# DENDRITIC PATHOLOGY IN A MOUSE MODEL OF MULTIPLE SCLEROSIS

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# List of Abbreviations

| | |
|---|---|
| (s)gRNA | (Single) Guide RNA |
| Aβ | Amyloid beta protein |
| AAV | Adeno-associated virus |
| aCSF | Artificial cerebrospinal fluid |
| AD | Alzheimer's disease |
| ALS | Amyotrophic lateral sclerosis |
| AMPA(R) | α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (receptor) |
| ASD | Autism spectrum disorder |
| BBB | Blood-brain-barrier |
| BTK | Bruton's tyrosine kinase |
| Cas9 | CRISPR-associated nuclease 9 |
| CCL2 | Chemokine ligand 2 |
| CFA | Complete Freund's adjuvant |
| CIS | Clinically isolated syndrome |
| c-MS | Cortical MS model |
| CNS | Central nervous system |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CRISPRa | CRISPR activation |
| CRISPRi | CRISPR inhibition |
| CSF | Cerebrospinal fluid |
| CXCR4 | Chemokine receptor type 4 |
| dCas9 | (catalytically) dead Cas9 |
| DMT | Disease modifying therapy |
| DSB | Double strand break |
| dsRNA | Double strand DNA |
| EAE | Experimental autoimmune encephalomyelitis |
| EBNA1 | EBV transcription factor EBV nuclear antigen 1 |
| EBV | Epstein-Barr virus |
| EDSS | Expanded disability status scale |
| EPSP | Excitatory postsynaptic potential |
| FAD | Focal axonal degeneration |
| FLAIR | Fluid-attenuated inversion recovery |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GWAS | Genome wide association study |

| | |
|---|---|
| HAD | HIV-associated dementia |
| HDR | Homology-directed repair |
| hiPSC | Human induced pluripotent stem cell |
| HLA | human leukocyte antigens |
| ID | Intellectual disability |
| IFNGR | Interferon-gamma receptor |
| IFN-γ | Interferon-gamma |
| IL | Interleukin |
| Indel | Insertion and/or deletion |
| KO | Knock-out |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| MAP | Microtubule-associated protein |
| MBP | Myelin basic protein |
| mGluR5 | Metabotropic glutamate receptor 5 |
| MHC | Major histocompatibility complex |
| MOG | Myelin oligodendrocyte glycoprotein |
| MOGAD | MOG antibody-associated disease |
| MRI | Magnetic resonance imaging |
| mRNA | Messenger RNA |
| MS | Multiple sclerosis |
| mTOR | Mammalian target of rapamycin |
| NAGM | Normal-appearing grey matter |
| NAWM | Normal-appearing white matter |
| NfL | Neurofilament light chain |
| NHEJ | Non-homologous end joining |
| NMDA(R) | N-methyl-D-aspartate (Receptor) |
| NMOSD | Neuromyelitis optica spectrum disorder |
| NO | Nitric oxide |
| NPC | Niemann Pick type-C disease |
| OCB | Oligoclonal bands |
| PAF | Platelet-activating factor |
| PAM | Protospacer-adjacent motive |
| PD | Parkinson's disease |
| PLP | Proteolipid protein |
| PPMS | Primary progressive MS |

| | |
|---|---|
| PRMS | Progressive relapsing MS |
| PSAP | Prosaposin |
| PSD-95 | Postsynaptic density protein 95 |
| RER | Rough endoplasmic reticulum |
| RIS | Radiologically isolated syndrome |
| RNAi | RNA interference |
| RRMS | Relapsing-remitting MS |
| SER | Smooth endoplasmic reticulum |
| SNPH | Syntaphilin |
| SPMS | Secondary progressive MS |
| TALEN | Transcription activator-like effector nuclease |
| TNF(R) | Tumour necrosis factor (receptor) |
| VLA4 | Very late antigen-4 |
| ZFN | Zinc-finger nuclease |

# Abstract

With nearly 3 million individuals affected worldwide, multiple sclerosis (MS) stands as a significant global health concern. This disease is marked by the formation of inflammatory and demyelinating lesions within both the grey and white matter of the central nervous system (CNS). In the initial relapsing phase of MS, treatment strategies primarily focus on mitigating inflammation by targeting the adaptive immune system. However, as the disease progresses and disability accumulates, these therapies often prove insufficient, as they do not effectively address the neurodegenerative processes underlying disease progression. Consequently, there is a pressing need for studies aimed at elucidating the mechanisms driving neurodegeneration in MS, with the ultimate goal of identifying targets that can halt or slow down the progression of MS.

The neurodegenerative mechanisms occurring within the grey matter remain poorly understood. Neurons are cells with highly specialized compartments like dendrites and axons. While axons have garnered significant attention as targets of an immune attack, dendrites have been relatively understudied in the context of MS. Hence, my doctoral project centred on visualizing and characterizing dendritic pathology in the inflamed CNS, as well as elucidating possible molecular mechanisms underlying this pathology.

In the first part of this thesis, I examined dendrites within the lumbar spinal cord of mice with experimental autoimmune encephalomyelitis (EAE), a common animal model of MS. Notably, dendrites within the grey matter appeared to maintain their structural integrity during peak of disease. Intriguingly, a proportion of dendrites were observed extending into the white matter, predominantly originating from motor neurons. At acute disease stages, these dendrites exhibited a beaded appearance, indicative of potential distress due to their proximity to lesions. Interestingly, the dendritic density restored to healthy levels after resolution of initial inflammation. Furthermore, thorough examination revealed no evidence of neuronal loss, synaptic density reduction, or nanoruptures allowing calcium influx – as found in axons – thus ruling out these possibilities for dendritic damage. Consequently, I conclude that dendritic pathology in the context of neuroinflammation occurs independently of neuronal cell loss, possibly depending on inflammatory signals, underscoring a possible link between preceding neuroinflammation and resulting neurodegeneration.

To explore whether soluble molecules, possibly diffusing from lesions within the EAE tissue, contribute to dendritic beading and to identify the specific molecules involved, I developed a method to manipulate motor neurons in the spinal cord. The goal was to deplete their

membrane receptors for these extrinsic signals while preserving the diseased environment. To achieve this, I employed a combination of CRISPR-engineering and AAV-PHP.eB viral vectors. This innovative approach enabled DNA-editing of specific receptors and assessment of their impact within the EAE model. The development of this technique offers a rapid means of manipulating multiple candidates in neurons *in vivo*. This approach has the potential to accelerate experimentation and enhance our ability to dissect the intricate molecular mechanisms underlying dendritic pathology in MS and EAE. Subsequently, I employed this technique to investigate the involvement of several receptors in dendritic pathology, focusing on receptors for molecules that are typically found within or near inflammatory lesions. By targeting the receptor for the inflammatory cytokine IFN-γ, as well as the glutamate receptors NMDAR and AMPAR, I sought to determine whether depletion or blocking of these receptors could prevent or alleviate the observed dendritic pathology. However, the results within this thesis suggest that none of the interventions lead to changes in dendritic fate, and the pathology remained similar to that observed in unedited wild type neurons.

Overall, this research underscores the presence of dendritic pathology within the inflamed spinal cord. However, it did not unveil a specific molecular mechanism underlying the observed dendritic damage. During this work, I also established a versatile pipeline for *in vivo* gene editing of adult motor neurons using CRISPR/Cas9 technology that can help to improve our molecular understanding of neurological disorders.

# Zusammenfassung

Die Multiple Sklerose (MS), von der weltweit fast 3 Millionen Menschen betroffen sind, stellt ein erhebliches globales Gesundheitsproblem dar. Diese Krankheit ist gekennzeichnet durch die Bildung von entzündlichen und demyelinisierenden Läsionen sowohl in der grauen als auch in der weißen Substanz des zentralen Nervensystems (ZNS). In der anfänglichen schubförmigen Phase der MS konzentrieren sich die Behandlungsstrategien in erster Linie auf die Eindämmung der Entzündung, indem sie auf das adaptive Immunsystem abzielen. Wenn die Krankheit jedoch fortschreitet und die Behinderungen zunehmen, erweisen sich diese Therapien oft als unzureichend, da sie die neurodegenerativen Prozesse, die dem Fortschreiten der Krankheit zugrunde liegen, nicht wirksam verhindern können. Daher besteht ein dringender Bedarf an Studien, die darauf abzielen, die Mechanismen zu ergründen, die entzündliche Schädigung der Nervenzellen vorantreiben, mit dem Ziel, Therapiestrategien zu identifizieren, die das Fortschreiten der MS aufhalten oder verlangsamen können.

Die neurodegenerativen Mechanismen, die in der grauen Substanz ablaufen, sind nach wie vor nur unzureichend bekannt. Neurone sind Zellen mit hochspezialisierten Kompartimenten wie Dendriten und Axonen. Während die entzündliche Schädigung von Axonen schon vergleichsweise intensiv untersucht wurde, ist die Pathologie der Dendriten im entzündeten Nervensystem noch relativ wenig erforscht. Daher konzentrierte sich mein Promotionsprojekt auf die Visualisierung und Charakterisierung der dendritischen Pathologie sowie auf die Erforschung möglicher molekularer Mechanismen, die diesem Phänomen zugrunde liegen.

Im ersten Teil dieser Arbeit untersuchte ich Dendriten im lumbalen Rückenmark des Tiermodells der Multiplen Sklerose, der experimentellen autoimmunen Enzephalomyelitis (EAE). Bemerkenswerterweise schienen die Dendriten in der grauen Substanz ihre strukturelle Integrität zu bewahren. Zudem untersuchten wir auch die Dendriten, die sich in die weiße Substanz erstreckten und überwiegend von motorischen Neuronen stammen. Diese Dendriten wiesen Schwellungen auf, was auf eine entzündliche Schädigung aufgrund ihrer Nähe zu den Läsionen hindeutet. Interessanterweise erreichte die dendritische Dichte nach Abklingen der anfänglichen Entzündung wieder ein gesundes Niveau. Darüber hinaus ergaben sich bei den nachfolgenden Untersuchung keine Anzeichen für einen neuronalen Verlust, eine Verringerung der synaptischen Dichte oder Nanorupturen, die einen Kalziumeinstrom ermöglichen (wie sie in Axonen zu finden sind), so dass diese Möglichkeiten für den dendritischen Rückgang ausgeschlossen werden können. Daraus ergibt sich die Schlussfolgerung, dass die dendritische Pathologie im Zusammenhang mit

MS ein vom neuronalen Zelltod unabhängiger Prozess ist, der möglicherweise von Entzündungssignalen abhängt und eine mögliche Verbindung zwischen vorausgehender Neuroinflammation und daraus resultierender Neurodegeneration unterstreicht.

Um zu untersuchen, ob lösliche Moleküle, die möglicherweise aus Läsionen im EAE-Gewebe diffundieren, zur dendritischen Schädigung beitragen, und um die spezifischen Moleküle zu identifizieren, die daran beteiligt sind, habe ich eine Methode entwickelt, um Motoneurone im Rückenmark zu manipulieren. Ziel war es, Membranrezeptoren für spezifische entzündliche extrinsische Signale zu deaktivieren und gleichzeitig die entzündliche Umgebung zu erhalten. Dazu habe ich eine Kombination aus CRISPR-Engineering und AAV-PHP.eB-Virusvektoren eingesetzt. Dieser innovative Ansatz ermöglichte DNS-Editierung spezifischer Rezeptoren und die Bewertung ihrer Auswirkungen auf die neuronale Pathologie im EAE-Modell. Die Entwicklung dieser Technik bietet einen vielseitig einsetzbaren Ansatz zur Manipulation molekularer Kandidaten in Neuronen *in vivo*. Dieser Ansatz hat das Potential Experimente zu beschleunigen und unsere Fähigkeit zu verbessern, die molekularen Mechanismen, die der dendritischen Pathologie bei MS und EAE zugrunde liegen, zu untersuchen. Anschließend setzte ich diese Technik ein, um die Beteiligung des Rezeptors für das entzündliche Zytokin IFN-γ sowie die Glutamatrezeptoren NMDAR und AMPAR an der dendritischen Pathologie zu untersuchen. Die Ergebnisse dieser Arbeit deuten jedoch darauf hin, dass keiner der Eingriffe zu einer Veränderung des dendritischen Schicksals führte und die Pathologie derjenigen ähnelte, die bei unbehandelten Wildtyp-Neuronen beobachtet wurde.

Insgesamt unterstreicht diese Untersuchung das Vorhandensein einer dendritischen Schädigung innerhalb des entzündeten Rückenmarks. Einen spezifischen molekularen Mechanismus, der der beobachteten Degeneration zugrunde liegt, konnte ich in dieser Arbeit noch nicht aufdecken. Im Rahmen dieser Arbeit habe ich aber eine Pipeline für das genetische Editieren erwachsener Motoneuronen *in vivo* mit der CRISPR/Cas9-Technologie entwickelt. Dieser Ansatz bietet die Möglichkeit, die Untersuchung der molekularen Mechanismen neurologischer Erkrankungen zu beschleunigen.

# Chapter I - Introduction

## I.1. Multiple Sclerosis

With its complex pathophysiology and a wide spectrum of clinical manifestations, multiple sclerosis (MS) challenges our understanding of the human central nervous system (CNS) and its interactions with the immune system. Although its aetiology remains elusive, it is believed to arise from an interplay of genetic susceptibility and environmental triggers. This multifaceted chronic disease, affecting millions worldwide, is typically considered an autoimmune disorder in which a dysregulated immune system targets the myelin sheath. This attack triggers a cascade of neuroinflammatory and neurodegenerative responses, leading to the formation of demyelinated lesions in the brain and spinal cord. As these lesions accumulate in the white and grey matter, they lead to a range of neurological symptoms, such as paralysis and impaired cognitive function. The clinical manifestations of MS are as diverse as the individuals it affects. Heterogeneity in its clinical presentation, progression, and response to treatment have confounded attempts to understand the disease and find cures for affected patients.

### I.1.1. Epidemiology and Aetiology of MS

In 2023, it is estimated that 2.9 million people worldwide are living with MS. Among them, 280.000 MS patients live in Germany, affecting roughly 1 in 300 inhabitants, which represents one of the highest incidences of the disease globally. Interestingly, the incidence of MS is higher among women, who account for 70% of cases (Federation, 2020). While women have a higher risk of developing MS, men tend to have worse disease prognosis (Runmarker et al., 1994). Although MS can manifest itself at any age, it is typically diagnosed between the ages of 20 and 40, making it the most common neurological cause of disability in young adults (Stroke, 2023; Wallin et al., 2019). Rarely, paediatric MS is diagnosed when the onset of the disease starts before the age of 16, accounting for up to 5% of the MS population (Yeh et al., 2009).

The aetiology of MS is considered to be multi-factorial, resulting from a complex interplay of genetic, immunological, and environmental factors. While the exact cause of MS remains elusive, various risk factors have been proposed:

1. *Genetic susceptibility:*

   While MS itself is not an inheritable disease, its susceptibility can be inherited. A Swedish registry-based study brought to light that monozygotic twins display the highest familial risk of developing MS. The unaffected twin in such pairs has a 17%

age-adjusted risk of developing MS, underscoring a significant heritable influence (Westerlind et al., 2014).

Moreover, the complexity of MS genetics is underscored by the identification of over 200 independent risk loci associated with the condition. Many of these loci are linked to functions of the immune system, particularly within the human leukocyte antigens (HLA) genome in the major histocompatibility complex (MHC) and overlap with genetic variants associated with other autoimmune diseases (Consortium*† et al., 2019; Farh et al., 2015).

2. *Infectious factors and viruses:*

   Among the many infections proposed to play a role in MS pathogenesis, the most consistent findings point to the accumulating evidence for the Epstein-Barr Virus (EBV) as a potential trigger of MS. Recent studies have shed light on the epidemiological and mechanistic links between EBV and MS. In individuals genetically predisposed to the disease, MS appears to be triggered by an infectious agent, with EBV emerging as the primary candidate. Bjornevik et al. conducted a study utilizing data from US military recruits monitored over two decades, revealing a robust correlation between EBV infection and the subsequent development of MS. Following an EBV infection, the risk of MS increased by 32-fold, in stark contrast to infections by other viruses, including the similarly transmitted cytomegalovirus (Bjornevik et al., 2022). From a mechanistic perspective, Lanz et al. proposed that molecular mimicry between the EBV protein EBNA1 and the CNS associated protein GlialCAM leads to the production of cross-reactive antibodies, thereby providing a potential link between EBV and MS (Lanz et al., 2022).

3. *Environmental and lifestyle factors:*

   MS prevalence and incidence rise with increasing distance from the equator, with higher latitudes being associated with an earlier onset of MS symptoms (Tao et al., 2016). This geographical pattern may be at least partly attributed to decreased sun exposure and subsequently reduced vitamin D levels in individuals residing in countries closer to the poles. Consequently, research has demonstrated that vitamin D supplementation can effectively reduce the rate of relapses in individuals with relapsing-remitting MS (RRMS, see below) (Laursen et al., 2016).

   Lifestyle factors, such as smoking and diet, also play a significant role in MS risk. Studies indicate that smokers are at an increased risk of developing MS, and both active and passive smoking contribute to more rapid disease progression, as

measured by the Expanded Disability Status Scale (EDSS) (Hedström et al., 2009; Wu et al., 2023). Obesity and adherence to a Western diet are similarly associated with a heightened risk of developing MS. A study conducted by Munger et al. revealed that women with a BMI ≥ 30 kg/m2 at age 18 had a 2.25-fold higher risk of developing MS compared to those with a BMI in the normal range (Manzel et al., 2014; Munger et al., 2009).

Migration studies further support the role of environmental and lifestyle factors in shaping MS risk. These studies demonstrate that individuals who migrate from regions with low MS prevalence to high-prevalence areas—and vice versa—acquire the risk profile of their new location (Pugliatti & Ferri, 2020). Recent studies suggest that the risk of developing MS increases with the duration of exposure to a high-MS-risk environment, irrespective of age at migration, suggesting that the adoption or acculturation to a "Western" lifestyle and environment may play a critical role (Rotstein et al., 2019).

### I.1.2. Symptoms and Clinical Course

Flares, also known as, attacks or relapses (if recurring), are a characteristic feature in the clinical course of MS. They refer to the occurrence of new symptoms, recurrence, or sudden worsening of neurological symptoms in individuals with MS. Typically, flares manifest swiftly, reaching their peak within hours or days, and gradually resolve over the next days to several months. On average, relapses take about eight weeks from onset to recovery (Rolak, 2003). Several commonly proposed factors that trigger relapses include infections, sleep disturbance and emotional stress (Kamel, 2019; Polick et al., 2023; Sahraian et al., 2017).

Patients with multiple sclerosis commonly experience a range of motor, cognitive and psychiatric symptoms. Occurrence and severity of symptoms vary vastly within MS patients, giving rise to profound heterogeneity in clinical course. Physical MS symptoms include fatigue, pain, paralysis, balance dysfunction and tremors. Additionally, they may face challenges with bladder and bowel function, as well as encounter vision problems. In the early stages of the disease, cognitive problems may be mild or non-existent. However, as MS progresses, an estimated 50% of patients will experience cognitive dysfunction, which usually worsens in advanced cases (Rao et al., 1991). Common cognitive symptoms of MS include deficits in complex attention, verbal fluency, executive functioning, as well as slowed processing speed, and long-term memory decline (Benedict et al., 2006; Chiaravalloti & DeLuca, 2008). Furthermore, many patients suffer from neuropsychological disorders, such as depression and anxiety, which are likely to result

both from pathological changes in the brain as well as social consequences and insecurities that accompany the condition (Di Filippo et al., 2018; Weerasinghe-Mudiyanselage et al., 2024). Taken together, these deficits can affect many aspects of daily life, such as the ability to run a household, participate in society, and maintain employment. Patients with MS report lower health-related quality of life compared to other chronic disease populations, which mainly correlates with disease disability and depression. These findings highlight the importance of providing effective therapies and implementing support measures to improve well-being of MS patients (Berrigan et al., 2016).

