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Mechanisms of synaptic pathology in an animal model of cortical multiple sclerosis

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Å	Ångström, 10^{-10} m
AAV	adeno-associated virus
aEAE	actively induced EAE
aHSCT	autologous haemopoietic stem cell transplantation
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
AP	action potential
APP	amyloid precursor protein
ASIC1	acid-sensing ion channel-1
ATP	adenosine triphosphate
Aβ	amyloid beta
BBB	blood brain barrier
BFP	blue fluorescent protein
BG	background
BSA	bovine serum albumin
BT	bleedthrough
C1q	complement component 1q
C3	complement component 3
CaM	calmodulin
CASK	calmodulin-dependent serine protein kinase
CCL2	C-C chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CD	cluster of differentiation
CFP	cyan fluorescent protein
CIS	clinically isolated syndrome
c-MS	cortical MS (animal model)
CNS	central nervous system
CR3	complement receptor 3
CSF	cerebrospinal fluid
CSF1	colony stimulating factor 1
CSF1R	colony stimulating factor 1 receptor
CX3CR1	C-X3-C motif chemokine receptor 1
dH_2O	distilled water
DNA	deoxyribonucleic acid
dX	dayX
E. coli	Escherichia coli
e.g.	for example
EAAT	excitatory amino acid transporter
EAE	experimental autoimmune (allergic) encephalomyelitis
EF hand	helix-loop-helix structural domain or motif found in a large family of calcium-binding proteins
EGTA	ethylene glycol tetraacetic acid
EPSC	excitatory postsynaptic current
ER	endoplasmic reticulum
EPSP	excitatory postsynaptic potential
F1	first filial generation
F2	second filial generation

FRET	fluorescence resonance energy transfer
GaAsP	gallium arsenide phosphide
GECI	genetically encoded calcium indicator
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GS	goat serum
GTP	guanosine-5'-triphosphate
h	hours
hi	high
hsyn1	human synapsin I gene promoter
i.e.	id est
IFNγ	interferon gamma
IL-1β	interleukin 1 beta
IL-34	interleukin 34
iNOS	inducible nitric oxide synthase
IP3	inositol trisphosphate
IP3R	inositol trisphosphate receptor
IVC	individually ventilated cage
JC	john cunningham virus
K_d	dissociation constant
LAMP 1	lysosomal-associated membrane protein 1
lo	low
Ly6c	lymphocyte antigen 6 complex
MAC	membrane attack complex
MBP	myelin basic protein
mGluR	metabotropic glutamate receptor
MGV	mean gray value
MHC	major histocompatibility complex
MMF	midazolam - medetomidin – fentanyl
MOG	myelin oligodendrocyte glycoprotein
MRI/MRT	magnetic resonance imaging/tomography
MS	multiple sclerosis
mtDNA	mitochondrial DNA
n	number of experimental animals
NAGM	normal-appearing gray matter
NAWM	normal-appearing white matter
NBQX	2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline; AMPA receptor antagonist
NCX	Na^+/Ca^{2+} exchanger
NDDS	non-descanned detector
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NMSOD	neuromyelitis optica spectrum disorders
NO	nitric oxide
ns	not significant
OCT	optical coherence tomography
OPCs	oligodendrocyctes progenitor cells
PBS	phosphate buffered saline
pEAE	passively induced EAE
PET	positron emission tomography
PFA	paraformaldehyde
PLP	proteolipid protein

PMCA	plasma membrane Ca^{2+} ATPase
PPMS	primary progressive multiple sclerosis
PSD95	postsynaptic density protein 95
Ptx	pertussis toxin
PUFA	poly-unsaturated fatty acids
rAAV	recombinant adeno-associated virus
RFP	red fluorescent protein
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROI	region of interest
ROS	reactive oxygen species
RRMS	relapsing remitting multiple sclerosis
RyR	ryanodine receptor
SCoRe	specimen in a corrected optical rotational enclosure
SD	standard deviation
SEM	standard error of the mean
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SNR	signal-to-noise ratio
SPMS	secondary progressive multiple sclerosis
TCR	T-cell receptor
Ti:sapphire	Titanium-sapphire
TnC	Troponin C
TNFα	tumor necrosis factor alpha
TPBS	Triton X-100 in PBS
TRPM4	transient receptor potential cation channel subfamily M member 4
VGCC	voltage-gated calcium channel
XFP	stands for any fluorescent protein
YFP	yellow fluorescent protein
ΔR/R	fractional ratio change
2PLSM	two-photon laser-scanning microscopy

Zusammenfassung

Die Multiple Sklerose (MS) ist eine chronisch-entzündliche Erkrankung des zentralen Nervensystems. Kennzeichnend für die Erkrankung sind herdförmige ZNS Läsionen, die durch Demyeliniserung der weißen Substanz sowie axonale Schäden charakterisiert sind. Während die akuten Phasen der Erkrankung therapeutisch behandelt werden können, bleiben progrediente Phasen der MS weitestgehend therapierefraktär. Hierbei zeigt sich zunehmend, dass inflammatorische sowie neurodegenerative Prozesse innerhalb der grauen Substanz inklusive des Kortex entscheidend für die Progression der Erkrankung sind und gleichzeitig mit chronischer kognitiver Beeinträchtigung der Patienten korrelieren. Trotz dieser wesentlichen klinischen Bedeutung ist das Verständnis über die Mechanismen der kortikalen Pathologie in progredienter MS gering. Wesentliche Herausforderungen sind hierbei, die kortikale Inflammation akkurat zu modellieren und diese präzise zeitlich sowie räumlich bildgebend darzustellen.

Ein wesentlicher Bestandteil der kortikalen Pathologie ist ein ausgedehnter Synapsenverlust, der in Patienten mit MS nachgewiesen werden kann. Dieser tritt unabhängig vom Grad der Demyelinisierung der grauen Substanz auf und kann daher den Ort der Initiierung eines neuronalen Schadens darstellen. Bisher waren die Mechanismen, welche den Synapsenverlust initiieren und die Frage, ob der Verlust irreversibel ist, weitestgehend unerforscht.
Um die Mechanismen der synaptischen Pathologie, insbesondere die Rolle von Kalzium, genauer zu untersuchen, setzte ich *in vivo* Zwei-Photonen Mikroskopie in einem Mausmodell der kortikalen Multiplen Sklerose ein. Mittels transgener Mauslinien, welche ein ratiometrisches, genetisch enkodiertes Kalzium-Sensorprotein exprimieren, maß ich *in vivo* dendritische sowie postsynaptische Kalziumkonzentrationen. Zunächst bestätigte ich einen ausgedehnten Verlust dendritischer Dornenfortsätze in unserem kortikalen MS Modell. Anschließend wies ich nach, dass postsynaptische Kalzium Akkumulationen in kortikaler MS zur nachfolgenden Entfernung jener Dornenfortsätze mit Kalzium Überschuss führten. Meine Ergebnisse zeigen somit, dass lokale Kalzium Akkumulationen im Prozess des Synapsenverlustes maßgeblich involviert sind. Um die Rolle von Glutamat für die synaptische Pathologie zu untersuchen, blockierte ich mittels der Glutamat Antagonisten Memantin und NBQX die NMDA und AMPA/Kainat Rezeptoren, welche ebenso als mögliche Quelle des Kalziums fungieren könnten. Unter dieser Behandlung konnte weder der Verlust dendritischer Dornenfortsätze verhindert werden, noch waren die Kalzium Akkumulationen reduziert.
Mittels transgener Mauslinien sowie konfokaler Mikroskopie, charakterisierte ich anschließend die zelluläre Inflammation: Ich zeigte, dass der Verlust dendritischer Dornenfortsätze von einer

ausgedehnten Aktivierung residenter Mikroglia ($CX3CR1^{GFP}$) sowie der Infiltration von Monozyten abstammenden Makrophagen ($CCR2^{RFP}$) begleitet wird. Diese zelluläre Inflammation sowie der Synapsenverlust in unserem kortikalen MS Modell waren reversibel. Zusammenfassend deuteten die Ergebnisse auf eine Phagozyten-vermittelte Entfernung jener Dornenfortsätze mit Kalzium Überschuss hin.

In nachfolgenden Studien wurden die Rolle des mononukleär-phagozytären Systems für den Synapsenverlust sowie die Mechanismen der Kalzium Akkumulation genauer untersucht. Darüber hinaus ist es unser Ziel, neue immunomodulatorische Therapien zu entwickeln, welche in der Lage sind den Synapsenverlust und dadurch die Progression kognitiver Beeinträchtigungen von MS Patienten zu verhindern.

Summary

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system. The disease is characterized by focal CNS lesions in which demyelinated white matter and axonal damage are found. While acute phases of the disease can be targeted therapeutically, later progressive phases remain largely refractory to therapeutic interventions. It is becoming increasingly evident, that inflammatory and neurodegenerative processes of the gray matter including the cortex are central for the progression of the disease and correlate simultaneously with the chronic cognitive impairment of patients with MS. Besides this fundamental clinical relevance, our understanding of the mechanisms of cortical pathology in progressive MS is poor: Major challenges include an accurate model of cortical inflammation and a sufficiently precise temporal and spatial imaging process of the pathology.

A main feature of cortical pathology in MS is a widespread synapse loss in the gray matter of patients with MS. This occurs independently of the degree of demyelination and thus, could represent the initiation site of neuronal damage. So far it has remained unknown how the synapse loss is initiated and whether it is irreversible.

In order to investigate possible mechanisms of synaptic pathology, in particular the role of calcium, I applied two-photon *in vivo* microscopy in a mouse model of cortical MS. Using transgenic mice that express a ratiometric, genetically encoded calcium indicator, I measured dendritic and postsynaptic calcium levels. First, I confirmed the widespread dendritic spine loss in our cortical MS model. Secondly, I showed that spines destined for removal showed local calcium accumulations in the cortical MS model. Therefore, my results indicated that local calcium accumulations are directly involved in synapse loss. In order to investigate the role of glutamate for synaptic pathology, I blocked the NMDA and AMPA/kainate receptors, which also might function as sources of calcium by using the glutamate antagonists Memantine and NBQX. My results showed neither an increase in dendritic spine density, nor a decrease in calcium accumulations under the antiglutamatergic therapy.

In order to characterize the cellular inflammation in our cortical MS model, I applied confocal microscopy in transgenic mice. My findings demonstrated that dendritic spine loss was accompanied by a widespread activation of resident microglia ($CX3CR1^{GFP}$) and an infiltration of monocytes-derived macrophages ($CCR2^{RFP}$). The cellular inflammation as well as the spine loss were reversible in our c-MS model. Taken together our findings suggest that a phagocytic-mediated engulfment of those spines displaying calcium overload results in reversible synapse loss in the inflamed gray matter.

In subsequent studies in our lab the specific role of the mononuclear-phagocytic system for synaptic removal and the mechanisms of local calcium accumulations were investigated. Beyond this, we aim to develop new immunomodulatory therapies that are able to prevent synaptic loss and thus prevent the progression of cognitive impairments of patients with MS.

1. Introduction

1.1 Motivation

The typical presentation of multiple sclerosis is dominated by the presence of disseminated demyelinated lesions of the white matter and resulting motoric and sensoric symptoms (M. Calabrese et al., 2015). As the disease progresses, MS patients often face cortical symptoms including cognitive impairment and fatigue (Compston & Coles, 2008). It is becoming increasingly evident that a widespread gray matter pathology, which already occurs during early phases of the disease (Lucchinetti et al., 2011), is associated with these cortical symptoms and the conversion to progressive MS (M. Calabrese et al., 2012).

A key feature of such gray matter pathology is a pronounced synapse loss which can be found independently from cortical demyelination and axonal loss in patients with MS (Albert et al., 2017; Jürgens et al., 2015). Moreover, synapse loss is a hallmark of other neurodegenerative, neuropsychiatric and neuroinfectious diseases (Di Liberto et al., 2018; Olmos-Alonso et al., 2016; Vasek et al., 2016). This indicates that similar mechanisms, inflammatory and neurodegenerative ones, may lead to a wide range of cortical pathologies.

In previous studies, our laboratory induced cortical neuroinflammation in mice by using cortically targeted models of experimental allergic encephalomyelitis (Gardner et al., 2013; Merkler, Ernsting, Kerschensteiner, Brück, & Stadelmann, 2006). Such cortical MS models reproduced the severe synapse loss (Jafari et al., 2021) also observed in MS patients.

The aim of my study was to unravel the mechanisms that contribute to gray matter pathology and lead to synapse loss in cortical neuroinflammation. With these findings, we expect to develop early interventions which reduce cortical inflammatory processes, maintain synapses and synaptic connectivity and do, counteract the progression of MS. Towards this goal, I researched the following mechanisms: (1) the role of local disturbances in calcium homeostasis for spine loss, (2) the role of glutamate-mediated excitotoxicity, and (3) the identification of the cells which execute the removal of spines.

The following chapter will give an introduction into the disease multiple sclerosis; it will emphasize the differences between white and gray matter pathology in the progressive phases of the disease, demonstrate potential mechanisms of synaptic pathology in MS and in animal models of MS, and introduce imaging methods as well as the use of biosensors required to study calcium levels in dendritic spines.

1.2 Multiple Sclerosis

1.2.1 Definition

Multiple sclerosis is the most prevalent chronic immune-mediated disease of the human central nervous system (CNS) and affects approximately 2.5 million people worldwide (Dendrou, Fugger, & Friese, 2015).

It is characterized by disseminated lesions within the CNS which cause heterogeneous clinical manifestations including motor, sensory, cognitive and neuropsychiatric deficits. (Chiaravalloti & DeLuca, 2008; Dendrou et al., 2015; Geurts & Barkhof, 2008). The lesions are pathologically characterized by the infiltration of immune cells across the blood brain barrier which results in inflammation, demyelination, neuroaxonal damage and gliosis (M. Calabrese et al., 2015). The disease is autoimmune and is at this point incurable.

1.2.2 Clinical manifestation

1.2.2.1 Disease courses

The disease course of patients with multiple sclerosis is heterogeneous and depends on both the location of lesions within the CNS and the type of disease onset (progressive or relapsing). The vast majority of patients who develop multiple sclerosis begin with a monophasic clinically isolated syndrome that reflects focal or multifocal demyelinating lesions which usually involve the optic nerve, brainstem or spinal cord (Thompson, Baranzini, Geurts, Hemmer, & Ciccarelli, 2018). It is commonly followed by a clinical recovery and subsequently by recurring periods of relapse and remission (relapsing remitting multiple sclerosis (RRMS)) (Dendrou et al., 2015). Typical clinical presentations include optic neuritis, double visions, sensory symptoms, cerebellar ataxia and symptoms affecting the autonomous nervous system such as incontinence (Brownlee, Hardy, Fazekas, & Miller, 2017). In up to 50 percent of untreated patients with RRMS, the disease becomes progressive after 10-20 years and is then named as secondary progressive multiple sclerosis (SPMS) (Correale, Gaitan, Ysrraelit, & Fiol, 2017). Here, inflammatory lesions are no longer characteristic. Instead, the progressive neurological decline is accompanied by increasing axonal loss and decreasing brain volume (Dendrou et al., 2015). About 15% of patients show a constant deterioration of symptoms from the onset of the disease which assigns them to the group of primary progressive multiple sclerosis (PPMS) (Miller & Leary, 2007). In this case, clinical manifestation is usually characterized by progressively evolving asymmetric paralysis, cerebellar ataxia and neurocognitive deficits (Brownlee et al., 2017).

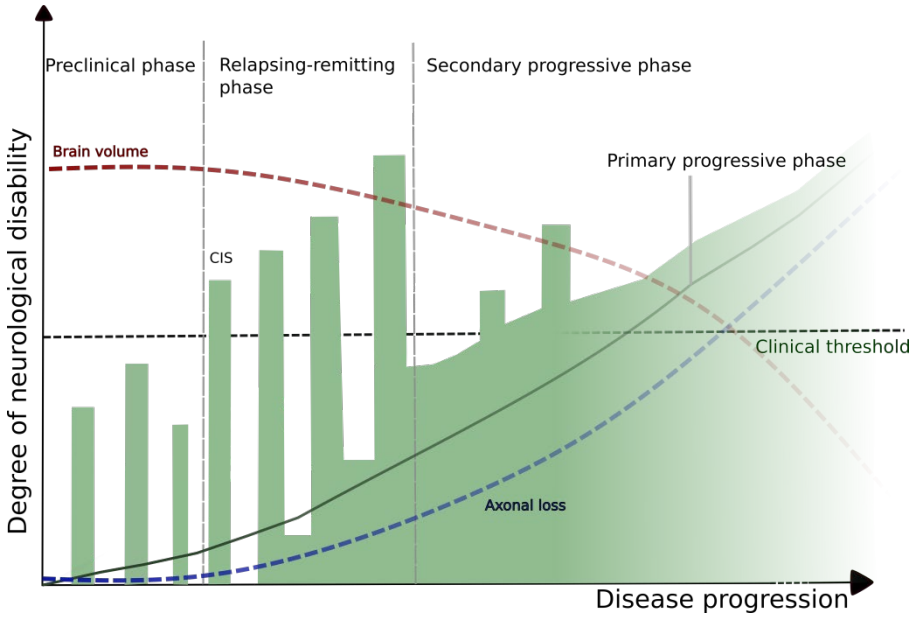


Figure 1: Disease courses of multiple sclerosis

Schematic illustration of different clinical courses in MS. The green bars represent frequent inflammatory relapses that first do not reach clinical relevance. By exceeding the clinical threshold, a monophasic clinically isolated syndrome (CIS) becomes manifest which is followed by the phase of relapsing remitting disease (RRMS) and goes over to the phase of secondary progressive disease with increasing resting disability and neurological dysfunction. The primary progressive disease shows an evolving increase in disability from the onset. The disease progression is accompanied by CNS atrophy, i.e. the brain volume decreases and the axonal loss increases. Figure modified from (Dendrou et al., 2015).

1.2.2.2 Cognitive and neuropsychiatric symptoms

About 40% to 70% of patients with MS suffer from cognitive deficits (Damjanovic et al., 2017) which likely correlate with gray matter damage in the form of cortical lesions (M. Calabrese et al., 2009; M. Calabrese et al., 2012).

Main aspects of cognitive functioning which are affected during the disease course are efficiency and speed of information processing, long term memory, executive functioning, visuo-spatial perception as well as complex attention (Chiaravalloti & DeLuca, 2008; Piras et al., 2003). Overall patients with SPMS show greater cognitive dysfunction compared to patients with RRMS or PPMS (Denney, Sworowski, & Lynch, 2005; Huijbregts et al., 2004). Other factors impairing neurocognitive functions include highly prevalent neuropsychiatric problems such as depression (Arnett, Barwick, & Beeney, 2008) and fatigue which is one of the most common symptoms of MS patients (Chiaravalloti & DeLuca, 2008). These might have effects on cognitive performances which for example require consistent mental effort, verbal and visual memory or conceptual planning (Krupp & Elkins, 2000). Overall, the cognitive impairments

impede the patient's ability to meet the challenges of daily life such as running a household or maintaining employment and thus have a huge impact on the quality of life of the patient (Chiaravalloti & DeLuca, 2008).

1.2.3 Diagnostic criteria

The most recent diagnostic criteria of multiple sclerosis are the 2017 McDonald criteria from the International Panel on Diagnosis of Multiple Sclerosis. They are based on the combination of clinical symptoms and clinical history, MRI results, and laboratory findings (Thompson, Banwell, et al., 2018). In MS, MRI imaging of the brain and spinal cord typically show highly sensitive chronic T1- hypointense (so-called black holes) and T2- hyperintense lesions in the white matter which are located in distinctive areas (periventricular, juxtacortical, infratentorial and in the spinal cord). An abnormal brain or spinal cord MRI occurs in almost all patients with diagnosed MS and in a vast majority of those with a CIS developing MS (Thompson, Banwell, et al., 2018). MRI imaging is also of great value to exclude differential diagnoses such as acute disseminated encephalomyelitis or diseases being allocated to the neuromyelitis optica spectrum disorders (NMSOD) (Brownlee et al., 2017).

In the field of neurodegeneration and gray matter pathology, the role of cortical imaging is arising as well (Forslin et al., 2018): gray matter atrophy predominantly caused by neuroaxonal loss (Rocca et al., 2017) can be measured; by applying double-inversion recovery and ultra-high-field (7T) MRT, cortical lesions may be discovered (Ontaneda, Thompson, Fox, & Cohen, 2017) and optical coherence tomography (OCT) allows to assess the retina at micron level resolution and measure retinal thinning due to retinal ganglion-cell axon loss (Saidha et al., 2015). Also for predicting the risk of evolving neurological disability, OCT may be applied (Martinez-Lapiscina et al., 2016).

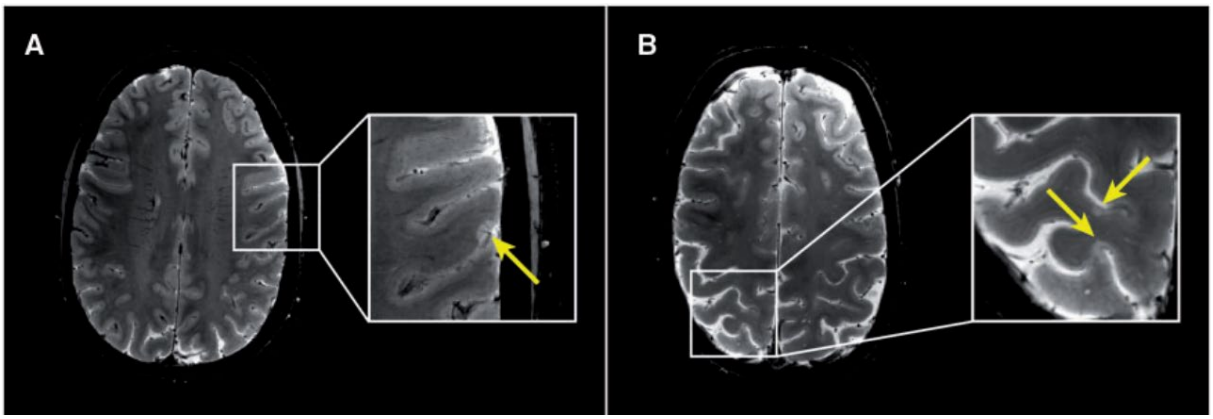


Figure 2: Examples of cortical lesions detected with ultra-high-field (7T) MRT

(A) Female patient (36-year-old) with a disease duration of nearly 2 years and a disability score of 0. (B) Female patient (45-year-old) with a disease duration of nearly 3 years and a disability score of 3. Figure adapted from and caption modified from (Granberg et al., 2017).

Next to MRI imaging results, biomarkers which are regularly used in the clinical setting include oligoclonal bands in the CSF and JC viral titers which are required for an immunomodulatory therapy (e.g. with natalizumab). Other potential biomarkers such as neurofilament, GFAP, various cell markers and antibodies may in future be able to measure and reflect the grade of neurodegeneration. Further, genetic testing may be a predictor for MS vulnerability and progression (Housley, Pitt, & Hafler, 2015; Novakova et al., 2018).

It has been demonstrated that early treatments improve long-term progression of the disease and that inflammatory processes which may be undetected during the early phases of the disease drive the progression of the disease (Gunnarsson et al., 2011; Hutchinson, 2015; Jones et al., 2010). The progress of useful prognostic biomarkers may thus enable predicting disease progression and therapy response and therefore, contribute to the prevention of early irreversible neurological disabilities.

1.2.4 Therapeutical approaches

As of today, several disease-modifying, anti-inflammatory treatments are licensed for the therapy of patients with relapsing remitting multiple sclerosis, e.g. B-cell depletion agents such as rituximab and ocrelizumab (Hauser et al., 2017; Hauser et al., 2008). In general, there are two long-term therapeutical options in the clinical setting: (1) starting with a first-line treatment and then escalating to a more potent but also less safe drug (escalation strategy), or (2) starting with a highly potent therapy such as natalizumab to obtain remission (induction strategy). The latter is in particular applied in patients with a more fulminate course of the disease. Generally, more potent medications involve a higher risk of complications such as infections or the

development of progressive multifocal leukoencephalopathy caused either by a reactivation of the JC virus or new infection with it (Thompson, Banwell, et al., 2018). Another therapeutical approach, particularly suitable for young patients with inflammatory MS activity, is immunoablation followed by an autologous haemopoietic stem cell transplantation (aHSCT). This procedure was demonstrated to suppress disease activity in up to 80% of MS patients for several years (Muraro et al., 2017).

So far, therapies which target the progressive phases of the disease are still limited. The only agent approved as a therapy in patients with primary progressive multiple sclerosis is the B-cell depleting agent ocrelizumab (Montalban et al., 2017). As for the treatment of secondary progressive MS, mainly those groups which show inflammatory activity benefit from a treatment with anti-inflammatory drugs (Ontaneda et al., 2017). There has been some progress in the treatment of progressive MS: For instance, Siponimod showed a reduction in disability progression (Kappos et al., 2018) and treatment with simvastatin led to reduced progression of brain atrophy (Chataway et al., 2014). Cell-based repair promoting strategies, for example the transplantation of oligodendrocyctes progenitor cells (OPCs) or mesenchymal stem cell (MSC), do represent potential approaches for prospective treatment of patients with progressive MS (Ontaneda et al., 2017; Petrou et al., 2020; Scolding, Pasquini, Reingold, & Cohen, 2017; Windrem et al., 2008).

Acute relapses are treated with intravenous or oral steroids. Patients who have steroid-resistant relapses usually are treated by plasma exchange. To maintain the patients quality of life, active management in MS therapy also includes treating the consequences of the disease such as spasticity, impaired ambulation (e.g. with fampiridine), incontinences, pain and depression (Thompson, Baranzini, et al., 2018).

1.2.5 Pathological features

The pathological features of multiple sclerosis are characterized by demyelinated areas in the white and gray matter of both the brain and the spinal cord. These areas, so-called lesions or plaques, mainly display a loss of myelin sheets and oligodendrocytes, acute axonal damages and reactive astrogliosis. While acute lesions are characterized by dense inflammatory infiltrates and active demyelination, chronic lesions show gradual neuroaxonal loss and brain atrophy which correlate with increasing patient's disability (Dendrou et al., 2015).

The following chapter will outline the main pathological features of MS with focus on the progressive phases: (1) the differences between focal and diffuse white/gray matter lesions, (2)

the putative mechanisms of neuroaxonal damage/loss, and (3) the involvement of inflammatory and non-inflammatory processes in neurodegeneration.

1.2.5.1 White versus gray matter damage

"Is then, the process that attacks the cortex different in its nature and origin from that which affects the rest of the central nervous system?" (Dawson, 1916).

Lesions of the myelin-rich white matter throughout the CNS are characteristic for multiple sclerosis and the resulting clinical symptoms. Moreover, gray matter including the cortex is affected during the disease and has emerged as an important contributor to both physical and cognitive disability. As pathological patterns of white and gray matter damage differ, Dawson's question remains of great importance today. In the following, the main characteristics of focal and diffuse white and gray matter lesions will be demonstrated.

White matter damage

Active lesions of the white matter mainly occur in early phases of the disease and display dense inflammatory infiltrates, while progressive phases of MS are mainly characterized by focal chronic active and inactive lesions, and a diffuse injury of the otherwise macroscopically normal-appearing white matter (NAWM) (Frischer et al., 2015; B. F. Popescu & Lucchinetti, 2012). The following section will first outline the focal pathological features of the white matter, i.e. active lesions, chronic active lesions, inactive lesions, and then continue to the diffuse features of white matter damage (Schumacher, Mahler, & Kerschensteiner, 2017).

