secreted under physiological conditions by adipocytes and many other tissues. It links nutritional status with neuroendocrine and immune functions. Leptin regulates inflammatory and immune responses by especially

affecting T-cells (Lord et al., 1998; Kuchroo and Nicholson, 2003; Otero et al., 2005). The change in body weight have been shown to correlate with the inflammatory response and the accompanying leptin surge as a result of the increased inflammatory cells (Sanna et al., 2003). Inhibition of calpain that leads to suppression of the inflammatory reaction appears to be a possible factor for the changes in body weight associated with the inflammatory response.

Of great interest is the observation that the orally administered diethyl acetal prodrug is as effective as the free aldehyde injected intraperitoneally (Fig. 28). The diacetal is not capable of calpain inhibition in vitro and must be treated with acid in order to release the free aldehyde (Fig. 42). This suggests that some metabolic pathways must be capable of converting the diacetal prodrug into an active aldehyde form in vivo or it appears that the HCL in the stomach of these mice is capable of cleaving the prodrug after oral administration. Further studies attempting to define this reaction are necessary.

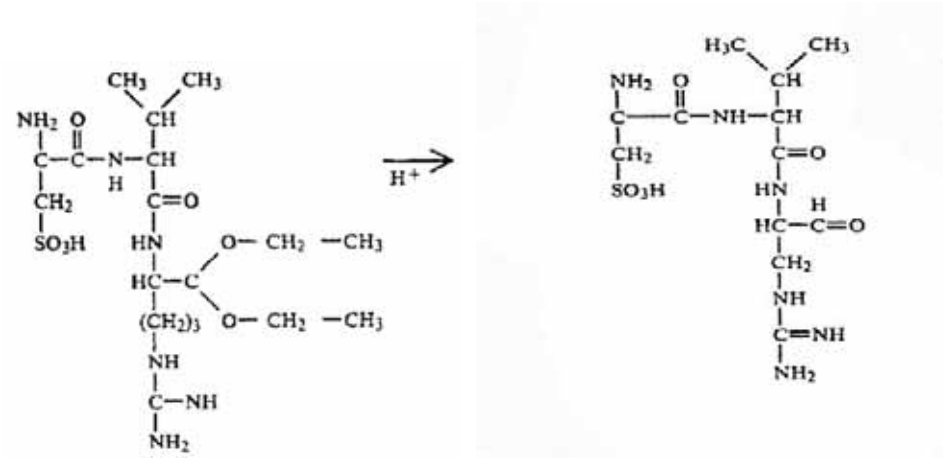


Figure 42 Steps in converting the diacetal prodrug into its active form, deacetylated CYLA, prior to ip administration. The prodrug is cleaved in to its active form using HCl in vitro. The free aldehyde is the active protease inhibiting part of the drug.

After the end of each independent study, tissues were collected (on day 21pi for acute EAE and on day 31pi for chronic EAE) to study the correlation of the clinical signs of EAE with histopathological findings. Different histopathological methods were used to study tissue inflammatory infiltration, demyelination, and calpain content including calpain activation.

H&E was done to evaluate the normal cellularity and inflammatory infiltration of the CNS tissue (Fig. 29). In general, very little cellular infiltration takes place prior to the onset of the neurological deficit. The infiltration of the inflammatory cells in to the CNS did not appear until day 9pi. The infiltration was present in animals with grades of at least stage one. It has been reported that mononuclear cells that infiltrate the CNS tissue around day 9pi showed qualitative increase in calpain expression relative to the cells of day 8pi (Schaecher et al., 2002). In the above mentioned experiment, animals exhibited clinical signs of the disease starting from day 9pi, unlike in our experimental groups symptoms appeared around day 11-12pi. The greatest cellular infiltration and increased calpain expression is observed on days 10-11pi. There might be a minor discrepancy in the time course of cellular infiltration and calpain expression following EAE induction in our experimental setting compared with that reported above. This might have its origin in the type of antigen our animal used for the experiment. It is well-known that the course of the disease differs depending upon the strain and the antigen used (Fuller et al., 2004; Rao and Segal, 2004). The treatment with CYLA decreased the cellular infiltration into the CNS to a greater extent following the challenge with the MOG peptide. The degree in which the cellular infiltration decreased correlated with the amount of CYLA used. The

higher the dose of CYLA used to treat the mice, the lesser the cellular infiltration observed in the CNS tissue.