MS is typically classified into four main types, each representing a distinct pattern of disease progression and symptom presentation (Fig. 1) (Lublin et al., 2014):

1. *Clinically Isolated Syndrome (CIS)* involves a single episode where one or multiple neurological symptoms associated with MS manifest. On MRI scans, lesions are identifiable in a single area within the CNS. Usually, there is complete or partial recovery following this initial attack.
2. *Relapsing-Remitting Multiple Sclerosis (RRMS)* is characterized by the occurrence of new symptoms or the recurrence of previous ones, known as relapses. These relapses are accompanied by multiple lesions in different areas of the brain and spinal cord. Following each relapse, a period of remission ensues, which can last for months or even years until interrupted by another relapse.
3. *Secondary Progressive Multiple Sclerosis (SPMS):* After an initial period of relapsing-remitting MS, approximately 70% of patients transition into SPMS. In this stage, the disease progresses steadily without distinct relapses or remissions. Disability tends to accumulate over time, attacks persist more chronically and remit less completely. This leads to a pattern of steady deterioration rather than episodic flares.
4. *Primary Progressive Multiple Sclerosis (PPMS)* is less common than RRMS and SPMS. It is characterized by a steady accumulation of disability from the onset of the disease, without distinct relapses or remissions.

While these categories provide a helpful framework for understanding the disease's progression and clinical patterns, MS is in reality more likely to be a highly dynamic condition with a spectrum of manifestations. This view proposes that MS is not a strictly compartmentalized disease but rather exists on a continuum with overlapping features (Vollmer et al., 2021). The transitions between the different stages, particularly between RRMS and SPMS, can be challenging to differentiate and may not always be clear-cut. The disease can exhibit considerable variability among individuals, and the pace and nature of its progression can vary significantly.

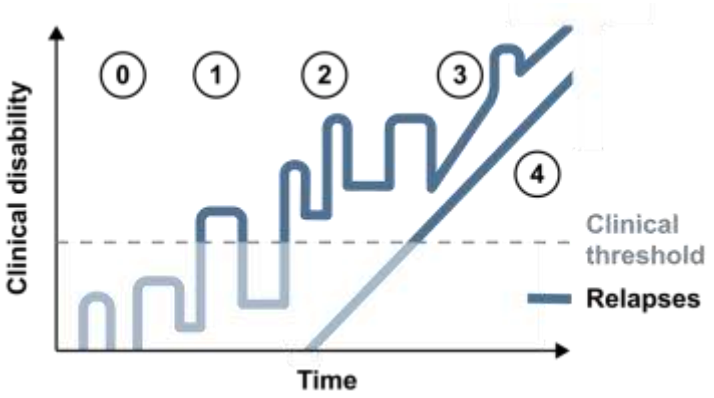



**Figure 1 | Clinical categories of MS.** **(0)** Before clinical symptoms are perceived, lesions can pre-exist in an asymptomatic prodromal phase (radiologically isolated syndrome; RIS). **(1)** First neurological symptoms associated with MS arise in a stage termed clinically isolated syndrome (CIS), followed by a period of recovery. **(2)** Subsequent relapses and remission periods together with accumulating lesions characterize the relapsing-remitting type of MS (RRMS). During remission, patients can recover fully or partially from clinical disability. **(3)** RRMS can transition into secondary progressive MS (SPMS), featuring incomplete recovery and disability progression in between relapses. **(4)** Primary progressive MS (PPMS) is a type of MS where a steady increase of disability, independent of relapses, occurs from disease onset. Figure created by Adinda Wens, inspired by (Lublin et al., 2014) and (Filippi et al., 2018).

### I.1.3. Diagnosis

Diagnosing MS is a complex process, as there is no single definitive test to confirm the condition. Instead, specific criteria must be met to establish an MS diagnosis. The McDonald criteria are a set of guidelines that incorporate clinical and laboratory evaluations, aiding in the diagnostic process. They require the presence of lesions that are disseminated both in space and time. This means there should be two or more separate lesions in different areas of the CNS that have occurred in two or more distinct episodes (Thompson et al., 2018).

Magnetic Resonance Imaging (MRI) is a non-invasive imaging technique that enables clinicians to visualize areas of pathology in the CNS, follow the formation of new lesions over time and assess their distribution. Most commonly used MRI sequences are T2-weighted, fluid-attenuated inversion recovery (FLAIR) and proton density weighted, capable of detecting MS lesions in different CNS compartments (Trip & Miller, 2005) (Fig. 2, A and C). Gadolinium-enhanced contrast in T1-weighted MRI scans are useful to distinguish between active and inactive lesions, valuable for indicating lesion age and in monitoring disease progression (Fig. 2, D) (Lublin et al., 2014).

A supporting diagnostic tool is the testing for IgG oligoclonal bands (OCB). OCBs signify the production and presence of antibodies within the CSF, indicating chronic immune activation. Because OCBs are detected in 95% of patients diagnosed with MS, but can also be found in a variety of other inflammatory diseases, it is not a definitive test for MS (Deisenhammer et al., 2019; Graner et al., 2020).

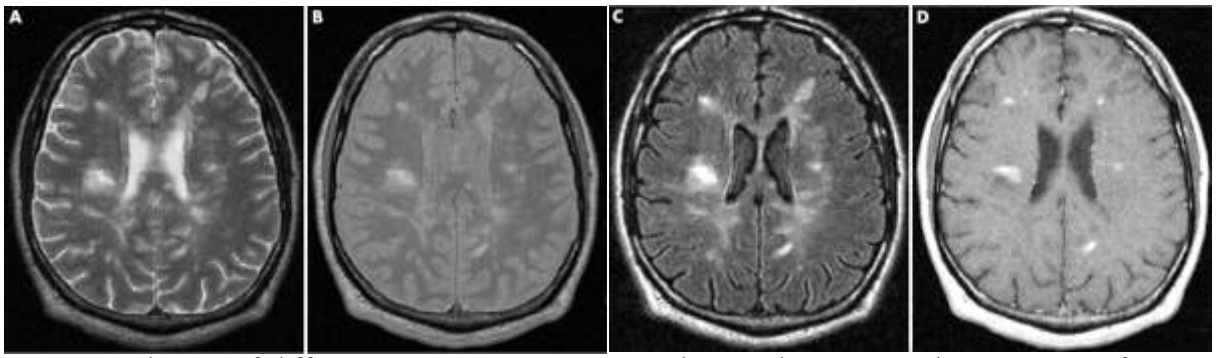



**Figure 2 | The use of different MRI sequences to visualize MS lesions. Axial MRI images of an MS patient with RRMS. (A)** T2-weighted image, **(B)** proton density weighted image, **(C)** FLAIR image. **(D)** Gadolinium-enhanced T1-weighted image. Reproduced from (Trip & Miller, 2005) with permission from BMJ Publishing Group Ltd.

The duration, availability and costs related to an MRI limit its feasibility for frequent monitoring. Obtaining CSF through lumbar puncture for OCB testing is invasive and can be an uncomfortable procedure. Consequently, researchers have sought to identify alternative, less invasive biomarkers in the blood. Numerous studies have explored potential biomarkers, including antibodies, cytokines, chemokines, and complement system proteins, all of which are involved in the processes of damage and repair in MS (Deisenhammer et al., 2019). While a definitive diagnostic biomarker for MS has yet to be identified, neurofilament light (NfL) has garnered considerable attention as a monitoring biomarker. It is important to note that NfL is not a diagnostic biomarker, as elevated levels of NfL reflect any type of neuronal damage and are not specific to MS. Rather, NfL is emerging as a biomarker for monitoring disease activity and progression once the diagnosis has been made. Its potential clinical utility lies in providing valuable insights into prognosis and therapeutic efficacy in MS (Ferreira-Atuesta et al., 2021).

Finally, the diagnostic process involves ruling out other potential alternative diagnoses, an approach known as differential diagnosis. This is essential as several other disorders can present with syndromes that overlap with MS. For example, conditions like myelin oligodendrocyte glycoprotein (MOG) antibody-associated disease (MOGAD) and aquaporin-4 antibody-positive neuromyelitis optica spectrum disorder (NMOSD) can mimic MS symptoms and should be excluded during the evaluation (Miller et al., 2008; Solomon et al., 2023). For this purpose, serological tests and assessment of lesion location can be used to differentiate between diseases (Cortese et al., 2023; Hyun et al., 2019; Patel et al., 2022).

To improve the accuracy of MS diagnoses, it is crucial to deepen our understanding of the disease's pathology and its potential mimicking conditions. By leveraging advancing diagnostic tools and adopting consensus approaches, medical professionals can better

differentiate and diagnose patients with MS, ensuring they receive appropriate care and management.

### I.1.4. Pathogenesis

MS is an inflammatory CNS disorder marked by demyelination and neurodegeneration. As previously discussed, both its clinical trajectory and symptoms exhibit heterogeneity, paralleling the diversity of its underlying pathology (Lassmann et al., 2001). Given the elusive aetiology of MS and the often-obscure onset of the disease, unravelling the precise sequence of events has proven to be a formidable challenge. Many of the involved cells and mechanisms display interdependence, potentially perpetuating themselves and each other.

MS is thought to start before the first clinical manifestation. Most patients presenting with CIS already show pre-existing, quiescent lesions evident in their MRI scans. This occurrence can be categorized as a radiologically isolated syndrome (RIS) or asymptomatic MS, often detected as an incidental MRI finding. Lesions can be found both in the brain and spinal cord, often characterized by the presence of a central vein. They typically occur in the periventricular and juxtacortical white matter, as well as distributed along the spinal cord (Fig. 3) (Matthias Bussas et al., 2022). Furthermore, the use of FLAIR MRI allows for the identification of grey matter lesions, present in cerebral and cerebellar cortex, as well as spinal cord grey matter (Pretorius & Quaghebeur, 2003). Interestingly, although MS has classically been seen as a disease of the white matter, it became apparent most of the pathology in progressive cases can be found within the grey matter (C. P. Gilmore et al., 2009).

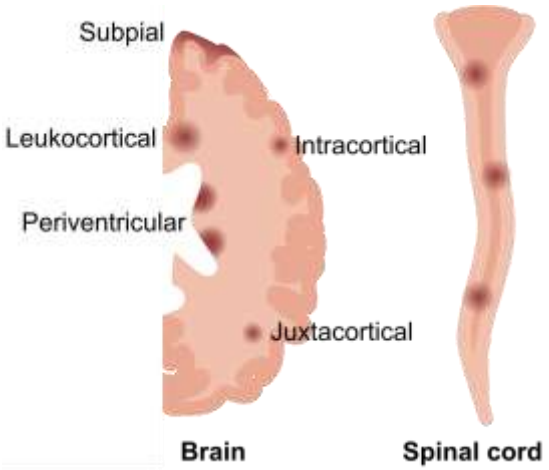



**Figure 3 | Typical locations of MS lesions.** Red foci depict inflammatory demyelinating lesions, forming in the brain and spinal cord of MS patients. Lesions can be found in both the grey and white matter. Figure created by Adinda Wens.

#### *I.1.4.1. Immune Cells in MS Lesions*

Over time, MS lesions may undergo changes in size and activity. Initial active lesions can persist in a chronically active state or transition into an inactive state. The terms "active" and "inactive" refer to the cellular composition of the lesion. Lesions are characterized by demyelination and the presence of immune cells which can vary over time (Stadelmann et

al., 2011). The trigger prompting the infiltration of immune cells remains unidentified. Two complementary paradigms, termed as "outside-in" and "inside-out", are leading the discussions surrounding origins of MS. The outside-in paradigm poses an initial autoimmune attack where immune cells infiltrate the CNS and target myelin, whereas the inside-out theory proposes a primary CNS cytodegenerative process that subsequently triggers a secondary autoimmune reaction (Stys et al., 2012). Which of the two theories more accurately reflects the initial pathological changes in MS is debated and will be discussed more in-depth in chapter I.1.4.2. “Inflammation or Degeneration: what comes first?”.

The elicited immune reaction involves peripheral activation of CD4+ and CD8 + T-cells, presumably against myelin epitopes, which cross into the CNS through a permeable blood-brain-barrier (BBB) (Wekerle et al., 1994). Once within the CNS, these T-cells undergo local activation by antigen-presenting cells (APCs), initiating tissue damage, secretion of cytokines, and ultimately resulting in demyelination and axonal damage. Additionally, B cells and plasma cells are recruited, accumulating perivascularly, while infiltrating macrophages and monocytes are present within and surrounding the lesion parenchyma (Lassmann, 2018b). This composition is indicative of an active lesion with ongoing inflammation in its core (Fig. 4, left).

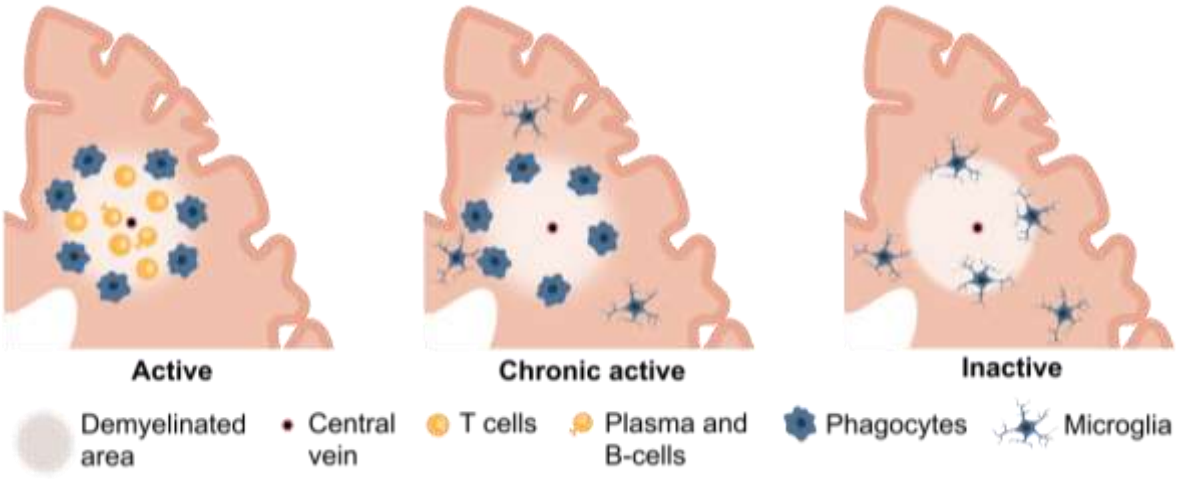



**Figure 4 | Cellular composition of MS lesions. (Left)** Active lesions have a demyelinated centre with B- and plasma cells surrounding the central vein, and activated T cells as well as infiltrating macrophages and monocytes within and surrounding the lesion parenchyma. **(Middle)** Chronic active lesions are characterized by an acellular demyelinated core and a rim lined with phagocytosing microglia. **(Right)** Inactive lesions are areas of demyelination with reactive gliosis and scar tissue, mainly devoid of cells, and probable remyelination. Figure created by Adinda Wens.

Simultaneously, it is postulated that a parallel process unfolds. Lymphocytes gather in the meninges and perivascular spaces of the CNS, potentially forming structures reminiscent of B-cell follicles. These structures are associated with the formation of subpial lesions in the cerebral and cerebellar cortex. Throughout progressive disease stages, these structures contribute to the gradual expansion of diffuse inflammation, where T-cells become diffusely distributed within the parenchyma. This phenomenon is believed to underlie neurodegeneration in areas of normal-appearing grey and white matter (NAGM, NAWM), areas with no apparent demyelination, in progressive stages. Notably, in these cases, active demyelination and diffuse tissue injury occurs at a distance from the lymphocytic infiltrates, suggesting the potential propagation of these processes through a soluble demyelinating or neurotoxic factor (Lassmann, 2018b).

Chronic active lesions feature a demyelinated core with decreased axonal density, which is surrounded by a rim of phagocytosing microglial cells, which is responsible for the expanding myelin breakdown at the edge (Fig. 4, middle). Inactive lesions are sharply demarcated areas of demyelination with reactive gliosis and scar tissue, largely devoid of immune cells (Fig. 4, right) (Lassmann, 2018a). These inactive lesions can turn into so-called shadow plaques, wherein partial remyelination by surviving oligodendrocytes or newly differentiated oligodendrocyte precursor cells (OPCs) takes place (Mezydlo et al., 2023). The degree of remyelination is variable between patients, with some individuals showing no sign of remyelination whereas others almost fully remyelinate (95% of global lesion area) (Patrikios et al., 2006).

### *I.1.4.2. Inflammation or Degeneration: What comes first?*

Two opposing paradigms are proposed to reason the underlying mechanisms of the initial MS trigger(s): the "inside-out" and "outside-in" models, which respectively suggest neurodegenerative and neuroinflammatory triggers for MS.

The prevailing notion suggests that the inflammatory demyelination typifies the initial phases of MS, accompanied by concurrent axonal and neuronal loss that contributes to long-term physical and cognitive impairment. Current most effective therapies target the adaptive immune system and show significant efficacy during the relapsing-remitting phases of the disease. However, their impact diminishes in the progressive stages, where neurodegenerative processes become more prominent (see also chapter I.1.5. Available and Emerging Treatments). Most genetic variations linked to MS are associated with immune functions, particularly HLA class II alleles, which are believed to drive greatest disease risk (Consortium*† et al., 2019). As discussed earlier, auto-reactive T-cells cross the permeable

BBB, culminating in the destruction of myelin and axons (Wekerle et al., 1994). Experimental autoimmune encephalomyelitis (EAE), an animal model of MS, has highlighted that T-cells reactive to myelin antigens primarily localize to white matter lesions. Intriguingly, T-cells targeting the neuronal protein β-synuclein appear to drive grey matter inflammation in a cortical EAE model. These T-cells are in close proximity to neurons, instigating glial activation, leading to synaptic spine reduction and apoptotic neurons. Given the presence of auto-reactive T-cells with similar targets in the CSF of humans with MS, it is conceivable that they may possess analogous functions and exert comparable effects (Lodygin et al., 2019).

Inflammation can provoke a secondary degeneration of CNS-resident cells, like oligodendrocytes and neurons. This degeneration may arise from phagocytosis by macrophages/microglia or due to cell death following exposure to a toxic environment. In particular, demyelination in the cortex seems to happen distant from inflammatory hubs, (Magliozzi et al., 2007). This raises the hypothesis that an array of soluble secreted factors could be at the base of this degeneration. The release of cytokines produced by immune cells, such as GM-CSF, TNF-α, IFN-γ, IL-2, CXCR4 and VLA4, but also accumulation of nitric oxide (NO) and glutamate may trigger neuronal decline and perpetuate microglial migration and activation, thereby sustaining tissue injury (Galli et al., 2019). Although microglia are initially considered to have protective functions, persistent activity might shift them to a disease-promoting role, becoming the source of aforementioned excessive glutamate and NO in the CNS, and the cause of diffuse axonal pathology in NAWM (Kamma et al., 2022; Kutzelnigg et al., 2005; Yong, 2022). Additionally, microglia can directly drive neuronal pathology, for example by phagocytosing synapses (Jafari et al., 2021). Another piece of compelling evidence highlighting the involvement of soluble factors is the ability of IgG immunoglobulins isolated from the CSF of PPMS patients to induce motor disability and spinal cord pathology in mice (Wong et al., 2023).

In individuals with RIS, MRI reveals cortical and overall brain volume loss comparable to that seen in RRMS patients. The prevalence of cognitive impairment in RIS mirrors that of RRMS, implying early neurodegeneration (Amato et al., 2012). Supporting this hypothesis, spinal cord atrophy is evident at CIS, and worsens during disease progression, but is not correlated to lesion number or volume. This suggests early neurodegeneration independent of focal inflammation, as it does not correlate with increasing lesion burden (Matthias Bussas et al., 2022). Remarkably, the biomarker NfL, indicative of neuronal degeneration, can be detected up to 6 years before clinical onset, signalling early neuronal loss, consistent with RIS (Bjornevik et al., 2020). Pathological examination of initial disease stages reveals inner-

myelin sheath impairment, while the outer myelin wraps remain intact, challenging the notion of immune cells attacking from the outside (Rodriguez & Scheithauer, 1994). Such impaired myelin and swollen axons have been found in NAWM, without surrounding inflammation (Luchicchi et al., 2021). Areas of speculated initial demyelination show minimal T-and B-cell presence, but pronounced oligodendrocyte loss (Barnett & Prineas, 2004; Prineas & Parratt, 2012). This might suggest that peripheral lymphocyte infiltration only occurs after the demyelinating process has begun (Henderson et al., 2009). Furthermore, inducing EAE in antigen-experienced marmoset monkeys requires the exposure of myelin antigens without additional immunological danger signals, usually required for the induction of EAE in mice (Jagessar et al., 2010). Immunomodulatory drugs and hematopoietic stem cell transplantations, effective in modulating or resetting inflammatory activity during RRMS, can delay but do not mitigate progressive stages with neurodegeneration (Bergamaschi et al., 2016). These treatments cannot avert progression to progressive MS, illustrating that at least inhibiting peripheral inflammation alone is insufficient to prevent oligodendrocyte and neuronal degeneration. These findings point towards a possibly primary and immune-independent cytodegeneration in MS.