In general, it is unclear whether multiple sclerosis is triggered in the CNS or in the periphery. Potential mechanisms such as molecular mimicry, bystander activation or CNS viral infections are assumed to activate autoreactive T-cells which migrate across the blood brain barrier to the CNS along with monocytes and activated B-cells. Among the invading peripheral immune cells, macrophages and CD^{8+} T-cells dominate, however CD^{4+} T-cells, B-cells and plasma cells as wells as activated astrocytes can be found in the affected CNS (Dendrou et al., 2015). The massive infiltration of immune cells is concentrated on focal lesions in early MS and leads to a pronounced demyelination of the white matter, loss of oligodendrocytes and acute axonal damages. Four different patterns of white matter demyelination have been described based on the loss of myelin, the distribution and extension of lesions, the patterns of oligodendrocyte destruction, and the activation of the complement system. The most frequent patterns I and II are characterized by the infiltration and activation of mononuclear phagocytes, which are accompanied by parenchymal and perivascular T-cell infiltrates. In pattern II, additionally, specific demyelinating antibodies and complement deposition can be found. In these patterns,

demyelination resembles a T-cell–mediated or a T-cell plus antibody–mediated autoimmune response. In the patterns III and IV, where oligodendrocytes apoptosis due to a primary oligodendrocyte dystrophy is characteristic, demyelination appears similar to virus- or toxin-induced processes (Lucchinetti et al., 2000). Focal demyelinated lesions of the white matter are mainly found in patients with acute and relapse remitting multiple sclerosis. Although invading immune cells are concentrated on focal lesions in early multiple sclerosis, general brain atrophy can already be apparent (Chard et al., 2002).

Focal chronic active lesions of the white matter are nearly exclusively attributed to primary and secondary progressive phases of MS. In these 'smoldering' lesions only few inflammatory cells can be found in the center of the lesion, which is surrounded by a rim of activated microglia. Inactive lesions represent the 'burned out' end stage of an inflammatory lesions. These lesions lack activated inflammatory cells, and instead display a gliotic appearance (Frischer et al., 2015). In addition to focal white matter lesions, progressive MS also displays a diffuse injury of the NAWM. This injury is characterized by the widespread accumulation of T-cells in the CNS, perivascular infiltrates of mainly mononuclear cells, and a diffuse activation of microglia (Kutzelnigg et al., 2005; Lassmann, van Horssen, & Mahad, 2012). In addition to the migration of inflammatory cells and the activation of microglia, neuronal damage can be found in the NAWM. In particular, focally swollen or fragmented axons are found throughout the NAWM and are not necessarily correlated to the quantity of focal white matter lesions (Kutzelnigg et al., 2005).

Gray matter damage

Gray matter damage is part of the pathological and clinical manifestation of multiple sclerosis, and in particular correlates with the progression of the disease and the appearance of cognitive deficits. Pathological changes of the gray matter include focal lesions, i.e. cortical demyelination and meningeal infiltrations, and diffuse changes which result in neurodegeneration and synapse loss. The following section will outline the main pathological features of gray matter damage in MS.

Focal demyelinated lesions can be found not only in the white but also in the gray matter and can affect the neocortex and deeper gray matter areas such as the thalamus, basal ganglia and hippocampus (Kutzelnigg et al., 2005; Vercellino et al., 2005). They can occur already during the relapsing-remitting phase, but are mainly correlated to progressive phases of the disease (M. Calabrese et al., 2012; Lucchinetti et al., 2011). In comparison to white matter lesions, cortical demyelinated lesions in MS are less inflammatory and relatively lack infiltrated

lymphocytes and microglia, the disruption of blood-brain barrier and complement deposition (M. Calabrese et al., 2015). By now, three different patterns of cortical demyelination have been defined: (1) subcortical lesions, which are most inflammatory, (2) intracortical perivascular lesions, and (3) subpial lesions which extend from the pial surface up to cortical layer IV and represent the most common and specific lesions in MS (Peterson, Bo, Mork, Chang, & Trapp, 2001). Intracortical and subpial lesions are both categorized as relatively 'non-inflammatory', which suggests that other factors contribute to the damage process of gray matter such as meningeal inflammation and the formation of meningeal follicles (Howell et al., 2011). These follicles consist mainly of proliferating B-cells, plasma cells, T-cells and macrophages and correlate with the presence of subpial lesions (Fischer et al., 2013; Haider et al., 2016). Indeed, patients with meningeal B-cell follicles show greater local inflammation and tissue damage, are younger on average at the onset of the disease and progress earlier into disability (Howell et al., 2011; Magliozzi et al., 2007). B-cell follicles can activate inflammatory cells, such as macrophages and microglia, by releasing pro-inflammatory factors. These inflammatory cells can subsequently promote cortical demyelination and neuroaxonal damage, e.g. via promoting oxidative stress and thus, drive pathological processes of the gray matter during progressive phases of the disease (Fischer et al., 2013; Haider et al., 2016).

A diffuse atrophy of the cortical gray matter, which is independent of cortical myelin density (V. Popescu et al., 2015), correlates clinically with increased cognitive deficits of MS patients (Damjanovic et al., 2017). Likewise, atrophy of the gray matter in the spinal cord is associated with the extent of motor deficits and predicts the progression of the disease independently from white matter atrophy (Schlaeger et al., 2015). The underlying pathological feature of this gray matter atrophy is mainly the loss of neurons and axons, i.e. neurodegenerative processes which, at least in part, appear independent from local demyelinated lesions and white matter damage (V. Popescu et al., 2015). A marked synapse loss, which can be found in both demyelinated and normal-appearing gray matter (NAGM) of MS patients, is another indicator for the presence of such diffuse processes of neurodegeneration of the MS gray matter (Albert et al., 2017; Jürgens et al., 2015).

In summary, both white and gray matter damage, stress the importance of diffuse neurodegenerative processes for the progression of MS. However, the exact pathogenesis of neurodegeneration in MS is still unexplored and most likely based on the combination of inflammatory and non-inflammatory mechanisms.

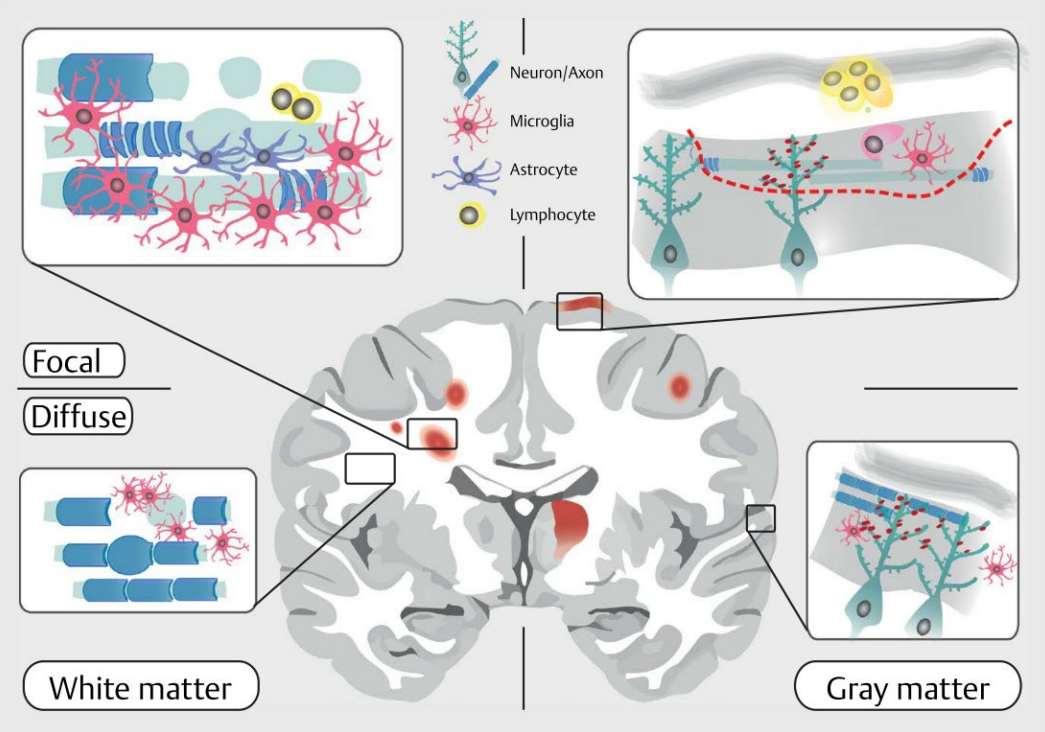


Figure 3: Focal and diffuse changes in the white and gray matter in progressive MS

Patients with progressive MS exhibit focal white matter lesions, mainly chronic active lesions, which are characterized by only few inflammatory cells in the center of the lesion and a rim of activated microglia. In addition to these focal lesions, there is evidence of diffuse inflammatory activity in the white matter, which is mainly characterized by a diffuse activation of microglia. Focal pathologies of the gray matter in progressive MS are characterized by cortical demyelination and meningeal infiltrates, such as B-cell follicles. Diffuse pathological processes within the gray matter of MS patients lead to the loss of synapses, axons, and neurons which is reflected in the increasing atrophy of gray matter. Figure adapted from (Schumacher et al., 2017).

1.2.5.2 Pathogenesis of neuroaxonal damage and loss

Neuroaxonal damage and loss are key pathological features of multiple sclerosis–most prominently in patients with progressive forms of MS. These pathologies correlate with the cumulative neurological disability in MS patients, and can progress independently from signs of direct inflammatory demyelination (Bjartmar, Kidd, Mörk, Rudick, & Trapp, 2000; V. Popescu et al., 2015).

The pathogenesis of neurodegeneration in MS is most likely based on the combination of (1) inflammatory processes, which result in a cascade of pathological events leading to neurodegeneration, and (2) primary neurodegenerative processes, that are correlated to the respective genetic and metabolic background and which may be influenced by inflammatory responses. The following section will focus on the inflammatory processes.

The release of soluble factors, e.g. antibodies, cytokines and chemokines, by immune cells is one possible cause for the diffuse damage of neurons and axons. Meningeal inflammatory infiltrates and meningeal B-cell follicles can be the producers of such soluble factors (Howell et al., 2011). Indeed, increased concentrations of pro-inflammatory mediators such as the cytokines TNFα and interferon-γ can be detected in the CSF of progressive MS patients (Gardner et al., 2013; Rossi et al., 2014). Moreover, resident cells of the brain parenchyma, such as activated microglia which release cytokines and chemokines, can maintain a chronic inflammatory environment.

A second mechanism of diffuse neurodegeneration is the accumulation of reactive oxygen and nitrogen species (ROS/RNS), which have high oxidative potential and can directly damage nerve and glial cells. ROS/RNS are mainly released by infiltrating macrophages and activated microglia. However, oxidized DNA and phospholipids can also be found in chronically active lesions which show less inflammatory cells. This indicates the presence of amplification mechanisms. One of them is a progressive mitochondrial dysfunction due to oxidative injury (Haider et al., 2011; Nikic et al., 2011). Mitochondrial damages, in the form of mtDNA mutations, promote protein misfolding in the endoplasmic reticulum (ER) and as such, they cause a lack of energy and reduced neuronal capability. As axonal transport processes are highly energy demanding, further energy deficiency and metabolic stress cause a vicious circle of tissue damage, which is further amplified by iron ions that accumulate in the aging brain (Correale et al., 2017). The energy imbalance leads to the redistribution of various neuron ion channels, such as the Na^+ channels along demyelinated neurons (Craner et al., 2004; Friese et al., 2007; Schattling et al., 2012). This leads to the rise of axonal Na^+. Via the operating Na^+/Ca^{2+} exchanger, the axoplasmatic Ca^{2+} concentration rises. This in turn initiates neurodegenerative processes by activating Ca^{2+}-dependent catabolic enzymes (such as proteases and calpains) which eventually leads to axonal injury (Stys, 2005). Indeed, the axonal injury marker APP colocalizes with the redistribution of Na^{2+} channels in both EAE and MS (Vergo et al., 2011).

A third mechanism which is potentially involved in diffuse neurodegeneration is glutamate-mediated excitotoxicity, meaning neuronal over-excitation due to increased extracellular concentrations of the transmitter glutamate. Glutamate is the main excitatory neurotransmitter of the CNS and is excessively released by cells of the nervous system as well as by immune cells during inflammation. It is directly toxic to neurons and axons by activating glutamate receptors (NMDA and AMPA/kainate glutamate receptors) which are preferably located

postsynaptically. The activation can result in a massive influx of calcium into neurons and lead to the activation of Ca^{2+}-dependent catabolic enzymes (Matute, Sanchez-Gomez, Martinez-Millan, & Miledi, 1997; Ouardouz et al., 2009). Further details on the role and mechanisms of glutamate-mediated excitotoxicity in MS will be discussed in the context synaptic pathology in gray matter in section 1.3.3.

1.2.5.3 Brief excursus: mononuclear phagocyte system of the CNS

Given the significant role of the mononuclear phagocyte system of the CNS for neurodegeneration, the following section will give a short overview of its structure and function.

The mononuclear phagocyte system of the CNS is partly formed by the population of bone marrow–derived myeloid cells. These cells circulate as monocytes in the blood and reside as macrophages in the CNS during inflammatory processes to remove myelin debris and inflammatory secondary products. Depending on the differential expression of chemokine receptors, highly mobile inflammatory monocytes (Ly-$6C^{hi}$ CCR^{2+} $CX3CR1^{lo}$) can be distinguished from resident monocytes (Ly-$6C^{lo}$ CCR^{2-} $CX3CR1^{hi}$).

In contrast to the population of monocytes, parenchymal or juxtavascular local microglia ($CX3CR1^{hi}$) engraft the brain during early embryogenesis. Their function is amongst others to sense pathological changes such as inflammatory stimuli in the environment, phagocytose debris, produce growth factors and inflammatory cytokines and possibly to control the maintenance of synapses (Prinz, Priller, Sisodia, & Ransohoff, 2011).

The specific role of monocytes and microglia in MS and the question of whether the role is protective, pathogenic or both remain undetermined. Experiments in animal models of MS (EAE) have suggested that monocyte-derived macrophages rather initiate demyelination at disease onset, whereas microglia may be more important for clearing debris (Yamasaki et al., 2014). Monocytes are recruited continuously even in patients with long-standing and progressive multiple sclerosis. Further, they are required for cortical demyelination (Lagumersindez-Denis et al., 2017). In primary neurodegnerative diseases such as Alzheimer's disease, failed neuroprotective microglial function is considered to be a major component of the disease (Heneka, Kummer, & Latz, 2014). In progressive phases of MS, the diffuse and continuous activation of microglia is proposed to maintain a chronic inflammatory environment and by this, to promote cortical demyelination and neuroaxonal damage.

1.2.5.4 Inflammatory vs. primary neurodegenerative processes

It has been proposed that the events leading to neurodegeneration are triggered by inflammatory demyelinating processes affecting neurons and axons and strengthened by pathogenic mechanisms related to brain aging and the accumulated burden of disease, finally resulting in axonal and neuronal death due to chronic cell stress and an imbalanced ionic homoeostasis (Mahad, Trapp, & Lassmann, 2015).

However, the question of whether inflammation or neurodegeneration is the primary process is still under debate. Also, the exact interplay between inflammation and neurodegeneration over the course of the disease is unknown.

On one hand, there is evidence that neurodegeneration can, at least in part, evolve independently from an acute inflammatory environment and that it is influenced by the genetic and metabolic background. Also, efficient therapies which target long-term disability and progressive forms of MS are still lacking. These arguments would therefore support the idea of an autoimmune triggered neuroaxonal injury with self-supporting neurodegenerative processes (Louapre & Lubetzki, 2015).

On the other hand, there is evidence that inflammation is a predictor of secondary neurodegeneration in MS and that chronic (possibly undetected) inflammatory signaling in the gray matter contributes to gray matter atrophy. Chronic inflammatory signaling, for example originating from lymphoid aggregates in the meninges, may maintain and promote neurodegenerative processes (Haider et al., 2011; Howell et al., 2011; Hutchinson, 2015; Magliozzi et al., 2018). Neurodegeneration can also result from chronic inflammatory lesions of the white matter which lead to the degeneration of neuronal connections and therefore, to progressive degeneration in the gray matter, i.e. the loss of neurons, axons, and synapses. Finally, highly effective immunosuppressive therapies do not only diminish the amount of relapses but also the risk of evolving increased disability (Jokubaitis et al., 2016).

Overall, the aforementioned findings indicate that an optimal therapy for patients with progressive MS needs to be based on a combination of immunomodulatory and neuroprotective strategies in order to address these manifold pathophysiological mechanisms of MS.

1.2.6 Experimental animal models

Experimental autoimmune encephalomyelitis (EAE), or experimental allergic encephalomyelitis, is the most widely used animal model for multiple sclerosis. It represents a condition in which the interplay between neuro- and immunopathological mechanisms results

in an approximation of the pathophysiological signs of multiple sclerosis: inflammation, loss of myelin, neuroaxonal damage and gliosis (Constantinescu, Farooqi, O'Brien, & Gran, 2011). Originally, it was observed from using anti-rabies vaccinations, which still contained brain tissue, that the sensitization of humans with brain cells can be followed by neuroparalytic symptoms and induce a MS-like disease (Lassmann & Bradl, 2017). Based on this observation, efforts were made to reproduce the disease in an animal model. First active immunization was performed by using emulsions of brain tissue resolved in saline. To stimulate the immune response, strong adjuvants, most often Freund's adjuvant, which allows to release the sensitizing antigen slowly and uses inactivated mycobacteria, have been added to the protocol (Kabat, Wolf, Bezer, & Murray, 1951). The effect was the massive stimulation of antigen presentation by phagocytes and thus, the activation and expansion of CD^{4+} T-cells (Billiau & Matthys, 2001). Therefore, a CD^{4+} mediated T-cell response dominates in almost all of the models used so far (Lassmann & Bradl, 2017).

Today, depending on the underlying question, a wide range of different models exists to mirror different aspects and mechanisms of MS pathology.

(1) EAE can be induced actively (aEAE) by immunizing experimental animals with a CNS antigen together with a strong adjuvant such as the complete Freund's adjuvant. The most frequent antigen used for sensitization is the myelin oligodendroglial glycoprotein (MOG) (Mendel, Kerlero de Rosbo, & Ben-Nun, 1995); other CNS antigens are the myelin basic protein (MBP) or the proteolipid protein (PLP) (Steinman, 1999). Mice are additionally required to be treated with pertussis toxin to augment the sensitization process (Bernard, 1976). Major advantages of this aEAE are the simple induction, the solid and fast results provided by it, and the possibility to study the encephalitogenic response of myelin and neuronal autoantigens. A major limitation, however, is the mode of induction, which requires large amounts of adjuvants and thus, may globally modulate the immune reactivity in experimental animals (Krishnamoorthy & Wekerle, 2009). (2) EAE also can be induced passively (pEAE) by transferring *in vitro*-activated autoimmune T-cell lines (Ben-Nun, Wekerle, & Cohen, 1981; Wekerle, Kojima, Lannes-Vieira, Lassmann, & Linington, 1994). This passive transfer model allows in particular to study the mechanisms of a T-cell mediated inflammation of the CNS (Lassmann & Bradl, 2017). (3) As both aEAE and pEAE only offer limited possibilities to study the mechanisms initiating the disease or controlling relapses, models of spontaneous EAE have been created. They were induced by transgenically expressing a T-cell receptor (TCR) of encephalitogenic T-cells, which detects brain antigens (Bettelli et al., 2003). (4) To investigate

the role of human TCR on the disease, humanized mice, which carry TCR from human T-cell clones, have been developed (Ben-Nun et al., 2014).

(5) All of the models described above induce inflammatory demyelinated lesions which are mainly located and randomly distributed within the spinal cord. Often, a precise and reproducible localization of lesions is required, e.g. for studying molecular mechanisms in a specific area of the CNS *in vivo*. To induce reproducible inflammatory and demyelinated lesions in the cerebral cortex, one approach was to stereotactically inject the pro-inflammatory cytokines tumour necrosis factor (TNFα) and interferon gamma (IFNγ) into the cortices of rats which were pre-immunized with myelin oligodendrocyte glycoprotein $MOG_{1–125}$ (Merkler et al., 2006). The latter induces the anti-myelin antibody response and the encephalitogenic T-cell response, whereas the focal injection of cytokines leads to localized inflammatory and demyelinated lesions at the desired site of the CNS (Lassmann & Bradl, 2017). This procedure opened the possibility to study the evolution and resolution of cortical pathology (Merkler et al., 2006). Also, both TNFα and IFNγ were found to be increased in the meninges of patients with secondary progressive multiple sclerosis and therefore, might be crucial for driving inflammation in progressive MS and contributing to neuronal damage (Gardner et al., 2013). Therefore, to pursue our aim of studying cortical pathology and to induce neuroinflammation in the cortices of mice, we adapted these previous targeted models of cortical demyelination from rats (Gardner et al., 2013; Merkler et al., 2006) to mice in our studies (c-MS model) (Jafari et al., 2021).

(6) Moreover, autoimmune CNS gray matter pathology has been recently induced passively, by directing T–cells against the neuronal protein β-synuclein. The resulting inflammation led to typical patterns of gliosis, neuronal destruction and brain atrophy and thus made it possible to study T-cell mediated processes that are directed against the gray matter (Lodygin et al., 2019).

1.3 Synaptopathy in MS

Gray matter damage in MS is associated with rising disability, increasing cognitive and neuropsychiatric symptoms, and the conversion to progressive MS (Damjanovic et al., 2017; Eshaghi et al., 2018; Ontaneda et al., 2017; Scalfari et al., 2018). Next to demyelination, axonal transsection and neuronal loss, synaptopathy, i.e. dysfunctional synapses and the loss of synapses/dendritic spines, is a key pathological feature in gray matter lesions in MS.

The following chapter will first introduce the structure and function of dendritic spines, which were the target object of my thesis. Secondly, it will demonstrate the evidence of synapse loss

in MS and in animal models of MS, and finally outline the present findings about the mechanisms involved in synaptic pathology and loss.

1.3.1 Structure and function of dendritic spines

In the mammalian brain, most neuronal synaptic connections occur on dendritic spines which represent the postsynaptic terminals of mainly excitatory synapses and are found as specialized structures protruding from the dendritic shaft (Higley & Sabatini, 2012). Being prominent features of neuronal morphology, spines were stained and imaged in fixed human brain tissues of patients with neurological disorders to investigate the correlation between dendritic spine morphology/density, and the progression of diseases (Mancuso, Chen, Li, Xue, & Wong, 2013). The number and functionality of dendritic spines is correlated proportionally to the number and functionality of synapses. Indeed, in many neuronal populations such as hippcampal pyramidal cells, spine number complies with synapse number (Alvarez & Sabatini, 2007; Nimchinsky, Yasuda, Oertner, & Svoboda, 2004). A reduced spine density, usually to be equated with the loss of excitatory synapses, is observed in many neurological diseases and is in particular correlated to cognitive impairment in neurodegenerative conditions (Bittner et al., 2010; Moolman, Vitolo, Vonsattel, & Shelanski, 2004), including MS (Albert et al., 2017; Dutta et al., 2011; Jürgens et al., 2015). Overall, the number and physiological dynamics of dendritic spines are great indicators of synaptic connectivity and thus functionality of neuronal circuits (B. Calabrese, Wilson, & Halpain, 2006) which made them target objects of my thesis to study synaptic pathology in MS.

1.3.2 Synapse loss in MS and in animal models of MS

Widespread synaptic loss has been identified in the forebrain, hippocampus, cerebellar dentate nucleus and spinal cord of patients with multiple sclerosis (Albert et al., 2017; Dutta et al., 2011; Jürgens et al., 2015; Petrova et al., 2020). As all neuronal compartments, i.e. neuronal cell bodies, axons and synapses are affected in the gray matter of patients with MS, finding the order of degeneration during the disease course would have a great clinical implication as treatments should keep the neuron, but also their axons and synapses alive (Friese, 2016). In Alzheimer's disease, for example, neuronal death is the result of synaptic dysfunction and consequent synaptic loss (Selkoe, 2002). Therefore, also in MS, synaptic loss may initiate neuronal death and drive the disease progression.

Several findings support the idea that synaptopathy and synaptic loss are primary processes during neurodegeneration in MS: (1) synaptic proteins (e.g. synaptotagmin, synaptophysin, PSD-95 and CASK), which maintain synapses and synaptic functions, were found to be reduced

in demyelinated hippocampi of patients with progressive MS, while hippocamal neuronal loss was minimal (Dutta et al., 2011). (2) The loss of dendritic spines occurs independently of cortical demyelination. The loss is apparent throughout the demyelinated and the non-demyelinated, the so-called 'normal-appearing' gray matter (NAGM) in patients with MS. (3) Moreover, dendritic spine loss is independent of axonal loss (Albert et al., 2017; Jürgens et al., 2015).

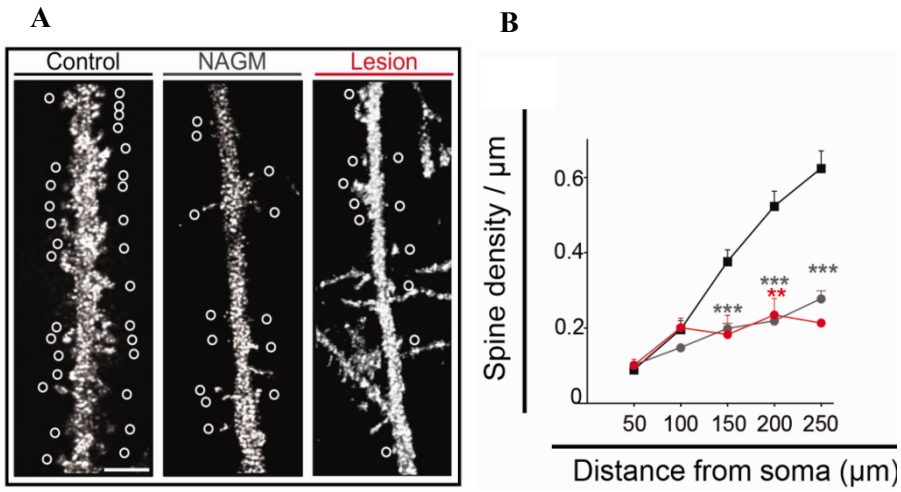


Figure 4: Dendritic spine density of individual cortical neurons in patients with MS

(A) Confocal projection images demonstrate distal segments of Golgi-Cox impregnated dendrites of frontotemporal cortical layer IV-VI neurons located in: the control cortex (left); in the normal appearing gray matter (NAGM, middle) of a MS cortex; in a lesion, i.e. demyelinated (right) area of a MS cortex. Hollow circles mark spines along those apical dendrites. (B) Quantification of spine density along the main apical dendrite from cortical layer IV-VI neurons, which were located in the insular lobe, reveals a reduction in spine density in both NAGM and lesion areas of multiple sclerosis cortices. Figure adapted from and caption modified from (Jürgens et al., 2015).

Also in animal models of MS, synaptic alterations and the loss of dendritic spines have been demonstrated. In EAE, inflammation was shown to lead to dendritic spine loss which can occur independently of demyelination or the amount of neuronal somata and dendrites. These findings suggest that synapses are highly sensitive to homeostasis changes such as inflammation, and that apoptosis can be limited to synapses. Therefore, inflammatory processes can result in synaptic failure without inducing neuronal cell death (Centonze et al., 2009).