The extent of demyelination was evaluated with the basic dye luxol fast that stains for myelin (Fig. 30). Observations from serial studies of EAE mice showed that minor demyelination occurred at the onset of the clinical symptoms. It progresses with the increased infiltration and calpain expression (Schaecher et al., 2002). The treatment with CYLA reduced the demyelination. EAE mice that received no treatment exhibited extensive demyelination with increased vacuolation of the white matter in concentric areas where the cellular infiltration was the greatest. CYLA was capable of reducing the infiltration of the inflammatory cells into the CNS and calpain expression within the infiltrating cells, which resulted in less myelin damage and better outcome of the treated animal groups.

Over the period of time that the mice were observed, we did not observe any weight loss or neurological impairment in the CYLA control group. The weight change pattern and the clinical scores showed similar results as those of the naïve controls. We also conducted histological evaluation of the spinal cords of this group of animals and did not observe any abnormality. We believe the drug is not toxic at least for the doses we used during the course of the treatment. From this we believe that there is room for increasing the CYLA doses which might lead to a complete suppression of the disease onset or total recovery from the disease. More tests are required to determine the effect of CYLA in terms of toxicity studies.

The idea for the dose response is that we are trying to get more CYLA to inhibit enough calpain so that the subsequent inflammation could be suppressed. In the initial

phase of the disease a small amount of autoreactive T-cells cross the BBB and initiate the disease(Karin et al., 1993; Brocke et al., 1996). This leads to the degradation of myelin and recruitment of more inflammatory cells from the periphery, leading to sever injuries (Kawakami et al., 2004; Kawakami et al., 2005) . This phenomenon was also reflected in our experiments in which a better outcome was observed when CYLA was given in higher doses before the onset of the clinical signs. CYLA was less effective when administered in lower doses and after the onset clinical signs. This may mean that we inhibit much less calpain than is present in the tissue and the degradation of myelin and the subsequent recruitment of inflammatory cells is kept going.

The role of microglia in demyelination has been investigated and their role in MS has been extensively studied (Ford et al., 1995). It has been reported that microglia become a competent antigen presenting and effector cell that contribute to the inflammation of the CNS tissue in MS and animal models of MS. Their activation and proliferation occurs before the onset of the clinical signs and the infiltration of the CNS with activated lymphocytes and blood-born macrophages (Mack et al., 2003; Ponomarev et al., 2005).

Their activational status has been investigated by means of surface markers and cytokine and protease production. Some of the activation markers that have been studied are ICAM1, MHC-II molecules, B7-1, B7-2 and CD40 (Mack et al., 2003). It has been reported that freshly isolated microglia from naïve C57BL6 mice express low levels of ICAM, CD40 and B7-2 immediately ex vivo, but failed to express B7-1 and MHC-II molecules and were not competent APCs for T-cells (Carson et al., 1998). The possibility of discrepancy for expression of activation markers and significant differences in

constitutive expression of antigen-presenting molecules between different mouse species was reported (Carson et al., 1998). During inflammatory settings triggered either by antigenic peptides or viral infection, microglia and peripheral macrophages, increase the expression of the above mentioned activation markers (Mack et al., 2003). The suppression of these activation markers detected using Fluorescence-activated cell sorter (FACS), can give an indirect clue for the suppression of the disease progression using therapeutic agents such as CYLA in our case.

The FACS data showed increased expression of the activation markers from cells isolated from EAE mice while cells isolated from CYLA treated mice showed significantly reduced expression of the activation markers (data not shown). This finding further indicates that the treatment with CYLA reduced tissue damage, which in turn leads to fewer antigens that could be processed and presented. The fewer antigens are, in part, the result of calpain inhibiton and decreased tissue destruction.

Immunofluorescent staining was performed to analyze changes in calpain content, inflammatory cell infiltration and calpain expression within the inflammatory cells (Fig. 31 and 32). Tissues from EAE mice demonstrated increased calpain content, activated peripheral inflammatory cells and increased activated microglia within the lesion (Fig. 31F-J). Treatment with CYLA reduced the tissue calpain content and the inflammatory infiltration. In addition to the reduced infiltration with inflammatory cells, there were less calpain within these cells (Fig. 31K-O).