In summary, both paradigms offer compelling arguments, yet the initial trigger for MS remains enigmatic. It is evident that inflammation and cytodegeneration can mutually influence each other. Once the disease is established, these processes can amplify and perpetuate each other.

### *I.1.4.3. Pathology of Grey and White Matter Degeneration*

Grey matter is distinct from white matter: it predominantly consists of cell bodies and relatively few/sparsely myelinated axons, whereas the white matter comprises primarily myelinated axons with fewer cell bodies. As MS lesions can develop both within the grey and white matter, they could exert different effects on the local cellular structures. The pathological changes are not limited to lesions, but also extend diffusely within the parenchyma of the CNS.

#### White Matter Pathology

During the early stages of disease, focal demyelinating lesions often form surrounding a central vein within the white matter. These lesions exhibit myelin and axon loss in presence of infiltrating immune cells (Lassmann, 2018a). Over a span of 30 years of disease progression, the axonal density in the spinal cord's white matter is estimated to be reduced by 60% (Petrova et al., 2018). This inflammatory loss of axonal connections could lead to a retrograde secondary degeneration of neurons, initiating the neurodegenerative processes

associated with progressive disease courses (M. Bussas et al., 2022; Stys, 2004). The relationship between demyelination and axonal loss is a subject of debate. Some studies suggest a dependency between myelin and axonal damage, while others propose that axonal injury can occur independently of demyelination (Dziedzic et al., 2010; Kornek et al., 2000; Rahmanzadeh et al., 2021; Schäffner et al., 2023).

In the EAE mouse model, axonal damage is observed in the form of focal swellings (focal axonal degeneration, FAD), which can eventually lead to axon fragmentation. Interestingly, some swollen axons recover spontaneously, indicating a reversible process. Similar pathological changes have been observed in MS tissue, making axons a compelling therapeutic target for progressive disease. Several mechanisms have been implicated in the induction of immune-mediated axonal damage: (i) FAD can occur independently of demyelination but seem to be triggered by preceding mitochondrial dysfunction (Buonvicino et al., 2023; Nikić et al., 2011; Tai et al., 2023). Inflammatory lesions in MS show an increased number of mitochondria in demyelinated axons. In mouse models, demyelination induces the recruitment of mitochondria from the neuronal soma, initially serving a protective role (Licht-Mayer et al., 2020). However, over time, the accumulation of unhealthy mitochondria within demyelinated axons seems detrimental, leading to axonal degeneration (Joshi et al., 2015). Whether mitochondrial recruitment and functional changes are compensatory or pathological is debated. (ii) Another potential mechanism involves the role of calcium buffering in axonal integrity. Studies in EAE have demonstrated that overexpression of the PPargc1a gene, a master regulator of mitochondrial activity, improves mitochondrial function, thereby enhancing neuronal calcium-buffering capacity and promoting neuronal survival. Effective calcium regulation within axons is crucial in determining the fate of FAD, dictating whether the axon fragments or recovers. (Rosenkranz et al., 2021). (iii) Disrupted ion channel activity and altered distribution in MS axons have also been linked to calcium accumulation. Voltage-gated calcium and glutamate channels are suggested mediators in this process, contributing to increased intracellular calcium levels, which can exacerbate axonal damage. (Correale et al., 2020; Kornek et al., 2001; Tisell et al., 2013). (iv) Finally, calcium influx may also result from extracellular accumulation due to nanoruptures in the axonal membrane. These small tears could allow calcium to enter the axon, further disrupting homeostasis and promoting degeneration (Witte et al., 2019). While each of these mechanisms has been implicated in axonal injury, their relative contribution may vary depending on the stage of lesion development. Further research is needed to clarify the precise roles of these processes in progressive MS.

## Grey Matter Pathology

Grey matter pathology in MS is characterized by demyelination, synaptic damage, and irreversible neurodegeneration. While some of the grey matter damage is secondary to white matter pathology, it is evident that grey matter can undergo primary damage independent of white matter changes (Bö et al., 2007; Kutzelnigg et al., 2005). For instance, a widespread spine loss has been observed independent of demyelination and axon loss, suggesting a primary synaptic pathology in MS (Jürgens et al., 2015). The presence of grey matter lesions at disease onset suggests a poorer prognosis. Disability and cognitive decline are strongest correlated with grey matter atrophy. A higher number of cortical lesions is associated with a greater risk of early conversion compared to fewer cortical lesions (Filippi et al., 2013; Fisniku et al., 2008; Pravatà et al., 2017; Scalfari et al., 2018; Schlaeger et al., 2014). With disease progression, grey matter lesions become more prevalent, eventually surpassing white matter lesions in progressive stages (C. P. Gilmore et al., 2009).

In the cortex, lesions are categorized by their location: (i) intracortical lesions are confined to the cortex, (ii) leukocortical lesions contact both grey and white matter, and (iii) subpial lesions extend from the pial surface, being the most common type of cortical lesion (see Fig. 3). Subpial lesions are strongly associated with meningeal inflammation, leading to a gradient of neuronal loss in both demyelinated and NAGM (Lucchinetti et al., 2011). As cortical lesions are not associated with increased lymphocyte infiltration, demonstrated by the lack of MRI enhancing lesions, it is proposed that neuronal loss could be mediated by cytotoxic factors, like interferon gamma (IFN-$\gamma$) and tumour necrosis factor alpha (TNF-$\alpha$). These factors diffuse from the meningeal compartment into the brain parenchyma or can be produced locally by activated microglia and infiltrated lymphocytes (Bø et al., 2003; Kutzelnigg et al., 2005; Lodygin et al., 2019; Magliozzi et al., 2010; Sastre-Garriga et al., 2005). Additionally, neuronal and axonal loss is comparable in subpial lesions and NAGM (~30%) suggesting that neurodegeneration might be independent of demyelination, further supporting the hypothesis of diffuse soluble players (Klaver et al., 2015). Other than cytokines, it has been shown that glutamate levels are elevated in CSF of MS patients, and glutamate neurotransmission and homeostasis are impaired, suggesting a role for glutamate excitotoxicity (Dutta et al., 2011; Sarchielli et al., 2003; Srinivasan et al., 2005). Calcium influx through dysregulated neuronal glutamate receptors can lead to elevated calcium levels, causing mitochondrial dysfunction and ultimately neuronal degeneration (Campbell et al., 2011). Concurrently, ongoing synapse removal occurs in the cortex, hippocampus and spinal cord (Dutta et al., 2011; Jürgens et al., 2015; Petrova et al., 2020). A previous study in the EAE model demonstrated that microglia remove synapses with

elevated calcium levels (Jafari et al., 2021). Moreover, microglia have been observed in close proximity to apoptotic neurons and areas of neuritic beading (Peterson et al., 2001). Dendritic and synaptic pathology in neurological diseases, including MS, will be discussed in further detail in the following chapter (I.2. Dendritic pathology).

### I.1.5. Available and Emerging Treatments

To this date, there is no cure for MS. Most MS therapies aim to increase quality of life, managing symptoms and improving everyday life, striving to a status known as "freedom from disease". The concepts of "no evidence of disease activity" (NEDA) and "no evidence of progression or active disease" (NEPAD) are a set of goals, adopted from cancer and rheumatoid arthritis treatment assessment, which were recently introduced to evaluate treatment efficacy in clinical trials and therapy (Giovannoni, 2018; Lorefice et al., 2023; Wolinsky et al., 2018). The endpoint measures include:

- No relapses (NEDA-3, NEDA-4, NEPAD)
- No increase in disease disability measured by EDSS (NEDA-3, NEDA-4, NEPAD)
- No new or active MRI lesions (NEDA-3, NEDA-4, NEPAD)
- No or slowing down of brain atrophy (NEDA-4, NEPAD)
- No worsening in the performance of upper and lower extremity motor tests (NEPAD)

Nowadays, many disease modifying therapies (DMTs) are available for the relapsing remitting phase characterized by ongoing inflammation. These drugs primarily target the adaptive immune system through depletion of effector T cells, enhancement of regulatory T cells, B cell suppression, inhibition of cytokine secretion, and blocking immune cell trafficking into the nervous system (Table 1). However, most drugs for RRMS are generally not effective in treating progressive disease stages as they do not address the degenerative elements underlying progression.

Recently, the first drug has been approved for treatment of PPMS: Ocrelizumab. The results from a phase III study demonstrated that participants receiving Ocrelizumab exhibited a 24% lower likelihood of experiencing disease progression compared to the placebo group. Surprisingly, Ocrelizumab, an anti-CD20 monoclonal antibody, functions by targeting and eliminating B-cells, further expanding the array of drugs aimed at the adaptive immune system (Montalban et al., 2016). As the need for medications that slow down or halt progression is still unmet, researchers are exploring therapeutic options targeting innate immune responses.

**Disease-Modifying Therapies for Multiple Sclerosis available in Germany**

| Drug | Target | Mechanism of Action |
|---|---|---|
| High efficacy | | |
| Ocrelizumab | Anti-CD20 | Depletion of CD20+ B cells |
| Ofatumumab | | |
| Natalizumab | Anti-CD49d (VLA-4) | Inhibition of migration T and B cells |
| Alemtuzumab | Anti-CD52 | Depletion of T and B cells |
| Good efficacy | | |
| Cladribine (Oral) | Adenosine deaminase inhibitor | Depletion of lymphocytes, ↓of T cells, transient ↓of B cells |
| Dimethyl fumarate | Nrf2 pathway | ↓ oxidative stress, inhibition inflammatory products |
| Diroximel fumarate | | |
| Fingolimod | S1P receptor agonists | Inhibition of lymphocyte migration |
| Ozanimod | | |
| Ponesimod | | |
| Siponimod | | |
| Moderate efficacy | | |
| IFN-β 1a | Type I IFN receptor agonists | ↓ Proinflammatory cytokines, ↑ anti-inflammatory cytokines, Inhibition T cell division, ↑ Tregs, ↓ BBB migration |
| IFN-β 1b | | |
| Peginterferonβ-1a | | |
| Glatiramer acetate | MBP analogue | Competition binding to MHC, ↑ Tregs |
| Teriflunomide | Dihydroorotate dehydrogenase inhibitor | ↓ T- and B-cell activation and proliferation, ↓ response to autoantigens |
| Off-label DMTs | | |
| Azathioprine | Purine analogue | T cell apoptosis |
| Cladibrine (subcutaneous or IV) | Adenosine deaminase inhibitor | Depletion of lymphocytes, ↓ T cells, transient ↓ B cells |
| Mitoxantrone | DNA repair | Inhibition of lymphocyte and monocyte migration, B cell function, secretion of TNF-α, IL-2 and IFN-γ |
| Methotrexate | General immunosuppressant | Impairs T and B cell activity |
| Cyclophosphamide | Alkylating metabolite | ↓ T and B cells |
| IV Immunoglobulin | Immune modulation | Idiotypic regulation of B cells |
| Rituximab | Anti-CD20 | Depletion of CD20+ B cells |

**Table 1 | Disease modifying therapies to treat MS in Germany.** Adapted from (Baecher-Allan et al., 2018).

A promising category of emerging drugs encompasses Bruton's tyrosine kinase (BTK) inhibitors. BTK is a signalling molecule, selectively important in B-cells, macrophages, and microglia, thereby addressing both the adaptive and innate immune mechanisms. Preclinical studies have highlighted that BTK inhibitors can inhibit B-cell activation, prevent lymphocyte infiltration, and suppress meningeal inflammation, microglial activation and demyelination. Currently, several BTK inhibitors are undergoing phase III clinical trials in patients with RRMS and progressive MS (Krämer et al., 2023).

Moreover, Ibudilast and Simvastatin are being evaluated for their potential in treating progressive MS. So far, they have shown a capacity to decrease the rate of brain atrophy, suggesting a potential neuroprotective effect. However, the precise mechanisms underlying their neuronal protective properties remain incompletely understood (Fox et al., 2018; Kmietowicz, 2014).

Lastly, a group of drugs proposed for promoting myelin repair, is currently undergoing evaluation in various phase II studies ("Assessment of Clemastine Fumarate as a Remyelinating Agent in Acute Optic Neuritis (ReCOVER)," ; "CCMR Two: A Phase IIa, Randomised, Double-blind, Placebo-controlled Trial of the Ability of the Combination of Metformin and Clemastine to Promote Remyelination in People With Relapsing-remitting Multiple Sclerosis Already on Disease-modifying Therapy," ; "Metformin Treatment in Progressive Multiple Sclerosis,"). A compound of particular interest, Clemastine, has demonstrated improvement in visual evoked potential latency among patients with RRMS, indicative of enhanced axonal myelination (Green et al., 2017). A follow-up study on these patients showed, for the first time, imaging-based evidence of myelin repair in humans, particularly within the corpus callosum (Caverzasi et al., 2023). Animal studies have s a synergistic effect through the combined administration of Clemastine and Metformin, a strategy currently being tested in a phase II trial ("CCMR Two: A Phase IIa, Randomised, Double-blind, Placebo-controlled Trial of the Ability of the Combination of Metformin and Clemastine to Promote Remyelination in People With Relapsing-remitting Multiple Sclerosis Already on Disease-modifying Therapy," ; Mezydlo et al., 2023).

### I.1.6. EAE as an Animal Model of MS

Numerous animal models of MS have been developed to dissect the pathological mechanisms implicated in MS. Employing animal models allows for enhancing our understanding of mechanisms implicated in disease initiation and progression, but also guide the development of therapeutic agents and strategies. The notable achievements of translating discoveries from animal models into clinical applications are exemplified by the

success stories of disease-modifying therapies (DMTs) such as IFN-β and Natalizumab (Abreu, 1982; Yednock et al., 1992).

Animals do not naturally develop MS, therefore, any pathology is artificially induced within a laboratory setting (Gran et al., 2007). Although no single animal model can fully emulate the intricate complexity of MS, these models can replicate certain disease stages or mechanisms of the disease. For that reason, it is important to select the fitting animal model for the research question at hand.

EAE is the most studied inflammatory neurodegenerative model, and can be induced in a panel of laboratory animals such as zebrafish, rabbits, and marmosets, however, most commonly studied are mice and rats (Brenner et al., 1985; Kulkarni et al., 2017; Liu et al., 1998; Massacesi et al., 1995). The EAE model mirrors the "outside-in" model, replicating the neuropathological manifestations driven by autoimmune processes. Importantly, it captures the essential attributes of MS pathology: inflammation, demyelination and neurodegeneration (Constantinescu et al., 2011; Martin et al., 1992). EAE can be induced either actively or passively. Active induction of EAE involves immunization with myelin peptides, such as myelin basic protein (MBP), MOG or proteolipid protein (PLP), along with a sensitizing adjuvant, often a bacterial component known to activate the innate immune system, like complete Freund's adjuvant (CFA) (Libbey et al., 2010). The addition of pertussis toxin (PTx) for permeabilizing the BBB further increases induction efficiency (Arimoto et al., 2000). In passive EAE, activated T-cells from an animal with active EAE, which are autoreactive against myelin self-antigens, are transferred into the recipient animal. These transferred cells initiate the inflammatory-driven demyelination within the recipient (Stromnes & Goverman, 2006). Beyond these acutely disease inducing methods, researchers have also engineered transgenic animals that spontaneously develop EAE by genetically manipulating T and B cell receptors (TCR, BCR) to react against myelin peptides. These models have proven particularly valuable in elucidating the roles and mechanisms of autoreactive T cells during the initial stages of the disease (Bettelli et al., 2003; Litzenburger et al., 1998).

Even the different disease courses seen in MS patients can be replicated in animals with EAE. By altering the mouse strain or the specific myelin peptide used for inducing inflammation, researchers can influence the type of disease manifested in the animal model. For instance, when MOG is administered to C57BL/6 mice, a monophasic or chronic form of EAE is elicited, while induction of EAE in SJL/J mice with PLP results in a relapsing-remitting disease course. In both of these models, initial symptoms typically emerge approximately 10 days after immunization, peaking after 2-3 days after disease-onset. Eventually, the disease

course transitions into a phase of remission or sustained chronicity (Day, 2005; Mattner et al., 2013; Vanderlugt et al., 2000). The pathological processes observed in EAE likely parallel those seen in MS. Autoreactive T cells migrate into the CNS and get re-activated by myelin-presenting APCs (Kawakami et al., 2004). Subsequently, these T cells release cytokines, triggering the recruitment of additional lymphocytes and the activation of microglia, ultimately leading to demyelination and loss of oligodendrocytes and neurons (Glatigny & Bettelli, 2018). The lesions in EAE predominantly emerge disseminated along the spinal cord (Lassmann & Bradl, 2017). This observation prompted the development of models in which (i) lesions are intentionally targeted to an area of interest within the spinal cord, and subsequently in which (ii) lesions are induced within the cortex through stereotaxic injection of IFN-$\gamma$ and TNF-$\alpha$ in MOG-sensitized animals (Jafari et al., 2021; Kerschensteiner et al., 2004; Merkler et al., 2006).

While EAE models have significantly advanced our understanding of MS pathogenesis and have facilitated the discovery of drug targets, it is important to acknowledge their limitations. No animal model can fully recapitulate the complexity of MS in humans. However, when chosen carefully and applied appropriately, EAE and other models remain indispensable tools in MS research, providing valuable insights into disease mechanisms, progression, and potential therapeutic interventions. As research continues, these models will be crucial in driving forward our understanding of MS and informing the next generation of treatments

## I.2. Dendritic Pathology

### I.2.1. Neuronal Dendrites

Neurons come in a great variety of shapes and sizes. They have rootlike extensions called dendrites, which serve as specialized post-synaptic structures responsible for receiving and processing incoming signals from other neurons. These branching neurites collectively form complex dendritic trees, which can take on various forms ranging from relatively simple to intricately elaborate patterns (Fig. 5). The extension of dendrites from the neuronal soma offers an expanded surface area without significantly increasing the cell's volume, thereby enhancing the potential for establishing numerous connections and receiving a multitude of inputs. The neuronal architecture achieved by these branching trees enables networks of neurons to perform specialized cognitive and motoric functions. The formation of these complex trees and their features is far from trivial. Aspects such as dendrite diameter, distance from the soma, and the number of branchpoints that signals must cross to reach the soma all contribute to a neuron's function by influencing the likelihood that an excitatory postsynaptic potential (EPSP) generated at a specific synapse will contribute to the neuron's firing activity.

The formation of dendritic trees initiates during the perinatal stages of development after postmitotic neurons have migrated to their final destinations and the initial extension of their axon (Redmond et al., 2002; Rosário et al., 2012; Zolessi et al., 2006). In human development, the childhood period is characterized by substantial structural refinement within dendritic arbours, a critical process for establishing functional and adaptable synaptic circuits. This dynamic process involves the continual growth and extension of dendrites during the initial two years of life. Subsequently, dendrites undergo a simultaneous and continuous process of extension, retraction, and pruning, allowing dendrites to explore potential synaptic partners, until they reach maturity during adolescence (Forrest et al., 2018; Jan & Jan, 2010).

The development of dendritic trees is orchestrated by a combination of intrinsic and extracellular cues, including autolytic stimuli and extracellular gradients (Horton et al., 2006; Polleux et al., 2000). This process of dendrite morphogenesis involves an interplay of various molecules. These molecules encompass transcription regulators, secreted proteins and cell surface receptors, cytoskeletal regulators, motor proteins, secretory, and endocytic pathway proteins, cell adhesion proteins, and proteins associated with the mammalian target of rapamycin (mTOR) pathway (Jan & Jan, 2010).