In previous studies, our laboratory induced cortical neuroinflammation in mice (cortical MS, c-MS) by using cortically targeted models of experimental autoimmune encephalomyelitis (Jafari et al., 2021). In this model of c-MS, the density of dendritic spines was significantly reduced as well. Moreover, the loss of dendritic spines was reversible and dendritic spine density recovered over time. By using electron microscopy, the transient reduction of cortical synapses was

confirmed and quantitative immunofluorescence analysis showed a preferential loss of excitatory synapses (Jafari et al., 2021).

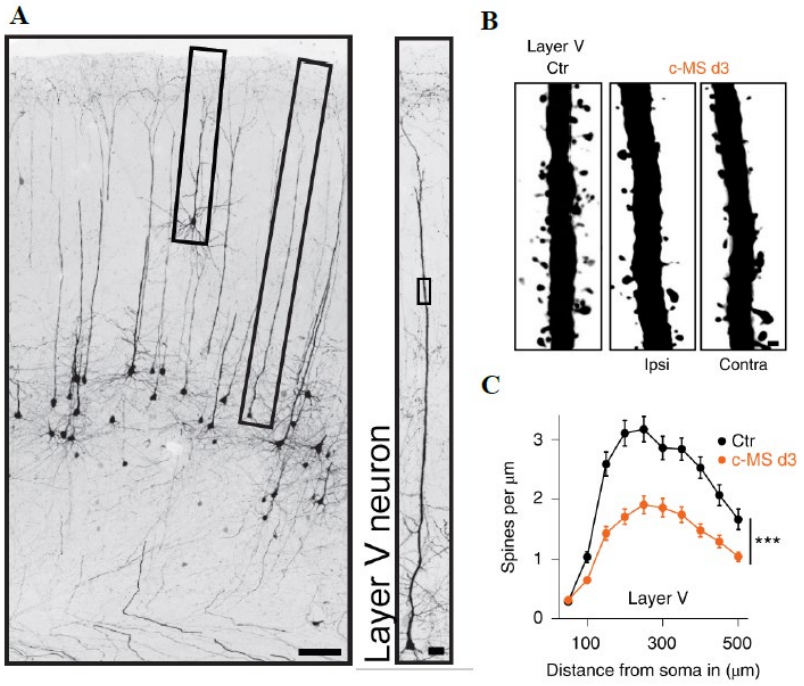


Figure 5: Reduced spine density in the cortical MS model

(A) Confocal projection images demonstrate reconstructed layer V cortical neurons. (B) Representative images of deconvoluted apical dendrites of layer V pyramidal neurons demonstrate spines along dendrites in healthy controls and in mice with c-MS in both hemispheres (ipsilateral and contralateral to cytokine injection). (C) Quantification of dendritic spine density of layer V pyramidal neurons in control and c-MS mice reveals a spine loss in c-MS animals. Figure adapted from and caption modified from (Jafari et al., 2021).

1.3.3 Mechanisms of synapse loss in MS

The above described neuropathological findings raise the question about the underlying pathophysiological mechanisms driving synapse loss. Indeed, several possible mechanisms for synapse loss in MS have been reported.

1.3.3.1 Role of glial cells

First, there is evidence of a direct inflammation-mediated synaptopathy which is triggered by the infiltration of macrophages and T-cells, or the chronic activation of microglia (Albert et al., 2017; Kreutzfeldt et al., 2013; Ziehn, Avedisian, Tiwari-Woodruff, & Voskuhl, 2010). Several findings support the idea of a glia-mediated synaptic removal in the inflamed cortex. First, in the cerebellum of MS patients, an increased surveillance of synapses by astrocytes and microglia, with or without active synaptic removal, is observed (Albert et al., 2017). Secondly,

activated microglia or astrocytes release proinflammatory cytokines such as IL-1β and TNFα which alter synaptic transmission and induce synaptic loss in EAE (Centonze et al., 2009; Haji et al., 2012; Nistico et al., 2013; Rossi et al., 2012; Verderio et al., 2012; G. Yang, Parkhurst, Hayes, & Gan, 2013). Thirdly, microglia can remove synapses, not only in the context of neuronal injury (termed 'synaptic stripping'), but also during development, learning and memory consolidation (termed 'synaptic pruning') (Kettenmann, Kirchhoff, & Verkhratsky, 2013; Mandolesi et al., 2015). During the development of the nervous system, complement proteins are significant mediators of synapse removal: complement-tagged synapses are detected and subsequently removed by micrgolia cells (Schafer et al., 2012; Stevens et al., 2007). An increased activation of the complement system was shown in the gray matter of progressive MS patients and was spatially related to synaptic pathology and microglia cells (Michailidou et al., 2015). These findings suggest that similar mechanisms of the developing nervous system are reactivated in the inflamed cortex and are responsible for pathological elimination of synapses in multiple sclerosis.

1.3.3.2 Degeneration of neuronal connections

A second potential mechanism of synaptic loss is the degeneration of neuronal connections. This degeneration could be both antero- and retrograde. Anterograde, often referred to as 'transsynaptic' degeneration, is caused by a loss of afferent input due to the degeneration of afferent neurons and the transsection of their axons (M. Calabrese et al., 2015; Peterson et al., 2001). Retrograde degeneration is usually the result of axonal injury in the white matter of MS (Geurts & Barkhof, 2008; Park, Park, Cho, & Park, 2013). Cortical neurons with signs of retrograde neurodegeneration can be traced by measuring the amount of cytoplasmic phosphorylated neurofilaments which accumulate when an axon is transected. This type of neurodegeneration can mainly be found in close relation to demyelinated lesions and axonal loss in the white matter (Haider et al., 2016). Several observations support the idea of a propagated neuronal degeneration in MS and the contribution to spine loss. In the cerebellum for example, where spine loss has been found, reduced Purkinje cell numbers within areas of cortical cerebellar demyelination have been reported (Kutzelnigg et al., 2007; Redondo et al., 2015) and would be compatible with the idea of a reduced afferent which leads to an anterograde degeneration. Moreover, considering the common presence of lesions in deeper gray matter areas, such as the thalamus, a partial contribution of retrograde degeneration commonly resulting from axonal transection in white-matter tracts cannot be excluded (Haider et al., 2016). However, a solely degenerative mechanism of spine loss appears unlikely, because (1) gray matter and white matter pathologies occur, at least in part, independent from one

another, and (2) therapies which prevent the formation of white matter lesions do not necessarily prevent progressive neuronal degeneration.

1.3.3.3 Calcium dyshomeostasis and glutamate-mediated toxicity

Impaired calcium homeostasis has been shown to be the initiation signal for loss of axons or dendrites in neuroinflammatory lesions (Siffrin et al., 2010; Witte et al., 2019). Also in Alzheimer's disease, disturbances in calcium homeostasis were shown to disrupt the structure and function of neuronal networks (Kuchibhotla et al., 2008). These findings render calcium dyshomeostasis a likely role in synaptic pathology in MS.

A potential source of calcium in spines is the influx from the extracellular compartment via ionotropic glutamate receptors, including NMDA, AMPA or kainate receptors. As glutamate can be excessively released in neuroinflammatory conditions, this can result in an increased postsynaptic transmission and in cell damage caused by the accompanying Ca^{2+} influx.

Glutamate levels are shown to be increased in the brains and CSF of patients with MS, whereas glutamate receptor expression and clearance are reduced. Increased glutamate levels can mainly be found in active lesions, but also in the NAWM, where the amount of glutamate concentration predicts the extent of neuroaxonal damage. Moreover, glutamate receptor antagonists show beneficial effects in MS and in animal models of MS by limiting neuronal and oligodendrocytic damage (Azevedo et al., 2014; Centonze et al., 2009).

The exact distribution and origin of glutamate in MS is unknown. At least in the setting of neuroinflammatory lesions, a main source is considered to be the release of glutamate by immune cells such as T-cells, dendritic cells, macrophages and microglia. There is also evidence for an increased presynaptic release of glutamate from neurons under inflammatory conditions (Macrez, Stys, Vivien, Lipton, & Docagne, 2016). At the same time, the uptake of glutamate in glial cells is impaired due to the loss of excitatory amino acid transporters (EAAT) in MS lesions (Pitt, Nagelmeier, Wilson, & Raine, 2003). The resulting increased and constant availability of glutamate leads to an upregulation of glutamate receptors such as NMDA and AMPA receptors which are preferably located postsynaptically and thus, could particularly contribute to synaptic pathology in the gray matter (Bolton & Paul, 2006; Centonze et al., 2009; Grasselli et al., 2013). Indeed, the loss of glutamate transporters (EAATs) is correlated with the presence of activated microglia and with signs of synaptic damage in cortical lesions in MS—an observation which links inflammation, glutmate-mediated excitotoxicity and synaptic damage in cortical lesions (Vercellino et al., 2007).

In view of the above, glutamate receptor antagonists such as AMPA/kainate or NDMAR antagonists were assumed to counteract these mechanisms and by this, to have protective effects on neurodegeneration and on synaptopathy. In animal models of MS, the application of those antagonists ameliorated the disease course and prevented synaptic degeneration (Centonze et al., 2009; Pitt, Werner, & Raine, 2000; T. Smith, Groom, Zhu, & Turski, 2000). Overall, this makes glutamate an interesting target structure for prospective therapy.

In summary, synapse loss is a key feature of gray matter pathology in MS and can be reproduced in cortically targeted models of experimental allergic encephalomyelitis (cortical MS). Several mechanisms can be involved in the processes leading to synapse loss, among these are calcium dyshomeostasis, inflammatory glia-mediated mechanisms, neuronal antero-/retrograde degeneration, and glutamate-mediated excitotoxicity. The following considerations emphasize the significance of studying the mechanisms leading to synapse loss in cortical neuroinflammation: (1) synapse loss is a significant feature of gray matter damage in MS, but also a hallmark of other neurological diseases, (2) synapses play a major role for the maintenance of neuronal connectivity and their loss can induce neuronal death, and (3) synapse loss can be reversible, thus it can be restored by early interventions and by this, synaptic plasticity can be maintained.

Overall, a better understanding of the mechanisms that lead to synapse loss and synaptic dysfunction could have huge clinical impacts, not only for MS, but also for other neurological disorder where a correlation between inflammation and neurodegenerative processes is suggested. The questions, (1) if synaptic calcium disturbances drive dendritic spine loss in the c-MS model, (2) if glutamate-mediated excitotoxicity is involved in calcium disturbances and synapse loss, and (3) which cells finally execute synapse removal, remain unanswered. Finding the answers to these questions motivated the experiments described in my thesis.

1.4 Intravital imaging of calcium

1.4.1 Imaging calcium in single dendritic spines *in vivo*

The classical dendritic spine consists of a bulbous head with a diameter of approximately 0.5µm. The head is connected to the parent dendritic shaft by a thin neck and thereby creates an isolated signaling compartment of biochemical and electrical signals which read out and regulate synaptic activity (Higley & Sabatini, 2012). Today, the use of animal models together with technical advances allow to examine changes in the number, size and structure of dendritic spines, and correlate those to functional and biochemical aspects of individual dendritic spines (Mancuso et al., 2013).

Intracellular calcium which accumulates in spines is the most studied biochemical signal. However, imaging calcium in single dendritic spines in the living brain has been quite a daunting challenge in the past. Reasons are: the small size of dendritic spines, the fact that the living brain is highly scattering and absorbent to visible photons, and finally, the sheer complexity of the mammalian brain and the extremely high density of synapses.
In general, the understanding of calcium signaling in a single dendritic spine was achieved by three fundamental technical developments: (1) the development of two-photon laser-scanning microscopy (2PLSM), (2) the progresses in the design and application of calcium indicators which provide bright, fast and high-dynamic ranged calcium sensitive fluorophores, and (3) the development of photosensitive derivatives of neurotransmitters which directly stimulate spines and by this, allow to image synaptic signaling in dendritic spines without the need to electrically activate axons (Higley & Sabatini, 2012). My thesis aimed to reveal *continuous* dysregulated calcium accumulations in single dendritic spines due to inflammatory signaling. In view of this, the following will focus only on the main considerations of the first two technical approaches.

1.4.2 *In vivo* two-photon microscopy of the CNS

Over the past two decades, the development of two-photon laser-scanning microscopy (Denk, Strickler, & Webb, 1990), combined with *in vivo* fluorescence labeling techniques, have allowed researchers to study real-time cellular, subcellular and molecular mechanisms of the CNS in rodent models of neurological diseases (Misgeld & Kerschensteiner, 2006).
In general, optical microscopy includes two forms of excitation: linear and nonlinear one. In traditional techniques, such as confocal microscopy, the process of generating contrast uses only one single photon and thus, depends linearly on the light intensity. In contrast, in non-linear techniques, the excitation depends on the almost simultaneous absorption of multiple photons of longer wavelength (near-infrared wavelength) (Helmchen & Denk, 2005; Misgeld & Kerschensteiner, 2006). For biochemical imaging, mainly two-photon absorption is used: two photons which arrive almost simultaneously at a molecule promote the molecule to an excited state by merging their energies. This is followed by normal fluorescence emission of the excited molecule (Denk et al., 1990; Helmchen & Denk, 2005).

As transition probabilities are very low at normal light intensities in most nonlinear processes, excitation light has to be concentrated in space and time. Femtosecond lasers, e.g. the commonly used Titanium-sapphire (Ti:sapphire) oscillators, generate ultrashort pulses with high peak intensities to ensure the concentration in time. By focusing such a laser beam through a high numerical aperture (NA) objective, spatial concentration is achieved (Helmchen & Denk,

2005). Thereby, multiphoton absorption is limited to a defined perifocal region and the need for a pinhole to exclude out-of-focus fluorescence light (as applied in confocal microscopy) is circumvented (Helmchen & Denk, 2005; Misgeld & Kerschensteiner, 2006). Even in strongly scattering tissue, emitted photons can be detected as all fluorescence photons are known to originate in the perifocal region. This advantage along with the benefits that (1) longer wavelengths allow to penetrate deeper into scattering tissue (imaging depth can reach up to 1000 µm (Theer, Hasan, & Denk, 2003)), (2) near-infrared light is less phototoxic due to the reduced absorption by endogenous chromophores (Svoboda & Yasuda, 2006), and (3) reduced phototoxicity raises tissue viability needed for long-term imaging (Squirrell, Wokosin, White, & Bavister, 1999) account for the practicality of 2PLSM for *in vivo* imaging.

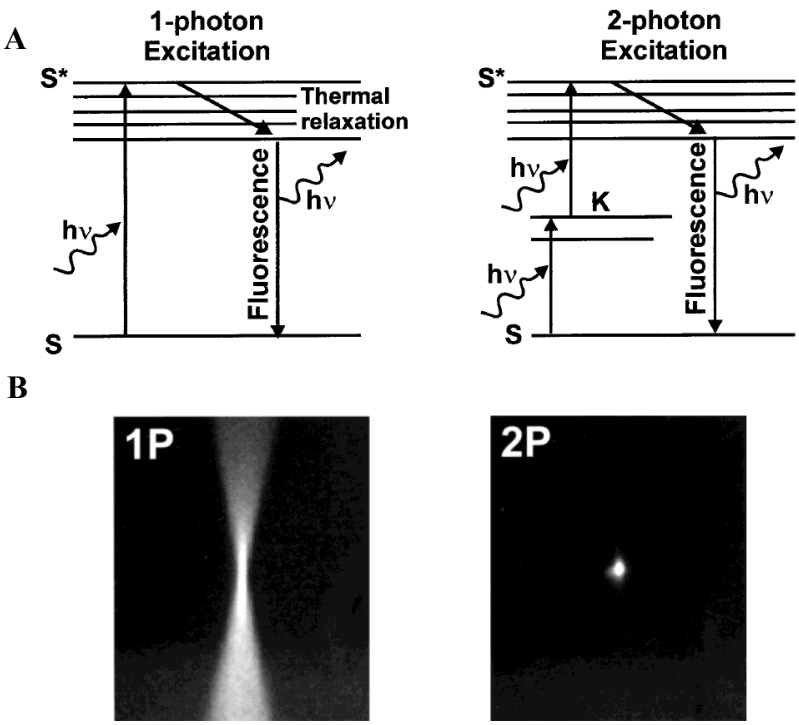


Figure 6: Basic principles of one- and two-photon excitation

(A) Jablonski diagram of one-photon and two-photon excitation. Fluorescence emission from an excited state (S*) can be evoked by the excitation with one photon (left) or simultaneous absorption of two photons with longer wavelengths who merge their energies and thus, have lower energy per photon (right). After achieving the excited state S*, the way of fluorescence emission is equal in both forms. The emitted photon has a longer wavelength than the excited one, due to the energy loss which is associated with thermal relaxation from the excited state. (B) Fluorescence emission in a solution of fluorescein. In linear/single-photon microscopy, the cone of excitation results in fluorescence emission throughout the depth of the sample (left), whereas in multiphoton, nonlinear microscopy excitation is highly localized. Figure adapted from and caption modified from (Soeller & Cannell, 1999).

1.4.3 Fluorescence labeling techniques

Along with the progresses of multiphoton microscopy, the development of *in vivo* fluorescence labeling techniques has been crucial for the performance of intravital imaging studies of the CNS. The following will demonstrate the main milestones which made *in vivo* microscopy of the nervous system possible.

The labeling of single neurons was achieved by the generation of transgenic mice which selectively express different spectral variants of fluorescents proteins (XFPs) under the control of a modified Thy1-promoter element in their neurons (Feng et al., 2000). Depending on differences in the chromosomal integration site, the reporter expression could be restricted to a small number of neuronal subsets and by this, an intravital Golgi-like staining pattern could be

created (Feng et al., 2000). This made sparse labeling of selected structures in selected sites possible. Since the introduction of Thy1-XFP lines by Feng and colleagues (Feng et al., 2000), numerous Thy1-transgenic lines have been generated and enabled imaging of dendrites, neuronal subtypes, and entire axons in specific sites of the nervous system. Even subcellular structures (e.g. mitochondria) or basic cell biological processes mechanisms (e.g. synaptic transmitter release or neuronal calcium concentrations) can be assessed (Bareyre, Kerschensteiner, Misgeld, & Sanes, 2005; Feng et al., 2000; Keller-Peck et al., 2001; Lee et al., 2006; **Error! Hyperlink reference not valid.**). To label glial cells, such as astrocytes, microglia and oligodendrocytes, other transgenic lines, where fluorescent proteins are expressed under the control of a specific promoter, have been generated (Fuss et al., 2000; Jung et al., 2000; Nimmerjahn, Kirchhoff, & Helmchen, 2005; Nolte et al., 2001). By using knock-in strategies and differential labeling, macrophages along with microglia can be imaged under neuroinflammatory conditions (Saederup et al., 2010). Moreover, for specific components of the nervous system, vital dyes can be added *in vivo* and viral labeling, which can be coupled with transgenic mouse lines, has been described for most cell types as of today (Dittgen et al., 2004; Flügel, Willem, Berkowicz, & Wekerle, 1999; Romanelli et al., 2013).

1.4.4 Principles of genetically encoded calcium indicators

In the CNS, intracellular calcium signals regulate several processes: triggering neurotransmission in presynaptic terminals, inducting synaptic plasticity, and regulating gene transcription in the nuclei (Berridge, Bootman, & Roderick, 2003; Zucker, 1999). Moreover, overload in cellular calcium or disturbances of intracellular calcium compartmentalization can result in cytotoxicity and eventually lead to cell death (Orrenius, Zhivotovsky, & Nicotera, 2003). Therefore, many investigations have been made to develop techniques which allow the visualization and quantitative assessment of neuronal intracellular calcium signals.

A breakthrough of measuring intracellular calcium signals has been the introduction of protein-based genetically encoded calcium indicators (GECIs) by the laboratory of Roger Tsien (Miyawaki et al., 1997), which subsequently have been diversified. Today, imaging with genetically encoded calcium indicators has become a common method in neurosciences. By making use of modern 2PLSM together with improved GECIs, *in vivo* imaging of calcium in small neuronal compartments such as dendritic spines has become possible and has been performed in my thesis.

The term ‘genetically encoded’ means that the sensors are only composed of amino acids and no synthetic compound or cofactors are added. This makes it possible to apply those sensors in

the living brain. In other words, sensors are encoded by a section of DNA which can be modified. When the sequence which codes for the sensor is delivered into a cell, either via production of a transgenic mouse, or postnatally via a viral vector such as lenti- or adeno-viruses (Akerboom et al., 2012), the sensor is formed within the cell in situ. This approach offers the possibility to implant calcium indicators deeply within the tissue of living organisms. As GECIS use XFPs as fluorophores, the signal strength of the fluorescent probes is always linked to progresses on the biosensor side (Mank & Griesbeck, 2008).

In general, there are two different pathways to design GECIs: (1) single-wavelength, non-ratiometric calcium indicators, and (2) ratiometric indicators which are based on fluorescence resonance energy transfer (FRET) between two different mutants of an autofluorescent protein. The physical phenomenon fluorescence resonance energy transfer (FRET) occurs when two fluorophores change their orientation, conformation and distances in scales below 100 Å. In these cases, an excited donor will transfer its energy to an acceptor fluorophore which leads to the emission of a photon by the acceptor. In order for this phenomenon to occur, there has to be a spectral overlap between the shorter wavelength donor (which dominates in a calcium-free state) and the longer wavelength acceptor (which dominates after binding of calcium) (Jares-Erijman & Jovin, 2003; Mank & Griesbeck, 2008). The transfer efficiency E_{FRET} is described by the so-called Förster equation and E_{FRET} decreases with increasing distance r between the fluorophores.

$$E_{\mathrm{FRET}} = \frac{R_0^6}{R_0^6 + r^6}$$

Formula 1: Förster equation

The term r describes the actual distance between the fluorophores. The Förster radius R_0 is the distance at which the efficiency of energy transfer is halfmaximal. R_0 represents a specific value for each individual donor–acceptor pair. Formula adapted from (Mank & Griesbeck, 2008).

In both approaches described above, fluorescent proteins complex with calcium binding properties (such as calmodulin). If calcium binds, the emission and excitation behavior of the fluorescent proteins is altered. In general, binding kinetics and the affinity of a sensor depend on the calcium binding domain, whereas fluorophore variants influence the signal intensity (Mank & Griesbeck, 2008).

In ratiometric FRET-based calcium indicators, calcium binding brings together donor and acceptor fluorophores and induces FRET. Therefore, FRET-based sensors allow to determine calcium levels by the ratio of two fluorophore intensities and by this, provide a relative measurement. In contrast, the readout of single fluorophore, non-ratiometric Ca^{2+} indicators is a single-channel measurement, where the signal intensity depends proportionally to the amount of bound calcium ions (Mank & Griesbeck, 2008).

Single fluorophore Ca^{2+} indicators, such as GCamPs, show large response amplitudes if calcium rises and display a high dynamic range. In terms of signal-to noise level, they appear superior to FRET-based sensors (Garaschuk, Griesbeck, & Konnerth, 2007; Mank & Griesbeck, 2008). However, the low baseline fluorescence of those indicators only allows measuring changes in calcium. Lacking baseline values impede using them in animal models of neurological diseases (Mao, O'Connor, Scheuss, Nakai, & Svoboda, 2008; Schumacher, 2015). Moreover, single fluorophore Ca^{2+} indicators often lead to misreadings during movement artifacts. Also, activity-dependent autofluorescence cannot be excluded as a confounder. In particular, under *in vivo* imaging conditions, where one has to face movement artifacts regularly, those sensors appear less suitable. In contrast, FRET-based sensors, in which both emission channels are equally affected, signals are not influenced by changes in either the sensors expression level or movement artifacts. Next to this benefit of ratiometric imaging, FRET sensors offer more pH-resistance and photostability in comparison to single fluorophore Ca^{2+} indicators (Garaschuk et al., 2007; Griesbeck, 2004; Mank & Griesbeck, 2008). These factors together with the higher baseline fluorescence make FRET-based sensors the more eligible choice for intravital imaging.

Early FRET-based sensors, so-called ‘cameleons’, consisted of fusions of a blue- or cyan-emitting mutant (BFP or CFP) of the green fluorescent protein (GFP) and a green- or yellow-emitting (YFP or GFP) GFP. The calcium binding domain consisting of calmodulin (CaM) and its binding peptide M13 was inserted between two of those fluorescent proteins (Miyawaki et al., 1997). CaM is a ubiquitous signal transduction protein and is itself regulated by the host cell’s biochemical processes. Intracellular interactions which cannot be controlled might explain the lack of full functionality of various CaM-based Ca^{2+} sensors when expressed transgenically (Garaschuk et al., 2007; Hasan et al., 2004; Nagai, Yamada, Tominaga, Ichikawa, & Miyawaki, 2004). To overcome these limitations, sensors which had Troponin C from cardiac and skeletal muscle as the Ca^{2+} binding domain were generated (Heim et al., 2007). As the regulation of muscle contraction is the only known function of TnC, the TnC-based

sensors are less likely to interfere in the cell's biochemical processes and are therefore, well suited for transgenic expression (Garaschuk et al., 2007).

Both calmodulin and TnC consist of two globular domains which are connected by a central linker. As each domain has two calcium-binding EF hand motifs, up to four calcium ions can bind per sensor. This highly nonlinear mechanism contributes to calcium buffering during long term expressed GECIs. To overcome this limitation, minimal domain 'Twitch' sensors, based on a TnC variant from the toadfish Opsanus, which offers only two or one remaining calcium binding site and is at the same time highly affine to calcium, were created (Thestrup et al., 2014). The results were a reduced calcium buffering, larger dynamic ranges and linear response properties. Furthermore, diversifications in the linker optimized FRET changes of these sensors and enhanced donor/acceptor variants of fluorescent proteins increased brightness and photostability.

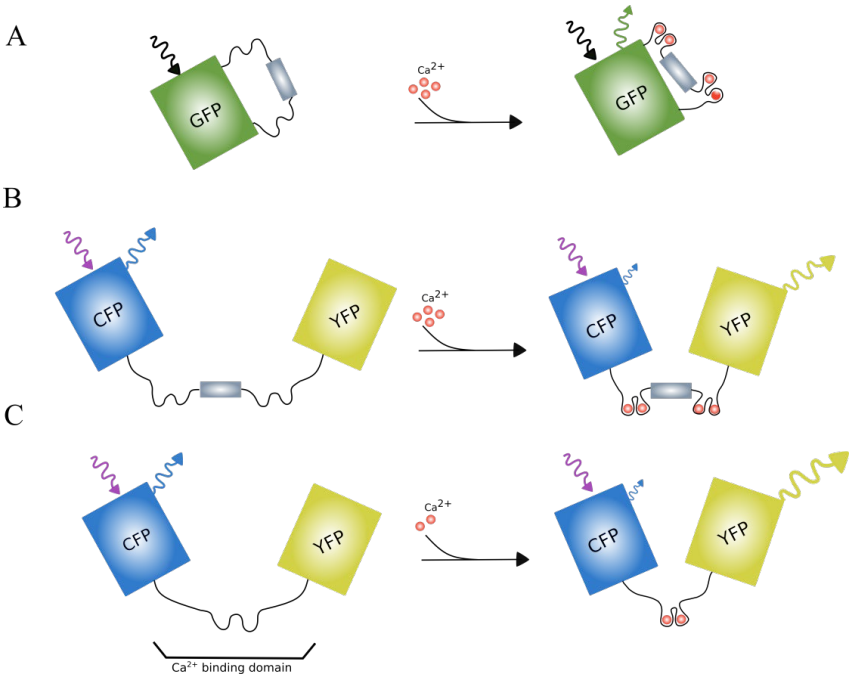


Figure 7: Genetically encoded calcium indicators

(A) Single-fluorophore, non-ratiometric genetically encoded calcium indicator. Binding of calcium to the Ca^{2+} binding domain leads to conformational intramolecular changes. This results in an increase in the emitted green fluorescence. (B) Ratiometric genetically encoded calcium indicator based on fluorescence resonance energy transfer (FRET). Binding of calcium to the Ca^{2+} binding domain brings together the donor fluorophore CFP and the acceptor fluorophore YFP. This enables fluorescence resonance energy transfer (FRET) and thus, leads to the decrease of the blue fluorescence and an increase of the yellow fluorescence. The ratio change of blue and yellow fluorescence intensities allows calcium readout independently of the volume or indicator concentration. In (A) and (B) the calcium binding domain (CaM or TnC) consists of two globular domains which are connected by a central linker. Each domain has two calcium-binding EF hand motifs- thus, up to four calcium ions can bind per sensor. (C) FRET-based Twitch sensor. Here, the calcium binding domain (TnC) was reduced to one domain consisting of just two EF hands. This minimal calcium-binding motif was fused with cyan and yellow fluorescent proteins to yield Twitch. FRET induction occurs as described in (B).