Changes in tissue calpain content under CYLA treatment was studied using a fluorogenic in vitro calpain assay. The overall increase in tissue calpain reflected the severity of the disease. The higher the ACS of the mice, the higher the overall tissue

calpain content as evidenced by the increased substrate degradation and increased fluorescence in the in vitro assay. The suppression of the calpain production/activation corresponded to the doses applied during the experiments. A 1mg CYLA/mouse/day doses reduced the elevated calpain content within the spinal cord tissue by 46% whereas 2mg CYLA/mouse/day reduced the tissue calpain content by 96% (Table 7).

Days after MOG 35-55 Challenge	ACS (n=6)	CYLA Dose (n=6)	% Calpain content	% Reduced	P-value
21	2.83 ± 0.74	1mg/mouse/day	203	46	0.05
21	4.00 ± 0.17	2mg/mouse/day	219	96	0.001

Table 7 Inhibition of calpain by CYLA showed dose-dependency. Calpain content as determined from the in vitro fluorogenic enzyme activity assay. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 1mg/mouse/day (0.5mg per injection twice a day) or a total of 2mg/mouse/day (1mg per injection twice a day) was applied i.p. twice a day. Spinal cord homogenates were incubated with a calpain specific fluorescent substrate in the presence of activation and inhibition buffer separately. The net activity of calpain is obtained by subtracting the fluorescent units with the inhibitor from that of the activator. (A) The net values of are given as percentages compared to naïve control (n=4). Treatment with a total of 1mg/mouse/day (n=6) reduced the elevated total spinal cord calpain content by 46%. Treatment with a total of 2mg/mouse/day (n=6) reduced the elevated total spinal cord calpain content by 96%. Similar results were observed from day 12pi groups (n=6). Data from three independent experiments was expressed as mean ± S.E.M. Differences in calpain content was compared by one-way ANOVA followed by Tukey post hoc test and was considered significant at $*P<0.05$.

The results of this assay demonstrated that CYLA treatment reduces the elevated calpain content of the CNS tissue significantly for the higher doses (Fig. 33; Table 7). However, it could not reduce the calpain content to a normal level. Possible reasons for this result are the following: 1. the amount of CYLA used was not enough to suppress the increased calpain in the inflamed tissue; 2. CYLA is an active site-directed inhibitor, so it binds and inhibits the active form of calpain. Since the inflammatory cells express a lot of

calpain during the disease state and possibly not all calpain is active at the same time, CYLA may not inhibit the entire calpain produced by the inflammatory cells. The double immunofluorescent staining supported this assumption by demonstrating the two different states of calpain (membrane bound active and cytosolic inactive; Fig. 43 and Fig. 31J).

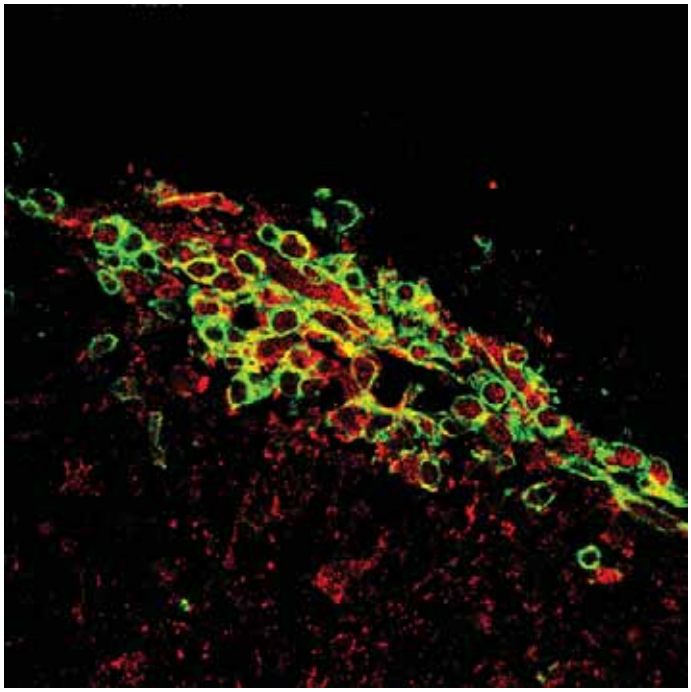


Figure 43 Colocalization of calpain within the membranes of the inflammatory cells as well as within these cells. Double IF staining with CD45 and calpain was performed on 50μm cryosections of spinal cord sections to identify the distribution f calpain within the cells (green). EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection twice a day) was applied i.p. The membrane bound form of calpain is believed to be the active form of the enzyme (yellow color), while the non active form is the one inside the cells (red dots) inside the green labled cells.