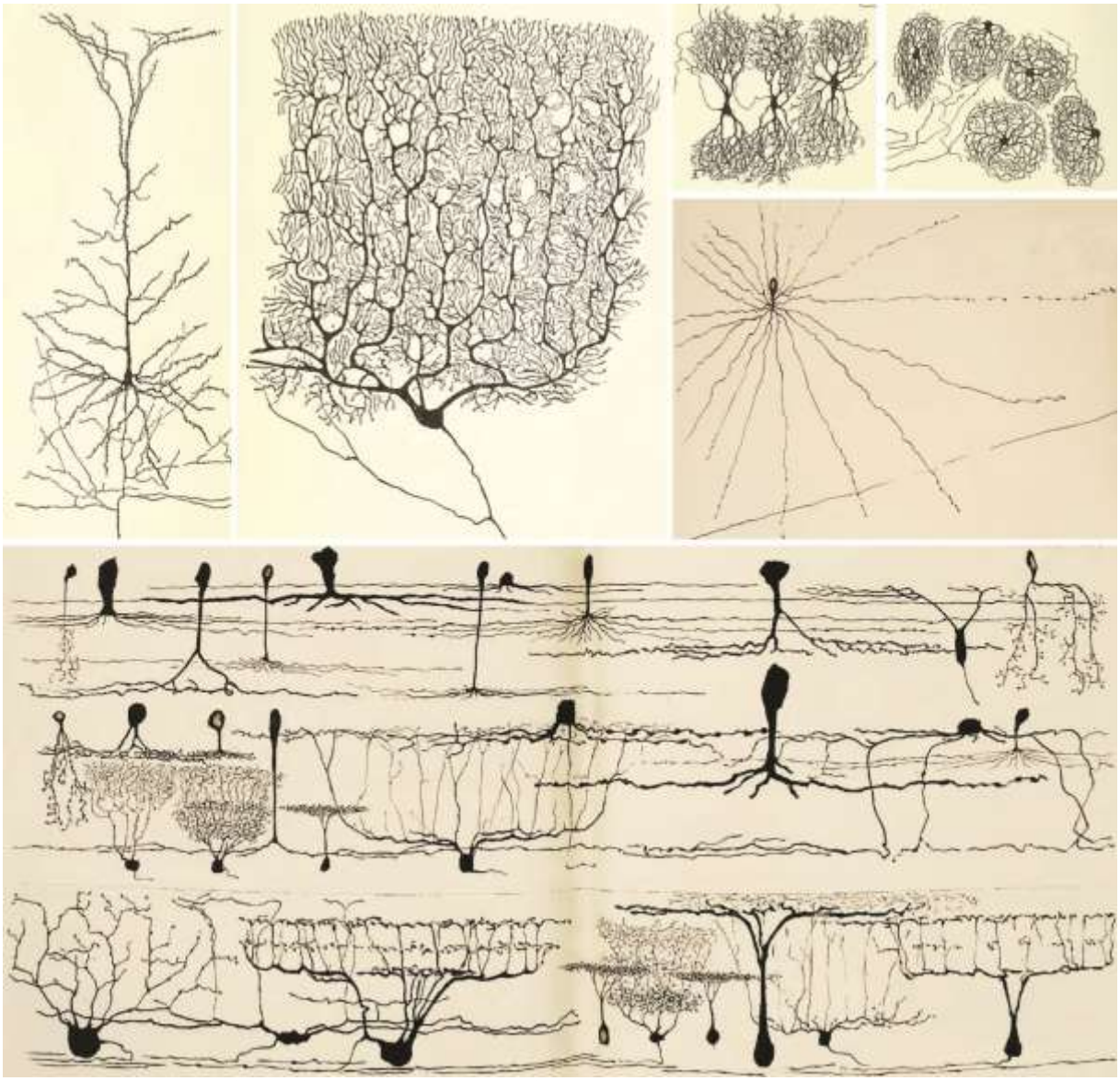


**Figure 5 | Variety in the architecture of neuronal dendritic trees.** Drawings from Ramón y Cajal. Used with permission from (Lefebvre et al., 2015). Permission conveyed through Copyright Clearance Center, Inc.

Dendritic arbours are highly dynamic structures. Their branching is linked to the growth, breakdown and reorganization of the cytoskeleton. The cytoskeletal framework of dendrites primarily comprises (i) microtubules bridged by microtubule-associated proteins (MAPs), which are necessary for intracellular transport, and (ii) actin filaments, crucial for rapid shape changes (Hlushchenko et al., 2016; Kapitein et al., 2010). Additionally, (iii) neurofilaments can also be found in dendrites, although they are more abundant in axons (Yuan et al., 2012). The reorganization required for dendrite ramification involves microtubule dynamics where actin filaments undergo growth and subsequent destabilization. During this process, transient membranous protrusions called filopodia extend. Eventually, microtubules infiltrate these filopodia, thereby stabilizing the developing dendrite. The establishment of these microtubule networks serves as a

transport route, facilitating the import and export of various intracellular organelles essential for the constant remodelling and maintenance of dendritic structures.

Dendrites house subcellular organelles that provide resources at a distance from the cell soma (Fig. 6). In the proximity of the soma, dendrites contain smooth and rough endoplasmic reticulum (SER, RER), Golgi apparatus, and mitochondria. More distally, mitochondria can still be found alongside Golgi outposts. The RER within dendrites allows for on-site protein translation, while the SER provides them with posttranslational modifications and contributes to calcium regulation. Both the Golgi apparatus and its outposts synthesize the plasma membrane and guide locally produced proteins to the plasma membrane (Sun et al., 2021). Mitochondria located within dendrites are highly mobile as they are required to localize to sites of energy demand, particularly areas of synaptogenesis or synapse enlargement (Li et al., 2004). These organelles are essential to dendritic growth and maintenance, ensuring an adequate supply of proteins and energy to support synaptic plasticity demands (Sun et al., 2021).

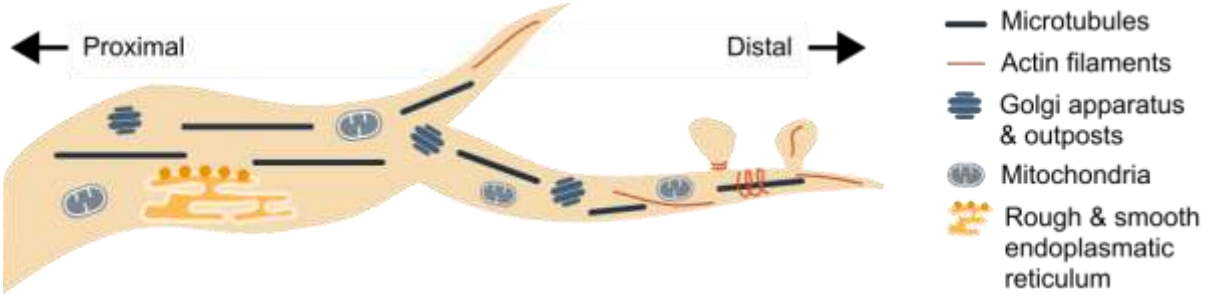



**Figure 6 | Main components of dendrites.** Dendrites are structurally supported by microtubules and actin filaments. They contain local Golgi apparati and outposts, rough and smooth endoplasmic reticula and mitochondria. Figure created by Adinda Wens.

Synaptic inputs can be received either directly on the dendritic shaft or in specialized compartments known as spines. Much like dendrites, spines are highly dynamic structures susceptible to sprouting and pruning. In rodents, the growth of dendrites and spines is closely intertwined during early development. As development progresses, dendritic branches become more stabilized, while spines retain their dynamism (Koleske, 2013). Notably, in mature neuronal progenitor cells that give rise to non-spiny neurons, the dendritic arbour retains its inherent dynamism for much longer (Mizrahi, 2007).

In addition to intrinsic signals and extracellular gradients, the development and maintenance of the dendritic arbour are influenced by synaptic activity (Niell et al., 2004). *In vivo* imaging of hippocampal slices revealed that high frequency synaptic stimulation induces enhanced growth of small filopodia like protrusions (Engert & Bonhoeffer, 1999). Research in zebrafish has shown that the establishment of a synapse on a filopodium

stabilizes it and can guide the formation of the dendritic tree (Niell et al., 2004). Conversely, the loss or absence of synaptic input can trigger the retraction of dendritic structures (Lohmann et al., 2002). The consequences of activity-dependent dendritic and spinal plasticity seem to be mediated by calcium, a known modulator of the dendritic cytoskeleton (Lohmann et al., 2002; Redmond & Ghosh, 2005). Synaptic calcium can originate from intracellular calcium stores (e.g. the ER), or the influx of calcium through calcium-permeable channels, including glutamate receptors such as NMDA and AMPA receptors, as well as voltage-gated calcium channels (Yuste et al., 2000). Especially AMPA and NMDA receptors play a central role in activity-dependent mechanisms that shape dendrites and spines. In the context of the visual system, neural activity promotes stabilization of more active dendrites, while pruning weaker ones, processes that rely on NMDA receptors and/or local rises in calcium (Engert et al., 2002; Lohmann et al., 2002). The phenomena of long-term potentiation and long-term depression (LTP, LTD) are two well-studied forms of synaptic plasticity that are activity-dependent. LTP and LTD are orchestrated through the neurotransmitter glutamate which binds to ionotropic receptors such as NMDA, AMPA and kainite receptors as well as metabotropic glutamate receptors. During LTP and LTD, the number of NMDA and AMPA receptors on the postsynaptic neuron in- or decreases, respectively, thereby strengthening or weakening the connection of the synapse, as quantified by the output EPSPs (Bliss & Collingridge, 1993).

### I.2.2. Dendrites in Disease

Dendrites have been implicated in a wide variety of diseases. Dendritic pathology can manifest itself in various structural and functional alterations. At a broader scale, dendritic trees may exhibit alterations, such as hyper- or hypo-branching, or faulty directionality and wiring (Takashima et al., 1981). Zooming in, we might observe instances where the dendritic tree is correctly established, but the overall length of dendritic branches is increased or decreased. Furthermore, there can be modifications in dendritic morphology, with dendrites taking on a beaded shape, potentially leading to fragmentation and degeneration (Greenwood et al., 2007). On the level of individual spines, differences in spine numbers, density, and shape can both cause or result from ageing or pathological brain alterations (Glausier & Lewis, 2013).

The disturbance of dendrites can disrupt neural communication and contribute to the pathophysiology of several neurological, psychiatric and developmental disorders. The nature and extend of these alterations are disease-specific, occurring at the level of the dendrite or their spines (Fig. 7). It is important to note that changes observed in these

disorders are distinct from the normal dendritic and synaptic adaptations that occur during development, learning and memory formation.

Changes in dendritic structure or function can arise from improper dendritic formation during development or can be inflicted later in life. Early-onset neurodevelopmental conditions like autism spectrum disorders (ASDs) and intellectual disabilities (IDs) are characterized by reduced dendritic branching and variable spine density (increased in ASDs, decreased in IDs, Fig. 7) (Hutsler & Zhang, 2010; Purpura, 1974; Raymond et al., 1996). Post-mortem brain analysis of ID patients shows remaining spines are long and thin, suggestive of immaturity and dysfunctional synaptic connectivity (Purpura, 1974). Furthermore, neuropsychiatric disorders such as schizophrenia and bipolar disorder, which typically manifest later in life, have been associated with distinct dendritic structural alterations, including simplified dendritic arbours and decreased spine numbers. These modifications in neuronal connectivity, stemming from compromised dendritic structure and function independent of neuronal or axonal loss, are believed to contribute to the cognitive difficulties experienced by affected individuals.

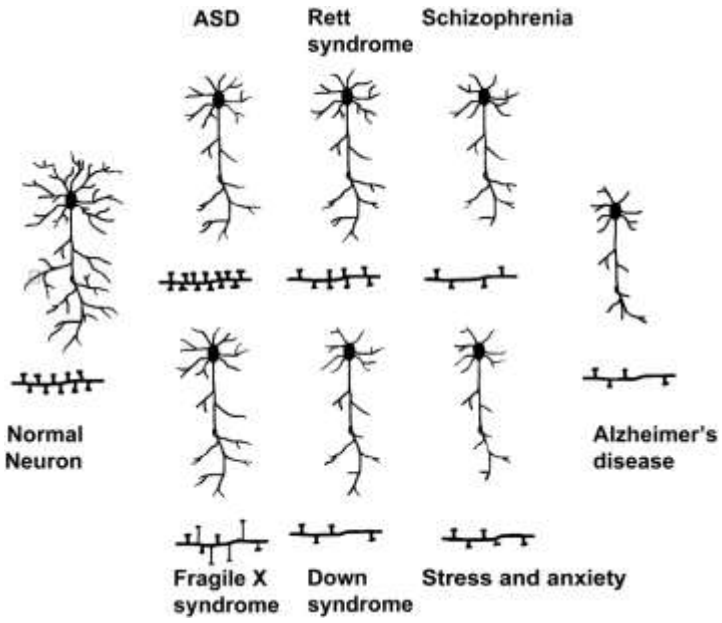



**Figure 7 | Dendrite and spine changes across various neurological disorders.** Reprinted from (Kulkarni & Firestein, 2012) with permission from Elsevier

Genome-wide association studies (GWAS) conducted across a spectrum of psychiatric disorders have identified shared risk loci, often linked to genes involved in neurogenesis, nervous system development and neuron differentiation. This supports the notion that these disorders have a neurodevelopmental basis. Pleiotropic risk loci have also demonstrated enrichment in genes linked to glutamate and calcium channel signalling, suggesting a shared role of these pathways in the pathogenesis of diverse neuropsychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium. Electronic address & Cross-Disorder Group of the Psychiatric Genomics, 2019). For instance, Grin2B, encoding a subunit of the NMDA receptor, has emerged as the strongest susceptibility candidate for developing ASDs (C Yuen et al., 2017). Likewise, Gria1 and Grin2A, responsible

for subunits of the AMPA and NMDA receptors respectively, have been identified as genome-wide significant susceptibility genes in the largest GWAS of schizophrenia (Ripke et al., 2014). Numerous mutations in ion channel genes have been uncovered in patients with neuropsychiatric conditions, and experimental validation has confirmed their capacity to induce the dendritic alterations observed in these diseases (Johnston et al., 2016). For example, a point-mutation within the gene encoding for Ca(V)1.2, a subunit of the L-type voltage-dependent calcium channel, is associated with ASD and has been shown to trigger activity-dependent dendritic retraction in rodent neurons and iPSC-derived neurons from patients carrying the mutation (Krey et al., 2013). These findings collectively suggest that the morphological and functional defects in dendrites or spines in neuropsychiatric disorders are the consequence of altered postsynaptic signalling pathways.

Neurodegenerative disorders are characterized by the progressive loss of neurons. Common neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD), share related pathological mechanisms involving the aggregation of misfolded proteins, ultimately resulting in neuronal loss (Ross & Poirier, 2004). Also, in these diseases, abnormal spine and dendrite morphology have been observed. In AD, a major correlate of cognitive decline is the loss of synapses (Terry et al., 1991). Dendrites exhibit dystrophic characteristics and reduced complexity. These changes are widespread and occur in the early stages of the disease (Cochran et al., 2014). The accumulation of amyloid beta peptides (Aβ) disrupts dendritic signalling by selective binding to various receptors and ion channels within dendrites. Importantly, the mislocalization of tau protein into dendrites is a prerequisite for Aβ-induced NMDAR-mediated excitotoxicity (Ittner et al., 2010). In PD, Golgi-impregnation studies have revealed a reduction in dendritic length, along with the loss of dendritic spines and formation of dendritic varicosities in the dopaminergic neurons of the substantia nigra, which are selectively degenerating in the condition (Patt et al., 1991). Research has also shown that the overexpression of α-synuclein, a hallmark of PD, leads to spine loss and the development of beaded dendrites in mice (Blumenstock et al., 2017). However, the direct connection between protein aggregation and dendritic dystrophy remains unclear, and it is debated whether this effect is acting directly on dendrites or results from retrograde degeneration due to damage in the cell body or axons. Beyond the dendritic changes associated with protein aggregation, emerging studies suggest that ion channel signalling plays a role in the degeneration of neurons in both AD and PD. These ion channels modulate dendritic function and firing properties, contributing to the overall neuronal degeneration observed in these disorders (Johnston et al., 2016).

Injury to dendrites and synapses is also observed in neuroinflammatory diseases. In HIV-associated dementia (HAD), neurological impairment is strongly associated with dendritic and synaptic pathology, rather than neuronal loss, and manifests itself as focal swellings, spine loss, and reduced complexity (Bellizzi et al., 2016).Also the neurodegenerative Niemann Pick type-C disease (NPC) is associated with chronic neuroinflammation and microglial activation (Cologna et al., 2014). In the mouse model of NPC, engulfment of Purkinje cell dendrites by microglia has been identified to precede the disease-typifying neurodegeneration (Kavetsky et al., 2019). A similar involvement of inflammation-mediated dendritic damage has also been noted in other neuroinflammatory conditions, such as ischemia and AD, where phagocyte-mediated engulfment of postsynaptic structures has been demonstrated (Hong et al., 2016; Shi et al., 2021). These findings help illustrate how neuroinflammatory conditions can contribute to dendritic pathology across different neurological diseases. The mechanisms of dendritic and spine pathology under neuroinflammatory conditions will be discussed in more detail in the following chapters.

### I.2.3. Dendritic Pathology in MS and EAE

As previously discussed, MS is characterized as an autoimmune inflammatory disorder which also exhibits key features of classical neurodegenerative diseases. Given what is known from other neurological conditions described above, it is unsurprising that dendritic pathology is also apparent in MS.

One of the earliest indications of dendritic pathology in MS came from a study examining cortical lesions in MS patients. In these lesions, researchers discovered transected neurites (dendrites and axons), often exhibiting terminal swellings Additionally, a significant reduction in dendritic branching density was observed in demyelinated cortical regions, reflecting a simplified dendritic structure. Interestingly, this structural simplification was also detected, though to a lesser extent, in the cortical NAGM (Jürgens et al., 2015). Concurrent with these dendritic alterations, substantial synaptic loss was documented within lesioned cortical areas of MS patients (Wegner et al., 2006). Further studies using higher-resolution techniques confirmed that, like dendrites, the reduction of dendritic spines is not only found in lesioned regions but also in the NAGM. These results indicate that dendrite and spine loss can occur independently of cortical demyelination or axonal injury (Jürgens et al., 2015). Beyond the cortex, synaptic loss has also been reported in the hippocampus, cerebellum, and spinal cord of individuals with MS (Albert et al., 2017; Dutta et al., 2011; Petrova et al., 2020).

Further insight into dendritic and synaptic pathology has been gained through research using the EAE animal model. In both the brain and spinal cord of EAE models, reductions in spine density and dendritic length have been observed. Notably, these reductions appear to correlate with the inflammatory burden, as studies consistently report that dendritic length and spine density are diminished during periods of heightened inflammation (Jafari et al., 2021; Tambalo et al., 2015; Zhu et al., 2003). Alongside dendrite and spine loss, acute EAE episodes and relapses are characterized by extensive dendritic beading, particularly within the lumbar and sacral regions of the spinal cord. Notably, dendritic beading was predominantly observed in the white matter, while only mild swelling occurred in the grey matter. Despite the severity of these changes, dendrites exhibited the capacity for recovery during periods of remission. A similar dynamic was observed in synaptic markers, which showed a reduction during EAE episodes, followed by partial recovery as the disease entered remission (Bannerman et al., 2005; Zhu et al., 2003).

### I.2.4. Mechanisms of Dendrite Damage in the Inflamed CNS

There are several mechanisms through which the dendrites and their spines could be compromised during neuroinflammation. This section will explore the various pathways through which dendritic injury can occur, examining how these mechanisms are activated in the context of neuroinflammation, and how they may contribute to the pathology of MS and EAE.

#### Soluble Mediators causing Dendrite and Spine Loss

As previously established, the inflamed CNS of MS patients contains infiltrated lymphocytes, as well as activated microglia and astrocytes. These immune and glial cells are known to secrete soluble mediators, such as cytokines, and can directly or indirectly contribute to elevated glutamate levels (Bø et al., 2003; Kutzelnigg et al., 2005; Magliozzi et al., 2010; Sastre-Garriga et al., 2005).

Cytokines are produced in response to invading pathogens to stimulate, recruit, and proliferate immune cells. Beyond these classical functions, cytokines can also exert direct effects on neurons, as neurons express the necessary receptors to respond to these signalling molecules (Conti et al., 2008). For example, upon neuronal exposure, IFN-γ has been shown to induce dendritic beading (Kim et al., 2002). Both IFN-γ and TNF-α can cause dendritic beading through their interaction with, or potentiation of, the AMPAR (Bernardino et al., 2005; Mizuno et al., 2008). Additionally, in animal models of HAD, microglial secretion of platelet-activating factor (PAF) has been shown to induce neurotoxicity, in a way where physiological synaptic activity becomes excitotoxic (Bellizzi

et al., 2005). The interaction of PAF with its receptor on neurons could lead to synaptic loss, as treatment with a PAF receptor antagonist has been demonstrated to prevent spine loss in EAE models (Bellizzi et al., 2016). In a novel mouse model of MS, it was shown that CSF from patients with PPMS is uniquely capable of inducing motor disability and spinal cord pathology, mediated by the presence of IgG (Wong et al., 2023). Although this particular study did not address dendritic pathology, another investigation into prion disease demonstrated that immunoglobulins can indeed trigger dendritic beading (Wu et al., 2017). These findings suggest that immunoglobulins may also contribute to dendritic pathology in neuroinflammatory diseases like MS.