1.4.5 The choice of Twitch-2B

Twitch-2B was one of the sensors generated in the screening for minimal domain and high dynamic ranged FRET-based indicators. It incorporates the two fluorescent proteins mCerulean3 (derivate of cyan fluorescent protein (CFP)) and $cpVenus^{CD}$ (derivate of yellow fluorescent protein (YFP)) (Thestrup et al., 2014). The following section will outline the main reasons which led to us to choose Twitch-2B to image calcium levels in dendrites and dendritic spines in the c-MS model.

First, the following parameters have to be taken into account when choosing a sensor: A key parameter is the dissociation constant K_d which represents the concentration of calcium (in molar units) where half of the senor protein is bound to it and thus, represents a measure for the calcium affinity of a sensor (Grienberger & Konnerth, 2012). Another parameter is the maximal fractional ratio change (ΔR/R). It is calculated by dividing the maximal change in ratio (ΔR) by the baseline ratio (R) and represents the maximal FRET ratio change from zero calcium to calcium saturation. ΔR/R is thus an indicator for a sensor's dynamic range and ability to display small changes in calcium. Twitch-2Bs has a K_d of 200 nM and a fractional ratio change of 800%, thus is highly affine to calcium and has a high dynamic range (Thestrup et al.). These properties of Twitch-2B allow to resolve calcium fluctuations in the nanomolar range. Several additional advantages compared to other synthetic indicators or Twitch variants were observed in *in vitro* and in *in vivo* investigations on Twitch-2B performed by Thestrup and colleagues (2014): (1) at basal condition, Twitch-2Bs donor fluorescence intensity was shown to be twofold higher, (2) the signal amplitude as a function of the number of underlying neuronal action potentials was approximately linear, (3) under *in vivo* conditions, signals were stronger and had higher signal-to-noise ratio, and (4) signals did not change during long-term imaging experiments (Thestrup et al., 2014).

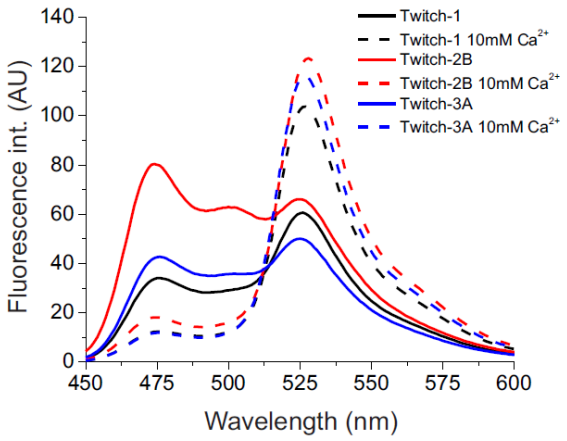


Figure 8: Emission spectra of purified 'Twitch' variants *in vitro*

The graph displays the emission spectra of equimolar amounts of purified Twitch variants at basal condition and at calcium saturation (10mM Ca^{2+}). Twitch 1: black, Twitch-2B: red, Twitch 3: blue. Note that Twitch-2B's donor fluorescence intensity is twofold higher at resting state than in other Twitch variants. Figure adapted from and caption modified from (Thestrup et al., 2014).

Considering these benefits along with the advantages of ratiometric imaging of YFP/CFP derived fluorophores for which all filters were already available, made the FRET-based Twitch-2B the sensor of choice for our purposes.

To target Twitch-2B to a subset of cortical neurons, while avoiding the need to generate a transgenic mouse line, it can be introduced into rAAV under the neuron-specific human synapsin 1 gene (hsyn1) promoter and injected into the desired neuronal population (Akerboom et al., 2012; Kügler, Kilic, & Bähr, 2003; Thestrup et al., 2014). Together with the usage of 2-photon-microscopy, we thereby achieved calcium imaging in local dendritic shafts and single dendritic spines of selected neuronal populations under the neuroinflammatory condition.

2. Objectives

Focal and diffuse alterations of the gray matter contribute to the pathological and clinical manifestations of multiple sclerosis, and in particular, correlate with the progression of the disease and with the appearance of cognitive deficits. A key feature of gray matter pathology in MS is widespread synapse loss which can be found independently of cortical demyelination and axonal loss in patients with MS. This widespread synapse loss can be reproduced in animal models of cortical neuroinflammation (cortical MS). The aim of my thesis was to unravel the mechanisms which lead to synapse loss in the cortical MS model.

The first part of my thesis aimed to characterize calcium levels in dendrites and dendritic spines in the cortical MS model. Specifically, the following questions were addressed:

- How is intracellular calcium in apical dendrites and dendritic spines affected in cortical MS?
- Can changes of intracellular calcium be detected in cortical MS? If so, what are the dynamics of these changes?
- Do changes of calcium in dendritic spines influence the fate of those spines?

The second part of the thesis aimed to explore the effector mechanisms that induce spine loss in cortical MS and, specifically, aimed to answer the following questions:

- Is the loss of dendritic spines preventable by pharmacological inhibition of glutamate receptors?
- Do the activation of local microglia and the infiltration of monocytes correlate with the spatial and temporal characteristics of spine loss in cortical MS?

3. Materials and methods

3.1 Materials lists

3.1.1 Reagents

Induction of cortical multiple sclerosis and neuronal calcium labeling

Incomplete Freund's adjuvant	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Mycobacterium tuberculosis H37 Ra, desiccated	BD Difco Laboratories Becton, Dickinson and Company, Maryland, USA
Myelin Oligodendrocyte Glycoprotein (MOG), N1-125, expressed in E. coli	Stock solution, produced by laboratory of Martin Kerschensteiner, Planegg, Germany
Pertussis toxin (Ptx) from Bordetella pertussis, inactivated	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Recombinant Mouse TNF-alpha protein	R&D Systems, Minneapolis, MN, USA
Recombinant Murine IFN-γ	PeproTech Germany, Hamburg, Germany
AAV1.hSyn1.Twitch2b.WPRE.SV40	Addgene, catalog no. 100040-AAV1

Antiglutamatergic therapy

Memantine hydrochloride	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
NBQX disodium salt	Abcam, Cambridge, UK

Tissue processing and immunohistochemistry

Alexa Fluor 633 Goat Anti-Rat IgG	Life Technologies GmbH, Darmstadt, Germany
Gibco Goat Serum	Invitrogen GmbH, Darmstadt, Germany

NeuroTrace 435/455 Blue Fluorescent Nissl Stain	Invitrogen GmbH, Darmstadt, Germany
PFA (paraformaldehyde) 4%	8% PFA (Sigma-Aldrich) in dH_2O, heated up to 55 °C (maximum 60°C) and stirred additional 10 minutes, filtrated and mixed in a 1:1 ratio with 0,2 M PB (Phosphate buffer), pH adjusted to 7,2-7,8 (if necessary NaOH added)
Phosphat Buffer (PB) 0,2 M	27,598 g $NaH_2PO_4 \cdot H_2O$, 35,598 g $Na_2HPO_4 \cdot 2H_2O$ dH_2O ad 1l
Phosphate Buffered Saline (PBS), 10x	103,23 mg $Na_2HPO_4 \cdot H_2O$ 26,52g $Na_2HPO_4 \cdot 2H_2O$ 40g NaCl dH_2O added to 1l
Purified rat anti-mouse I-A/I-E	BD Biosciences, CA, USA
Sodium hydroxide solution	Life Technologies GmbH, Darmstadt, Germany
Sucrose	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Triton X-100	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Vectashield Mounting Medium, Fluorescence H-1000	Vector Labs, Burlingame, CA USA

Surgical procedures

Agarose	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Bepanthen Augen- und Nasensalbe 5g (eye ointment)	Bayer Vital GmbH, Leverkusen, Germany

Cutasept F Lösung 250ml (disinfectant spray)	Bayer Vital GmbH, Leverkusen, Germany
Dulbecco's phosphate buffered saline	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Ethanol 70%	CLN GmbH, Niederhummel, Germany
Fentanyl	B. Braun Melsungen AG, Melsungen, Germany
Forene (Isoflurane)	Abbott AG, Baar, Switzerland
Ketamine hydrochloride 10 %	Bremer Pharma GmbH, Warburg, Deutschland
Local scalp anesthesia with Xylocain Gel 2%	AstraZeneca GmbH, Wedel, Germany
Domitor (Medetomidin) 1mg/ml	Orion Pharma Corporation, Espoo, Finland
Midazolam	B. Braun Melsungen AG, Melsungen, Germany
Ringerlösung Fresenius KabiPac (Ringer´s solution)	Fresenius KaBI Germany, Bad Homburg, Germany
Xylazine 2%	Riemser Arzneimittel AG, Greifswald-Insel Riems, Germany

3.1.2 Tools and materials

Immunization and intracortical injections

51500D Digital New Standard Stereotaxic, Rat and Mouse	Stoelting Europe, Terenure, Dublin, Ireland
AutoClip Clips 9mm	Fine Science Tools GmbH, Heidelberg, Germany
BD Plastipak 20ml sterile disposable graduated concentric luer lok lock syringe	Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA

BD Plastipak Hypodermic luer slip syringe 1 ml	Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA
Diamant drill head (0.8mm)	Hager & Meisinger GmbH, Neuss, Germany
Discofix 3SC	B.Braun Melsungen AG, Melsungen, Germany
Dumont Mini Forceps – Inox Style 3 (Small forceps)	Fine Science Tools GmbH, Heidelberg, Germany
Dumont Mini Forceps – Inox Style 5 (Small forceps, tip smaller than Inox style 3)	Fine Science Tools GmbH, Heidelberg, Germany
Feather stainless steel blade (surgical blade)	pfm medical ag, Cologne, Germany
Hypodermic Needles BD Microlance 3 23 Gauge (0,6 mm, blue)	Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA
Hypodermic Needles BD Microlance 3 30 Gauge (0,3 mm, yellow)	Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA
Metal plate	Custom-made
Micromotor High-Speed Drill for stereotaxic surgery	Stoelting Europe, Terenure, Dublin, Ireland
Noyes Spring Scissors (Large spring scissors)	Fine Science Tools GmbH, Heidelberg, Germany
P-30 Vertical Micropipette Puller	Sutter Instrument, Novato, California, USA
Reloadable Skin Stapler	
Safety-Multifly-Set 21Gx3/4” TW (0,8 x 19 mm)	Sarstedt AG & Co. KG, Nümbrecht, Germany
Sugi 17mmx8mm (absorbent triangles)	Kettenbach GmbH & Co. KG, Eschenburg, Germany
Thin wall Borosilicate Glass Micropipettes	Sutter Instrument , Novato, CA, USA

Vannas-Tübingen Spring Scissors (Small angled spring scissors)	Fine Science Tools GmbH, Heidelberg, Germany
Wella contura W7807 (hair clipper)	Wella, Darmstadt, Germany

Cranial window surgery

Biopsy Punch, 4mm	Kai medical GmbH Solingen, Germany
Dental Cement: Paladur, denture acrylic for repairs, cement liquid and cement powder	M+W Dental GmbH, Büdingen, Germany
Glass coverslips: 4mm, round	Warner Instruments, LLC Hamden, CT, USA
Spongostan Special (7x5x0,1cm), absorbable haemostatic gelatin sponges	Ethicon Inc., Somerville, New Jersey, USA
Stainless steel drill head (0.5mm)	Hager & Meisinger GmbH, Neuss, Germany
Steel bar	Custom-made
Vetbond 3M Kleber: tissue adhesive	3M Science, Minnesota, United States

Tissue processing and immunohistochemistry

12- and 24-well cell culture plates	Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA
15 and 50 ml Falcon tubes	Greiner Bio-One GmbH, Frickenhausen, Germany
Microscope cover slips 24x60 mm	Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany
Microscope slides 76x26 mm	Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG, Braunschweig, Germany
Paper filters (185 mm Ø circles)	Whatman Schleicher & Schuell GmbH, Dassel, Germany

Parafilm	Brand GmbH & Co. KG, Wertheim Germany
Pipettes, pipette tips and tubes (1.5 and 2 ml)	Eppendorf AG, Hamburg, Germany
Transparent nail varnish	

3.1.3 Technical devices

Surgery

FST 250 Hot Bead Sterilizer (for surgical instruments sterilization)	Fine Science Tools GmbH, Heidelberg, Germany
Olympus KL 1500 LCD (cold light source for stereomicroscopy)	Olympus GmbH, Hamburg, Germany
Olympus SZ51 stereo microscope (microscope for surgery)	Olympus GmbH, Hamburg, Germany
T/Pump (Heating pad)	Gaymar Industries, Orchard Park, New York, USA

Tissue processing and immunohistochemistry

Vortex- Genie 2	Scientific Industries, Inc., Bohemia New York, USA
Analytical balance KERN EW 150-3M	Kern & Sohn GmbH, Balingen-Frommern, Germany
HISTO LEICA Vibratome VT1200S	Leica Microsystems GmbH, Wetzlar, Germany
Laboratory pH meter inoLAB	Wissenschaftlich-Technische Werkstatten, Weilheim, Germany
Magnetic stirring hotplate MR 3001K and stirring bars	Heidolph Instruments GmBH & Co. KG, Schwabach, Germany
Olympus IX71 inverted fluorescence microscope (initial evaluation of immunohistochemistry)	Olympus GmbH, Hamburg, Germany

Pumps used for perfusions: Ismatec IP high precision multichannel pump	ISMATEC SA, Labortechnik - Analytik, Glattbrugg, Switzerland

Microscopy

MaiTai Deep See® Titanium:sapphire Laser	Newport/ Spectraphysics, Irvine, California, USA
380 FM XY-shifting table	Luigs & Neumann Feinmechanik und Elektrotechnik GmbH, Ratingen, Germany
Olympus FV1000 confocal system mounted on BX61 vertical microscope, equipped with x10/0.4 water, x20/0.85 oil and x60/1.42 oil immersion objective	Olympus GmbH, Hamburg, Germany
Olympus FV1200-MPE two-photon microscopy system with a x25/1.05 water immersion objective	Olympus GmbH, Hamburg, Germany
SP8 confocal laser-scanning microscope equipped with a 20x/0.75 NA oil immersion HC PL APO CS2 objective	Leica Microsystems, Wetzlar, Germany

3.1.4 Data analysis and software

Adobe CS 6 (Photoshop, Illustrator)	Adobe Systems, Inc., San Jose, California, USA
Graphpad Prism	GraphPad Software, La Jolla, California, USA
ImageJ/ FIJI	General Public License http://rsbweb.nih.gov/ij/download.html
Inkskape	General Public License https://inkscape.org/
Microsoft Office (Powerpoint, Excel, Word)	Microsoft Corporation, Redmond, Washington, USA

3.2 Experimental animals

All experiments were performed on animals with a F1 and F2 background of *BiozziABH* (strain designation *BiozziABH/RijHsd*, Harlan Laboratories). *CCR2^{RFP}×BiozziABH* mice (derived from *B6.129(Cg)-Ccr2tm2.1Ifc/J*, Jackson Laboratory) and *CX3CR1^{GFP}×BiozziABH* animals (*B6.129P-Cx$_3$cr-1tm1Litt/J*, Jackson Laboratory) or a F2 crossbreeding of both lines were used to characterize the activation and infiltration of phagocytes. To image calcium in dendrites and dendritic spines and to study the effects of an antiglutamatergic therapy, viral labeling was performed in *CCR2^{RFP}×BiozziABH* mice.

All experimental animals were kept and bred under standard conditions in the animal facilities of our institution. They were held in Eurostandard Type II long cages 365x207x140mm (Tecniplast, Hohenpreißenberg, Germany). Cages were stored in an IVC rack system at a temperature of 22° ±2°C, a relative humidity of 55% ± 10%, and under a 12h light/dark cycle. A maximum of five mice were held in each cage. Animals had free access to autoclaved food (regular food 'Maus' from Ssniff, Soest, Germany) and sterilized water. For husbandry, one male was housed with one or two females. At postnatal day 21, the mice were weaned. Both male and female mice, which were aged between 6 to 14 weeks, were included into the experiments. All animal work was performed in accordance with regulations of the relevant animal welfare acts and protocols approved by the local animal ethics committee of the Regierung von Oberbayern (reference: 55.2-1-54-2532-173-13).

3.3 Methods

3.3.1 Model of cortical multiple sclerosis (c-MS)

To induce cortical MS (c-MS), adult mice were immunized with 200µl of an emulsion containing 30µg of purified recombinant rat myelin oligodendrocyte glycoprotein (MOG, N1-125, expressed in E. coli) and complete Freund's adjuvant, consisting of incomplete Freund's adjuvant and mycobacterium tuberculosis H37 Ra (10mg/ml). For the immunization procedure, each mouse was anesthetized with ketamine-xylazine (ketamine 87mg/kg and xylazine 13mg/kg body weight) and received four subcutaneous injections of 50µl of the emulsion in each flank. On the day of immunization and the day after, Pertussis toxin (200ng diluted in 100µl of sterile Saline) was administered intraperitoneally. The immunization procedure was repeated after one week. To induce cortical lesions three weeks after the first immunization, mice were stereotactically injected 2µl of a cytokine mixture containing 0.25µg/µl TNF-α and 750U/µl interferon-γ dissolved in PBS/0.1% BSA into their cortices. The animals were anaesthetized by intraperitoneal injection of MMF (a mixture consisting of medetomidin (0.5 mg/kg), midazolam (5 mg/kg) and fentanyl (0.05 mg/kg)) and then transferred to a heating pad

for approximately 20-30 min to ensure deep anesthesia. Their head was shaved and stabilized in a stereotactic frame. The eyes were protected from dehydration by applying an eye-ointment. The scalp was washed and disinfected four to five times with swabs of 70% ethanol. By using surgical blades, the skin was cut open, covering the skull of both hemispheres. Any bleeding was staunched with sterile cotton swabs. A fine hole was drilled through the skull to give access to the surface of the brain (coordinates: 1.2mm lateral and 0.6mm caudal to the Bregma). A finely calibrated glass capillary which was produced with a vertical pipette puller was filled with the cytokine mixture and was inserted and targeted to the cortex (depth: 0.8mm). The cytokine mixture was injected over a time period of 2-3 minutes. After injection, the glass capillary was carefully removed, and the operation site was sealed with clips. Mice were given buprenorphine (0.05 - 0.1 mg/kg) subcutaneously for analgesia every 12h on the days following the surgery.

All experimental mice were weighed and scored daily from the day of initial immunization according to an standardized EAE scoring scale which mainly mirrors locomotor deficits caused by spinal white matter lesions (Nikic et al., 2011): 0, no detectable clinical signs; 0.5, partial tail weakness; 1, tail paralysis; 1.5, gait instability or impaired righting ability; 2, hind limb paresis; 2.5, hind limb paresis with partial dragging; 3, hind limb paralysis; 3.5, hind limb paralysis and forelimb paresis; 4, hind limb and forelimb paralysis; 5, death. Mice which did not show clinical signs of systemic EAE were also included in the study.

3.3.2 Cranial window surgery

For acute *in vivo* imaging of calcium of dendrites and spines during acute neuroinflammation, the FRET-based calcium sensor Twitch-2B was employed and delivered by AAV-*hsyn1* viral infection: 0,5µl of 10^{12} *AAV1.hSyn1.Twitch2b.WPRE.SV40* (Addgene, catalog no.100040 - AAV1) was injected 0.7mm deep into the somatosensory cortex, ten days before the day of imaging (experimental protocol established by Adrian-Minh Schumacher). The intracortical injection and the application of analgesia after surgery were performed as described above. The coordinates of viral injections were 2mm lateral and 2mm caudal to the Bregma.

To gain optical access to the animal's cortex on the day of imaging (=three days after cytokine injection), cranial windows above the somatosensory cortex of experimental animals were implanted. Surgery was performed as previously described (Holtmaat et al., 2009).

The preoperative preparation and anesthesia (MMF intraperitoneally) of experimental animals was performed as described above. After stabilizing the head in the stereotactic frame, the clips were removed, and the scalp was washed and disinfected four to five times with swabs of 70% ethanol. Next, a flap of skin covering the skull of both hemispheres was removed using scissors.

Any bleeding was staunched with sterile cotton swabs. Local anesthesia (Xylocain, 2%) was applied to the periosteum of the skull and the exposed muscles at the lateral and caudal sides of the wound. The cortical area of interest was gently marked with a biopsy punch having the same diameter as the size of the circular cover glasses (4mm).

Craniotomy was performed slowly using a 0.5mm stainless steel drill head (Meisinger). During drilling, saline was regularly applied to avoid heating. An island of skull with a diameter of approximately 4mm was left intact in the center. The bone was removed under a drop of saline by horizontally inserting the tip of a hypodermic needle (BD Microlance 3, 30 G) into the bone on the side of the groove. The central bone island was lifted and gently loosened laterally until the bone flap could be removed. The dura was covered with a circular coverglass (4mm diameter, 0.15mm thickness), flush with the skull. The surface of the skull around the coverglass was dried with sterile cotton swabs. The skull, the edges of the coverglass and the wound edges were covered, first with tissue adhesive and afterwards with dental cement. A shallow well was formed out of the dental cement for the water immersion objective. A stainless steel bar was embedded over the caudal intact skull into the cement to stabilize the animal for subsequent imaging sessions. The bar was placed at a level with the glass surface, so that the window could be positioned horizontally under the microscope and vertically to the optical axis of the microscope. Afterwards, mice were transferred to a heating pad for another approximately 20-30 minutes to let the cement dry.

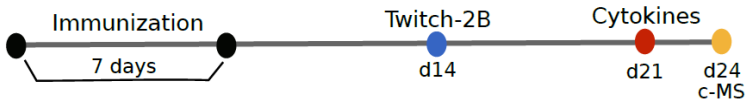


Figure 9: Experimental design of the cortical MS model

Schematic diagram shows the experimental design to study calcium in dendrites and spines in cortical MS: Mice were immunized at d0 and d7. Cortical viral labeling with AVV-Twitch-2B was performed at d14. Cytokines (TNF-α and IFN-γ) were intracortically injected at d21. Craniotomy and *in vivo* imaging of mice was performed at d24.

3.3.3 *In vivo* microscopy of calcium of dendrites and spines

To perform acute *in vivo* imaging, experimental animals were anesthetized intraperitoneally with MMF and cranial windows were implanted as described above. For *in vivo* microscopy, a Olympus FV1200-MPE two-photon microscopy system, equipped with a femto-second pulsed Ti:Sapphire laser (Mai Tai HP-DS, Spectra-Physics) was used. The laser power was attenuated by acousto-optical modulators. To image the FRET-based genetically encoded calcium

indicator Twitch-2B, a two-photon wavelength of 840nm was applied (Thestrup et al., 2014). Both cpVenus^{CD} and mCerulean3 were simultaneously excited and their fluorescence emissions were collected in a yellow channel ('YFP') and a cyan channel ('CFP'), respectively, using emission barrier filters of 526-557nm and 455-490nm. Emissions were detected with non-descanned gallium arsenide phosphide (GaAsP) detectors. Images were acquired in 12 bit with a 25x/1.05 dipping cone water-immersion objective (Olympus), a laser power of 30-50mW, which was measured in the back focal plane, a pixel size of 124nm/ pixel and a dwell time of 2.0µs/pixel. Volume stacks were acquired with a Z-spacing of 1µm and were located up to 60µm from the surface (cortical layer I). To explore calcium dynamics over time, experimental animals were re-imaged after two hours. Within this time frame, they were transferred to heating pads to ensure a stable body temperature. Under these imaging conditions, no signs of photodamage in both c-MS and healthy animals over the imaging period were observed. Animals which had signs of traumatic damage after cranial window implantation were excluded from the analysis (Jafari et al., 2021).

3.3.4 Application of antiglutamatergic drugs

The c-MS model was induced as described above. To analyze the role of glutamate-mediated excitotoxicity for spine loss, experimental animals were given a daily antiglutamatergic treatment from the day of cytokine injection to the day of imaging (cumulative four days). They were treated by intraperitoneal injection of the AMPA/kainate antagonist NBQX (30 mg/kg body weight, twice daily) and the NMDA antagonist memantine (60 mg/kg body weight, once daily) (Centonze et al., 2009; T. Smith et al., 2000; Sulkowski, Dabrowska-Bouta, Chalimoniuk, & Struzynska, 2013) or were treated with PBS as a vehicle. Viral labeling, craniotomy and *in vivo* microscopy of dendrites and spines were performed as described above.

3.3.5 Tissue processing and immunofluorescence

For the analysis of the phagocyte activation and infiltration, c-MS was induced as described above. At d24, experimental animals were euthanized with isoflurane. They were transcardially perfused with 4% (wt/vol) paraformaldehyde (PFA) in PBS. The head and the upper cervical spine were dissected and post-fixed in 4% PFA at 4° for 24-48 hours. Afterwards, the brain and upper cervical spinal cord were micro-dissected under the stereomicroscope. To prepare cutting, the sections in the proximity to the injection area were embedded into 4% agarose for approximately 30 minutes. Using a vibratome, 80 µm thick sections were cut and collected in well plates with PBS for subsequent immunohistochemical staining. MHCII and NeuroTrace staining were performed according to the protocol outlined in Table 1. After staining, brain

sections were mounted on microscope slides. A drop of Vectashield was applied and the sections were covered with microscope cover slips. The edges were sealed with nail varnish.

Procedure	**Temperature**	**Time**
Wash in PBS	Room temperature	3x10 minutes
Block with 10% goat serum (GS) in 0.5% (vol/vol) Triton X-100 in PBS (TPBS)	Room temperature	60 minutes
Incubate with rat anti-major histocompatibility complex (MHC)-II (I-A/I-E) (1:300) in 1% GS/TPBS	4°C	Overnight (12-24 hours)
Wash in PBS	Room temperature	3x15 minutes
Incubate with Alexa Fluor-594 goat anti-rat (MHC II) antibodies (1:500) in 1% GS/TPBS and counterstain with the Nissl-like nucleic acid stain NeuroTrace 435/455 (1:500)	4°C	12-24 hours

Table 1: Staining protocol of MHCII and NeuroTrace

3.3.6 Confocal microscopy

To characterize the spatial and temporal characteristics of infiltration and activation of phagocytes in cortical MS, stained tissue samples were scanned with upright confocal laser-scanning microscopes (SP8 Leica or FV1000 Olympus) equipped with standard filter sets and laser lines. Total volumes of 40 µm were imaged with a 20x/0.75 NA oil immersion HC PL APO CS2 objective (Leica), a Z-spacing of 5 µm and a 1.0x zoom in tile scan mode. The NeuroTrace signals were acquired with a 20x/0.85 NA oil immersion UPLSAPO Objective (Olympus). The overview scans were then overlaid on the NeuroTrace scans.