Two different mechanism of calpain activation have been proposed (Molinari and Carafoli, 1997). The second hypothesis states that the increased cytosolic Ca^{2+}-ion leads to the dissociation of calpain from its natural inhibitor calpastatin and translocates

towards the membrane. The activation of calpain occurs at the membrane of the inflammatory cells in the presence of calpain activating proteins and membrane phospholipids (Molinari and Carafoli, 1997; Ray and Banik, 2003). In our in vitro calpain activity assay as an indirect measure of the tissue calpain content of all of the tissue calpain present in the tissue at the time of the assay is being activated artificially, thus yielding more calpain activity as measured by the free fluorescent released from the substrate than in vivo, however the enzyme assay is performed under idealized conditions and does not necessarily reflect the absolute in vivo situation. Thus, calpastatin and phospholipid interactions are not taken into account as well as the in vivo calcium concentration, all of which influence calpain activity.

Calpain is one of the proteolytic enzymes that are upregulated during the disease process in MS and EAE (Shields et al., 1998). It degrades a variety of myelin proteins and is believed to contribute to the subsequent inflammatory reaction following induction of EAE using known myelin peptides or peptide components in a susceptible animal strain (Shields et al., 1998). Calpain degrades myelin components within the CNS releasing more encephlitogenic peptides that promote the inflammatory reaction leading to the progression of the disease process (Shields et al., 1998). Thus, calpain has been a target in the treatment of those disease states that involve abnormal and prolonged calpain activation. Scientists have tried to develop many calpain inhibitors and tested in an attempt to make calpain inhibitors available for therapeutic use. One limiting factor has been the inabilities of the current calpain inhibitors to cross the BBB.

CYLA is not specific for calpain; it most likely inhibits other cysteine proteases such as cathepsin B, just as leupeptin and other leupeptin derivatives inhibit cathepsin B

in vitro (Hashida et al., 1980; Kominami et al., 1980). Although, as a derivative of leupeptin, CYLA has the potential to inhibit cathepsin B, in vitro results may not reflect the in vivo situation. It has been shown that leupeptin showed a paradoxical effect on cathepsin B when tested in vivo (Sutherland and Greenbaum, 1983). Administration of leupeptin in the experiment conducted by Sutherland et al. increased the cathepsin B activity to different levels in different organs including heart, kidney, liver and skeletal muscle. We assume that similar results might happen in the CNS as well, since CYLA crosses the BBB and inhibits cathepsin B. However, if cathepsin B was upregulated within the CNS by CYLA in vivo, it would have resulted in more tissue damage, which clearly did not take place in this case. Our experiments showed improvement of EAE signs during the course of treatment with CYLA. Cathepsin B may not have been activated in vivo by CYLA. Furthermore, inhibition of calpain was more effective in prevention of myelin degradation when compared with the inhibition of other proteases in vitro (Deshpande et al., 1995; Smith et al., 1998). Thus, we believe that the improvements of EAE signs in the treated group most likely resulted from calpain inhibition, further strengthening our hypothesis that the inhibition of calpain is responsible for the amelioration and suppression of EAE.

In addition, cathepsin B is involved in protein degradation and antigen processing within the lysosomes (Nakanishi, 2003). In case of MS inhibition of cathepsin B and suppression of the antigen processing appears to be of important to prevent disease onset and progression. Inhibition of cathepsin B, if it occurs in vivo, might be desirable and could bring additional effects in ameliorating MS.

We further investigated the role of CYLA in suppressing axonal injury in chronic EAE. Our results from the chronic EAE study demonstrated that the long term application of CYLA significantly reduced the clinical disability, the inflammatory response and prevented axonal injury as compared to the non-treated groups. Compared to control groups, there was significant axonal injury in the EAE mice as evidenced by the silver staining evaluation of the morphological changes within the axons (Fig. 35). CYLA treated mice exhibited staining patterns similar to that observed in control group. Furthermore, we analyzed the tissues of all animal groups with APP, a well-known marker for early axonal injury. In the acute phase of the disease damage to the axons leads to impairment of axonal transport. This leads to the accumulation of some neuronal markers of axonal injury within the axon. Results from the APP-staining method clearly showed an extensive accumulation of this marker in areas of axonal injury in EAE mice (Fig. 36). The control group and CYLA treated group showed no significant APP staining. These results are consistent with results from other group groups studying axonal injury in EAE (Gilgun-Sherki et al., 2003b; Waxman et al., 2004). This result further indicate that there is a disturbance of axoplasmatic transport as a result of cytoskeletal injury witin the axons that had led to the accumulation of markers that are normally transported to the axonbulbs.