Glutamate is a crucial neurotransmitter that plays a significant role in synaptic plasticity and dendritic development; however, excessive concentrations of glutamate can lead to neurotoxicity [188]. Research indicates that levels of glutamate are markedly elevated in the CSF and brains of patients with MS (Sarchielli et al., 2003; Srinivasan et al., 2005). In both MS and EAE, persistent increases in pro-inflammatory cytokine levels, coupled with elevated glutamate availability, can upregulate the expression of neuronal glutamate receptors and exacerbate synaptic dysfunction, thereby amplifying local glutamate excitotoxicity (Centonze et al., 2009; Kim et al., 2002; Mizuno et al., 2008; Newcombe et al., 2008; Prieto & Cotman, 2017). Abnormal release of glutamate can result in excessive activation of NMDA and AMPA receptors, leading to an overabundant influx of sodium and calcium ions, which ultimately contributes to excitotoxicity. Elevated synaptic activity, such as that occurring with hyperstimulation, may induce dendritic beading(Bellizzi et al., 2005), while hypostimulation can cause dendritic arbors to atrophy (Anderson & Flumerfelt, 1986). Studies have shown that *in vivo* blockade of the AMPAR can improve clinical outcomes and restore dendritic spine density, providing evidence for the involvement of excitotoxicity in dendritic pathology in EAE (Centonze et al., 2009). The precise mechanisms through which excitotoxicity leads to dendritic beading remain unclear. Glutamate-induced excitotoxicity can trigger the formation of dendritic beads, which often localize to areas containing dysfunctional mitochondria (Greenwood et al., 2007). A recent study has demonstrated that dendritic blebbing following NMDA exposure is associated with a calcium-dependent, spatially restricted increase in chloride levels at the sites of the beads, suggesting a role for chloride in dendritic beading (Weilinger et al., 2022). Additionally, it has been proposed that the sodium influx, rather than calcium influx, contributes to dendritic beading in response to excitotoxic stimuli (Greenwood et al., 2007).

### Engulfment of Postsynaptic Structures by Microglia and Macrophages

In addition to secreting various molecules, activated glial cells also exhibit phagocytic functions. In the cortex of MS patients, activated microglia have been found in close proximity to neuronal bodies and proximal dendrites, suggesting direct interactions that may lead to dendritic destruction (Peterson et al., 2001). Evidence from EAE demonstrates that microglia engulf injured excitatory postsynaptic structures within the hippocampus (Bellizzi et al., 2016). Further, it has been shown that microglia and infiltrating macrophages in EAE can engulf synapses primed for removal due to calcium accumulation (Jafari et al., 2021). The "Déjà vu" model of viral encephalitis shows that microglia-mediated synaptic stripping is dependent on CD8+ T-cell-derived IFN-γ and neuronal IFN-γ signaling via the STAT1 pathway and downstream CCL2 expression. Blocking this pathway has been effective in preventing synaptic loss (Di Liberto et al., 2018; Kreutzfeldt et al., 2013). In AD, fibrinogen induces spine elimination by microglia through the microglial integrin receptor CD11b-CD18 (Merlini et al., 2019). In EAE, fibrinogen has been shown to cause axonal damage by microglia (Davalos et al., 2012). This suggests that a similar mechanism may occur in MS, where the presence of fibrinogen could lead microglia to target dendrites and spines.

The complement system, traditionally associated with immune defense, has also been implicated in synaptic and dendritic pathology in neurodegenerative diseases. Complement proteins such as C1q and C3 are deposited on spines in AD, marking them for removal by microglia (Hong et al., 2016). Elevated expression of the C1qA transcript has been observed in neurons, with an increased proportion of C3b-labeled neurons noted in the cortical and deep grey matter of MS patients (Watkins et al., 2016). In EAE, heightened levels of C1q and C3 on dendrites and postsynaptic structures in the hippocampal grey matter have been documented (Hammond et al., 2020). Studies have shown that blocking C3 in EAE models prevents early dendritic loss and reduces microglial engulfment of spines, indicating a role of the complement system in dendritic pathology (Bourel et al., 2021).

In addition to microglia, astrocytes are also capable of phagocytosis. Following transient brain ischemia, reactive astrocytes are seen to engulf pre- and postsynaptic structures (Morizawa et al., 2017; Shi et al., 2021). However, the potential phagocytic role of astrocytes in MS and EAE remains to be elucidated. While significant strides have been made in understanding the mechanisms underlying dendritic damage in neuroinflammatory diseases like MS, the complexity of these processes leaves many questions unanswered. Further exploration of the interplay between immune signalling, excitotoxicity, and dendritic integrity is needed to identify therapeutic targets that may preserve or restore dendritic function.

## I.3. Manipulating Neuropathology with CRISPR/Cas9

### I.3.1. CRISPR/Cas9 Gene Editing

To gain insight into a gene's function, researchers have developed methods to modulate or disrupt the expression of the gene of interest. They deduce its function by studying the resulting loss- or gain-of-function phenotype. Genome engineering technologies provide an entry point into understanding and controlling the function of genetic elements in health and disease. Genome editing has facilitated research aimed at elucidating the contribution of specific genes to disease.

For instance, the introduction of target-specific double-stranded RNA (dsRNA) can silence gene expression by degrading the complementary messenger RNA (mRNA), preventing its translation into a functional protein. This phenomenon is known as RNA interference (RNAi) (Fire et al., 1998). While this method can achieve gene silencing relatively quickly, it has confounding aspects such as transient efficiency, off-target effects and the inability to achieve a full knock-out (KO) (Birmingham et al., 2006; Khan et al., 2009).

The further development of genome editing technologies using DNA nucleases has made it possible to completely disrupt genes and their functions because they act on the level of DNA rather than mRNA. Programmable nucleases, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), can be designed to target specific gene loci of interest. Localizing these nucleases to the target DNA sequence results in the introduction of double-strand breaks (DSB), leading to the accumulation of insertions or deletions (indels) during the repair process. These indels result in frame-shift mutations causing loss of gene function (Joung & Sander, 2013; Urnov et al., 2010). Despite their increased efficacy compared to RNAi, manufacturing of ZFNs and TALENs is complicated and labour-intensive, as de novo engineering of nuclease proteins is required for each target gene (Li et al., 2020).

Recently, the development of the clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated nuclease 9 (Cas9) gene editing method has emerged as a powerful genome editing tool. Originally, CRISPR/Cas systems are part of the bacterial immune system, used to defend against phage DNA or mobile genetic elements. Like other nucleases, the RNA-guided Cas9 cleaves the target genomic locus, resulting in a DSB. In the absence of a repair template, the cell attempts to repair the DSB by non-homologous end joining (NHEJ), which is error-prone and results in the introduction of indel mutations. These indels create frameshifts or premature stop codons, leading to the disruption of the targeted gene (Jinek et al., 2012; Ran et al., 2013). Directing the Cas9 nuclease to the DNA

sequence of interest requires a 20-nucleotide long guide RNA (gRNA) and a protospacer-adjacent motive (PAM) adjacent to the target sequence on the target DNA (Anders et al., 2014). To customize the CRISPR/Cas9 system for each gene of interest, researchers have to engineer and synthesize a suitable gRNA which guides the nuclease protein (Ran et al., 2013). This adaptability of CRISPR/Cas9 has made it widely accessible as a genome-editing tool. It can be employed for the KO of a single gene or can be multiplexed by the simultaneous use of multiple gRNA. Additionally, it is valuable for screening a high number of candidate genes with the help of genome-wide gRNA libraries (Cong et al., 2013; McCarty et al., 2020).

The CRISPR/Cas9 system, widely recognized for its versatility in scientific research, has been adapted for various applications. Template-based repair allows for precise mutations at specific sites by utilizing a repair template for homology-directed repair (HDR) instead of non-homologous end joining (NHEJ). Additionally, catalytically dead Cas9 (dCas9) can bind to DNA without cleaving it and can be fused with modifying proteins for several functions: CRISPR inhibition (CRISPRi) reduces gene expression through the transcriptional repressor KRAB, while CRISPR activation (CRISPRa) increases gene expression by linking dCas9 to transactivator domains. dCas9 also facilitates base editing by recruiting DNA deaminases to induce specific point mutations, which is valuable for addressing mutations in monogenic diseases. Furthermore, dCas9 can be combined with epigenome editors to manipulate transcription through acetylation, demethylation, or methylation. Other Cas proteins, such as Cas12 and Cas13, enhance the system's capabilities by broadening target sequence recognition and enabling transcriptome engineering for transient gene knockdown (Braun et al., 2022).

### I.3.2. Applications of CRISPR in Neuropathology

CRISPR/Cas genome engineering has proven to be useful in research across several neurological diseases. To study neuronal pathology *in vitro*, within a context specific to patients, the use of patient-specific human induced pluripotent stem cells (hiPSCs) offers a practical way of studying live human cells. hiPSCs can be reprogrammed into various cell types, including several neuronal lineages (Chambers et al., 2009). These hiPSC-derived neurons carry disease-specific genetic alterations, rendering them a suitable platform to recapitulate specific disease aspects *in vitro*, a concept often referred to as “disease in a dish modelling” (Vogel, 2010). This approach has been used to model several neurological diseases, such as AD, PD and schizophrenia (Chung et al., 2013; Penney et al., 2020; Powell et al., 2020). Furthermore, hiPSC-derived neurons can be manipulated *in vitro* using the CRISPR/Cas9 system to identify or correct essential disease-related mutations. For

instance, a study involving hiPSC neurons derived from a schizophrenia patient demonstrated that CRISPR-based manipulation of a prominent schizophrenia risk locus resulted in improved dendrite arborization and synapse maturation compared to unaltered neurons displaying disease-specific dendritic alterations (Forrest et al., 2017). In another example, hiPSC-derived neurons from an AD patient were engineered using CRISPR to mutate the APP C-terminus, which attenuated Aβ production, a key pathological hallmark of AD (Sun et al., 2019).

However, to assess whether these cellular changes translate into functional improvements, *in vivo* studies become necessary, especially when CRISPR manipulations aim to investigate changes in cells that cannot be manipulated *in vitro* before being transplanted into a living model organism, such as mature neurons. Several mouse lines and vector delivery methods have been developed to facilitate CRISPR manipulations in living organisms. Initially, a mouse line constitutively expressing Cas9, in conjunction with a Cre recombinase-dependent conditional Cas9 mouse line, were developed by the lab of Feng Zhang (Platt et al., 2014). Subsequently, mice expressing dCas9 variants for epigenome editing, like CRISPRi and CRISPRa, were developed (Gemberling et al., 2021). In addition to transgenic mouse lines, researchers have successfully introduced the Cas9 nuclease through non-viral vectors, viral vectors or physical delivery (Asmamaw Mengstie, 2022; Duan et al., 2021). These advancements enable the manipulation of virtually any tissue using CRISPR, including the CNS. Indeed, the CRISPR/Cas9 system has been effective in editing neurons *in vivo*. As a proof-of-concept experiment, the neuronal-specific RNA-splicing factor NeuN could successfully be deleted from cortical neurons using the Cre-dependent Cas9 mouse line (Platt et al., 2014). Similarly, the KO of MECP2, a protein highly enriched in neurons, was achieved in the mouse cortex using a dual virus approach involving adeno-associated viruses (AAVs) carrying either Cas9-encoding DNA or the gRNA (Swiech et al., 2015). Santinha et al. recently developed an AAV-PHP.eB virus-mediated single-cell CRISPR screening, termed AAV-Perturb-seq, a method for transcriptional linkage analysis as well as high-throughput and high-resolution phenotyping of genetic perturbations in neurons *in vivo* (Santinha et al., 2023).

In the context of CNS diseases, CRISPR has contributed to our understanding of various pathological molecular mechanisms. For example, reducing metabotropic glutamate receptor 5 (mGluR5) levels in the striatum of a mouse model of Fragile-X-Syndrome, a neurodevelopmental ID, via CRISPR modification led to the amelioration of repetitive behaviours (Lee et al., 2018). CRIPSR-editing the Bace1 gene in the 5XFAD mouse model of familial AD, which interferes with Aß production, resulted in improved associative learning

and a reduction in AD-associated phenotypes (Park et al., 2019). In a mouse model of Huntington's disease, the suppression of mutant Huntingtin protein expression via CRISPR attenuated early neuropathology (Yang et al., 2017). Similarly, in the spinal cord, CRISPR has been employed to modify motor neurons. Neonatal disruption of mutant SOD1 expression in a mouse model of amyotrophic lateral sclerosis (ALS) led to improved motor function and reduced muscle atrophy (Gaj et al., 2017). However, it's worth noting that manipulating mature neurons in the spinal cord presents certain challenges, unlike in the brain, where mature neurons can be more readily edited. Mature motor neurons in the spinal cord of adult animals are known to be difficult to target and manipulate (Towne et al., 2008). This highlights the need for further refinement of CRISPR techniques to study these neurons in the spinal cord in the context of neuropathological conditions, such as MS, which only occurs with advancing age.

# Chapter II - Aim of the Study

Multiple sclerosis (MS) is a complex disease characterized by inflammation, demyelination, and neurodegeneration, with its underlying causes still largely enigmatic. While the histopathological appearance in early stages of the disease is dominated by focal inflammatory lesions in the white matter, recent years have seen a growing interest in grey matter pathology, recognized increasingly as a primary correlate of disease progression and severity. Yet, treatments specifically targeting this aspect of MS, particularly during the progressive disease stage in which neurons are degenerating, are limited. This shortfall is largely due to incomplete understanding of the mechanisms that ultimately result in neurodegeneration at this disease stage.

In this thesis, I focus on the neurodegenerative aspects of MS and its experimental model, experimental autoimmune encephalomyelitis (EAE). In particular, I have studied inflammatory changes to dendrites, a neural compartment less studied in MS. To unravel dendritic pathology, I have investigated the dendrites within the spinal cord, which is believed to be a major contributor to motor symptoms observed in MS and EAE. **The initial objective of this thesis is to evaluate dendritic morphology within both the grey and white matter of the spinal cord.**

Additionally, I seek to delineate the mechanisms by which dendrites are affected in the context of demyelination and inflammation. To expand existing tools to study molecular impacts on dendritic structures without altering the disease milieu, t**he second aim of this thesis was to establish an approach based on CRISPR/Cas9 gene editing that allows to examine how inflammation-associated molecules impact dendritic pathology in the spinal cord.**

**Finally, I explore potential molecular mechanisms that could be implicated in dendritic degeneration, with a particular focus on inflammatory cytokines (IFN-γ) and glutamatergic neurotoxicity.** By selectively deleting or blocking their receptors (IFNGR1, NMDAR, and AMPAR) in neurons, I aim to investigate their impact on inflammatory dendritic degeneration. Through identifying specific mechanisms and pathways, this research hopes to contribute to the discovery of potential therapeutic targets for MS.

**Key Questions / Aims:**

1. How are dendrites affected within the demyelinating inflammatory CNS?
2. Is there any difference between dendrites found in the grey vs. white matter?
3. Establishment of technique which enables the study of molecular mechanisms that influence neuronal and dendritic health within motor neurons.
4. Do cytokines and/or glutamate signalling within the diseased environment exert an effect on dendrites?
5. More specifically: Does the elimination or blocking of the IFNGR, AMPAR and/or NMDAR prevent or rescue dendritic degeneration in acute EAE?

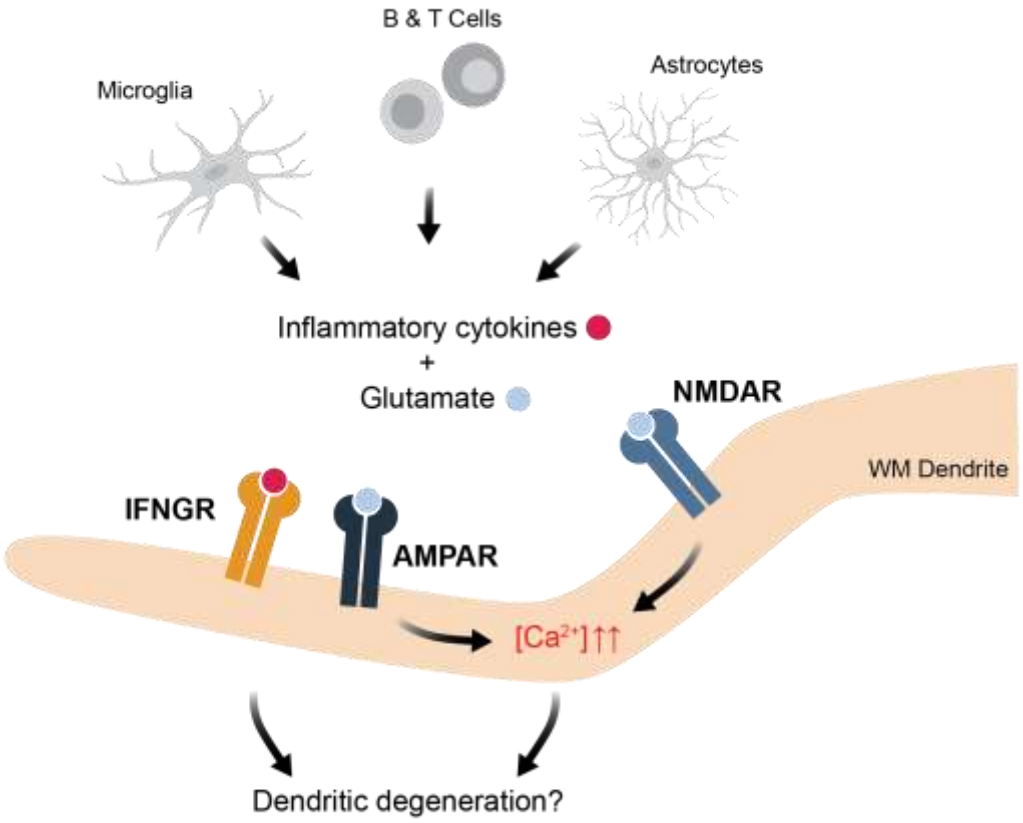



**Figure 8 | Graphical summary.** In this thesis, I want to explore dendritic pathology in the white matter of the spinal cord in the EAE model of MS. I developed a CRISPR/Cas9 based technique which allows to delete neuronal proteins in the adult mouse. With this technique, I explore the hypothesis that specific soluble factors – more specifically IFN-γ, and glutamate – contribute to the dendritic pathology.