3.3.7 Data Analysis and evaluation

The images were processed and analyzed using the open-source image analysis software, ImageJ/Fiji (http://fiji.sc), and Adobe Photoshop.

Analysis of spine densities

For the analysis of spine densities in the c-MS model *in vivo*, images were loaded into the FIJI software using the Bioformats Importer plugin. Each image, which is collectively formed by the CFP and YFP (FRET) channel, was opened in a grayscale look-up table. Per image, approximately ten suitable dendrites, i.e. dendrites which had a length of at least 50 µm, which were located in sparse labeled areas and which were traceable within their route were selected. Next, all spines on those selected dendrites were labeled and counted using the cell counter plugin in Fiji. The ratio *spines/µm dendrite length* was calculated for c-MS and control animals.

Analysis of calcium levels in dendrites and dendritic spines

For the subsequent analysis of calcium levels within the selected dendrites and corresponding spines, the two channels were opened separately in a grayscale look-up table. Regions of interest (ROI) were drawn around the spine heads and nearby non-neurite background (BG) areas for background correction. For the analysis of dendritic calcium levels, five ROIs were drawn in each selected dendrite and corresponding BG areas. The integrated fluorescence intensities within the ROIs were measured in both the CFP and the YFP channel. The measurements included the mean gray values (MGV), the minimum and maximum gray values and the corresponding standard deviations (SD). Microsoft Excel (2010 and higher) was used for data collection and calculations.

The calculation of calcium signals was performed as outlined in the following formulas. First, both channels were background (BG)-corrected (values were measured in respective channels):

$$CFP_{BG\text{-}corrected} = MGV_{ROI\ spine\ or\ dendrite\ CFP} - MGV_{ROI\ corresponding\ BG\ area\ CFP}$$
$$YFP_{BG\text{-}corrected} = MGV_{ROI\ spine\ or\ dendrite\ YFP} - MGV_{ROI\ corresponding\ BG\ area\ YFP}$$

Formula 2: Background corrections of CFP and YFP signals

One main factor that influences FRET changes is the spectral overlap of the channels, also known as ‘crosstalk’ or ‘bleedthrough’. As we detected crosstalk from the CFP to the YFP channel (not vice-versa), the YFP signal was corrected by subtracting the measured crosstalk-fraction of the CFP signal. The bleedthrough factor (BT of 0.22) was determined experimentally by Adrian-Minh Schumacher (Schumacher, 2015):

$$YFP_{BT\text{-}corrected} = YFP_{BG\text{-}corrected} - BT \times CFP_{BG\text{-}corrected}$$

Formula 3: Crosstalk correction of YFP signal

Finally, the ratio of both channels which is interpreted as a proxy of Ca^{2+} concentration was calculated according to the following formula:

$$Ratio_{YFP/CFP} = YFP_{BT\text{-}corrected} / CFP_{BG\text{-}corrected}$$

Formula 4: YFP/CFP ratio

The spines that exhibited a signal-to-noise ratio (SNR) < 5.2, as measured in the YFP channel, were excluded from the analysis:

$$SNR = MGV_{ROI\ spine\ or\ dendrite} / SD_{ROI\ corresponding\ BG\ area}$$

Formula 5: Signal-to-noise ratio

The spines or dendrites with an YFP/CFP ratio greater than mean plus three standard deviations of the ratios measured in healthy animals were considered to be Ca^{2+}-elevated ('high calcium').

The analysis of calcium levels in spines and dendrites after antiglutamatergic treatment was performed analogous. For analysis of dynamic calcium levels over time, per region ten dendrites including corresponding spines were selected and measured in the first (t_1=0 hours) and the second (t_2=2 hours) timepoint.

Ratiometric images shown in my thesis were created as follows: maximum intensity projections of single channels (YFP (FRET) and CFP) were created. To exclude background signals, a binary thresholded mask of dendritic structures was generated from the channel with a higher signal-to-noise ratio. Then projection images were multiplied by the binary mask. The masked images were divided by each other (FRET/CFP). This image was pseudocolored with a custom look-up table, which represents lower ratios as blue and highest ratios as yellow. Grayscale image projections from the FRET channel were despeckled in Photoshop. The generated ratiometric RGB images were blended with this grayscale FRET channel projection image.

Analysis of phagocyte activation and infiltration

The images were loaded into the FIJI software and opened separately into three channels ($CX3CR1^{GFP}$ signal, MHCII immunofluorescence signal, $CCR2^{RFP}$ signal). Cortical layers (layer I, layer II-IV and layer V) were defined using a nucleic acid stain (NeuroTrace). The analysis was performed ipsi- and contralaterally to the injection side of cytokines. Per layer and per hemisphere, counting frames of 100x100µm (for layer I and layer V), and 200x 200µm (for layer II-IV) were drawn at a distance of 1000µm, 1500µm and 2000µm lateral to the midline. Five ROIs were drawn in the background area of the MHCII channel and the mean and standard deviation of the intensity were measured for each image. Signals greater than mean plus three standard deviations of the background intensity were considered to be MHCII positive. In the

volume counted, numbers of $CX3CR1^{GFP}$ positive cells, numbers of $CCR2^{RFP}$ cells and co-labelling of MHCII immunofluorescence and $CX3CR1^{GFP}$ were analyzed using the cell counter plugin in Fiji. Absolute numbers were adjusted to a standard volume of $40x10^4$ μm^3. Microglial activation was presented as % of MHCII positivity of all $CX3CR1^{GFP}$ positive cells. For the presentation of confocal images, the images were exported as RGB images to Photoshop and despeckled.

Statistical analysis

Statistical significance was calculated using GraphPadPrism (version 7.01). Where normal distribution could be assumed, ANOVA and t-test were employed. Where non-normal distribution was suspected and confirmed by the Shapiro Wilk normality test, statistical significance was calculated using the Mann-Whitney U-test. Data are expressed as the mean ± standard error of the mean. Obtained p-values were corrected for multiple comparisons using Bonferroni's, Holm-Sidak's or Dunnett's procedure and stated as significance levels in the figure legends. The data was considered statistically significant when p-values were <0.05.

4. Results

Widespread synapse loss is a key feature of gray matter pathology in MS and can be reproduced in the murine model of cortical neuroinflammation (cortical MS) (Albert et al., 2017; Jafari et al., 2021; Jürgens et al., 2015). The goal of my thesis was to explore the mechanisms which contribute so synapse loss in cortical MS. Towards this goal, I researched the following mechanisms: (1) the role of calcium signals during spine loss, (2) the contribution of glutamate-mediated excitotoxicity to synapse pathology, and (3) the cells which execute synapse removal. The results of my thesis were published in:

> Jafari, M., Schumacher, A. M., Snaidero, N., Ullrich Gavilanes, E. M., Neziraj, T., Kocsis-Jutka, V., . . . Kerschensteiner, M. (2021). Phagocyte-mediated synapse removal in cortical neuroinflammation is promoted by local calcium accumulation. *Nat Neurosci*.

4.1 Calcium imaging of dendrites and spines in the c-MS model

4.1.1 *In vivo* visualization of dendrites and spines in the c-MS model

In the first part of my thesis, I aimed to characterize calcium in dendrites and spines *in vivo* in the cortical MS model.

To image dendrites and spines *in vivo* in acute neuroinflammation and to explore, whether intracellular calcium within these structures is affected in the neuroinflammatory environment, I used the genetically encoded calcium indicator Twitch-2B. I targeted Twitch-2B to layer V neurons in mice with cortical MS by rAAV-hsyn1 viral infection (experimental design outlined in Figure 9). Using two-photon microscopy, I collected the signal of these neurons and imaged apical dendritic tufts with corresponding dendritic spines in animals with c-MS at the peak of neuroinflammation (d3: three days after cytokine injection) and in healthy control animals.

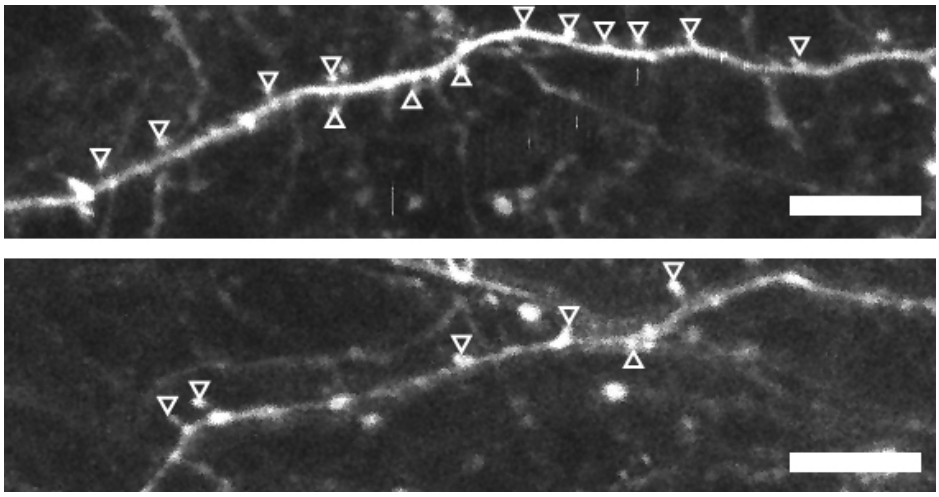


Figure 10: *In vivo* visualization of dendrites and spines in control and in c-MS animals

In vivo multiphoton projection images show apical tuft dendrites with corresponding spines in control animals (upper image) and acute (peak of the disease) c-MS model (d3) (lower image). Images are shown as grayscale images of YFP channel. Arrows highlight spines along the dendrites. Scale bar: 10 µm.

4.1.2 *In vivo* characterization of spine loss in the c-MS model

Subsequently, I explored the spine density of dendrites located in cortical layer I (between 30-60 µm from the surface).

Towards this goal, I analyzed the number of spines per µm dendrite length. My findings revealed a significant reduction of dendritic spines at the acute stage of the c-MS model (d3) compared to control animals (see figure 11). Spine loss was apparent ipsi- and contralateral (ipsi, contra) to the site of cytokine injection. These findings were in accordance with the initial experiments performed by Mehrnoosh Jafari which proved a widespread spine loss in Thy1-GFP-M transgenic mice with c-MS (Jafari et al., 2021).

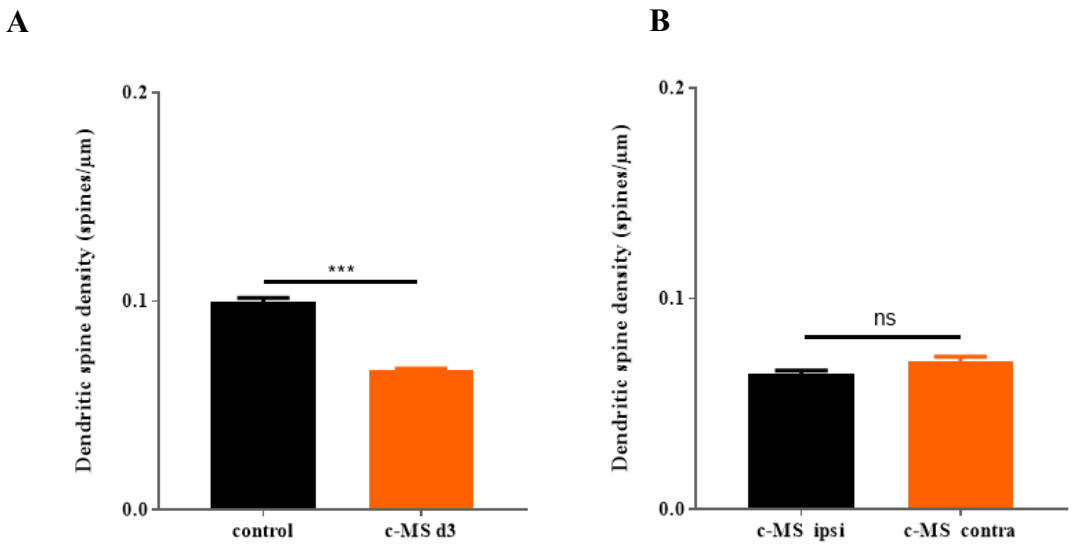


Figure 11: *In vivo* characterization of spine loss in the c-MS model

(A) Graph shows spine densities (spines/µm dendrite) in healthy controls (n=194 dendrites) and acute (peak of the disease) c-MS animals (d3, n=494 dendrites). (B) Spine loss is apparent ipsi- and contralaterally to the cytokine injection site in the c-MS animals (d3, c-MS ipsi, c-MS contra). Spine densities per animal are shown as mean ± SEM (tested per animal in n=8 control, n=17 c-MS d3 with n=10 c-MS ipsi and n= 7 c-MS contra, one-way ANOVA followed by t-test between (A) c-MS d3 and control, and (B) c-MS ipsi and c-MS contra). ***P < 0.001.

4.1.3 *In vivo* characterization of dendritic and spine calcium in the c-MS model

In the following, I aimed to explore how intracellular calcium in dendrites and spines is affected in cortical MS. Therefore, I investigated whether local calcium dysregulations existed in single spines and/or dendritic tufts at the peak of spine loss (d3, three days after cytokine injection).

Towards this goal, I calculated the YFP/CFP ratio as a proxy of Ca^{2+} concentration in the beforehand selected dendrites and corresponding spines. For the analysis of calcium in dendrites, five randomly chosen areas within the dendrite were selected. Spines or dendrites with an YFP/CFP ratio greater than mean plus three standard deviations of the ratios measured in healthy animals were considered to be Ca^{2+}-elevated (‘high calcium’).

My experiments revealed an increase of spines defined as ‘high calcium’ in c-MS mice compared to control animals. Overall, about 6% of tuft spines in cortical MS displayed a local calcium overload, whereas calcium levels in the large majority of dendrites remained unchanged.

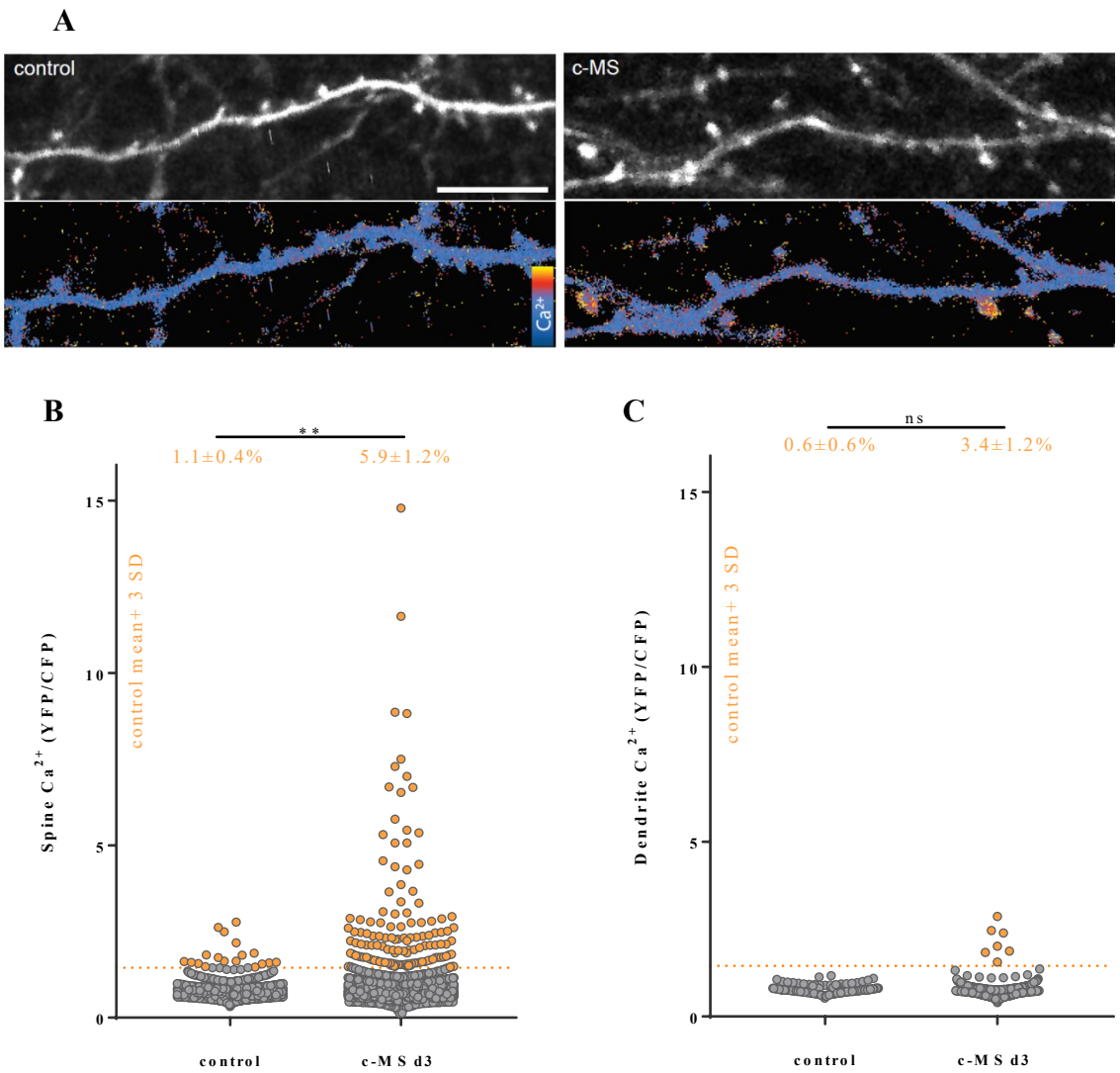


Figure 12: *In vivo* characterization of dendritic and spine calcium levels in the c-MS model

(A) *In vivo* multiphoton images show apical tuft dendrites with corresponding spines and their Ca^{2+} concentrations in healthy controls **(control, left)** and acute (peak of the disease) c-MS model (d3) **(c-MS, right)**. Images are shown as grayscale images of the YFP channel (top) and ratiometric (YFP/CFP) images (bottom) masked and color-coded for cytoplasmic Ca^{2+}. **(B)** Quantification of single spine Ca^{2+} levels (YFP/CFP channel ratios) in healthy controls (n=1287 spines) and c-MS (d3) animals (n=2368 spines) (dashed lines represent the high Ca^{2+} threshold, 3SD above control mean). **Top:** Percentage of 'high calcium' spines per animal is shown as mean ± SEM (tested per animal in n=8 control and n=17 c-MS mice, Mann-Whitney U test). **(C)** Quantification of Ca^{2+} concentration in focal areas of single dendrites in healthy control and c-MS (d3) animals, shown as YFP/CFP channel ratios (dashed lines represent high Ca^{2+} threshold, 3SD above control mean). **Top:** The percentage of dendrites per animal with a mean YFP/CFP ratio defined as 'high calcium' is shown as the mean ± SEM (tested per animal in n=8 control and n=17 c-MS mice, Mann-Whitney U test). Scale bar in **A**, 10 µm. **P < 0.01. Figure Panel A adapted from (Jafari et al., 2021).

4.1.4 *In vivo* calcium dynamics in dendritic spines in the c-MS model

Since I discovered an increase of 'high calcium' spines in c-MS compared to controls, I was interested in better understanding the relationship between spine loss and local calcium overload. More precisely, I aimed to explore, if 'high calcium' spines maintained their calcium levels, if their calcium levels decreased or increased, or if spines with local calcium overload were removed over a defined timeframe. Therefore, I tracked the fate of spines over time.

For this purpose, I reimaged identical cortical regions after two hours and calculated the corresponding YFP/CFP ratios of cortical spines in the first (t_1=0 hours) and the second (t_2=2 hours) timepoint. As the imaging pause and the need of re-imaging could distort the number of detectable dendritic spines and calcium levels, I first imaged control animals over the time course of two hours. Almost all spines could be detected in the second timepoint and moreover, they had a stable YFP/CFP ratio. No signs of phototoxicity, alterations in spine morphology/expression pattern or a decrease in the quality of acute cranial windows could be observed.

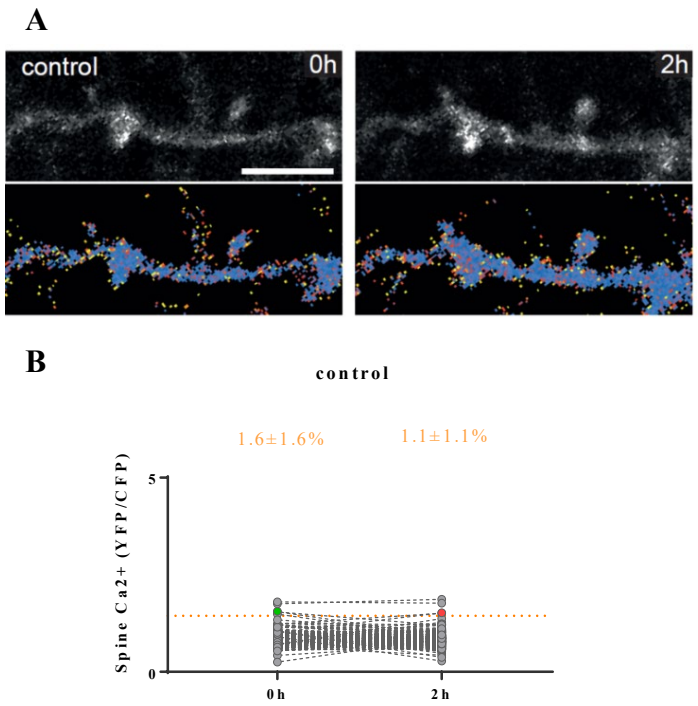


Figure 13: *In vivo* characterization of spine calcium dynamics in control animals

(A) *In vivo* multiphoton time-lapse images in control animals shown as grayscale images of the YFP channel (top) and ratiometric (YFP/CFP) images (bottom) masked and color-coded for cytoplasmic calcium. Grayscale was gamma-corrected. **(B)** Ca^{2+} concentration of spines in control animals (n=278 spines) over 2 hours shown as connected YFP/CFP channel ratios (dashed lines represent high Ca^{2+} threshold, 3SD above control mean). High Ca^{2+} spines (orange dots) disappearing within 2h are colored in green. Spines increasing in calcium level over two hours and becoming 'high' calcium spines are colored in red. **Top:** Overall percentages of spines per animal with high Ca^{2+} concentration is shown as mean ± SEM (n=4 control). Scale bar in **A**: 5 µm. Figure Panel A adapted from (Jafari et al., 2021).

Subsequently, I tracked the fate of spines in cortical MS at the peak of neuroinflammation and spine loss (between day 2 and 3 after cytokine injection). My results revealed that the probability for spine removal increased with rising calcium concentrations in the spines: about 25% of the calcium-overloaded spines were removed within the observation time of two hours. Almost all spines on the same dendrite with maintained calcium homeostasis survived. Interestingly, during the same observation time, several previously unaffected spines showed an increase in calcium levels. This created a stable fraction of 'high calcium' spines and suggested that highly dynamic and local calcium overloads in spines lead to the loss of approximately 1-2 % of cortical spines per hour. The corresponding daily rate matches the spine loss of approximately 30% observed in our c-MS model between day 2 and day 3 after cytokine injection, when most spine loss occurs (Jafari et al., 2021).

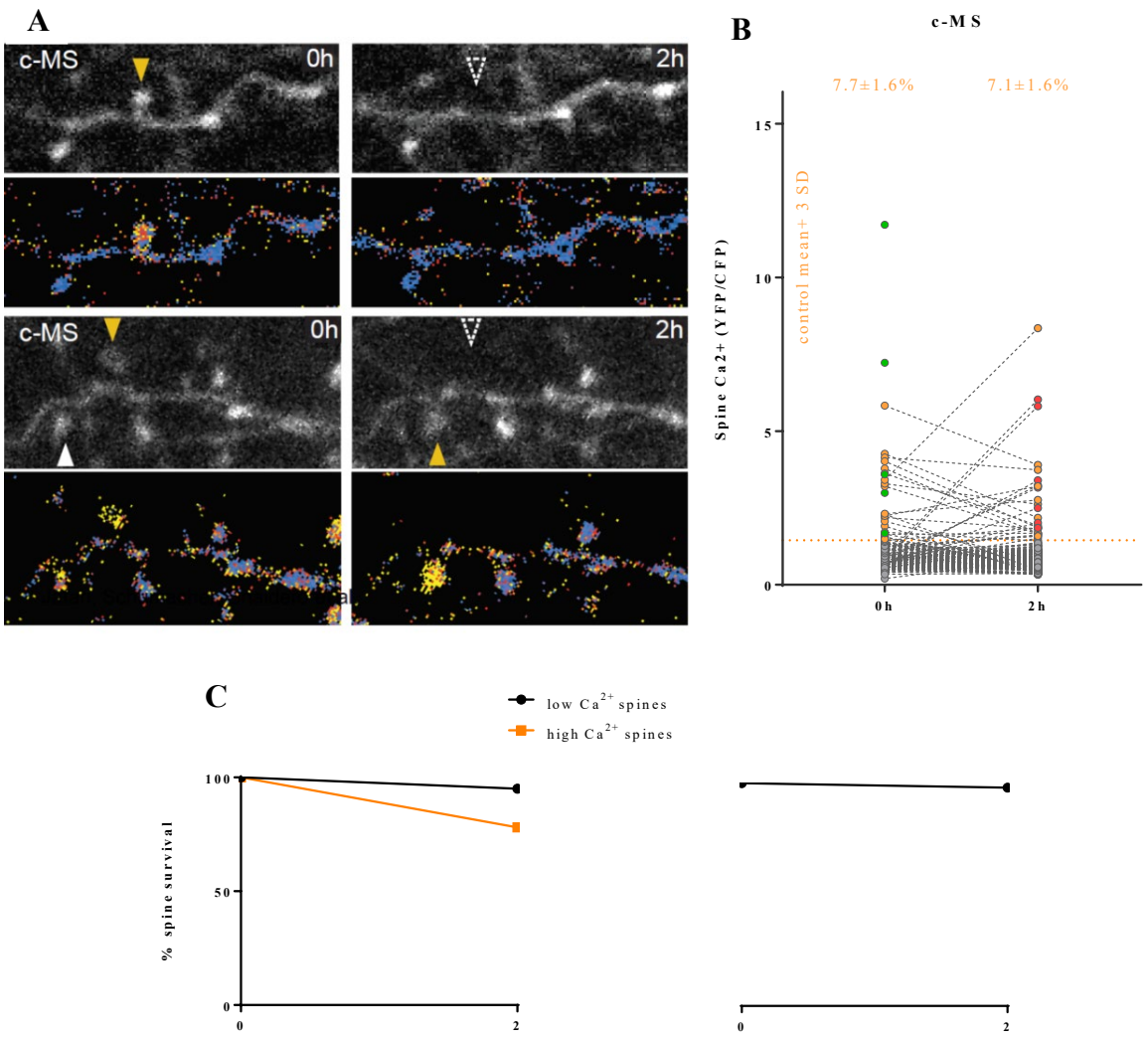


Figure 14: *In vivo* characterization of spine calcium dynamics in c-MS animals

(A) *In vivo* multiphoton time-lapse images in acute c-MS model (d2, d3) shown as grayscale images of YFP channel (top) and ratiometric (YFP/CFP) images (bottom) masked and color-coded for cytoplasmic calcium. **Upper images**: spine with high Ca^{2+} level (**left**, yellow arrow head) disappears after 2 hours (**right**, dashed arrow head). **Lower images**: spine with high Ca^{2+} level (**left**, yellow arrow head) disappears after 2 hours (**right**, dashed arrow head) next to a neighboring spine (**left**, white arrow head) that raises volume and Ca^{2+} level after 2 hours (**right**, yellow arrow head). Grayscale was gamma-corrected. **(B)** Ca^{2+} concentrations of spines in animals with acute c-MS (d2, d3, n=368 spines) over 2 hours shown as connected YFP/CFP channel ratios (dashed lines represent high Ca^{2+} threshold, 3SD above control mean). High Ca^{2+} spines (orange dots) disappearing within 2 hours are colored in green. Spines increasing in calcium level over 2 hours and becoming 'high calcium' spines are colored in red. **Top:** Overall percentages of spines per animal with high Ca^{2+} concentration is shown as mean ± SEM (tested per animal in n=8 c-MS mice, paired t-test) **(C)** Percentage of surviving spines after 2h in acute c-MS (**left**, n=8) and healthy control animals (**right**, n=4). The cohort of high Ca^{2+} spines is plotted in orange, neighboring spines with low calcium are plotted in gray. Figure Panel A adapted from (Jafari et al., 2021).