In addition, the increased expression of a specific type of sodium channel was observed on the western blot analysis. There was a 153% increase in sodium channel expression in spinal cord tissue of EAE mice compared to controls (Fig. 39). The IF staining with anti-$Na_v1.6$ antibodies demonstrated increased expression and abnormal distribution of these specific type of Na^+-channels in areas of demyelination of EAE mice

spinal cord cross sections (Fig. 37). The increased APP accumulation was observed at areas of increased $Na_v1.6$ channel expression, supporting the hypothesis that axonal injury occurs at areas of demyelination with underlying injured axons. The results of our study from the chronic EAE suggest that axonal injury is the result of increased expression and abnormal distribution of $Na^+1.6$ channels in areas of demyelination and and the subsequent increased expression and activation of calpain within the neurons that causes degradation of neuroskeletal structural proteins that apparently leads to increasing dysfunction and permanent disability.

We also made interesting additional observations regarding the distribution of calpain. In the acute EAE, we observed that calpain was increasingly expressed in the white matter of the spinal cord tissue within the inflammatory cells. The distribution of calpain within the neurons was similar with all groups as evidenced by the similar expression of calpain within NeuN positive cells. In contrast, in tissue sections of chronic EAE mice calpain was increasingly expressed within NeuN positive cells compared to control and CYLA treated mice tissues (Fig. 38B). This result is consistent with the proposed mechanism of calpain action in the pathophysiology of axonal injury in the chronic phase of MS and chronic EAE. The increased expression of $Na_v1.6$ channels in areas of demyelination leads to the increased influx of Ca^{2+}-ions into the axons. This process could lead to increased expression of calpain and prolonged action resulting in axon injury, the ultimate determinant of permanent disability (Fig. 44 and 40).

Over time axonal injury accumulates behind closed BBB in the chronic forms of MS, especially in RRMS where repeated flare ups lead to permanent neurological disability in the long term (Petzold et al., 2005).

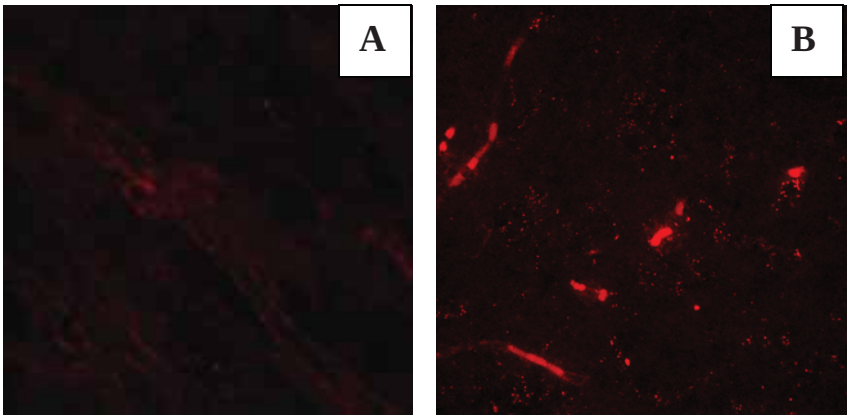


Figure 44 Immunofluorescent staining of a spinal cord section with anti-calpain antibody. Distribution of calpain within the axon in naïve control mice. EAE was induced in C57/Bl6 mice with MOG/CFA emulsion. CYLA, a total of 2mg/mouse/day (1mg per injection was applied i.p. twice a day). IF was performed on 50μm cryosections. Normal axonal morphology and normal distribution of calpain with clearly defined axonal tracts (red) was visible as red dots along axons of all control mice spinal cords [naïve (n=4), CFA (n=6) and CYLA (n=4)] (A). Increased calpain expression was observed along and within axonsof EAE mice. EAE mice exhibited extensive axonal calpain and disrupted axonal morphology as a result of injury to axoskeltal structures (B). The calpain distribution within the axons of CYLA treated mice (day 7pi) was similar to control groups. The results of day 7pi and day 12pi groups were similar.