# Chapter III - Materials and Methods

## III.1. Materials

### III.1.1. Induction of EAE

| Medication | | |
|---|---|---|
| KX anaesthesia | Ketamine 10% (Ketamine Hydrochloride) | CP-pharma GmbH, Burgdorf, Germany Bela-Pharm GmbH, Vechta, Germany |
| | Xylazine 20mg (Xylazine) | |
| Isoflurane | Isoflurane CP (Isoflurane) | CP-pharma GmbH, Burgdorf, Germany |
| Hydration solution | Ringerlösung Fresenius (Ringer's solution) | B. Braun AG, Melsungen, Germany |
| | Glucose 5% | |
| | Saline (NaCl 0.9%) | |
| Skin and eye protection/ disinfection | Bepanthen eye- and nose cream 5g | Bayer AG, Leverkusen, Germany |
| | Cutasept 250 ml (antiseptic spray) | Bode Chemie GmbH & Co, Hamburg, Germany |
| | Braunovidon Cream 10% (Povidon iod) | B. Braun AG, Melsungen, Germany |
| | Ethanol 70% | CLN GmbH, Niederhummel, Germany |

| Reagents | | |
|---|---|---|
| MOG emulsion | Purified recombinant MOG (N1- 125, from E. Coli) | Stock solution, produced in-house |
| | Mycobacterium Tuberculosis H37 RA | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | Incomplete Freund's adjuvant | |
| | Pertussis toxin from Bordetella pertussis, inactivated | |
| | Acetate buffer 20mM pH3.0 | |

| Tools and Materials | | |
|---|---|---|
| Syringes and needles | Hamilton 1 ml syringes | Hamilton, Bonaduz, Switzerland |
| | Hypodermic Needles BD, Microlance 3 23 Gauge (0-6 mm, blue) | Becton, Dickinson & Co, Franklin Lakes, USA |

### III.1.2. Perfusion and Immunohistochemistry

| Reagents | | |
|---|---|---|
| Perfusion | 4% Paraformaldehyde phosphate buffer (PFA), pH 7.4, ready-to-use | Morphisto GmbH, Frankfurt a.M, Germany |
| | Heparin-Natrium 25.000IE (Heparin sodium) | Ratiopharm GmbH, Ulm, Germany |
| Microdissection | Sodium azide 0.1% in 1x PBS | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | Sucrose 30% | |
| IHC | Tissue-Tek O.C.T. | Sakura Fintek Ltd, Tokyo, Japan |
| | Phosphate buffered saline (PBS) 10x pH7.4 | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | Triton X-100 | |
| | Horse serum | Invitrogen GmbH, Darmstadt, Germany |
| | Goat serum | |
| | Vectashield Mounting Medium | Vector Laboratories Inc., Burlingame, USA |

| Antibodies, Dyes and Tracers | | | |
|---|---|---|---|
| Primary antibodies | MAP2 | polyclonal chicken antibody, ab5392 | Abcam, Cambridge, UK |
| | EGFP | polyclonal goat antibody, ab6673 | |
| | Iba1 | polyclonal rabbit antibody, 019-19741 | FUJIFILM Wako, Osaka, Japan |
| | NeuN | polyclonal rabbit antibody, SAB4300883 | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| Secondary antibodies | anti-chicken AF647 | polyclonal donkey antibody, A21449 | Invitrogen GmbH, Darmstadt, Germany |
| | anti-goat AF488 | polyclonal donkey antibody, A11055 | |
| | anti-rabbit AF594 | polyclonal donkey antibody, A21207 | |
| | anti-rabbit AF 405 | polyclonal goat antibody, A31556 | |
| Dyes / Tracers | DAPI | | Invitrogen GmbH, Darmstadt, Germany |

| Tools and Materials | | |
|---|---|---|
| Perfusion | Noyes Spring Scissors (big) | FST GmbH, Heidelberg, Germany |
| | Dumont Mini Forceps Inox Style 3 (big) | |
| | Safety-Multifly 21 G 20 mm | Sarstedt AG & Co, Nümbrecht, Germany |
| IHC | Microscope slides 76x26 mm | Gerhard Menzel |

| | | |
|---|---|---|
| | Microscope cover slips 24x60 mm | Glasbearbeitungswerk GmbH, Braunschweig, Germany |
| | 96-well cell culture plate | Becton, Dickinson and Company, Franklin Lakes, USA |
| | Centrifuge tubes (15 and 50 ml) | Sarstedt AG & Co, Nümbrecht, Germany |
| | Pipettes, pipette tips and tubes (0.5, 1.5, 2 ml) | Eppendorf AG, Hamburg, Germany |

| **Devices** | | |
|---|---|---|
| Perfusion | Ismatec IP high precision multichannel pump | ISMATEC SA, Labortechnik-Analytik, Glattburg, Switzerland |
| IHC | Leica CM1950 (cryostat) | Leica Microsystem GmbH, Wetzlar, Germany |
| | Vortex-Genie 2 (vortex) | Scientific Industries Inc., Bohemia, USA |
| | Polymax 1040 (shaker) | Heidolph Instruments GmbH, Schwabach, Germany |

### III.1.3. Microscopy

All microscopic equipment listed below is from Leica Microsystem GmbH, Wetzlar, Germany.

| **Microscope** | **Objectives** |
|---|---|
| Leica TCS SP8 upright confocal microscope | HCX PL FLUOTAR 5x/0.15, dry |
| | PL Apo 20x/0.75, immersion correction CS2 |
| | HC PL Apo 40x/1.30, oil |
| | HC PL Apo 63x/1.40, oil |
| Leica SP8X WLL upright confocal microscope | HPCL FLUOTAR 10x/0.30 |
| | HC PL APO 20x/0.75 CS2 |
| | HC PL APO 20x/0.75 Immersion correction CS2 |
| | HC PL APO 40x/1.30, oil immersion CS2 |
| | HC PL APO 63x/1.30, glycerol correction CS2 |
| | HC PL APO 63x/1.40, oil immersion CS2 |
| Leica DMi8 inverted fluorescent microscope | HC PL FLUOTAR 5x/0.15 |
| | HC PL FL 10x/0.30 PH1 |
| | HC PL APO 20x/0.80 |
| | HC PL APO 40x/0.95 |

### III.1.4. Bath Application of Dyes

| Medication | | |
|---|---|---|
| KX aneasthesia | Ketamine 10% (Ketamine Hydrocloride) | CP-pharma GmbH, Burgdorf, Germany Bela-Pharm GmbH, Vechta, Germany |
| | Xylazin 20mg (Xylazine) | |
| **Tools and Materials** | | |
| Laminectomy | Hypodermic Needles BD Microlance 3 30 Gauge (0.3 mm, yellow) | Becton, Dickinson & Co, Franklin Lakes, USA |
| | Feather blade, surgical scalpel | Pfm medical AG, Cologne, Germany |
| | Noyes Spring Scissors (big) | FST GmbH, Heidelberg, Germany |
| | Vannas-Tübingen Spring Scissors (small) | |
| | Dumont Mini Forceps Inox Style 3 (big) | |
| | Dumont Mini Forceps Inox Style 5 (small) | |
| | AutoClip system | FST GmbH, Heidelberg, Germany |
| | Sugi wipes | Kettenbach GmbH&Co KG, Eschenburg, Germany |

| Reagents | | |
|---|---|---|
| Agarose Well | Agarose 3.5 % | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| Artificial CSF (aCSF) | 8.66 g NaCl | Merck Millipore, Darmstadt, Germany |
| | 0.224 g KCl | |
| | 0.206 g $CaCl_2 \cdot 2H_2O$ | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | 0.163 g $MgCl_2 \cdot 6H_2O$ | |
| | 0.214 g $Na_2HPO_4 \cdot 7H_2O$ | Merck Millipore, Darmstadt, Germany |
| | 0.027 g $NaH_2PO_4 \cdot H_2O$ | |
| | 500 mL $dH_2O$ | |
| Dye | Texas-red-conjugated Dextran 3kDa | Thermo Fisher Scientific, Planegg, Germany |

### III.1.5. CRISPR/Cas9 Editing

#### *III.1.5.1. gRNA Selection - In vitro*

| Cells and Plasmids | | |
|---|---|---|
| Cells | HEK293T cells | ATCC, Manassas, USA |
| | Cas9-expressing HoxB8-FL | Provided by dr. Seren Baygün (Laboratory of Prof. Dr. Marc Schmidt-Supprian) |
| | Flt3L-producing B16 melanoma cell line | |

| | | |
|---|---|---|
| Competent cells | Stellar Competent Cells | Clontech, Mountain View, USA |
| Plasmids | MSCV-pU6-(BbsI)-CcdB-(BbsI)-Pgk-Puro-T2A-BFP | Addgene, Watertown, USA |
| | MSCV-pklv2-U6-(Non-Targeting gRNA)-Pgk-Puro-T2A-tdTomato | In house cloning |
| | pCL-Eco | Provided by PD. Dr. Naoto Kawakami |

| **Reagents** | | |
|---|---|---|
| Cloning | FastDigest Bpil restriction enzyme | Thermo Fisher Scientific, Planegg, Germany |
| | Fast AP | |
| | FastDigest Buffer (10X) | |
| | Nuclease-free Water | Qiagen, Hilden, Germany |
| | QIAquick Gel Extraction Kit | New England Biolabs, Frankfurt am Main, Germany |
| | T4 DNA Ligase Reaction Buffer | |
| | T4 DNA Ligase | |
| | T4 Polynucleotide Kinase | |
| | Quick Ligation Kit | |
| Bacteria | NEB Stable Competent cells | New England Biolabs, Frankfurt am Main, Germany |
| | S.O.C medium | Invitrogen GmbH, Darmstadt, Germany |
| | Agar-agar | Merck, Darmstadt, Germany |
| | Ampicillin Sodium Salt | PanReac AppliChem, Darmstadt, Germany |
| | Rattler Plating Beads (4.5 mm) | Zymo Research, Freiburg im Breisgau, Germany |
| | Yeast extract | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | Sodium chloride | Merck, Darmstadt, Germany |
| | Peptone from Casein (Tryptone) | |
| Plasmid isolation | QIAprep Spin Miniprep Kit | Qiagen, Hilden, Germany |
| | EB buffer | |
| Retrovirus generation | DMEM, low glucose, GlutaMAX | Thermo Fisher Scientific, Planegg, Germany |
| | Penicillin Streptomycin (10,000 U/mL) | |
| | FBS SUPERIOR Stabil | Bio&SELL, Feucht, Germany |
| | TransIT-LT1 Transfection Reagent | MoBiTec GmbH, Göttingen, Germany |
| | Trypsin-EDTA (0.25%) with phenol red | Thermo Fisher Scientific, Planegg, Germany |
| | RPMI-GlutaMAX | |

| | | |
|---|---|---|
| Retrovirus infection of HoxB8 cells | β-estradiol | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | Recombinant murine IL-6 | Peprotech GmbH, Hamburg, Germany |
| | Recombinant murine IL-3 | |
| | Puromycin Dihydrochloride | Thermo Fisher Scientific, Planegg, Germany |
| | 2-Mercaptoethanol | |
| gDNA isolation and PCR amplification | DNeasy Blood & Tissue Kit | Qiagen, Hilden, Germany |
| | MinElute PCR Purification Kit | |
| | Q5 reaction buffer pack | New England Biolabs, Frankfurt am Main, Germany |
| | Q5 High-Fidelity DNA polymerase | |
| | Quick-Load® 1 kb Plus DNA ladder | |
| | Deoxynucleotide Solution Mix | |
| | Agarose 1% | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | DNA Gel Loading Dye | Thermo Fisher Scientific, Planegg, Germany |
| | peqGreen DNA/RNA Dye | Peqlab Biotechnologie GmbH, Erlangen, Germany |
| | TrisBase | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | Acetic acid | |
| | EDTA | |

| **gRNA's and Primers** | | |
|---|---|---|
| gRNA's | Ifngr1:<br>TTCAGGGTGAAATACGAGGA | Metabion, Planegg, Germany |
| | NeuN:<br>TCGGGGTCCCTGAACCGGA (Platt et al., 2014) | |
| | NR1:<br>AACATCACTGATCCACCGCG | |
| Primers | Hu6 primer:<br>GAGGGCCTATTTCCCATGATT | Metabion, Planegg, Germany |
| | Ifngr1 - gRNA specific:<br>F: CGAGGCAGAGTGTAGGTAAAG<br>R: CTTAATTGCCAACACTGGCC | |
| | NeuN - gRNA specific:<br>F: GTCTGCAGGACTACCTTACAAC<br>R: GCTGGGCTGTTCCTCTTT | |
| | NR1 - gRNA specific:<br>F: TCATCGGACTTCAGCTAATCAA<br>R: ACAGTGGTCCTGTCTTCCAGAT | |

| Tools and Materials | | |
|---|---|---|
| Cell culture | 6 and 12-well cell culture plate | Becton, Dickinson and Company, Franklin Lakes, USA |
| | 5 mL, 10 mL, 25 mL, 50 mL Corning® Costar ® Stripette ® serological pipettes, individually paper/plastic wrapped | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| Gel excision | X-TRACTA Generation II | Biozym Scientific GmbH, Oldendorf, Germany |

| Devices | | |
|---|---|---|
| Cloning and PCR | Biometra Trio Thermal Cycler | Analytik Jena, Jena, Germany |
| | Nanodrop 2000C/2000 | Thermo Fisher Scientific, Planegg, Germany |
| Gel electrophoresis and extraction | PerfectBlue Gel system | Peqlab Biotechnologie GmbH, Erlangen, Germany |
| | Power Pac 200 | Bio Rad, Feldkirchen, Germany |
| | High Performance Ultraviolet Transilluminator | UVP, Jena, Germany |
| | Quantum Vilber Lourmat | Vilber, Eberhardzell, Germany |

### *III.1.5.2. In vivo Editing and Labelling*

| Cells and Plasmids | | |
|---|---|---|
| Cells | HEK293T cells | ATCC, Manassas, USA |
| Plasmids | pAd-Delta-F6 packaging helper plasmid | Addgene, Watertown, USA |
| | pUCmini-iCAP-PHP.eB capsid | |
| | pAAV2/9n capsid | |
| | pAAV-U6-gRNA-hsyn-Cre-2a-eGFP-KASH-WPRE | |
| | pCMV-lox-mb-KOFP-SV40pA | Provided by Bareyre Lab |
| | pAAV-ITR-U6-NeuNgRNA-Cbh-mCherry-WPRE | In-house cloning |
| | pAAV-ITR-U6-gRNA-hSyn-DIO-KOFP-KASH | |
| | pAAV-ITR-U6-gRNA-CMV-DIO-KOFP-KASH | |

“gRNA” refers to the backbone plasmid, into which specific guide sequences for target genes can be cloned.

| Medication | | |
|---|---|---|
| MMF anaesthesia | Fentanyl 0.05mg | B. Braun AG, Melsungen, Germany |
| | Dormicum 5mg (Midazolam) | Cheplapharm Arzneimittel GmbH, Greifswald, Germany |
| | Dormilan 1mg/mL (Medetomidine hydrochloride) | Alfavet Tierarzneimittel GmbH, Neumünster, Germany |
| Anaesthesia antagonist | Atipazole 5mg (Atipamezole hydrochloride) | Prodivet Pharmaceuticals S.A., Raeren, Belgium |
| | Flumazenil 0.1 mg | Hameln GmbH, Hameln, Germany |
| | Naloxone 0.4 mg (Naloxone hydrochloride) | B.Braun AG, Melsungen, Germany |

| Reagents | | |
|---|---|---|
| AAV plasmid cloning | CutSmart buffer | New England Biolabs, Frankfurt am Main, Germany |
| | Gibson Assembly Master Mix | |
| gRNA cloning AAV backbone | SapI restriction enzyme | New England Biolabs, Frankfurt am Main, Germany |
| Plasmid isolation | QIAGEN Plasmid Maxi Kit | Qiagen, Hilden, Germany |
| AAV production | Poly-Lysine | Thermo Fisher Scientific, Planegg, Germany |
| | Opti-MEM | |
| | Pluronic F-68 Non-ionic | |
| | Surfactant | |
| | PEI MAX | Polysciences, Hirschberg an der Bergstraße, Germany |
| | DNase I, RNASE-free, recombinant | Roche, Basel, Switzerland |
| | SYBR mix | Bio Rad, Feldkirchen, Germany |
| | Polyethylene Glycol (PEG) 8000 | Selleck Chemicals, Houston, USA |
| | Magnesium chloride, anhydrous | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | Potassium chloride, powder, BioReagent | |
| | Trizma hydrochloride solution | |
| | Benzonase | |
| | Phenol red solution | |
| | OptiPrep™ | Serumwerk Bernburg AG, Oslo, Norway |
| gDNA isolation & amplification | QIAamp DNA Micro Kit | Qiagen, Hilden, Germany |
| Tail vein injection | Ringerlösung Fresenius (Ringer's solution) | B. Braun AG, Melsungen, Germany |

| gRNA's and Primers | | |
|---|---|---|
| gRNA's | See III.1.3.1. gRNA Selection - *In vitro* | |
| Primers | WPRE reverse primer:<br>CATACGGTAAAAGGAGCAAC | Metabion, Planegg, Germany |
| | Titer determination<br>F: GGA ACC CCT AGT GAT GGA GTT<br>R: CGG CCT CAG TGA GCG A | |

| Tools and Materials | | |
|---|---|---|
| Cell culture | Greiner CELLSTAR 14.5 cm diameter dish | Greiner Bio-One, Frickenhausen, Germany |
| | T75 flasks | Thermo Fisher Scientific, Planegg, Germany |
| Virus production | 0.45 µm filters | Merck Millipore, Darmstadt, Germany |
| | 100 kDA Amicon filters | |
| | Stainless steel 316 syringe needle, pipetting blunt 90° tip | |
| | 20 mL syringes (Luer lock) | Becton, Dickinson & Co, Franklin Lakes, USA |
| | qPCR plates | Bio Rad, Feldkirchen, Germany |
| | qPCR closing film | |
| | Cell scraper | Techno Plastic Products, Trasadingen, Switzerland |
| | Quick-Seal Centrifuge Tubes | Beckman Coulter Biomedical GmbH, Munich, Germany |
| Tail vein injection | Hypodermic Needles BD, Microlance 3 30 Gauge (yellow) | Becton, Dickinson & Co, Franklin Lakes, USA |
| | Tailveiner® Restrainer for Mice | Brain Tree Scientific, MA, USA |
| Cortical injection | Syringe 3pc 3 and 5ml Omnifix luer slip | B. Braun AG, Melsungen, Germany |
| | BD Plastipak Hypodermic luer slip syringe 1ml | Becton, Dickinson & Co, Franklin Lakes, USA |
| | Hypodermic Needles BD Microlance 3 30 Gauge (0.3 mm, yellow) | |
| | Feather blade, surgical scalpel | Pfm medical AG, Cologne, Germany |
| | Noyes Spring Scissors (big) | FST GmbH, Heidelberg, Germany |
| | Vannas-Tübingen Spring Scissors (small) | |
| | Dumont Mini Forceps Inox Style 3 (big) | |
| | Dumont Mini Forceps Inox Style 5 (small) | |

| | | |
|---|---|---|
| | Ethicon Ethilon monofil 6-0 size (skin) | Johnson & Johnson Medical GmbH, Norderstedt, Germany |
| | AutoClip system | FST GmbH, Heidelberg, Germany |
| | 0.4 mm and 0.5 mm stainless steel drill head | Meisinger, Neuss, Germany |
| | Blaubrand intraMark micropipettes (Ultrathin pulled glass pipette) | Brand GmbH & Co KG, Wertheim, Germany |
| | Sugi wipes | Kettenbach GmbH&Co KG, Eschenburg, Germany |

| **Devices** | | |
|---|---|---|
| Plasmid isolation | Avanti JXN-26 Centrifuge | Beckman Coulter Biomedical GmbH, Munich, Germany |
| Virus production | CFX Connect Real-Time PCR | Bio Rad, Feldkirchen, Germany |
| | Optima™ L-90K Ultracentrifuge | Beckman Coulter Biomedical GmbH, Munich, Germany |
| | Water bath WB-4MS | BioSan, Riga, Latvia |
| Cortical injection | Olympus KL 1500 LCD | Olympus Deutschland GmbH, Hamburg, Germany (Headquarters in Japan) |
| | Olympus Stereo Microscope SZ51 | |
| | Stereotaxic Frame | Stoelting CO, Wood dale, USA |

### *III.1.5.3. Nuclei Isolation and FACS Sorting*

| **Reagents** | | |
|---|---|---|
| Nuclei isolation | Nuclei EZ Lysis buffer | Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany |
| | Bovine Serum Albumin solution,10% in DPBS, low endotoxin, fatty acid free, suitable for cell culture, sterile-filtered | |
| | Protector RNase Inhibitor (10000U) | |
| | Dulbeccos Phosphate buffer saline modified without calcium chloride and magnesium chloride | |
| FACS antibodies | Mouse BD Fc block | BD Pharmingen, San Diego, USA |
| | Anti-NeuN monoclonal rabbit recombinant AlexaFluor 647 antibody | Thermo-Fisher Scientific, Planegg, Germany |
| | Nuclear Pierce DAPI | Merck Millipore, Darmstadt, Germany |

| **Tools and Materials** | | |
|---|---|---|
| Tissue isolation | 50 mL syringe with Luer-lock | Becton, Dickinson & Co, Franklin Lakes, USA |
| | Noyes Spring Scissors (big) | FST GmbH, Heidelberg, Germany |

| | | |
|---|---|---|
| | Vannas-Tübingen Spring Scissors (small) | |
| | Disposal Safety Scalpels | Aesculap AG, Tuttlingen, Germany |
| Nuclei isolation | MACS SmartStrainers 30µm | Miltenyi Biotec, Begisch Gladbach, Germany |
| | MACS SmartStrainers 70µm | |
| | Pestle for microtubes 1,5/2,0 ml | A. Hartenstein GmbH, Würzburg, Germany |
| | Pipettes, pipette tips and tubes (0.5, 1.5, 2 ml) | Eppendorf AG, Hamburg, Germany |
| FACS sorting | FACS tube 5 mL 75 mm x 12 mm | VWR, Ismaning, Germany |
| | FACS tube 5 mL 75 mm x 12 mm PS 125 | |
| | 5mL Polystyren Round-bottom tube with cell-strainer cap | Becton, Dickinson & Co, Franklin Lakes, USA |

| **Devices** | | |
|---|---|---|
| Nuclei isolation | Eppendorf Centrifuge 5417R | Eppendorf AG, Hamburg, Germany |
| FACS sorting | BD FACSAriaFusion<br>Equipped with 4 lasers (405, 488, 561, 640nm) | Becton, Dickinson & Co, Franklin Lakes, USA |
| | BD FACSAriaIIIu | |
| Perfusion | Ismatec IP high precision multichannel pump | ISMATEC SA, Labortechnik-Analytik, Glattburg, Switzerland |

### III.1.6. Software

| CRISPR/Cas9 engineering | | |
|---|---|---|
| Cloning | Snapgene software | www.snapgene.com |
| gRNA design | Broad Institute GPP Web Portal gRNA Designer | Broad Institute of MIT and Harvard, Cambridge, USA |
| Primer design | Benchling | Benchling Inc., San Francisco, USA |
| | IDT primer quest tool | Integrated DNA Technologies, San Diego, USA |
| TIDE | Tracking of Indels by DEcomposition | https://tide.nki.nl (Brinkman et al., 2014) |

| Data Analysis | | |
|---|---|---|
| Image processing and analysis | FIJI | https://imagej.net (Schindelin et al., 2012) |
| | Huygens Essentials | Huygens SVI, Hilversum, Netherlands |

| | | |
|---|---|---|
| Data processing and statistics | R Statistical Software | R Foundation for Statistical Computing, Vienna, Austria |
| | R Studio | Integrated Development for R, Boston, USA |
| Figures | Adobe Creative Suite CS6 (Photoshop, Illustrator) | Adobe Systems Inc., San Jose, California, USA |

## III.2. Experimental Animals

To establish the CRISPR/Cas9-editing pipeline, either constitutively or Cre-dependent (LSL) Cas9-expressing mouse lines were used (Gt(ROSA)26Sor$^{tm1.1(CAG-cas9*,-EGFP)Fezh}$/J or Gt(ROSA)26Sor$^{tm1(CAG-cas9*,-EGFP)Fezh}$/J, respectively, Jackson Laboratory). To assess dendritic pathology, I used F1 offspring from crossbreeding LSL-Cas9-EGFP (as above) and ChAT-Cre (B6.129S-Chattm1(cre)Lowl/MwarJ, Jackson Laboratory) mice. To determine the effects of overexpressing the GluA2 subunit of the AMPA receptor on dendrites, I used the EGFP-dnGluA2 mouse line (Rosa26-EGFP-Gria2, a gift from Dwight E. Bergles). Both male and female animals were used for all experiments and were equally distributed into control and experimental groups.