4.2 Effects of an antiglutamatergic therapy on spine pathology in the c-MS model

Glutamate-mediated excitotoxicity is assumed to be a significant contributor to synaptopathy. Therefore, glutamate receptor antagonists such as AMPA/kainate or NDMAR antagonists were assumed to have protective effects on neurodegeneration and on synaptopathy. Indeed, the application of glutamate receptor antagonists in animal models of MS was shown to ameliorate the disease course and to prevent synaptic degeneration (Centonze et al., 2009; Pitt et al., 2000; T. Smith et al., 2000). On the basis of those considerations, I decided to study the role of NMDA and AMPA receptors on calcium overload and subsequent spine loss in our c-MS model. For this purpose, I treated experimental animals daily from day of cytokine injection until the day of *in vivo* imaging with the AMPA/kainate receptor antagonist NBQX and the NMDA receptor antagonist memantine (Centonze et al., 2009; T. Smith et al., 2000; Sulkowski et al., 2013).

4.2.1 Effects of an antiglutamatergic treatment on spine loss in the c-MS model

First, I explored whether the antiglutamatergic treatment had an effect on the spine density in cortical MS. For this purpose, I imaged dendrites and spines in both c-MS mice that were given an antiglutamatergic treatment (NBQX and memantine) and in c-MS mice that were given a vehicle. I analyzed the number of spines per µm dendrite length. My findings showed that there was no significant difference in the spine density between both groups.

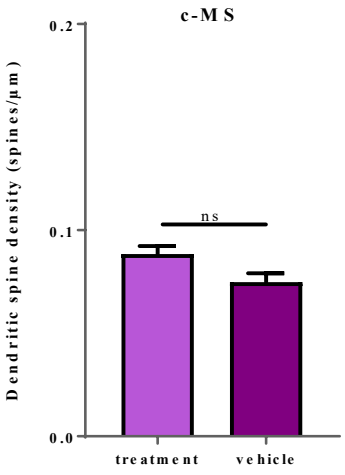


Figure 15: Effects of an antiglutamatergic therapy on dendritic spine density in c-MS animals

Quantification of dendritic spine densities (spines/µm dendrite) in c-MS (d3) mice that underwent an antiglutamatergic therapy (treatment, n=108 dendrites) and control c-MS mice (vehicle, n=95 dendrites). Shown as mean ± SEM (tested per animal in n=6 treatment, n= 6 vehicle, unpaired t-test).

4.2.2 Effects of an antiglutamatergic treatment on spine calcium in the c-MS model

Subsequently, I aimed to explore whether the antiglutamatergic treatment had an effect on local calcium overloads in dendritic spines in cortical MS. Towards this goal, I calculated the YFP/CFP ratio in dendritic spines in cortical MS mice that were treated with NBQX and memantine, and in c-MS mice that were given a vehicle. Dendritic spines with an YFP/CFP ratio greater than mean plus three standard deviations of the ratios measured in healthy animals were considered to be Ca^{2+}-elevated ('high calcium'). I found that c-MS mice which received the antiglutamatergic treatment (NBQX and memantine) displayed a higher amount of 'high calcium' spines compared to control c-MS mice that were given a vehicle. However, given the overall little amount of 'high calcium' spines in the vehicle group of c-MS mice, one has to question the formation of inflammatory and demyelinated cortical lesions within these mice in general. One approach to rule out lacking cortical lesion formation within the vehicle group might be to compare the amount of phagocytic activation, in this case the amount of infiltrating monocytes as a marker of cellular inflammation within both groups of $CCR2^{RFP}$ mice. Moreover, one must consider that the number of experimental animals (n=6 in each group) was lower than in the baseline analysis of calcium high spines in cortical MS (see figure 12, n=17 c-MS mice). A larger amount of experimental animals should give a more definitive answer.

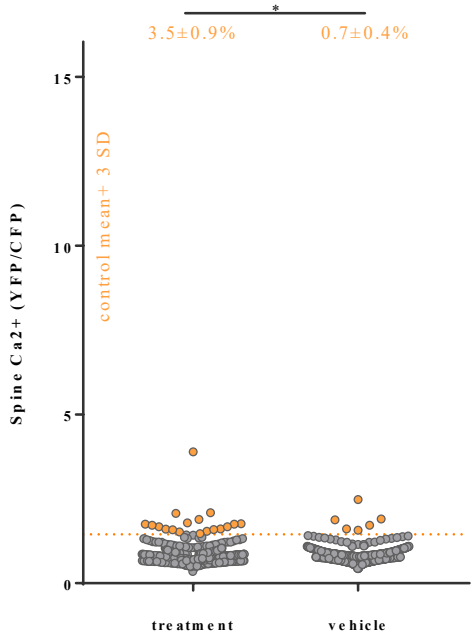


Figure 16: Effects of an antiglutamatergic therapy on spine calcium levels in c-MS animals

Quantification of single spine Ca^{2+} concentrations in c-MS (d3) mice that underwent an antiglutamatergic therapy (treatment, n=566 spines) and in control c-MS mice (vehicle, n=469 spines) shown as YFP/CFP channel ratios (dashed lines represent high Ca^{2+} threshold, 3SD above control mean). **Top:** Percentage of high calcium spines per animal is shown as mean ± SEM (tested per animal in n=6 treatment and n=6 vehicle, Mann-Whitney U test). *P<0.05.

4.3 Effector cells of spine removal

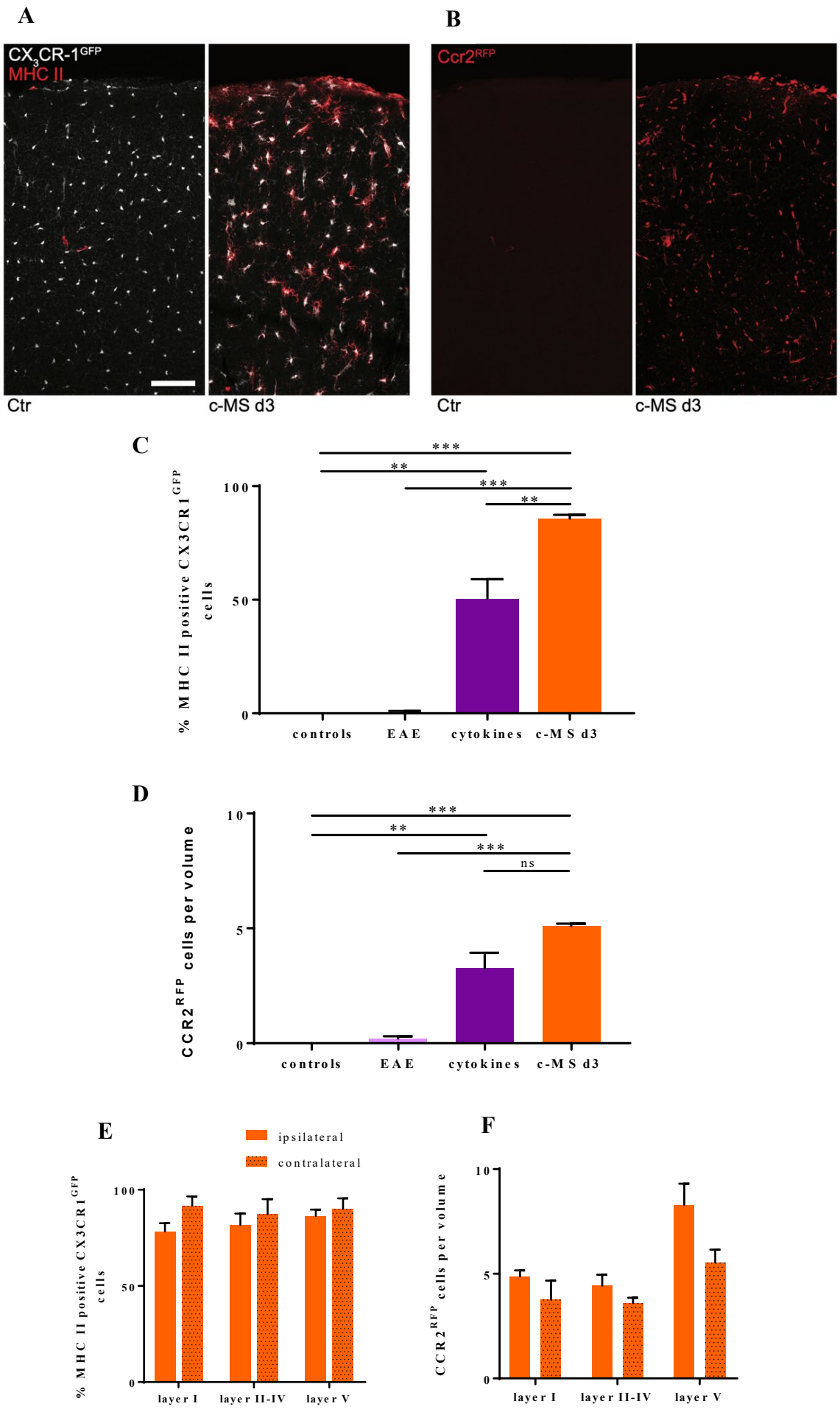
In the third part of my thesis, I aimed to characterize the involvement of the phagocyte system in cortical MS. More precisely, I explored the spatial and temporal activation of local microglia and infiltration of monocytes-derived macrophages in cortical MS in order to correlate the findings to the spatial and temporal characteristics of the spine loss in c-MS.

For this purpose, I induced cortical MS in mice with genetically labeled phagocytes (microglia, $CX3CR1^{GFP}$; invading macrophages, $CCR2^{RFP}$) (Yamasaki et al., 2014). Subsequently, I stained brain tissue from these mice for (1) MHCII as a marker for microglia activation and for (2) NeuroTrace to characterize the layers of the cortical column (layer I, layer II-IV and layer V). By applying confocal microscopy in the stained brain tissues, I analyzed the amount of locally activated microglia (presented as % of MHCII positivity of all $CX3CR1^{GFP}$ positive cells) and the number of infiltrated monocytes ($CCR2^{RFP}$ positive cells).

To assess the contributions of the different components of the c-MS model, i.e. MOG-immunization and cytokine injection, to the amount of phagocyte activation and infiltration in c-MS, I performed experiments in the following control groups: (1) healthy controls, (2) mice

which only were immunized with MOG (EAE), and (3) mice which only received intracortical injection of cytokines (cytokine). Spatial characterization was performed by comparing the amount of phagocyte activation/infiltration between (1) the hemisphere of cytokine injection (ipsilateral)/ the non-injected hemisphere (contralateral), and (2) within the cortical layers of each hemisphere.

My findings showed that the intracortical injection of cytokines had the greatest contribution to phagocyte activation and infiltration. However, cortical MS mice (which received MOG immunization and intracortical cytokine injection) displayed a significant higher activation of local microglia. Mice which were only immunized with MOG showed almost no phagocyte activation/infiltration similar to healthy control animals. The activation of microglia and infiltration of monocytes was apparent in both hemispheres, i.e. ipsi- and contralaterally to the site of cytokine injection, and throughout all cortical layers (layer I, layer II-IV and layer V).


A
B
CX3CR-1GFP
MHC II
Ctr
c-MS d3
Ccr2RFP
Ctr
c-MS d3
C
% MHC II positive CX3CR1GFP cells
100
50
0
***
**
***
**
controls
EAE
cytokines
c-MS d3
D
CCR2RFP cells per volume
10
5
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ns
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EAE
cytokines
c-MS d3
E
ipsilateral
contralateral
% MHC II positive CX3CR1GFP cells
100
50
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layer I
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layer V
F
CCR2RFP cells per volume
10
5
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layer I
layer II-IV
layer V

Figure 17: Characterization of microglia activation and monocytes infiltration in the c-MS model

(A, B) Confocal projection images of a cortical column in healthy control (Ctr, left) and acute cortical MS model (c-MS d3, right) in *CCR2*^{RFP} × *CX3CR1*^{GFP}*x BiozziABH* mice. **(A)** As a marker of microglial activation, immuno-staining for MHCII (red) was performed and overlayed on the GFP signal of resident microglia (grey) **(B)** The RFP signal of infiltrating monocytes-derived macrophages is shown in red. Images processed by Mehrnoosh Jafari. **(C)** Percentage of activated microglia (defined as the percentage of MHCII positive *CX3CR1*^{GFP} cells) in healthy control (controls), only immunized (EAE), only cytokine injected (cytokine) and acute cortical MS (c-MS d3) mice. **(D)** Number of infiltrating phagocytes (positive *CCR2*^{RFP} cells) per standardized volume in healthy control (controls), only MOG-immunized (EAE), only cytokine injected (cytokine) and acute cortical MS (c-MS d3) mice. **(E)** Distribution of activated microglia (in %) between the hemisphere of cytokine injection (ipsilateral) and the non-injected hemisphere (contralateral) and within the layers of the cortical column (Layer I, Layer II-IV and Layer V) in d3 c-MS mice. **(F)** Distribution of infiltrating phagocytes (positive CCR2^{RFP} cells) between the hemisphere of cytokine injection (ipsilateral) and the non-injected hemisphere (contralateral) and within the layers of the cortical column (Layer I, Layer II-IV and Layer V) in d3 c-MS mice. Shown as mean ± SEM (tested per animal in n=2 control, n=2 EAE, n=3 cytokine, n=3 c-MS d3; one-way ANOVA). Scale bars in **A**, **B**, 100 µm. ***P<0.001, **P<0.01. Figure Panels A and B adapted from (Jafari et al., 2021).

Subsequently, I performed the temporal characterization by analyzing microglia activation and monocytes infiltration in the c-MS model at different timepoints after the cytokine injection (d3, d7, d21). My findings revealed that phagocyte activation/infiltration was reversible and decreased to the level of healthy controls at day 21 after the injection of cytokines.

Overall, these results made mononuclear phagocytes prime candidates for synaptic removal. Both spatial and temporal characteristics correlated with the widespread and reversible spine loss observed in our cortical MS model (Jafari et al., 2021).

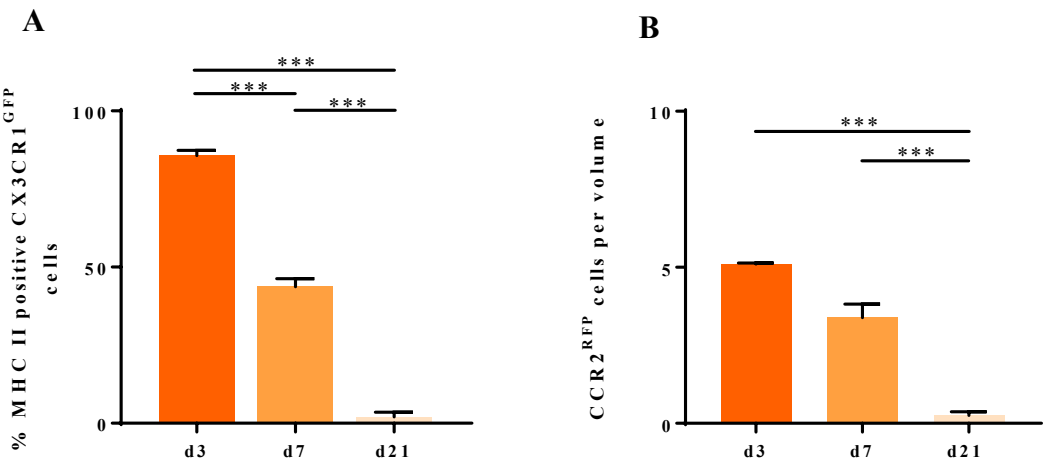

Figure 18: Temporal characteristics of microglia activation and monocytes infiltration in the c-MS model

Graph shows the amount of **(A)** activated microglia (in %) and **(B)** infiltrating phagocytes ($CCR2^{RFP}$ positive cells) at day 3, day 7 and day 21 after the induction of cortical MS model (d3, d7, d21). **(**Shown as mean ± SEM (tested per animal in n=3 c-MS d3, n=3 c-MS d7, n=3 c-MS d21; one-way ANOVA). ***P<0.001.

5. Discussion

5.1 Summary

In this thesis, I discovered new findings about dendritic and postsynaptic calcium dynamics and executive mechanisms of spine loss in a model of acute cortical neuroinflammation. By imaging calcium in dendrites and spines *in vivo* in a murine model of cortical MS, I found increased local calcium accumulations in dendritic spines. Moreover, I observed that these local postsynaptic calcium accumulations predicted spine loss. I found that an antiglutamatergic treatment neither led to an increase in dendritic spine density, nor to a decrease in calcium accumulations. Finally, I showed that spine loss was accompanied by a widespread activation of local microglia and infiltration of monocytes-derived macrophages. Overall, these results indicated local calcium accumulations induce phagocyte-mediated removal of spines in the acutely inflamed cortex.

In the following discussion, I will first outline the strengths and drawbacks of the applied methods. I will discuss the c-MS model and its ability to mimic cortical pathology of progressive MS and the methods used to detect calcium in spines and dendrites in acute cortical neuroinflammation. Secondly, I will assess the mechanisms and effectors of synapse loss. In the end, I will discuss therapeutical approaches for patients with progressive MS.

5.2 Cortical neuroinflammation

5.2.1 The c-MS model

Conventional rodent EAE models of multiple sclerosis only show limited inflammatory and demyelinated lesions in the cerebral cortex (Lassmann & Bradl, 2017; Storch et al., 1998). Therefore, our group adapted a focal EAE rat model of cortical demyelination (Merkler et al., 2006) to mice (c-MS model). The c-MS model, which is induced by the parenchymal injection of the proinflammatory cytokines TNFα and IFN-γ into the cortex of mice that were pre-immunized with MOG protein, reproduces the main features of human gray matter pathology in multiple sclerosis (Jafari et al., 2021). It leads to the formation of cortical lesions that are characterized by phagocyte activation and modest T-cell infiltrations which lead to similar extensive subpial demyelination as found in human cortical lesions (Lagumersindez-Denis et al., 2017; Lucchinetti et al., 2011). Moreover, it reproduces the widespread cortical synapse loss also observed in MS (Albert et al., 2017; Jürgens et al., 2015). Furthermore, the stereotactic intracortical injection enables a precise and reproducible localization of such cortical lesions. Overall these features allowed me to study MS-related spine loss and potential mechanisms of spine loss in this model (Jafari et al., 2021).

The parenchymal intracortical injection of cytokines is suggested to preferentially drain through subpial areas into the overlying CSF and thus, primarily cause a subpial distribution of lesions. This type of lesion is the most common seen pattern of lesions in gray matter damage in MS (Merkler et al., 2006). However, the parenchymal injection leads to a direct injury of the cortex and thus, to damages of the blood-brain barrier and glial limitans. This might lead to injury-related pathological changes in the cortex which may complicate to mirror specific cortical immunopathology resulting from the anti-myelin antibody response in conjunction with local effects of the cytokines. Alternative ways of modeling cortical neuroinflammation may be the injection of cytokines into the subarachnoid space of pre-immunized mice or the passive induction of an autoimmune T-cell directed gray matter damage (Gardner et al., 2013; Lodygin et al., 2019). Nevertheless, in our c-MS model, spine loss was only apparent in mice with cortically injected cytokines. Control groups of sham-injected mice which also displayed intracortical damage did not show any spine loss. Moreover, spine loss was only observed in mice in which an autoreactive immune response was triggered by pre-immunization with MOG. These findings indicated that both components of the adaptive immune response such as anti-myelin antibodies as well as the effects of cytokines are required to activate pathophysiological mechanisms which induce spine loss (Jafari et al., 2021).

5.2.2 Pathological features of the c-MS model vs. progressive MS

I found that inflammation, i.e. local microglia activation and the infiltration of monocytes-derived macrophages induced by a single intracortical cytokine injection in the c-MS model is completely reversible. Remarkably, our group observed that along with the decline of inflammatory cells, spine loss and, to a lesser extent, also cortical demyelination can be reversible. Indeed, even a hypoactivity of cortical projection neurons, which likely results from reduced excitatory synaptic inputs and correlates with arising functional imaging evidence of altered neuronal activity in human MS (Meijer, Eijlers, Geurts, & Schoonheim, 2018; Rocca et al., 2010), largely recovered along with the restoration of synaptic connectivity (Jafari et al., 2021). Importantly, this degree of reversibility observed in the c-MS model stands in contrast to (1) white matter lesions and to (2) the sustained gray matter damages observed in progressive MS. Indeed, EAE lesions in the white matter of spinal cords and EAE lesions which are targeted to the subcortical white matter persist with dense infiltrates of phagocytes and without significant remyelination (Kerschensteiner et al., 2004; Merkler et al., 2006). Also, if an axon has been transsected in a white matter lesion, no regeneration and only limited remodeling is possible (Kerschensteiner et al., 2004). Therefore, the two following questions arise: (1) Why are gray matter pathological features such as spine loss in the c-MS model transient whereas

lesions in the white matter persist? (2) As our c-MS model only displays acute inflammatory effects and thus, lacks mirroring the chronic phases of the disease, which mechanisms lead to the lasting cortical damages and deficits observed in patients with progressive MS?

When comparing the immunopathological features of gray and white matter lesions in MS, we find that both pathological processes differ in key respects: cortical demyelinated lesions in chronic progressive MS are less inflammatory and show less infiltrated inflammatory immune cells such as lymphocytes, less complement deposition and less blood-brain barrier disruption compared to white matter lesions (M. Calabrese et al., 2015; Vercellino et al., 2005). Merkler et al. (2006) suggested that the fast resolution of pathological features within the cortex explains the lack of inflammatory signs in chronically demyelinated cortical multiple sclerosis lesions (Merkler et al., 2006). Moreover, they suggested that transient cortical demyelination as observed in our c-MS model is followed by fast remyelination may thus, mask cortical demyelination in early MS. This is supported by the observation that patients with chronic multiple sclerosis show extensive cortical remyelination (Albert, Antel, Bruck, & Stadelmann, 2007). The exploitation of remyelination capacity might finally lead to sustained cortical demyelinated lesions found in patients with chronic MS.

Similarly, spine loss is mostly shown in patients with long standing RRMS or SPMS and thus, is likely observed in chronic and progressive phases of the disease (Jürgens et al., 2015). Our cortical MS model is induced by a single intracortical cytokine injection and thus, stands in contrast to the chronic sources of proinflammatory signaling in progressive MS. The formation of e.g. B-cell follicle like structures in the meninges together with the chronic activation of microglia may lead to the sustained spine loss (Howell et al., 2011; Magliozzi et al., 2007; Ziehn et al., 2010). This is correlated with permanently increased levels of proinflammatory cytokines in the CSF of patients with SPMS (Magliozzi et al., 2018). Overall, one could argue that the cortex has, in contrast to the white matter, a significant potential of endogenous neuronal repair, where even structural alterations such as synapse loss can be reversed (Jafari et al., 2021). This repair potential may at some point be exhausted due to chronic inflammatory signaling in MS and additional factors related to brain ageing and the accumulation of pre-existing brain damage (Mahad et al., 2015) which result in the lasting spine loss found in patients with progressive MS. On the basis of these considerations, one can argue that early therapy which prevents such a chronic, low-threshold neuroinflammatory signaling and by this, retains regenerative capacity, may decelerate progression in MS (Jafari et al., 2021).

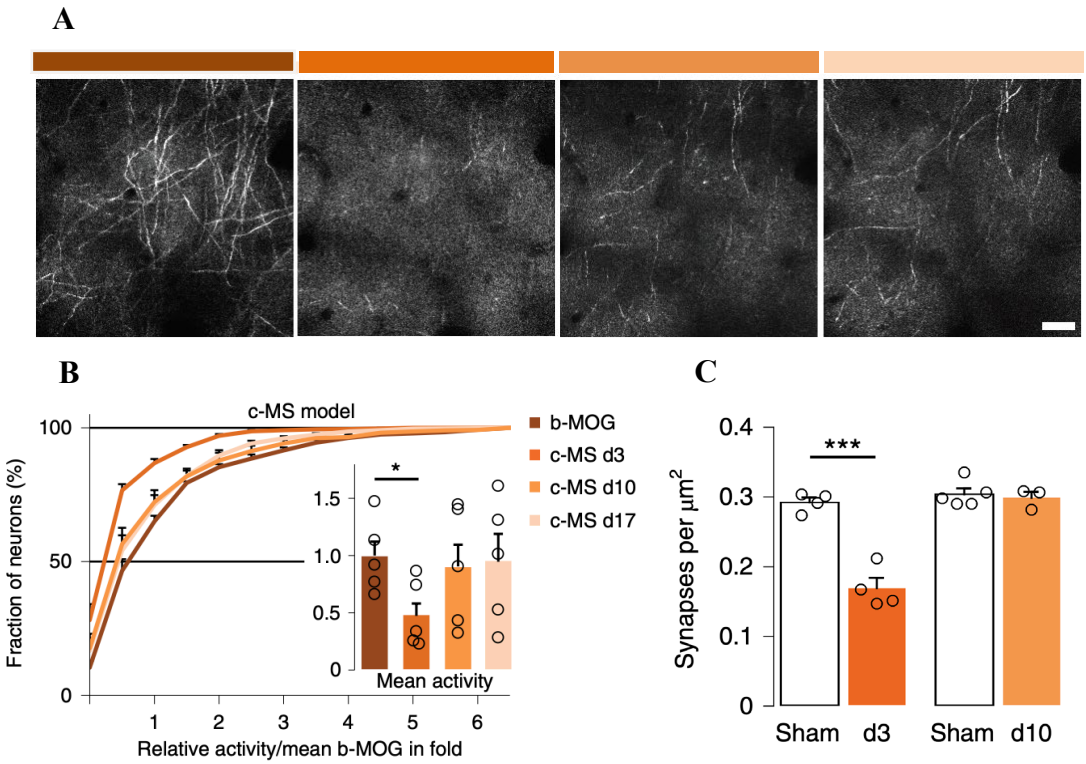

Figure 19: Reversibility of pathological features in the c-MS model

(A) *In vivo* SCoRe imaging projections show the recovery of myelin within the top 50 µm of layer 1 somatosensory cortex in c-MS. Timepoint of imaging from left to right: before cytokine injection, 3 days (d3), 10 days (d10) and 17 days (d17) after cytokine injection. **(B)** Diagram shows cumulative neuronal activity of a layer 2/3 neuronal population for selected timepoints (before cytokine injection (EAE), 3 days (d3), 10 days (d10) and 17 days (d17) after cytokine injection) in c-MS and reveals a significant reduction in mean neuronal activity in acute gray matter lesions (d3). Neuronal silencing is reversible. **(C)** Diagram shows synapse loss in c-MS animals compared to sham-injected control animals (electron micrograph analysis). Synaptic loss is reversible. Figure Panel A modified, Figure Panels B and C adapted from and caption modified from (Jafari et al., 2021).