The striking feature of CYLA in crossing the BBB, in addition to its calpain inhibitory effect, makes it a very suitable candidate in the suppression or reduction of axonal injury especially in the RRMS or chronic MS forms. Since all above mentioned processes happen behind closed BBB, applying a drug that is capable of crossing the BBB and inhibits CNS is of greater importance in treating this chronic disabling disease. RRMS represents the disease course of the majority of MS patients (80%-90%). In RRMS patients experience clearly defined flare ups followed by partial or complete recovery (Kieseier and Hartung, 2003).

One key factor for the tissue damage and the release of endogenous peptide antigen is calpain. Thus, calpain is a major target to suppress relapses resulting from epitope spreading in RRMS. Untreated 50% of the patients with RRMP will develop in

10years permanent disabilities (Weinshenker, 1994; Confavreux et al., 2000; Hafler, 2004), which is believed to be due to increased axonal injury in which abnormally activated calpain probably plays a key role. Use of CYLA with its calpain inhibition effect and ability to cross the BBB might give an enormous therapeutic privilege for this patient collection for the above mentioned reasons.

Additional observation was made from the ELISA antibody response test. There was less anti-MOG antibodies in the sera of CYLA treated mice whereas there was higher titer of anti-MOG antibody in the sera of the non-treated mice group.

MS is often described as a primarily T cell mediated disease, but there is increasing evidence for the role of antibodies in the pathogenesis of the disease. The following findings support the role of antibodies in the disease process. Immunoglobulins and complement activation products are found to have been deposited on the surface of myelin sheath at the active edge of demyelinating lesions (Storch et al., 1998; Lucchinetti et al., 2002). B cells are detected histopathologically in areas of active myelin break down (Genain et al., 1999; Raine et al., 1999). Indirect evidence comes from a trial in which a subset of MS patients were treated with plasma exchange which produced a dramatic reduction in clinical disease activity (Weinshenker, 1999; Weinshenker et al., 1999; Kieseier et al., 2003). Another clue for the role of myelin antigen antibodies in the pathogenesis of EAE comes from studies using B cell deficient mice. MOG-induced EAE was reduced but not absent in the B cell deficient mice. Demyelination rather than inflammation was affected in the B cell deficent mice (Svensson et al., 1998; Svensson et al., 2002). This phenomenon was also observed to some extent in our chronic EAE mice. The demyelinating potential of MOG-specific autoantibodies was confirmed in vivo by

adoptive transfer and subsequent application of the autoantibodies (Linington and Lassmann, 1987; Schluesener et al., 1987) and in vitro in aggregating brain cell cultures (Kerlero de Rosbo et al., 1990).

Intravenous administration of MOG-specific antibodies enhanced demyelination and inflammation resulting in a dramatic increase in disease severity. The response is mediated by combination of complement and antibody-dependent cell cytotoxicity effectors mechanism (Iglesias et al., 2001). More studies are needed to further the understanding of the mechanisms involving antibodies and cytokine switch during therapeutic intervention.

In summary, we showed that CYLA is capable of crossing the BBB, inhibiting calpain in vivo within the CNS and effectively reducing disease progression and severity in a mouse model of MS. Owing to its characteristics of calpain inhibition and ability to cross the BBB, CYLA may be considered as a supplement therapeutic agent in the treatment of MS, especially patients with RRMS preventing the worst course to secondary progression with permanent disability. Our results showed that application of CYLA in a medium dose (1 mg/day) before the onset of the symptoms showed better outcome. One possible application of this novel drug is in relapsing-remitting MS (RRMS). RRMS is characterized by a course of clearly defined flare ups interrupted by partial or complete remissions. CYLA could be beneficial in the treatment of RRMS patients during the remission phase in order to prevent the subsequent relapses. The relapses are believed to occur from epitope spreading, a phenomenon , in which calpain is believed to play a key role (Vanderlugt and Miller, 2002). Untreated, 50% of RRMS will progress to permanent disability resulting from axonal injury, another process involving

uncontrolled calpain action (Weinshenker, 1994). Given the role of calpain in the pathophysiology of RRMS and subsequent axonal damage, inhibition of calpain has the potential for improveing the quality of life of patients with MS.

Taking all results of the project together, we believe to have given additional evidence for the role of calpain in the pathophysiology of the inflammatory demyelinating disease such as EAE/MS and we have presented a potential therapeutic agent for the treatment of the above mentioned disease entities. Almost all known MS drugs act in suppressing the inflammatory phase of the disease leaving no therapeutic privilege for the neurodegenerative phase of the disease, which ultimately leads to permanent disability. This agent can be used as a supplement to the already existing anti-inflammatory drugs.