Mice were housed in social groups of a maximum of five in individual ventilated cage systems with standard bedding under a 12:12 light:dark cycle, at a temperature of 22 ± 2 °C and 55 ± 10% relative humidity with access to food and water ad libitum. The cages were provided with enrichment consisting of play tunnels, nestlets to be used as nesting material or a red plastic mouse house. For husbandry, one male was housed with one or two females. Mice were weaned at postnatal day 21. Experiments were performed on young adult (age 8–12 weeks) female and male mice. All animals were bred in our animal facility at the BMC, Planegg, Germany.

All animal experiments were performed in accordance with regulations of the relevant animal welfare acts and protocols approved by the local animal ethics committee of the state of Bavaria (Regierung von Oberbayern) in accordance with European guidelines.

# III.3. Methods

## III.3.1. Induction of EAE

Mice were anesthetized using ketamine-xylazine (87 mg/kg and 3 mg/kg, respectively). Afterwards, they were immunized subcutaneously at the flanks and tail base with an emulsion of in-house purified recombinant MOG1-125 (400µg) and CFA containing 650µg Mycobacterium tuberculosis. Pertussis toxin (PTX) dissolved in PBS (350ng) was administered intraperitoneally (i.p.) on day 0 and day 2 of immunization. Animals were weighed daily and evaluated based on an established EAE scoring system (Table 2). The first day in which any of the clinical signs could be observed was considered as onset of disease. Acute EAE mice were sacrificed two or three days after disease onset when they had a clinical score ≥ 2. Chronic EAE mice were sacrificed on day 30 following disease onset if they had a score ≥ 2 during the acute phase. Control animals were untreated littermates of immunized mice. In case of female mice, control and EAE animals were mixed within cages.

**Grading system for clinical assessment of EAE**

| Score | Clinical signs | Assessment |
|---|---|---|
| 0 | Normal behaviour | No changes in motor function or behaviour |
| 0.5 | Distal tail weakness | Absence of distal tail tension |
| 1 | Complete tail weakness | Absence of tail tension |
| 1.5 | Limp tail with slight hindlimb weakness | Unsteady gait, poor grip and jumping strength |
| 2 | Limp tail with unilateral hindlimb weakness | Unsteady gait, one limb is dragging, but moving, the other limb can move normally |
| 2.5 | Limp tail with bilateral hindlimb weakness | Both limbs are dragging, but moving |
| 3 | Complete bilateral hindlimb paralysis | Complete dragging of both hindlimbs without movement |
| 3.5 | Complete bilateral hindlimb paralysis with forelimb weakness | Dragging of both hindlimbs and poor grip with forelimbs |
| 4 | Quadriplegia | Mouse is unable to move around the cage |
| 5 | Moribund / Death | |

**Table 2 | Clinical disease scoring in EAE for the assessment of neurological and motoric deficits.** Light grey shaded area: termination criteria. When a score of ≥ 3.5 is reached, the experiment is terminated and the animal is immediately sacrificed according to the regulations of the Regierung von Oberbayern. Table adapted from hookelabs.com.

### III.3.2. Tissue Preparation and Immunofluorescence / Immunohistochemistry

Animals were lethally anaesthetized using isoflurane and perfused transcardially with 15-20mL PBS (1X)-Heparin (5000U/mL) followed by 25mL of 4% PFA in PBS using a peristaltic pump (velocity 6 mL/min). Afterwards, spinal cords and brains were isolated and post-fixed in 4% PFA at 4°C for 24 hours and 72 hours, respectively. After removal of excess bone and tissue by microdissection, the tissue was cryopreserved by immersion in a mixture of 30% sucrose with 0.1% PBS-sodium-azide at 4°C until the tissue sank to the bottom of the Eppendorf. Before cutting, the samples were fully submerged in Tissue-Tek O.C.T. and frozen at -20°C.

For evaluation of the dendritic pathology in the spinal cord, the upper (L1-3) and lower (L4-6) lumbar spinal cord were separated from the rest of the spinal cord and used for further processing. The spinal cord sections were cut in 30μm cross-sections using a Cryostat. Sections were collected in a 48 well plate, with multiple consecutive sections (4-5) per well. After cutting, sections were washed with 1xPBS on a shaker for 30 min. One section per well was mounted on a cover slip for lesion screening using polarization contrast imaging. After confirmation of lesions, remaining tissue from the corresponding wells was used for further staining. At least 4 wells per animal and area were selected. Control sections were chosen from untreated animals at similar levels of the spinal cord. Sections were blocked in a mixture of 0.5% horse serum and 1xPBS - 0.3% Triton X-100 for one hour on a shaker at room temperature before overnight incubation on a shaker at 4°C with the primary antibodies: chicken-anti-MAP2 (1:1000), goat-anti-GFP (1:1000) and rabbit-anti-IBA1 (1:500). After three washing steps of each 10 min in 1xPBS, the sections were again incubated overnight on a shaker at 4°C with the secondary antibodies: donkey-anti-chicken AF647, donkey-anti-goat AF488 and donkey-anti-rabbit AF594, each in a 1:500 concentration. Both, primary and secondary antibodies, were diluted in 1xPBS - 0.3% Triton X-100. The following day, 100μL of DAPI fluorescent dye dissolved in 1xPBS at a concentration of 1:10.000 was added to the wells and further incubated for 10 min on a shaker at room temperature. Sections were washed 3 more times. The sections were mounted on microscopy slides, covered with Vectashield and sealed with a coverslip.

For the dye experiment, the segment of the spinal cord that was exposed to the dye was isolated and cut in 30μm cross-sections using a Cryostat. Sections were incubated with chicken-anti-MAP2 (1:1000) and donkey-anti-chicken AF647 (1:500), as well as stained with DAPI (1:10000) as described above.

The validation of *in vivo* CRISPR editing of NeuN was performed in the mouse brain. Mice were perfused in the same way as described above. Subsequently, the brains were

embedded in Tissue-Tek medium and cut in 60µm thick coronal sections using a cryostat. Slices were collected in PBS and subsequently blocked for 1h at room temperature with 10% goat serum in 1xPBS - 0.5% Triton X-100. Slices were stained with rabbit anti-NeuN (1:400) in 1% goat serum in 1xPBS - 0.5% Triton X-100 overnight at 4°C. Afterwards, sections were washed three times with 1xPBS at room temperature. Finally, the tissue was incubated for 4 hours at room temperature with goat-anti-rabbit AF405 (1:500) in 1% goat serum in 1xPBS - 0.5% Triton X-100. Sections were mounted with Vectashield on microscopy slides and sealed with a coverslip.

### III.3.3. Microscopy

Screening of lesions in the spinal cord was performed at the Core Facility Bioimaging at the Biomedical Center of the Ludwig-Maximillian's-University in Munich. Polarized light microscopy of the myelin in the spinal cord was done on an inverted Leica DMi8 light microscope equipped with 2 linear polarizers at an angle of 90° to each other in the beam path. The condenser and aperture diaphragm were adjusted to achieve Köhler illumination. An HC PL FL 10x/0.3 objective was used to acquire single z-planes for all the sections.

Confocal microscopy of spinal cord sections for motor neuron and dendritic quantifications was also performed at the Core Facility Bioimaging. These images collected using a Leica SP8X WLL microscope equipped with 405 nm laser, WLL2 laser (470 - 670 nm) and acusto-optical beam splitter. For motor neuron counting, eight sections per animal were imaged using the HC PL APO 20x/0.75 IMM CORR CS2 objective, excitation light of 488 nm was used for GFP/AF488 and 548 nm for KOFP. The Z-stack was set as follows: 2 µm step size and a total number of 11 Z-steps. For dendrite density quantification, images of at least two spinal cord sections per animal and per upper / lower lumbar region were acquired with a 40x/1.30 Oil CS2 objective and the following fluorescence settings: DAPI (excitation 405 nm; emission 410-470 nm), GFP (AF488; 488 nm; 519 nm), IBA1 (AF594; 594 nm; 618 nm) and MAP2 (AF647; 647 nm; 671 nm). Recording was done sequentially to avoid bleed-through. GFP (AF488) and IBA1 (AF594) were recorded with hybrid photo detectors (HyDs), DAPI and MAP2 (AF647) with conventional photomultiplier tubes (PMT). The Z-stack was set for 11 Z-steps of a step size of 1 µm. The pixel size in x- and y-direction of the images is 271 nm. Polarized light microscopy images were captured using 2 polarization filters and a white light source under Köhler illumination.

Sections from the dye application experiment were imaged using the same set up as above. A 40x/1.30 Oil CS2 objective was used and the following fluorescence settings: DAPI (excitation 405 nm; emission 410-470 nm), MAP2 (AF647; 657 nm; 671 nm) and Texas Red

(595 nm, 615 nm) were recorded using PMTs. The Z-stack was set for 20 Z-steps of a step size of 1 µm. The pixel size in x- and y-direction of the images is 135 nm. Polarized contrast images were captured using 2 polarization filters and a white light source under Köhler illumination.

Brain sections for the validation of NeuN KO were imaged using an upright LeicaSP8 confocal microscope equipped with a standard filter set and laser lines. Sections were imaged using a 63x/1.40 NA oil objective. Single planes were recorded at a resolution of 0.18µm per pixel in both directions. NeuN and mCherry signals were acquired using following settings: NeuN (AF405, 405 nm, 420 nm) and mCherry (587 nm, 610 nm) with HyDs.

### III.3.4. Image Analysis

Open-source image analysis software FIJI was used to analyse all confocal microscopy images (Schindelin et al., 2012).

For counting of motor neurons, the ventral grey matter was divided into a left and right side using the central canal and the medial fissure as reference points. Maximum intensity z-projections of 10µm were created. The Cell Counter plugin was used to count all the GFP+, KOFP+ and GFP+KOFP+ cells in the ventral horn. Measuring the volume of motor neurons was automated using a script based on the 3D Objects Counter plugin. Tabulation of all results into an excel format was automated via a script as well.

For analysis of dendritic density, 6 slices of each Z-stack (5µm) from the middle of the stack were selected to create a maximum intensity Z-stack projection. Based on the channels with the polarization contrast and Iba1 staining of the projection, the border between the grey and white matter was traced, as well as the lesions of the white matter. For quantifications of the grey matter dendrites, an area of 100 by 100 µm in each ventral horn was analysed. For the analysis of white matter dendrites, the sections were divided as follows: The dorsoventral axis, the midsagittal axis and further divisions of the ventral side were drawn. The spinal cord cross section has 4 regions on each side: dorsolateral, ventrolateral, lateroventral and midventral. In each of the former mentioned regions, an area reaching from the grey-/white-matter border to the end of the spinal cord section with a width of ≥ 30µm was selected and saved as a region of interest (ROIs). The dendrites within these ROIs were skeletonized using a custom script in FIJI. Using another script, all ROIs were divided in steps of 25µm and the lengths of the skeletonized structures were measures and exported into excel data tables. The dendrites in the grey matter were skeletonized and quantified in

a similar way (without the division in 25µm sections). Additional script modifications were made to measure fluorescence intensity and area in the grey matter.

For quantification of dye uptake in dendrites, the grey-white matter border and lesions (when EAE) were identified based on the polarization contrast and DAPI channels. Dendrites were traced using the MAP2 signal and saved as separate ROIs. The mean grey values of the ROIs in the Texas Red (dextran 3kDa) channel were measured per dendrite.

For the quantification of synaptic density on dendrites in the grey matter, image slices were processed in the same way as above. Dendrites in similar areas of the white matter as previous analyses were selected. Dendritic structures were traced manually. Synapses were counted using the Cell Counter plugin.

### III.3.5. Dye Uptake Experiment

Animals were anaesthetized via intraperitoneal injection with KX and immobilized on a surgery plate. A laminectomy was performed to gain access to the lumbar spinal cord. A well was built around the spinal opening using 2%–4% agarose diluted in aCSF to form a reservoir for the dye. The dura was removed using a 30G needle. Texas-red-conjugated dextran 3kDa diluted in aCSF was applied to the well and left for 2 hours. After incubation, the spinal cord was washed 3 times with aCSF. The animals were euthanised and perfused as described above.

### III.3.6. CRISPR/Cas9

#### *III.3.6.1. Plasmid Design*

Plasmids used for CRISPR-editing *in vitro* and *in vivo* in this thesis were modified from following commercially available plasmids: MSCV-pU6-(BbsI)-CcdB-(BbsI)-Pgk-Puro-T2A-BFP (Addgene plasmid #86457 ; RRID:Addgene_86457; (Chu et al., 2016)) for use in retroviral vectors and AAV:ITR-U6-gRNA(backbone)-hSyn-Cre-2A-EGFP-KASH-WPRE-shortPA-ITR (Addgene plasmid #60231; RRID:Addgene_60231; (Platt et al., 2014)) for use in AAV vectors. The source for cloning of the Kusabira Orange Fluorescent Protein (KOFP) is pCMV-lox-mb-KOFP-SV40pA, kindly provided by PD Dr. Florence Bareyre. Cloning of preliminary testing plasmids was done in collaboration with Dr. Paula Sanchez. The creation of pAAV-ITR-U6-gRNA-hSyn-DIO-KOFP-KASH and pAAV-ITR-U6-gRNA-CMV-DIO-KOFP-KASH was done via Gibson cloning together with Dr. Arek Kendirli. MSCV-pklv2-U6-(Non-Targeting gRNA)-Pgk-Puro-T2A-tdTomato was previously cloned by Dr. Arek Kendirli. All steps and resources for cloning were designed using Snapgene software.

### *III.3.6.2. gRNA design and plasmid insertion*

The plasmids described above contain gRNA insertion sites which are cut by restriction enzymes BbsI and SapI within the retroviral and AAV plasmids, respectively. Therefore, oligonucleotides to be inserted should contain corresponding sticky ends: CACC (F) and AAAC (R) for retroviral vectors, ACC (F) and AAC (R) for AAV vectors (Fig. 9).

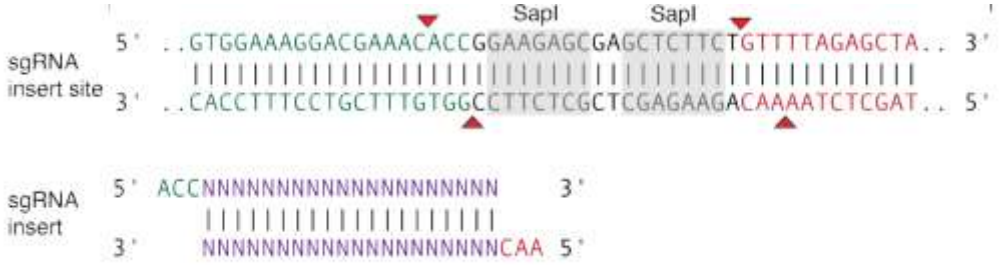



**Figure 9 | gRNA insert site in CRISPR plasmids. The CRISPR plasmids contain a gRNA insert site which can be digested by certain restriction enzymes. This digestion creates binding sites on which synthetic oligonucleotides containing the target locus sequence and matching sticky ends can bind. The plasmid suitable for use with AAV viral vectors can be cut with the SapI restriction enzyme, leaving TGG tails. The synthetic oligo should have ACC ends to be able to bind the plasmid. Image modified from https://www.addgene.org/60958.**

gRNAs for target genes were designed using the Broad Institute GPP gRNA Designer tool (https://portals.broadinstitute.org/gpp/public/analysis-tools/gRNA-design). Top 3 to 5 gRNA sequences were selected based on their score. The complementary and reverse complement DNA sequence with appropriate sticky ends where synthetized by Metabion, Planegg, Germany. To increase cloning efficiency, an additional G was added to the 5' end of the gRNA (if not already starting with a G).

Plasmids were digested with CutSmart buffer for 1 to 24 hours at 37°C and separated using a 1% agarose gel containing 1 µl Peq Green in TAE buffer for 30 minutes. The band at the expected size of the digested plasmid was excised from the gel and purified using the QIAquick gel extraction kit per manufacturer's instructions.

Complementary oligonucleotides were phosphorylated using T4 polynucleotide kinase in 10X T4 Ligation Buffer and annealed with a thermocycler protocol of 1 minute at 95°C followed by a gradual decrease of 5°C per minute until reaching 25°C. The annealed oligos were ligated to the digested retroviral or AAV plasmid using Quick Ligase for 5 minutes at room temperature.

The ligated plasmid was transformed into Stellar Competent cells via heat shock at 42°C for 55 seconds. The bacteria were cultured overnight at 37°C on an agar plate containing Ampicillin. The following day, surviving colonies were selected and grown overnight in LB medium with Ampicillin in a bacterial shaker at 37°C and 400 RPM. Subsequently, plasmid

DNA was isolated using the QIAprep Spin Miniprep Kit as per the manufacturer's instructions. Sanger sequencing using the hU6 promoter primer confirmed the correct insertion of the desired gRNA sequence.

### *III.3.6.3. Virus production Retroviruses*

Per gRNA to be tested, approximately 750,000 HEK cells were seeded in antibiotic free media in a well of a 6-well plate. The plasmids for target or non-target gRNAs (1.2-1.5 µg) were mixed with PcL-Eco plasmid (1.2-1.5 µg) and 7.5 µl TransIT in 500µl RPMI medium. The mixture was vortexed and incubated at room temperature for 30 minutes. Following incubation, the mixture was added dropwise to the seeded HEK cells using a P1000 pipette. The day after transfection, the medium was changed to HoxB8 cell medium (RPMI GlutaMAX supplemented with 0.1% 2-Mercaptoethanol, 1 µM β-oestradiol and supernatant from Flt3L-producing B16 melanoma cell line with a final concentration of 35 ng/ml). 48 hours after transfection, virus-enriched medium was collected from the HEK cells and centrifuged at 3000 rpm for 5 minutes. The medium containing the retroviruses was used freshly for spin-infection of HoxB8 cells.