5.3 *In vivo* two-photon imaging of calcium in single dendrites and spines

In this thesis, *in vivo* imaging of calcium in dendrites and single dendritic spines in the inflamed cortex was performed. For this purpose, the FRET-based genetically encoded calcium indicator Twitch-2B was used and targeted to a subset of cortical neurons (layer V neurons) by rAAV-hsyn1 viral infection. By applying two-photon microscopy in the murine cortex through implanted cranial windows, neuronal signals could be collected and cortical layer I dendritic tufts with corresponding spines could be imaged.

In the following, I am going to discuss my approach of characterizing calcium in single dendritic shafts and spines in cortical neuroinflammation. In particular, I will assess the chosen

calcium indicator. Moreover, I will explore potential confounding factors and limitations of the applied methods.

5.3.1 Characterization of calcium of dendrites and spines in c-MS and assessing the Twitch-2B calcium indicator

Dendritic spines were first described in 1888 by Cajal (Ramón y Cajal, 1888). Since then many of their functional properties and their role for synaptic signaling and plasticity have been revealed from work which was performed in acute brain slices and neuronal cultures. Hereby, confocal microscopy of calcium indicator dyes/fluorescent proteins was coupled with brain slice electrophysiology (Bloodgood & Sabatini, 2005; Engert & Bonhoeffer, 1999; Maletic-Savatic, Malinow, & Svoboda, 1999). However, imaging in fixed tissues only provided a snapshot of neuronal anatomy and imaged neurons lacked intact synaptic input or influences of external such as sensory information, thus these studies were not capable to fully address the dynamic nature of spine plasticity (Mancuso et al., 2013).

Since the groundbreaking study by Juste and Denk in 1995 (Yuste & Denk, 1995), a significant number of *in vivo* studies in which dendritic spine dynamics were imaged in the living brain over long time periods by using two-photon microscopy has been performed (Gray, Weimer, Bureau, & Svoboda, 2006; Spires-Jones et al., 2007; Svoboda & Yasuda, 2006). Often studies on spine calcium imaging were based on chemical calcium indicators (Jia, Rochefort, Chen, & Konnerth, 2011). Today, GECIs are successfully used to detect neuronal calcium signals (Heim et al., 2007; Kuchibhotla et al., 2008; Siffrin et al., 2010; Witte et al., 2019). For resolving calcium dynamics in dendritic spines *in vivo*, so far single fluorophore GECIs were applied (El-Boustani et al., 2018; Mao et al., 2008).

In my thesis, for the first time a FRET-based GECI, namely Twitch-2B (Thestrup et al., 2014), was applied to measure calcium in dendrites and dendritic spines *in vivo*. Several arguments led to the choice of Twitch-2B. First, Twitch-2B has FRET between two variants of YFP and CFP as its underlying mechanism of calcium detection. This goes along with the advantages of ratiometric imaging compared to single fluorophore GECIs. Among these advantages are the exclusion of movement artifacts, more pH-resistance and more photostability (Garaschuk et al., 2007; Mank & Griesbeck, 2008). Second, the TnC-based minimal domain of Twitch-2B leads to fairly linear response kinetics and moreover, implies reduced calcium buffering which can interfere in calcium measuring (Grienberger & Konnerth, 2012). Third, Twitch-2Bs two fluorophores cpVenus^{CD} and mCerulean3 offer high photostability and high quantum yields. This is in particular advantageous concerning the small volume of dendritic spines and their

limited ability to host larger amounts of the sensors. Finally, Twitch-2B has a high dynamic range (ΔR/R=800%) and is highly affine to calcium. Neuronal free intracellular calcium concentration is located in the range of 10–100nM. Twitch 2B has a dissociation constant (K_d) of 200nM and is thus able to resolve calcium changes in the nanomolar range, and also able to mirror putative calcium dysregulations in spines which lay above physiological neuronal calcium. These characteristics made Twitch-2B the sensor of choice.

Via rAAV-mediated expression of Twitch-2B and by applying two-photon microscopy, we were able to image single dendrites and dendritic spines with typical morphological structures in a dense environment. Thereby, I detected local calcium accumulations (i.e. change in the YFP/CFP channel ratio) in spines in cortical neuroinflammation. To study subcellular structures such as mitochondria or the ER which may represent confined structures of calcium overload, a specific targeting and a higher resolution in xy and z would be required.

Subsequently, I studied the effect of such *continuous* calcium dysregulations in spines on spine loss, independently of local synaptic activity. Therefore, I chose an imaging interval of two hours for characterizing calcium dynamics in spines to ensure a substantial spine loss within this timeframe. At this, I found that calcium accumulations in spines primed them for their removal. I excluded phototoxicity as the reason for observed changes in the cortical MS model by imaging control animals over the same timecourse. I did not detect any alterations in spine morphology or expression pattern. In principle a higher imaging frequency with shorter time intervals is possible, however this would go along with an increased risk of phototoxicity. Therefore, I focused on strong and slow calcium dynamics of large spine populations. Nevertheless, Ca^{2+} levels in spines can change quickly as calcium transients correlate with underlying neuronal action potentials (Denk, Yuste, Svoboda, & Tank, 1996; Kerr, Greenberg, & Helmchen, 2005; Wallace et al., 2008). In Alzheimer's disease e.g., which is also associated with synapse loss, neuronal hyperactivity as a function of increased calcium transients has been described (Busche et al., 2008; **Error! Hyperlink reference not valid.**). To detect potential fast calcium changes which may be apparent in normal or intact appearing spines but may also interfere in our spines defined as 'high calcium', higher imaging frequencies and lower laser power are required (Chen, Leischner, Rochefort, Nelken, & Konnerth, 2011; Chen et al., 2012). In subsequent studies, our group ruled out that the local calcium accumulations observed in our c-MS model were fluctuating or correlated with altered synaptic function. This was done by measuring the spiking frequency in 'high calcium' and 'low calcium' spines which was shown to be similar in both (experiments performed by Adrian-Minh Schumacher). These findings

confirmed that ‘high calcium’ spines showed a persistent elevation of basic calcium levels independently from synaptic activity in the c-MS model (Jafari et al., 2021).

5.3.2 Confounding factors and limitations in signaling detection

The following potential confounders have to be taken into account, when calcium is imaged *in vivo*.

First, the K_d value of Twitch-2B was determined *in vitro* by calcium titrations of purified Twitch-2B using fluorescence spectrophotometry (Thestrup et al., 2014). As the actual K_d depends on parameters such as temperature, pH and the presence of magnesium, it may differ in *in vivo* settings (Lattanzio & Bartschat, 1991; Oliver, Baker, Fugate, Tablin, & Crowe, 2000). These environmental factors can interfere with the signals obtained from FRET-based indicators by affecting either the calcium-responsive element or the fluorophores (Grienberger & Konnerth, 2012). To account for these potential influences, we set YFP/CFP ratios greater than mean plus three standard deviations of the ratios measured in healthy animals as the cut-off value for defining a region as ‘high calcium’. Values below this cut-off were thus defined to be ‘low calcium’. This binary method allowed us to have solid read-outs for calcium increases within spines.

Second, a main confounder in signal kinetics of all sensors is their intrinsic calcium buffering which reduces the amplitude of changes in intracellular calcium and prolongs the actual decay time (McMahon & Jackson, 2018). In general, calcium concentration is influenced by the amount of calcium influx and efflux and by the exchange of calcium with internal stores. Additionally, proteins such as calretinin, parvalbumin and calbindin-D28k do act as endogenous calcium buffers and calcium sensors add their own buffer capacity to the total capacity. Both govern the dynamics of free calcium which is measured by our indictors (Grienberger & Konnerth, 2012). Calcium buffering depends on the amount as well as on the affinity of the corresponding calcium indicator. Due to their smaller calcium buffer capacity, this gives low-affinity calcium sensors better signal kinetics. However, low-affinity calcium sensors go along with smaller fractional fluorescence changes (McMahon & Jackson, 2018). This ends in the main problem of high-Ca^{2+} affinity vs. fast decay kinetics (Wilms & Häusser, 2014). Smaller calcium binding domains with less binding sites were suggested to help reducing calcium buffering (Direnberger et al., 2012). In Twitch sensors this problem was addressed accordingly. Thestrup et al. (2014) created GECIs with a TnC-based minimal calcium binding domain from the toadfish Opsanus tau with a reduced number of calcium binding sites while maintaining high affinity for Ca^{2+}. Reduced calcium buffering resulted. However, due to its very high affinity for Ca^{2+}, Twitch-2Bs kinetics are still low (decay time of 2.8s) compared to

low-affinity Twitch sensors such as Twitch-5 (decay time of 0.16s) which may limit the use of Twitch-2B for imaging where resolving of fast events which occur at high frequencies is desired (Thestrup et al., 2014; Wilms & Häusser, 2014). Therefore, one may also combine low-affinity/fast-kinetics and high-affinity/slow-kinetic sensors with different spectral variants to image different sorts of calcium dysregulations in parallel within rodent models of neurological diseases (Tischbirek, Birkner, Jia, Sakmann, & Konnerth, 2015; Walker, Burrone, & Meyer, 2013).

5.4 Mechanisms of synapse loss in MS

5.4.1 Role of localized calcium accumulations for spine loss

Synapse loss is a pathognomonic feature of gray matter damage in MS and in particular, correlates with cognitive impairment. Therefore, identifying the pathways which contribute to synapse loss is essential. In focal gray matter lesions of MS, spine loss was accompanied by a reduced number of cortical axons and dendritic branches. However, selective spine loss was also observed in the NAGM with unaltered cortical axon density and dendrite morphology. These observations suggested a diffuse primary synaptic pathology (Albert et al., 2017; Dutta et al., 2011; Jürgens et al., 2015).

As synaptic loss can initiate neuronal death (Selkoe, 2002), and thus drive disease progression in MS, exploring the initiating causative mechanisms of synapse loss is of immense therapeutic value. The ability to model MS-related cortical neuroinflammation has allowed me to investigate these mechanisms. At this, I found that local calcium accumulations prime spines for subsequent removal. The assumption that these calcium accumulations are a primary signal for spine removal is in general plausible considering the following reasons. First, during development, synaptic connectivity is established in a diffuse process and subsequently refined in an activity-dependent, thus, likely refined in a calcium-dependent manner (Pan & Monje, 2020). Second, calcium signals can directly control the expression of ‘eat-me’ signals in neurons. This has been shown, e.g. for the membrane exposure of phosphatidylserine due to intracellular calcium dysregulations which triggered a microglia-mediated engulfment (Tufail et al., 2017). Third, calcium accumulations can activate calcium-dependent enzymes such as calpains or calcineurin which destabilize the cytoskeleton and might promote subsequent spine removal (Andres et al., 2013; Kuchibhotla et al., 2008). This might in particular occur, when calcium accumulations reach the dendritic shaft and cytoskeletal remodeling becomes more extended and may explain the observation that dendritic calcium overload correlates with a significant increase in the spine removal rate in the c-MS model (Jafari et al., 2021). Moreover, calcium accumulations can also target mitochondria, leading to mitochondrial dysfunctions and

by this, initiate apoptotic-like damages (Cai & Tammineni, 2017; Pivovarova & Andrews, 2010). Finally, cytoplasmic calcium increases have also been found to act as early predictors for the fate of axons or dendrites in the setting of contusion and in neuroinflammatory lesions of the spinal cord during EAE (Siffrin et al., 2010; Williams et al., 2014; Witte et al., 2019).

Overall, such calcium accumulations in spines could have a wide range of causes, e.g. the increased influx from extracellular or the release from intracellular stores. Mechanisms which can in theory be involved in these causes include glutamate-mediated toxicity, mitochondrial dysfunctions and the exposition to reactive oxygen and nitrogen species (ROS/RNS). After giving an overview of the sources of calcium in spines and how they can be assessed in experimental designs, I am going to discuss the proposed mechanisms of calcium overload in spines in the cortical MS model.

5.4.2 Assessing the sources of calcium in spines in the c-MS model

Neuronal free intracellular calcium concentration is located in the range of 10–100nM. Extracellular calcium levels are about 10.000 times higher than the intracellular ones and some intracellular organelles such as mitochondria or the ER contain calcium concentrations that are closer to extracellular levels. Therefore, these organelles are considered to be calcium stores (Segal & Korkotian, 2014). In spines, several extracellular and intracellular sources of calcium exist. A main source of calcium in spines is the influx from the extracellular space through voltage-gated calcium channels (VGCC) and through ionotropic glutamate receptors, including NMDA, AMPA or kainate receptors (Killestein, Kalkers, & Polman, 2005; Rochefort & Konnerth, 2012). VGCCs are mainly activated by action potentials that backpropagate into the dendrites of neurons and by synaptically mediated depolarization of spines (Bloodgood & Sabatini, 2007; Reid, Fabian-Fine, & Fine, 2001; Waters & Helmchen, 2004). Ionotropic glutamate receptors are non-specific-cation channels with a varying permeability for calcium (NMDA receptors are particularly permeable for calcium). Usually, spines are depolarized by the activation of AMPA receptors which removes extracellular magnesium. This results in the activation of NMDA receptors which leads to further depolarization and calcium entry. Spine depolarization can be subsequently strengthened by the activation of voltage-gated calcium or sodium channels (Rochefort & Konnerth, 2012). Additional forms of calcium signaling in spines include the release from internal stores, e.g. via ryanodine receptors or inositol trisphosphate (IP3) receptors which are distributed throughout the endoplasmic reticulum (ER) (Berridge, 1998). In addition to the ER, mitochondria can act as calcium buffers and thus, maintain calcium homeostasis (Grienberger & Konnerth, 2012). Extrusion mechanisms of

calcium involve the plasma membrane Ca^{2+} pumps (PMCAs), the Na^{+}/Ca^{2+} exchangers (NCX), and the uptake into intracellular stores through the smooth endoplasmatic reticulum Ca^{2+} ATPase (SERCA) (Scheuss, Yasuda, Sobczyk, & Svoboda, 2006).

Localizing the origin of calcium accumulations in dendritic spines in the c-MS model is of major importance. It would allow targeting calcium overloads in dendritic spines at their root in prospective therapeutical approaches of preventing spine loss.

For this purpose, one has to locate the source of calcium to the extracellular and/or the intracellular space. Calcium release from intracellular sources can originate from local internal calcium stores such as the ER or mitochondria (H. Li et al., 2014; Segal & Korkotian, 2014; Wu et al., 2017). ER calcium in the c-MS model could e.g. be monitored by performing *in vivo* imaging in transgenic mice which express an ER-targeted genetically encoded calcium indicator. This was previously performed in EAE to asses to contribution of ER calcium in axonal degeneration (Scheiter, 2020; Witte et al., 2019). To assess the contribution of calcium influx from the extracellular space, one can locally remove extracellular calcium by incubating the healthy and inflamed cortices with the Ca^{2+} chelator EGTA (Witte et al., 2019). In studies following my experiments, our group performed the above described experiment. They buffered extracellular calcium by local EGTA application to c-MS lesions *in vivo*. They found a reduced rate of new 'high calcium' spines, an improved reestablishment of calcium homeostasis in calcium-dysregulated spines and a significantly reduced rate of spine loss (Jafari et al., 2021). These findings indicated that local calcium indicators indeed are prime drivers for spine removal. Moreover, these results emphasized the significant contribution of extracellular calcium to spine loss.

5.4.3 Role of glutamate for spine loss and calcium accumulations in spines

5.4.3.1 Glutamate-mediated excitotoxicity in MS and in animal models of MS

An aim of my thesis was to assess the mechanisms involved in the above described sources of calcium accumulations in the c-MS model. Therefore, I investigated on the potential link between extracellular glutamate increase and calcium influx through glutamate receptors and subsequent spine loss.

The underlying assumption of the toxic effects of glutamate in neurological disorders is the following: constantly increased amounts of extracellular glutamate lead to the overactivation of glutamate receptors (in particular NMDA receptors). This results in Ca^{2+} influx, an increased postsynaptic transmission and cell damage caused by the activation of calcium dependent enzymes such as proteases, endonucleases, and nitric oxide synthases (Newcombe et al., 2008). Since glutamate receptors are preferentially located postsynaptically, this mechanism could in

particular contribute to synaptopathy as observed in the gray matter of MS and of the c-MS model. To assess the pathway of glutamate-mediated excitotoxicity in the c-MS model, the question of whether the above described cascade is in theory plausible to occur in our model needs to be answered.

First, there are several findings which underpin the presence of the glutamate-mediated cascade of excitotoxicity in MS and in EAE: (1) Glutamate availability is increased in both MS and EAE (Mandolesi et al., 2015). It is the result of the increased release by infiltrating inflammatory leucocytes and activated microglia, the failed reuptake into astrocytes, the dysfunction of glutamate transporters, and the downregulation of glutamate metabolizing enzymes (Castegna et al., 2011; Ganor & Levite, 2014; Gonsette, 2008; Hardin-Pouzet et al., 1997). (2) Moreover, there is evidence of upregulated ionotropic glutamate receptors in MS as the result of the increased glutamate availability (Newcombe et al., 2008). (3) Also, increased postsynaptic transmission in form of increased excitatory postsynaptic currents (EPSCs) mediated by the activation of AMPA receptors was shown in EAE (Centonze et al., 2009). (4) Finally, blocking AMPA receptors was reported to reduce dendritic spine loss in EAE, which linked glutamate-mediated excitotoxicity to spine loss in a neuroinflammatory condition (Centonze et al., 2009).

Second, the inflammatory pathological features observed in the c-MS model would in theory involve excessive increase of extracellular glutamate levels. In particular, activated microglia are assumed to regulate glutamate homeostasis. By producing RNS and ROS, they are able to impair glutamate uptake mechanisms (Bolton & Paul, 2006). Both, activated microglia and the release of TNFα were reported to correlate with the reduced expression of glutamate transporters in multiple sclerosis (Pitt et al., 2003; Vercellino et al., 2007) and thus, impaired glutamate uptake. Also, both mimicked the effects of EAE on postsynaptic currents and the sensitivity of AMPA receptors to synaptically released glutamate (Centonze et al., 2009). Our c-MS model, which displays (1) a widespread intracortical activation of microglia which also can release TNFα, and (2) involves the direct injection of TNFα into the cortices, is likely to result in increased extracellular glutamate levels. These increased glutamate levels could subsequently lead to the activation of postsynaptically located AMPA and NMDA receptors and calcium influx from extracellular into spines.

5.4.3.2 Blocking glutamate receptors in the c-MS model

On the basis of the aforementioned considerations, I decided to study the role of NMDA and AMPA receptors on subsequent calcium accumulations in dendritic spines and spine loss in our c-MS model. For this purpose, experimental animals were treated with the AMPA receptor

antagonist NBQX and the NMDA receptor antagonist memantine (Centonze et al., 2009; Sheardown, Nielsen, Hansen, Jacobsen, & Honore, 1990; T. Smith et al., 2000; Sulkowski et al., 2013). My results showed that there was no significant difference in spine density between c-MS mice which received the antiglutamatergic treatment (NBQX and memantine) and c-MS mice which received the vehicle. Moreover, the treatment did not lead to a decrease in calcium accumulations in the c-MS model. Also *in situ* no effect on spine density was found in an animal group which was treated with NBQX and memantine (experiments performed by Mehrnoosh Jafari, data not shown).

The following conclusions may be drawn: First, the dose or way of application did not ensure sufficient intracortical concentrations of the antagonists to prevent focal calcium overload in spines. Higher doses and eventually the direct application of the antagonists may help to rule out this possibility.

Alternatively, these results could mean that NMDA/AMPA receptors do not play the predominant role for spine loss or at least do not sufficiently account for calcium accumulations in spines which appear to initiate spine loss in acute cortical neuroinflammation. Interestingly, in axonal degeneration in EAE, where similar focal calcium overloads predicted axonal survival, glutamate toxicity was excluded as a contributor to focal calcium increase (Witte et al., 2019). However, in this context it should be noted, that ionotropic glutamate receptors are only found in confined nanocomplexes along axons, whereas they are plentiful synaptic locations on dendrites (Christensen, Samadi-Bahrami, Pavlov, Stys, & Moore, 2016; Ouardouz et al., 2009). In other neurodegenerative diseases with ongoing spine loss, similar observations concerning the involvement of ionotropic glutamate receptors have been made: in a murine Aβ-mediated Alzheimer's disease model, NMDA receptors were found to impair synaptic function, but were not capable of inducing spine loss (Müller, Jacobi, Sakimura, Malinow, & von Engelhardt, 2018). Aβ-mediated dendritic spine loss was suggested to occur due to metabotropic signaling of NMDA receptors combined with the intracellular increase of calcium due to the release from internal stores such as the ER (Birnbaum, Bali, Rajendran, Nitsch, & Tackenberg, 2015; Jensen et al., 2013).

Third, increased extracellular glutamate levels during *acute* neuroinflammation could be buffered, e.g. by auto-inactivating ionotropic glutamate receptors at high levels of extracellular glutamate or initially increased compensatory clearance mechanisms (Ehlers, Zhang, Bernhadt, & Huganir, 1996; Morimoto-Tomita et al., 2009; Sulkowski, Dabrowska-Bouta, Salinska, & Struzynska, 2014; Vallejo-Illarramendi, Domercq, Perez-Cerda, Ravid, & Matute, 2006). After

these adaptive, compensatory mechanisms have vanished, glutamate may have toxic effects on synapses and synaptic functioning in later, more chronic phases of the disease.

5.4.4 Effects of ROS/RNS on calcium homeostasis

Additional pathways of focal calcium accumulations have to be considered in our c-MS model and may be targeted in prospective studies. One of them is the influence of ROS/RNS on calcium homeostasis.

There is strong evidence of increased ROS/RNS levels, which are mainly produced by activated/infiltrating phagocytes, in multiple sclerosis (Haider et al., 2011; K. J. Smith & Lassmann, 2002). ROS/RNS can exert their effect on calcium homeostasis in two ways. First, in the context of 'virtual hypoxia' which was described in axons (Rizzuto & Pozzan, 2006; Stys, 2005), ROS/RNS have an indirect effect on calcium homeostasis by influencing mitochondrial energy production and thus, leading to an energy failure. The final pathway of it is the redistribution of ion channels such as sodium channels. This can lead to a reverse functioning of the Na^+/Ca^{2+} exchanger (NCX) and result in impaired calcium buffering, the dysregulation of calcium homoestasis and calcium overload (Craner et al., 2004; Friese et al., 2007; Schattling et al., 2012).

Second, ROS/RNS can induce calcium influx also via a direct effect on calcium channels/transporters or via the attack on neuronal plasma membranes. Indeed, ROS/RNS are able to induce damages to biological macromolecules such as proteins, DNA/RNA and polyunsaturated fatty acids which are found in membrane lipids (PUFA) (Fischer et al., 2012). As the CNS is rich in polyunsaturated fatty acids and shows high oxygen turnover, it is in particular at risk of lipid peroxidation (van Horssen et al., 2008). In addition to the lipid peroxidation, ROS/RNS can influence the rigidity and permeability of membranes, and impair membrane receptors (Di Domenico, Tramutola, & Butterfield, 2017; Gonsette, 2008). In axonal degeneration in EAE, where also focal calcium accumulations predicted the fate of axons, ROS/RNS were suggested to be one possible way of causing axonal membrane disruptions, which can be sufficiently stable to allow a significant calcium influx from extracellular into axons (Witte et al., 2019). In addition to the effect on membrane integrity, ROS/RNS can disturb calcium homeostasis by altering ion channel functions. So far, several calcium channels have been described to be influenced by ROS/RNS, among these are the sarco-/endoplasmic reticulum Ca^{2+} ATPase pump (SERCA), the plasma membrane calcium ATPases (PMCA), Na^+/Ca^{2+} exchangers (NCX), glutamate receptors such as NMDA receptors, voltage gated calcium channels (VGCC), IP3 and ryanodine receptors (Kiselyov & Muallem, 2016; Waring, 2005).

Experiments which investigate the effects of ROS/RNS application *in vivo* on spine morphology/spine calcium, and experiments, in which ROS/RNS are scavenged in cortical MS lesions (Nikic et al., 2011), may shed light onto the role of redox-mediated calcium accumulations in dendritic spines and spine loss in the c-MS model.

5.4.5 Phagocyte-mediated engulfment of spines

Irrespectively of the precise mechanism of calcium accumulation in spines, the effectors of spine removal in MS and in c-MS were also largely unknown. In this context, glia-mediated synaptic removal, in particular synaptic stripping by cortical microglia (Trapp et al., 2007), is likely to be significantly involved. Microglia are assumed to maintain and monitor synaptic function and to control synaptogenesis (Ransohoff, 2016; Roumier et al., 2004; Tremblay, Lowery, & Majewska, 2010). In EAE, the incubation of brain slices with activated microglia led to the induction of synaptic deficits and dendritic spine loss (Centonze et al., 2009; Nistico et al., 2013). Also in MS, a C1q-C3 complement-microglia axis was associated with synaptic alterations (Michailidou et al., 2015).

Phagocyte-mediated synapse engulfment has been suggested to be a hallmark of various neurological disorders including neuroinfectious, neuropsychiatric and neurodegenerative ones. In a murine model of Lupus, synapse loss is prevented by blocking the activation of microglia via type I interferon (IFN) signaling (Bialas et al., 2017). In chronic infections with the West-Nile Virus and in an Alzheimer's disease model, a complement-microglial axis has been shown to drive synapse loss (Hong et al., 2016; Vasek et al., 2016). Another microglia-related signaling pathway has been observed in the Alzheimer's disease model: the activation of the colony-stimulating factor 1 receptor (CSF1R) regulated the activity and proliferation of microglia and concomitant synaptic degeneration (Olmos-Alonso et al., 2016). In a model of viral encephalitis, chemokines such as CCL2 expressed by virally infected neurons acted as 'attack-me signal' to attract phagocytes which resulted in synaptic stripping (Di Liberto et al., 2018).

In our c-MS model, the spatial and temporal characterization of the phagocytic system revealed a widespread but reversible intracortical activation of local microglia (MHCII/CX3CR1^{GFP} positive cells) and infiltration of monocyte-derived macrophages (CCR2^{RFP} positive cells). The activation/infiltration of phagocytes matched with the spatial and temporal characteristics of spine loss observed in the c-MS model. Therefore, mononuclear phagocytes became the prime candidates of cells which execute the removal of spines in the inflamed cortex. In subsequent studies, our group assessed the contribution of microglia and blood-derived mononuclear phagocytes to synapse loss. Brain tissues from CCR2^{RFP} and CX3CR1^{GFP} mice (Yamasaki et

al., 2014) were stained for lysosomal (LAMP 1), presynaptic (PSD95 and Homer) and synaptic (Synapsin 1) markers (experiments performed by Mehrnoosh Jafari). Both, microglia and invading macrophages showed a pronounced increase in engulfed pre- and postsynaptic material. These findings indicated that both cell types are collaboratively involved in synapse removal in the setting of acute cortical neuroinflammation (Jafari et al., 2021). Subsequently, this phagocyte-mediated synapse removal was targeted by treating the c-MS mice with an inhibitor of CSF1 signaling (CSF1 receptor (CSF1R) antagonist; experiments performed by Mehrnoosh Jafari). Whereas high concentrations of the CSF1R antagonist lead to the depletion of microglial cells (Elmore et al., 2014; Nissen, Thompson, West, & Tsirka, 2018), lower concentrations have been reported to prevent phagocyte-mediated synapse loss in an animal model of Alzheimer's disease (Olmos-Alonso et al., 2016). The systemic treatment of c-MS animals with low-dosed CSF1R antagonist during the formation of cortical lesions fully prevented spine loss (Jafari et al., 2021). Moreover, the treatment resulted in the reduction of invading monocyte-derived phagocytes, along with the reduced expression of CCL2 which is the main chemokine responsible for the infiltration of monocytes to the CNS (Huang, Wang, Kivisakk, Rollins, & Ransohoff, 2001). The amount of locally activated microglia remained unaltered, but RNA-sequencing analysis revealed a pronounced change in the transcription profile within this phagocytic entity in response to the CSF1R inhibition. Locally activated microglia displayed a more homeostatic phenotype which implied a reduced ability to engulf synapses (Jafari et al., 2021).