The fact that calpain activation is part of the late phase of the disease, it makes calpain inhibitors better therapeutic agents since their use allows extended frame of window for their application. It has been shown that there was a better outcome in experimental brain injury when calpain inhibitors were applied up to six hours after the onset of the injury (Markgraf et al., 1998).

It has been reported that uncontrolled and prolonged activation of calpain plays a critical role in the pathophysiology of a number of other human diseases (Fig. 45, Table 8) (Redmond et al., 1998; Ray and Banik, 2003).

Calpain	Diseases
Calpain 1	Stroke, traumatic brain injury, Alzheimer's disease, cataracts
Calpain 2	Stroke, traumatic brain injury Alzheimer's disease, cataracts
Calpain 3	Limb-girdle muscular dystrophy 2A, cataracts
Calpain 9	Gastric cancer
Calpain 8/10	Type 2 diabetes mellitus

Table 8 Calpain family and various pathological conditions (Huang et al., 2000).

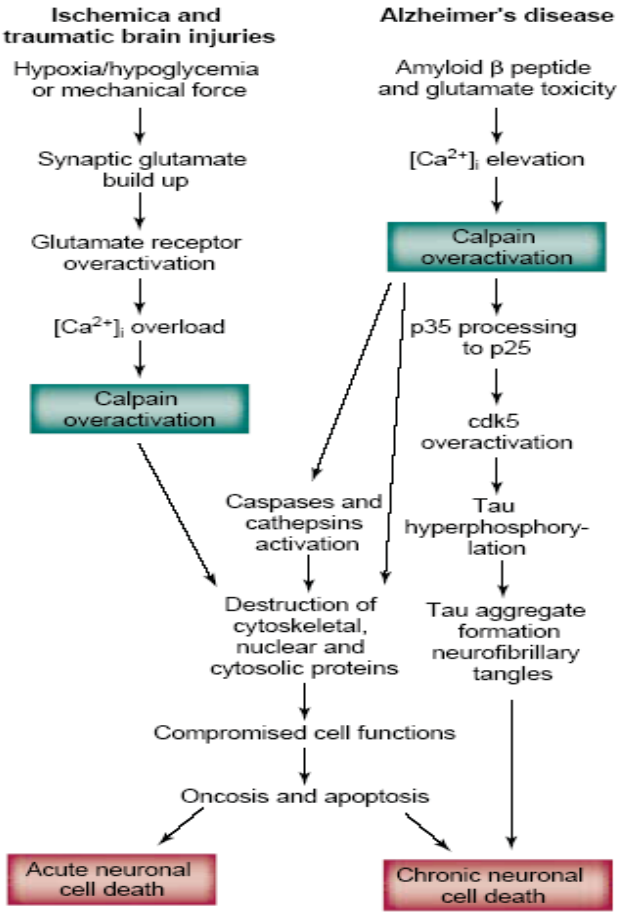


Figure 45 Mechanism of tissue damage by calpain. Several disease stated leads to the abnormal elevation of cellular calpain resulting in abnormal activation of calpain causing tissue damage. Examples are shown for ischemic stroke and Alzheimer`s disease (Huang et al., 2000).

The capacity inhibit CNS calpain and the ability to cross the BBB make CYLA a potential candidate for the treatment of other neurodegenerative disorder resulting from aberrant calapin activation such as amyotrophic lateral sclerosis (Ray and Banik, 2003), Alzheimer's disease (Nixon, 1989; Saito et al., 1993; Nixon, 2000), Huntington's disease (Gafni and Ellerby, 2002; Gafni et al., 2004), traumatic brain injury (Ray et al., 2003a) and cerebral stroke (Bartus et al., 1994b; Kampfl et al., 1996).

Animal models are good tools to study pathophysiology and treatment options of human diseases, but there is always a discrepancy between outcomes in animal studies and human trials (Steinman, 1992). Even if most valuable information came from animal studies, only few therapeutic interventions that showed good results in animal models were also successful in human trials. Other therapeutic interventions that were successful in pre-clinical trial were either less effective in MS patients, worsend the disease or caused severe side effects (Table 9 and 10).

	MS	**EAE**
Therapy		
γIFN, systemic	worsens	cures
anti-TNFα, systemic	worsens	cures
IL-4 transduced T cells	not done	cures
TNFα transduced T cells	not done	worsens
Copaxone	improves	cures
βIFN	improves	improves

Table 9 Comparison of effects of different therapeutic strategies in EAE and MS (Steinman, 1999).

Treatment approach	Based on clear hypothesis	Rationale confirmed	Efficacy in EAE	Efficacy in multiple sclerosis	Adverse event profile	Status of development
Glatiramer acetate	No	No	++	+	+++	Approved
Altered peptide ligand	Yes	No; i.d.	++	−; i.d.	±	No
Oral myelin	Yes	Yes	++	−	+++	Not continued after phase III owing to lack of efficacy
Anti-α_4 integrin	Yes	Yes	+++	+++	±†	Taken off the market
Anti-CD40L	Yes	Yes	+++	n.k.	±	No
Anti-CD4	Yes	No	+++	±	±	Halted in phase II
Anti-CD52	Yes	Yes	n.a.	++; i.d.	+	Approved for other indication
Anti-CD25	Yes	No	±	+++; i.d.	++	Approved for other indication
CTLA-4-Ig	Yes	No; i.d.	+++	n.k.	n.k.	In phase III
IFN-β	No	No	+	+	++	Approved
IFN-γ	No	No	++	−	−	Stopped in phase I
Anti-TNF antibodies	Yes	No	++(?)	−	−‡	Approved for other indication
TNFR-Ig fusion protein	Yes	No	++(?)	−	− ‡	Approved for other indication
TGF-β2	Yes	Yes	++	i.d.	−	Stopped in phase I
IL-10	Yes	No	±	i.d.	i.d.	Stopped in phase II
IGF-1	Yes	Yes	+(+)	−; i.d.	++	Phase IIa, not continued
PDE4 inhibitors	Yes	Yes	++	−; i.d.	+	Halted in phase II
PPARγ agonists	Yes	Yes	++	n.t.	n.a.	Not yet tested in multiple sclerosis
Statins	Yes	Yes	+++	++; i.d.	+	Approved for other indication
Mitoxantrone	No§	No§	++	++	±	Approved
Linomide	No	No	++	++	±	Phase III stopped due to cardiotoxicity
Laquinimod	No	No	++	++; i.d.	+	In phase II
FTY720/SP-1 agonist	No	No	+++	++(+); i.d.	+	In phase II
Deoxyspergualin	No	No	+	−	+	After phase II stopped owing to lack of efficacy
Sulphasalazine	Yes	No	±	±	+	In phase III
IVIG	No	No	±	±	++	In phase II
Haematopoietic stem cell transplant	Yes	Yes	+	++;i.d.	±; i.d.	In phase III

Table 10 Some immunomodulatory approaches of multiple sclerosis and their development from EAE or in vitro studies to clinical application*. i.d., insufficient data; n.a., not applicable; n.k., not known; n.t., not tested; *The table depicts whether a therapeutic approach was developed for multiple sclerosis on the basis of a clear and pre-formed hypothesis, whether the rationale for its clinical/EAE testing had later been shown and whether the therapy was effective in EAE and/or multiple sclerosis. Both the clinical efficacy and the tolerability and safety are depicted by + or - signs. In the context of the adverse events, + indicates a favourable profile. The relative weighting reflects the subjective perception of the authors either from own experience or the published literature; †Reasonable safety profile, but one specific severe adverse event (PML). ‡Development of demyelinating episodes and diseases in RA- and Crohn's patients; §Broad immunosuppressant; no specific target (Friese et al., 2006).

One has to bear in mind the fact that these results come from animal studies and the animals that are used for MS research are inbred whereas human population at large is out bred (Table 6). It is essential to modify current animal models to improve their use as a crucial tool to test drugs (Fig. 46).

- Validate therapeutic effect in more than one model
- Consider dissimilarities in immune systems of rodents versus humans
- Consider treatment after disease onset
- Record disease course for as long as possible (feasible)
- Establishment of spontaneous disease models
- Establishment of a two-stage disease course in EAE (first relapsing–remitting, later chronic-progressive)
- Incorporate human disease risk factors
- Incorporate human immune system and modifying human CNS factors

Figure 46 Furthur considerations necessary to improve animal models for a better use as tool to study pathogenesis or therapeutic interventions (Friese et al., 2006).

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