5.4.6 Role of the complement system for spine tagging

The question of how spines which are destined for removal are tagged for subsequent phagocytosis still needs to be answered. A mechanism which can be involved in spine tagging is the complement system: the classical complement proteins can act as 'eat-me signals' in the peripheral immune system and by this, promote the elimination of debris, pathogens and cellular structures (Hong & Stevens, 2016). This is in part achieved by phagocytes which express complement receptors, e.g. the CR3 (Schafer et al., 2012). The complement system was discovered to be fundamental for the activity-dependent synaptic pruning in the developing visual thalamus, a mechanism which is required for the formation of mature neural circuits (Hua & Smith, 2004; Stevens et al., 2007). More precisely, the initiation complement protein C1q and C3, which is a downstream protein, localized to immature synapses and likely tagged them for elimination. Tagged synaptic terminals can be subsequently engulfed by microglia through the C3-CR3 pathway (Hong & Stevens, 2016; Schafer et al., 2012; Stevens et al., 2007).

The reactivation of similar developmental mechanisms of complement-mediated synapse loss was suggested to drive progression in CNS diseases (Hong et al., 2016; Howell et al., 2011; Rosen & Stevens, 2010; Stephan, Barres, & Stevens, 2012). In an Alzheimer's disease mouse model, a complement-microglial axis was found to drive synapse loss (Hong et al., 2016). More specifically, the inhibition of C1q, C3 or microglial CR3, reduced the number of phagocytic microglia and along with this, the amount of early synapse loss. Similar observations were made in West Nile virus infection and frontotemporal dementia (Lui et al., 2016; Vasek et al., 2016). Also in MS, a C1q-C3 complement-microglia axis has been suggested to be involved in synaptic alterations (Michailidou et al., 2015). Moreover in progressive MS, complement pathways have been found to be activated in cortical gray matter lesions, in particular in areas of increased levels of complement receptor-positive microglia (Watkins et al., 2016).

In summary, phagocyte-mediated engulfment of complement-tagged spines represents a likely executive mechanism of synaptic removal in MS. To assess the role of complement to spine loss in our c-MS model, in an experimental series performed by Mehrnoosh Jafari (data not shown), c-MS mice were treated with an anti-C1q antibody (Hong et al., 2016). The results showed that there was no significant difference in spine density between c-MS mice which were treated with the anti-C1q antibody and c-MS mice which received a vehicle. Their findings could thus not verify an involvement of C1q in spine removal in the c-MS model.

Overall, the involvement of complement in gray matter pathology remains controversial. There are findings which demonstrate activated complement only at the edge of intracortical lesions (Schwab & McGeer, 2002) or no significant complement deposition in cortical lesions at all (Brink et al., 2005). Moreover cortical lesions are less inflammatory and autoantibodies directed against neuronal and axonal structures can only be found in a subset of patients with MS (Bø, 2009; Meinl, Derfuss, Krumbholz, Pröbstel, & Hohlfeld, 2011). However, in this context it should be noted that complement activation can also appear in the absence of antibodies or inflammatory responses (Flierman & Daha, 2007; Vanguri, Koski, Silverman, & Shin, 1982). In other neurodegenerative diseases such as the ALS, the suppression of C1q induction in mice did as well not protect against synaptic loss nor against motor neuron degeneration (Lobsiger et al., 2013). Similar observations were made in Parkinson's disease mouse models, where the C1q suppression did not protect against the dopaminergic neuronal degeneration (Depboylu et al., 2011). These findings suggested that the contribution of C1q induction depends on the specific neurodegenerative condition and the neuronal system which is involved (Lobsiger et al., 2013).

In the classical component cascade, C1q and downstream C3 localize to synapses and microglia that express the complement receptor CR3 engulf and eliminate synapses (Hong et al., 2016; Lui et al., 2016; Stevens et al., 2007; Vasek et al., 2016). However, it remains open if synapse loss in MS is mediated in the same way. Recently, Werneburg et al. (2020) demonstrated that microglial synaptic engulfment and synapse loss in the visual system of MS patients and in rodent models of MS coincided with increased complement component C3, but not C1q. Indeed, by inhibiting C3, microglial engulfment of synapses was reduced and the visual function was protected. These findings suggested that microglia engulf synapses through an alternative complement pathway which bypasses C1q and works directly through synapse-localized activated C3 (Werneburg et al., 2020). Alternative pathways of complement signaling may thus also be relevant for synaptic loss in our c-MS model.

What recruits complement proteins to synapses is an important question and remains unanswered so far. Candidates are apoptotic signals which are expressed on the surface of the damaged cellular structures (Bode et al., 2014). Recently, the exposure of phosphatidylserine (PS) was demonstrated to act as a neuronal 'eat-me' signal and to induce a complement-microglial-mediated removal of synapses (Scott-Hewitt et al., 2020). Importantly, the exposure of PS could be directly regulated by intracellular calcium dyshomeostasis, similar to those accumulations found in the dendritic spines of our c-MS model (Tufail et al., 2017).

Another question which remains open in this context is whether the synapse elimination in MS contributes to neurodegeneration or is a necessary mechanism to counteract external toxic effects on neurons. Increased extracellular glutamate levels for example can be detrimental for neurons and the engulfment of synapses may represent a mechanism to protect neurons from excessive excitatory activity (Michailidou et al., 2015). It was demonstrated that dendritic spines can be locally removed, e.g. via a caspase-3–dependent mechanism, thus that neuronal apoptosis can be initiated, but be spatially restricted without inducing the death of the target neuron. This local pruning pathway was described to be initiated by mechanisms such as mitochondrial oxidative stress and the activation of NMDA receptors (Ertürk, Wang, & Sheng, 2014). Both can go along with postsynaptic calcium overload as outlined in the sections 5.4.3 and 5.4.4. In this context, it should be furthermore noted that the prevention of spine loss by CSF1-R inhibition did not lead to signs of neuronal stress in the c-MS model und thus, argued that at least in regard to CSF1-R inhibition, the survival of neurons was not jeopardized (Jafari et al., 2021).

In the light of all discussed pathophysiological mechanism, the following pathway of spine loss in the acutely inflamed cortex could be imagined: molecular mechanisms acting alone or in

concert (e.g. glutamate-mediated toxicity, mitochondrial dysfunctions, the exposition to ROS/RNS) lead to calcium accumulations in spines, which control the expression of apoptotic signals and by this, activate alternative complement pathways. The complement system tags these synapses and attracts/activates phagocytes. Through the C3-CR3 pathway, synapses become engulfed by activated/infiltrated phagocytes. Along with the decline of inflammatory signaling and due to the endogenous repair potential in cortex, spine loss can recover. Chronic inflammatory signaling as observed in progressive MS may lead to the exhaustion of endogenous recovery mechanisms and thus, result in the lasting spine loss and cognitive decline observed in progressive MS.

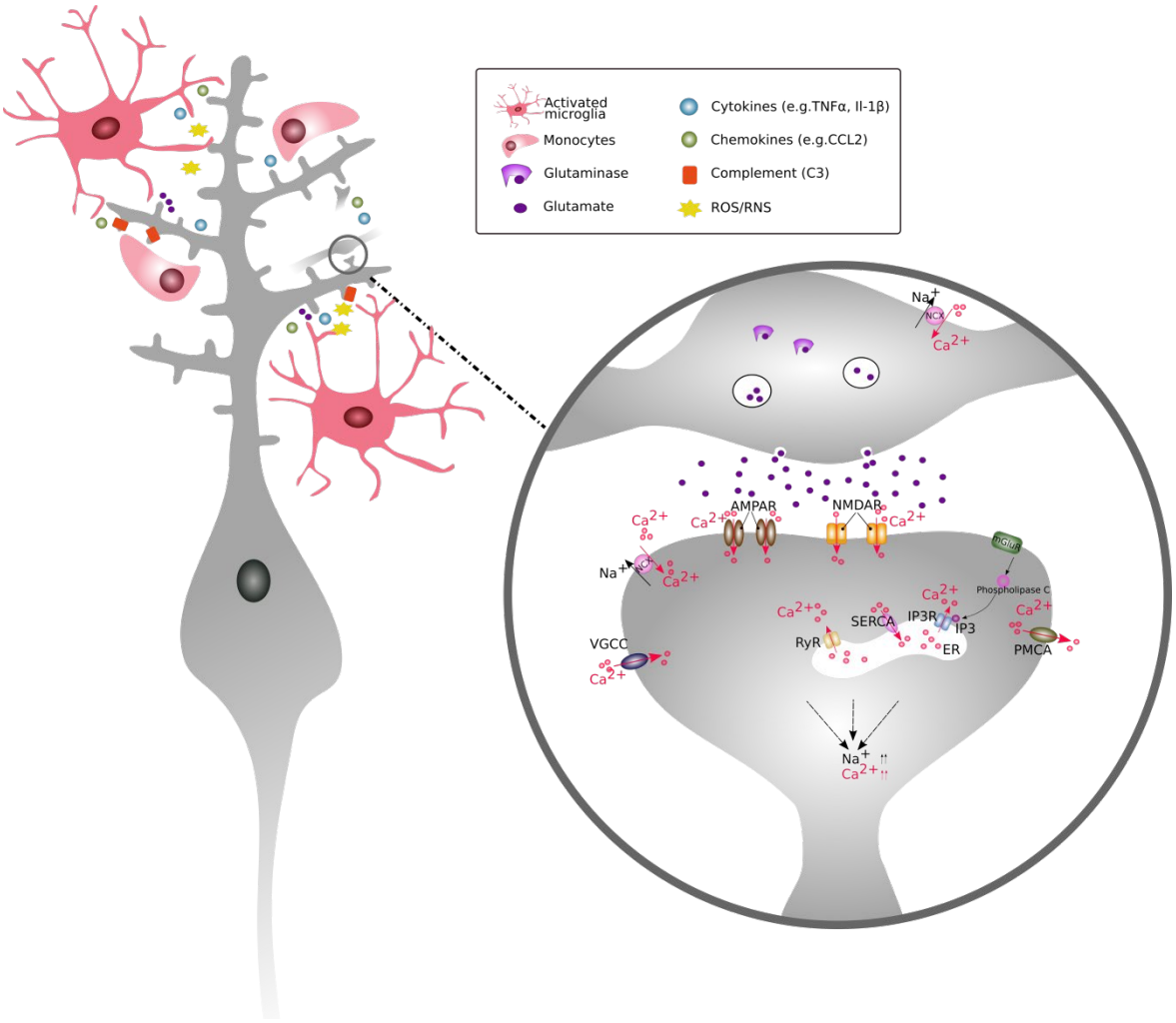

Figure 20: Summary: mechanisms of synaptic pathology in cortical MS

Schematic diagram illustrates potential neuroinflammatory and neurodegenerative mechanisms involved in synaptic pathology. These mechanisms include the chronic secretion of inflammatory mediators such as cytokines (TNFα, IL-1β) and chemokines (CCL2), the activation of the mononuclear phagocytic system (local microglia activation, monocytes infiltration), complement-mediated synaptic stripping as well as disturbances in calcium homeostasis. Several sources of calcium and molecular mechanisms can in theory be involved in synaptic calcium accumulations, such as glutamate-mediated toxicity, or the exposition to reactive oxygen and nitrogen species (ROS/RNS).

5.5 Therapeutical approaches for progressive MS

A central question is how pathogenetic ideas or new pathological findings such as the findings about synapse loss in the c-MS model can be translated into successful therapies for affected patients with progressive MS. This is in particular important, as numerous disease-modifying and anti-inflammatory treatments are licensed for the therapy of patients with relapsing remitting multiple sclerosis, but the later progressive phases remain largely refractory to therapeutic interventions (Schumacher et al., 2017). The assumption that the mechanisms

involved in synapse loss are a crucial therapeutic target in progressive MS is based on the widespread synapse loss observed in the gray matter of MS patients (Albert et al., 2017; Dutta et al., 2011; Jürgens et al., 2015), and the likely structural and functional consequences which can follow from it (Jafari et al., 2021). In this thesis, I searched and discussed the potential pathogenesis of synaptic pathology and neurodegeneration in progressive MS. Based on these discussed pathological concepts and previous studies, several targets emerge as therapeutical approaches for progressive MS: (1) glutamate-mediated excitotoxicity, (2) ionic disequilibrium, and (3) phagocytes.

5.5.1 Glutamate-mediated excitotoxicity

In my experiments, an anti-glutamatergic treatment did not prevent synaptic loss in the *acute* c-MS model. However, the involvement of glutamate-mediated excitotoxicity in synaptic pathology in *chronic* progressive MS is still likely as previous studies provided evidence of increased glutamate levels in MS to predict neuroaxonal damage and also postulated a promising link between inflammation, glutamate-mediated excitotoxicity and synaptic damage in cortical lesions (Azevedo et al., 2014; Mandolesi et al., 2015; Vercellino et al., 2007).

Most studies in clinics which investigated on glutamate-mediated excitotoxicity in MS were so far focused on inhibiting ionotropic glutamate receptors, in particular NMDARs (Macrez et al., 2016). One underlying reason was that NMDA and AMPA blockers had beneficial effects on neurological deficits in EAE (Centonze et al., 2009; T. Smith et al., 2000; Wallstrom et al., 1996). However in humans, studies which used these drugs either showed no effect or led to clinically intolerable side effects (Lovera et al., 2010; Peyro Saint Paul et al., 2016; Waubant et al., 2014). Indeed, the use of glutamate receptor antagonists in clinics is limited, as ionotropic glutamate receptors mediate normal synaptic transmission in the brain and thus, are required for the normal functioning of the nervous system. Therefore, blocking them can result in severe side effects, such as hallucinations and coma (Lipton, 2006b).

The previous failure of clinical trials tempts to assume that glutamate receptors are not suitable targets for treatment in MS. However, improvements in the design of trials as wells as refined mechanisms of preventing glutamate-mediated toxicity have to be considered. Concerning the design of trials, more precise selection criteria of patients which are included into the trials have to be defined. Patients should be selected by the subtype and their phase of disease. Moreover, the criteria should include objective signs of glutamatergic dysregulation. The selection of patients could be optimized by combining different diagnostic criteria such as imaging, biological fluids and genetic methods (Macrez et al., 2016). Techniques such as the use of

glutamate PET ligands present promising tools to visualize glutamate-mediated excitotoxicity as an index of neurodegeneration in MS (Fu, Chen, Josephson, Li, & Liang, 2019).

Regarding the refined antiglutamatergic treatment, the following aspects have to be taken into account. First, appropriate dosing is crucial for study outcomes as doses which are escalated to quickly might lead to serious side effects and therefore, lessen beneficial treatment effects. Secondly, CNS bioavailability plays a key role. Glutamatergic modulators with low bioavailability may help reducing side effects, as they are more likely to penetrate the CNS only at sites of active lesions (Macrez et al., 2016). Finally, antagonists that act more specifically and only block those glutamate receptors which are excessively activated have to be designed (Lipton, 2006b). Therefore, compounds that block pathological effects of glutamate receptors with a minimum effect on basic synaptic transmission can be considered, e.g., compounds that target extrasynaptic receptors and serine/glycine modulatory sites (LeMaistre et al., 2012; Lipton, 2006a, 2006b; Piña-Crespo et al., 2010). The role of those ligands and modulators of glutamate receptors should be investigated in future studies in order to design more effective and well tolerated antiglutamatergic drugs.

5.5.2 Ionic disequilibrium

A second therapeutical approach is to improve the ionic disequilibrium which can result from the chronic inflammatory reaction in progressive MS. In this context, the administration of sodium channel blockers was suggested to limit and prevent neuroaxonal degeneration in progressive MS by breaking through the vicious circle of tissue damage (Waxman, 2006). In EAE, the systemic administration of Na^+ channel-blocking anticonvulsants such as lamotrigine (Bechtold et al., 2006) or the sodium channel-blocking agent flecainide (Bechtold, Kapoor, & Smith, 2004) reduced axonal loss and neurological disability. In clinical trials, however, evidence about the efficiency of sodium channel blockers on neuroprotection in MS is lacking (Hayton et al., 2012; C. Yang, Hao, Zhang, Zeng, & Wen, 2015). Future strategies in this regard involve treatments which target certain subsets of Na^+ channels or which are selectively administrated into the inflamed tissue (Al-Izki et al., 2014). Moreover, other ion channels such as the proton-gated acid-sensing ion channel-1 (ASIC1) or TRPM4 which both can contribute to Na+ influx were suggested to serve as new targets (Friese et al., 2007; Schattling et al., 2012). However, in a recent trial, at least the treatment with amiloride, which blocks the ASIC1, did not protect from progressive brain atrophy (Chataway et al., 2020).

In conclusion, little evidence exists if and to what extent the inhibition of Na^+ conducting ion channels can reduce neuroaxonal damage and prevent progressive disability in patients with

MS. In regard to dendritic spine loss and synaptic alterations, their specific role is fully unexplored.

5.5.3 Phagocytes

The continuous recruitment of $CCR2^+$ monocytes into the CNS along with the chronic activation of local microglia are prominent pathological features in patients with long-standing and progressive multiple sclerosis (Howell et al., 2011; Lagumersindez-Denis et al., 2017; Magliozzi et al., 2007; Ziehn et al., 2010). In our c-MS model, both significantly contributed to synapse removal, and therefore, probably are involved in the synapse loss of progressive MS. Thus, a third therapeutical approach for progressive MS is, to target these phagocytic cells (Pinto & Fernandes, 2020).

5.5.3.1 Chemokine receptor antagonists

In this context, modulating chemokine signaling receptors was suggested to target the directed migration of phagocytes across the BBB (Hamann, Zipp, & Infante-Duarte, 2008). In particular, the CCR2/CCL2 axis was proposed to be a target for the treatment of multiple sclerosis (Mildner et al., 2009). Many studies have indicated that the chemokine receptor CCR2 and its ligand CCL2, which is the main chemokine responsible for the infiltration of monocytes to the CNS, have a fundamental role in the pathogenesis of EAE and multiple sclerosis (Huang et al., 2001; Mahad & Ransohoff, 2003).

In the gray matter of MS patients, the continuous infiltration of $CCR2^+$ phagocytes into the CNS is a prominent pathological feature (Lagumersindez-Denis et al., 2017). In our c-MS model, $CCR2^+$ monocytes were significantly involved in synapse removal (Jafari et al., 2021). Furthermore, is has been suggested that the integrity of circuit synaptic plasticity, at least in the case of peripheral nerve injury, depends on the CCR2 activation (Rotterman et al., 2019).

So far, in clinical trials the use of CCR2 and other chemokine receptor antagonists were largely disappointing which indicates the need to further study the intricate system of interactions between chemokines (e.g. CCL2) and chemokine receptors (e.g. CCR2) (Hamann et al., 2008; Mildner et al., 2009; Tschammer, 2015). In particular, the widespread expression of CCR2 in various other haematopoietic (e.g. basophils, dendritic cells, T-cells) and non-haematopoietic (e.g. brain endothelia, microglia, neurons, astrocytes) cells makes the understanding of CCR2's role during neuroinflammatory signaling more difficult (Mahad & Ransohoff, 2003; Mildner et al., 2009). Moreover, treatments which selectively block only one molecule can result in the organism finding mechanisms of compensating these blocked molecules, in particular as chemokines are redundant (Hamann et al., 2008). Indeed, most clinically approved treatments

for MS are mechanistically broad. The development of specific clinical markers which enable to stratify MS patients into chemokine receptor-specific and responsive subpopulations may help to successfully establish chemokine receptor antagonists as a MS therapy (Tschammer, 2015).

5.5.3.2 CSF1R inhibition

As of today, the progressive phases of MS appear largely non-responding to current immunomodulatory therapies which primary target the peripheral immune system (Hawker et al., 2009; Kapoor et al., 2018; Lublin et al., 2016; Wolinsky et al., 2007). However, they might respond to disease-modifying approaches which target inflammation in the CNS (Fox et al., 2018; Jafari et al., 2021). Among these, intervening in the CSF1R signaling pathway has arisen as a promising approach to alleviate detrimental functions of phagocytic cells in cortical neuroinflammation (Borjini, Fernández, Giardino, & Calzà, 2016; Hagan et al., 2020).

The CSF1R is as receptor tyrosine kinase and can be found on mircoglia and few peripheral immune cells, particularly monocytes and macrophages (Nandi et al., 2012; Nissen et al., 2018). The stimulation of CSF1R by the two independent ligands CSF1 and IL-34 regulates the differentiation, proliferation and survival of these cells (Lin et al., 2008; Nissen et al., 2018; Olmos-Alonso et al., 2016). Mice which lack CSF1R or a ligand were shown to have reduced amounts of macrophages in various tissues, whereas CSF1R knockout mice were completely devoid of microglia (Erblich, Zhu, Etgen, Dobrenis, & Pollard, 2011; Ginhoux et al., 2010; J. Li, Chen, Zhu, & Pollard, 2006). Although the functional consequences of CSF1R inhibitors in patients are not completely explored, numerous studies indicate that inhibitors of CS1FR can have beneficial effects in altering neuroinflammation (Hagan et al., 2020). In an Alzheimer's mouse model and in our model of cortical MS, low-dosed CSF1R inhibitors prevented phagocyte-mediated synapse removal (Jafari et al., 2021; Olmos-Alonso et al., 2016). In the c-MS model, this mechanism targeted the central and peripheral phagocyte reaction in parallel. CSF1R inhibition reduced chemoattraction of peripheral phagocytes on one hand and altered disease-associated phenotype of locally activated microglia of CNS on the other hand. Overall, the inhibition led to a reduced ability to engulf synapses. The suggestion that CSF1 signaling may be a promising way to target this synapse loss in MS is supported by the finding that the CSF1R is prominently expressed by microglial cells in cortex of patients with progressive MS (Schirmer et al., 2019).

Importantly, low dosed CSFR1 inhibitors do avoid the ablation of microglia, in contrast to high-dosed inhibitors of CSF1R (Elmore et al., 2014; Nissen et al., 2018). This is particularly important as microglia have a fundamental role for tissue homoestasis and responding to

pathological events in the CNS (Prinz et al., 2011). The transient ablation of these essential cells may have severe side effects and diminish the possibility of patients to react to CNS infections or damages (Nissen et al., 2018). In chronic EAE models, the ablation of microglia has divergent outcomes and can harbor adverse effects (Nissen et al., 2018; Tanabe, Saitoh, Miyajima, Itokazu, & Yamashita, 2019). In contrast, non-depleting CSF1R inhibition appear to have beneficial effects (Hagan et al., 2020) by reducing detrimental executive functions of microglia in neuroinflammation while maintaining their protective functioning (Jafari et al., 2021).

In a synopsis of all above discussed therapeutical approaches for progressive MS, the current findings indicate that treatments which target specific pathophysiological mechanisms do not appear to be the appropriate strategy to prevent disease progression in MS. In fact, the findings suggest that combinatorial therapeutical strategies together with new ways to stratify patients need to be developed in order to target the pathological mechanisms that are most prevalent in single patients.

5.6 Concluding remarks

Multiple sclerosis is the most prevalent chronic immune-mediated disease of the human central nervous system (CNS). Although a lot of progress has been made in the last years and decades of research to unravel the complex pathophysiology MS and to translate these findings into improved clinical therapies, many pathological features of MS remain unsolved.

While the acute pathological and clinical manifestation of MS is dominated by focal demyelinated white matter lesions and resulting sensorimotor deficits, progressive phases of MS are characterized by a diffuse and irreversible neuroaxonal damage/loss and increasing neurological disability. As of today, numerous immunomodulatory treatments exist to target the acute phases of the disease, but the progressive phases remain largely refractory to therapeutic interventions. In recent years of research, the pathological features of the gray matter in MS have emerged as fundamental contributors to both physical and cognitive disability in progressive MS. A major feature of such gray matter pathology in progressive MS is a diffuse synaptopathy, i.e. synaptic dysfunctions and synaptic loss, which has been revealed by previous histopathologcial and biochemical studies.

In preliminary work, our group reproduced gray matter inflammation and synapse loss in a murine model of cortically targeted EAE. This enabled me to study mechanisms of synapse loss in MS-related cortical neuroinflammation. The overriding goal of my thesis was to find

interventions which may reduce cortical inflammatory processes and maintain synapses and by this, find prospective therapeutic approaches which counteract the progression of MS.

Towards this goal, I imaged calcium in dendrites and spines *in vivo* in the mouse model of cortical MS and found increased calcium accumulations in dendritic spines in the cortical MS model. Moreover, I observed that these local postsynaptic calcium accumulations predicted spine loss. In conjunction with subsequent findings of our group which showed that the removal of extracellular calcium decreased the rate of spine loss, our results proved that local calcium accumulations in spines promote their subsequent removal in the acutely inflamed cortex. I have discussed the broad range of potential mechanisms of calcium influx/release in spines, among these are toxic effects of increased extracellular glutamate and/or the direct or indirect effects of ROS/RNS. Concerning a potential glutamate-mediated excitotoxicity, my experiments did not find a direct link between an antiglutamatergic treatment and postsynaptic calcium accumulations or spine loss in the c-MS model, however a correlation cannot be excluded. Although it is challenging to investigate the mechanisms of calcium accumulations, intervening in excessive calcium influx is a promising field for prospective therapeutical interventions.

Regarding the cellular inflammatory mechanisms, I found that dendritic spine loss was accompanied by a widespread activation of local microglia and an infiltration of monocytes-derived macrophages. These findings made them prime candidates for spine removal. In subsequent studies, our group found that both microglia and monocytes showed an increase in engulfed pre- and postsynaptic material in the c-MS model and thus, proved the shared contribution of both phagocytic entities for spine removal. Moreover, they found that the inhibition of the CSF1R, which reduced the recruitment of peripheral phagocytes and altered the disease-associated phenotype of local microglia, rescued spine loss. Overall, these results emphasize the role of a CNS targeted immunomodulation in future neuroprotective therapies for patients with progressive MS.

So far, progressive phases of MS remain largely refractory to current immunomodulatory treatments. The numerous failed clinical trials underline that still a lot of research is required to unravel the pathophysiological features of progressive MS. Moreover, ways of stratifying patients in further subgroups based on specific clinical biomarkers which predict the probability of being responsive to a specific treatment have to be developed. These approaches may enable us to find therapeutical strategies that are personalized and able to meet each individuals needs and finally, to achieve the superior aim: the prevention of disease progression and the reduction of severe personal consequences of the disease MS.

6. References

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8. Affidavit

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