### *III.3.6.4. Virus production AAV-PHP.eB viruses*

AAV-PHP.eB viruses for *in vivo* infection of neurons in mice were produced according to the following protocol. 2 x $10^6$ HEK cells (in standard medium, provided by Dr. Arek Kendirli) were seeded onto poly-lysine coated plates (diameter 15 cm) and kept in an incubator at 37°C with 5% CO2. The following day, the transfection mixture (16µg gRNA plasmid, 10µg PHP.eB capsid plasmid, 20µg pAdDeltaF6 packaging helper plasmid and 100µg Polyethyleneimine (PEI) in 4 ml Opti-MEM) was prepared and allowed to rest for 20-30 minutes at room temperature. The transfection mixture was added dropwise in a spiralling pattern to the HEK cells using a P1000 pipette for equal distribution. 24 hours after transfection, the HEK cells were checked under a fluorescence microscope to check transfection efficiency. The medium was replaced with FBS-free medium. 72 hours after transfection, the supernatant from the cells was harvested for a first time. Fresh HEK medium without FBS was again added to the HEK cells. Harvested supernatant was spun at 1500 RPM for 5 minutes at 4°C and was subsequently filtered through a 0.45µm filter into a sterile glass bottle that was kept at 4°C. After another 72 hours, the supernatant was collected, spun and filtered (same as before). The supernatant from both harvests was combined and concentrated using A 100 kDa AMICON filter. The filter was equilibrated with 15ml 1xPBS and spun at 4200 rpm for 2 minutes at 4°C (discard flow-through). The harvested supernatant was added onto the filter and spun at 4200 rpm for 30 minutes at

4°C. The spinning step was repeated until a final volume of 200-300µl remained. The virus was tittered following the standard Addgene titration protocol (https://www.addgene.org/protocols/aav-titration-qpcr-using-sybr-green-technology/).

### *III.3.6.5. In vitro infection of HoxB8 cells*

Screening for the most efficient gRNA for each target was done *in vitro* using various gRNA sequences complementary to different locations within the target gene. Freshly produced retroviruses in HEK medium were mixed in a 1:1 volume ratio with Cas9-expressing HoxB8-FL cells (approximately 200,000 cells, stock generously provided by Dr. Seren Baygün from the lab of Prof. Dr. Marc Schmidt-Supprian) in a well of a 12-well plate. The plate was centrifuged at 1200xG for 45 minutes at room temperature with slow acceleration and deceleration settings. After spin-infection, the HoxB8 cells were maintained in a humidified incubator at 37°C with 5% CO2. The following day, HoxB8 cells were checked under a fluorescent microscope for the expression of fluorescent marker proteins (BFP / tdTomato) and transferred to media containing 5µg/mL puromycin for cell selection. Successfully infected cells were allowed seven days for selection and growth.

### *III.3.6.6. In vivo viral delivery*

For systemic *in vivo* infection of neurons in adult mice, AAV-PHP.eB virus was administered intravenously via tail vein injection at a dosage of $2 \times 10^{12}$ vg (diluted in Ringer solution, max. 100µl) per mouse. Mice were restrained in a Tailveiner® Restrainer for mice. The tail was gently heated using a UV lamp to facilitate injections.

For the validation of *in vivo* CRISPR editing of NeuN, the virus was injected locally into the cortex of adult mice (right hemisphere). These surgery and injections were performed by Dr. Emily Melisa Ullrich Gavilanes. In short: Animals were anesthetized with MMF and head-fixed in a stereotaxic frame. A hole was drilled in the skull using a 0.5 mm stainless steel drill head. A 25µm diameter glass capillary was inserted through the hole into the cortex. 0.5µl AAV virus at a dosage of $10^{12}$ viral particles diluted in Ringer solution was injected at following coordinates: 1.2 mm lateral, 0.6 mm caudal to bregma and a depth of 0.8 mm. After injection, the skin of the skull was stitched and animals were injected with AFN antagonist. Several days following surgery, animals were treated with buprenorphine.

In both cases, the viruses were injected a minimum of 3 weeks before any following procedures.

### *III.3.6.7. Nuclei isolation and FACS sorting*

To check *in vivo* CRISPR-editing efficiency in neurons, mice were transcardially perfused with PBS. The spinal cord and/or cortex were isolated and placed on ice in an Eppendorf tube with cold Nuclei EZ lysis Buffer. The tissue was manually homogenized with a pestle, topped up with more cold Nuclei EZ lysis Buffer and left on ice for 5 minutes. The homogenate was filtered through a 70µm cell strainer and transferred into a clean tube. The mixture was centrifuged at 500xG for 5 minutes at 4°C (same conditions for all consecutive centrifugation steps). The supernatant was decanted, and remaining nuclei were resuspended in cold Nuclei EZ lysis buffer. Following an incubation of 5 minutes on ice, nuclei were centrifuged. The supernatant was discarded and 500µl of Buffer 1 (1x DPBS, 1.0% BSA and 0.2 U/µl RNase inhibitor) was added without resuspension and left to rest for 5 minutes. The tube was further filled with Buffer 1 and nuclei were resuspended, followed by centrifugation. The supernatant was decanted. Nuclei were incubated for 5 minutes in Fc block (1:1000 in Buffer 1). After blocking, anti-NeuN monoclonal rabbit recombinant AlexaFluor 647 antibody was added (1:500 in Buffer 1) and incubated for 10 minutes on ice. Following incubation time, samples were washed 2 times with Buffer 1. The nuclei were resuspended in 500 µl Buffer 2 (1x DPBS, 1.0% BSA, 0.2 U/µl RNase Inhibitor, 10µm/mL DAPI) and filtered through a 30µm cell strainer.

The nuclei were sorted using a BD FACS Aria sorter using following gating strategy: (1) granularity and size to remove debris, (2) size of the samples: single nuclei were selected removing doublets, (3) DAPI and NeuN staining: selection of neuronal nuclei, (4) selection based on marker protein from virus. Nuclei were kept in 500µl PBS at 4°C until further steps.

### *III.3.6.8. DNA isolation and TIDE analysis*

The efficiency and frequency in which a certain gRNA is capable of generating indel mutations at the desired genomic site was evaluated by comparing genomes exposed to targeted and non-targeted gRNA's using the "Tracking of Indels by Decomposition" (TIDE) method.

For the selection of gRNAs using the *in vitro* pipeline, the genomic DNA was isolated from HoxB8 cells. The cells were collected 7 days after spin-infection, spun down at 300xG for 5 minutes at room temperature and resuspended in PBS. The genomic DNA was isolated with the DNeasy Blood and Tissue Kit according to the manufacturer's instructions.

To assess CRISPR-editing efficiency *in vivo* in mouse neurons, their nuclei were isolated and sorted as described above. Genomic DNA was isolated using the QIAamp DNA Micro Kit following the manufacturer's instructions.

The genomic DNA from cells or tissue that received either targeted or non-targeted gRNAs were processed simultaneously. The target sequence was PCR amplified with primers specific for each gRNA. The PCR mixture contained 10µl 5xQ5 buffer, 0.5µl Q5 High-Fidelity DNA Polymerase, 1µl dNTPs, 100-200ng gDNA and nuclease free water up to a total volume of 50µl. The PCR cycler ran the following program: 30sec - 98°C, 10 sec - 98°C, 20 sec - 62°C, 30 sec -72°C and 2 min - 72°C for 50 cycles. The products were separated on a 1% agarose gel with 1µl PeqGreen in TAE buffer for 30 minutes. The bands at the expected amplicon size were excised and purified using the QIAquick gel extraction kit according to manufacturer's instructions. Extracted products were further purified using the MinElute PCR Purification Kit. The concentration of genomic DNA was quantified using a Nanodrop. 50ng of DNA were sent for Sanger sequencing using the same primers as used for the PCR reaction. Indels and frameshift mutations were observed through comparing the target sequences from samples which received targeted and non-targeted gRNAs using the tracking of indels by decomposition (TIDE) tool (https://tide.nki.nl, (Brinkman et al., 2014)).

### III.3.7. GluA2 Overexpression

For analysis of dendritic density using the EGFP-dnGluA2 transgenic line, mice were systemically injected with AAV-PHP.eB virus via the tail vein. The virus was produced in house following the same protocols as described above. The virus was left 3 weeks to incubate before immunization. Mice were scored daily and sacrificed at the peak of disease. Tissue was processed for immunohistological staining as in previous experiments quantifying dendritic density.

### III.3.8. Data and Statistical Analysis

All data was analysed (data transformations, statistical analysis) and visualized (creation of graphs) with R in RStudio. Analysis was performed using dplyr, tidyverse and rrstatix packages, graphs were made with the ggplot2. Results are given as mean ± sem or mean ± SD (specified in figure legends). Normality of data was checked with the Shapiro-Wilk method, density and QQ plots. A two-sample comparison was made using a student's t-test and comparisons of more than 2 samples was done using a one-way ANOVA. In the case of comparing the dendritic density, a repeated-measures ANOVA was used. In non-normal distributed samples, the nonparametric Mann-Whitney U-test was used when comparing 2 samples; and the Kruskal-Wallis followed by Dunn's multiple comparison test for comparing more than 2 samples. Dendritic densities were log transformed to normalize the data. Obtained p-values are stated as significance levels in the figure legends (**** $P<0.0001$, *** $P<0.001$; ** $P<0.01$; * $P<0.05$) Exact p-values can be found in figure legends.

# Chapter IV - Results

## IV.1. Dendritic Pathology in the EAE Spinal Cord

In this dissertation, I honed in on the significance of grey matter pathology in MS as a key predictor of disease prognosis and indicator of progression. I focussed on the features of neuronal pathology within the inflamed spinal cord, aiming to identify features that contribute to progression. Specifically, I focused on the potential involvement of dendritic injury in the grey matter pathology as observed in MS and EAE disease profiles. To achieve this, I have characterized several dendritic features within the spinal cord grey and white matter of acute and chronic disease stages in the EAE mouse model.

In MS patients, motor symptoms notably affect walking and balance. These symptoms are mirrored in the EAE mouse model, where initial motor impairments manifest caudally. Symptoms subsequently advance rostrally with increasing disease severity (Table 2, III.3.1. Induction of EAE). Previous work from our lab has shown that, at early stages, most lesions are formed in the lumbar spinal cord (unpublished, dissertation Stefan Syma). This observation could provide an explanation for the early onset of disability in the lower-body. Consequently, this thesis places an emphasis on the lumbar spinal cord.

In our model, mice generally start to display motor symptoms around 10 days after immunization, reaching a peak score within 2 or 3 days following the onset of symptoms (Fig. 10, C). In the following experiments, acute EAE (aEAE) refers to the peak of disease (onset + 2/3 days), while chronic EAE (cEAE) denotes the stage 30 days after onset (Fig. 10, A).

Demyelinated inflammatory lesions are a hallmark of MS, which can be recreated in the EAE mouse model. To visualize demyelination in the EAE spinal cord, I harnessed the birefringent properties of myelin, a characteristic that facilitates label-free visualization through polarization microscopy (Fig. 10, B) (Blanke et al., 2021; Morgan et al., 2021). This method not only allows for rapid lesion screening, but also minimizes the handling of delicate tissue. My observations reveal that, within our model, lesions are predominantly found beneath the pia mater, proceeding into the white matter and seldom reach the grey matter (Fig. 10, D). Furthermore, activated microglia and macrophages (Iba1+ cells) can be found within demyelinated areas and throughout the grey matter (Fig. 10, B).

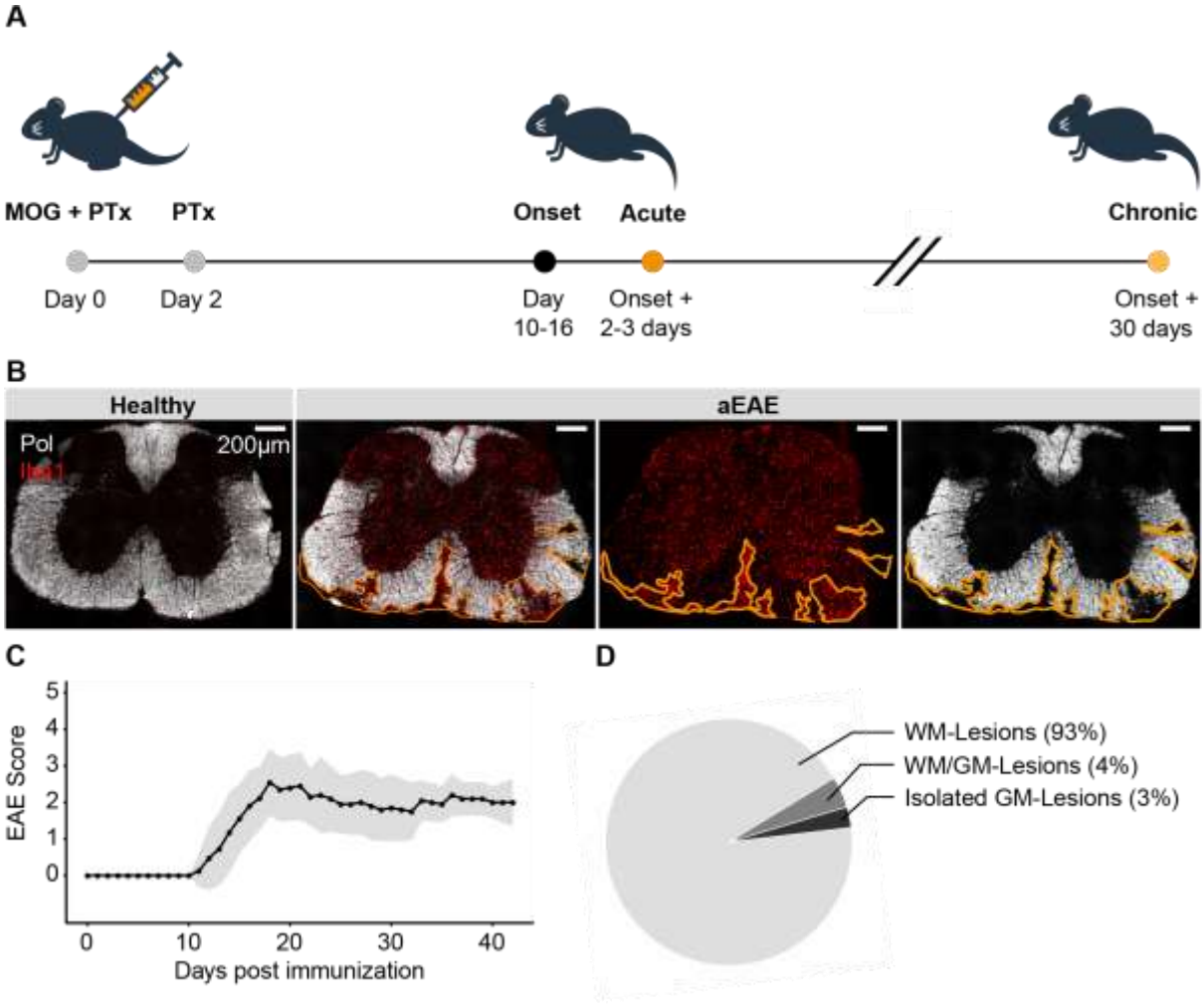



**Figure 10 | EAE in the spinal cord white matter manifests as demyelinated and inflammatory lesions.** **(A)** Schematic of EAE induction. Tissue was acquired at the peak of disease (acute EAE; onset + 2/3 days) or at a later time point (chronic EAE; onset + 30 days). **(B)** Confocal representative images of myelin (pol, grey) and microglia (Iba1, red) in healthy (left panel) and aEAE mice (right panels). **(C)** Typical EAE course in Bl6/J mice (n = 10 mice, mean ± sem). **(D)** Distribution of lesions over the white and grey matter (220 lesions in 4 animals). Figure created by Adinda Wens, includes data from Stefan Syma (panel D).

### IV.1.1. Histological Characterization of Dendrites within the Grey Matter

In the initial phase of my investigation, I examined dendrites within the grey matter of the spinal cord in both healthy and aEAE mice (Fig. 11, A). Utilizing MAP2 as a marker for dendrites, given its established association with dendritic structure (Hoskison et al., 2007) and its exclusion from axons (Goedert et al., 1991), I compared the percentage of MAP2-stained area (Fig. 11, C) and the total length of dendrites (Fig. 11, D) between healthy and aEAE mice. Notably, no significant differences were observed between the healthy and EAE conditions.

Recognizing that MAP2 fluorescence intensity can serve as an indicator of dendritic health (Hoskison et al., 2007), I compared the MAP2 fluorescence intensity of grey matter dendrites between the two conditions. However, this analysis did not reveal a significant difference either (Fig. 11, B). This collective data suggests that the dendrites within the grey matter of the spinal cord maintain their morphological integrity.

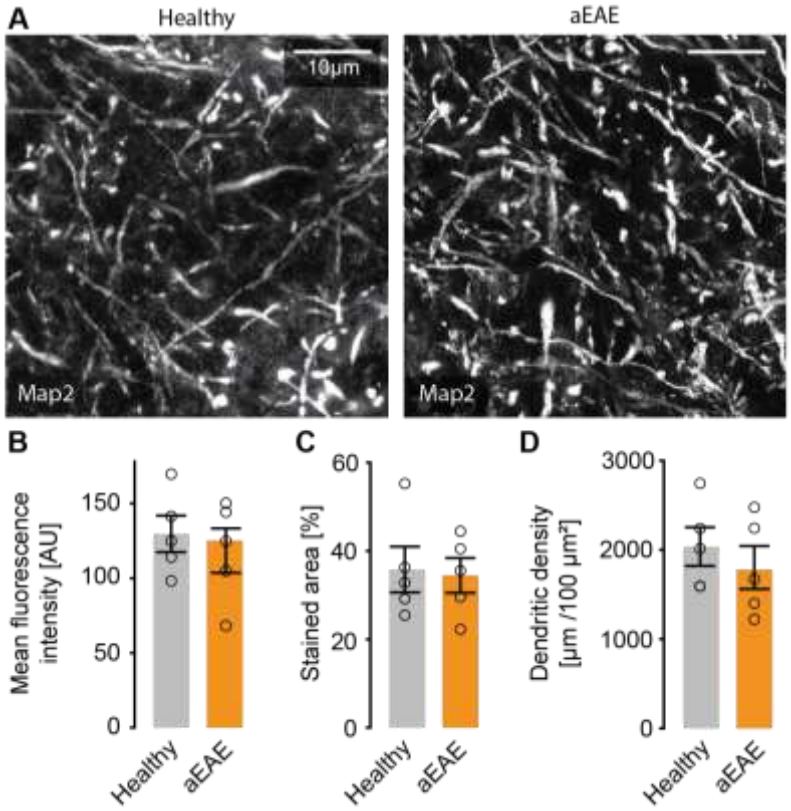



**Figure 11 | Dendrites within the ventral grey matter of the lumbar spinal cord. (A)** Confocal representative images of dendrites (MAP2) within the ventral horn of the spinal cord grey matter of healthy (left) or aEAE (right) Bl6/J mice. 5µm Z-projection **(B)** Mean fluorescence intensity measurement of MAP2 fluorescent staining in healthy vs. aEAE mice. **(C)** Percentage of stained area. **(D)** Dendritic density within the measured area. For all graphs: Measured area: 100µm x 100µm. Healthy: n = 5 mice, aEAE: n = 5 mice, mean ± sem. Figure created by Adinda Wens.

### IV.1.2. Histological Characterization of Dendrites within the White Matter

Interestingly, not all dendrites are confounded to the grey matter as we observed that some dendrites in the mouse spinal cord extend from the grey matter into the white matter (Fig. 12, C). This observation are in line with findings in previous publications where it has been established that motor neurons in various species, including rats, cats, chickens, and humans, exhibit dendrites crossing over into the white matter (Okado et al., 1990; Rose & Richmond, 1981; Schoenen, 1982; Zhu et al., 2003).

To check if the dendrites found in the white matter of mice also originate from motor neurons, I crossed genetically modified mouse lines that express Cre recombinase under the motor neuron-specific ChAT promoter (ChAT-Cre) with mice that express Cas9 and EGFP in a Cre-dependent manner (LSL-Cas9-EGFP) (Fig. 12, A). The resultant offspring exclusively expresses EGFP in the motor neurons (ChAT-Cre x LSL-Cas9-EGFP) (Fig. 12, B).

The necessity of Cas9 expression is relevant for the next part of the project and will become apparent in the next chapter.

Upon staining for MAP2, I observed that most of MAP2 dendritic structures in the white matter overlap with EGFP signal (87,2 ± 6,2 %, Fig. 12, D). This correlation leads to the conclusion that the majority of dendrites present within the white matter originate from motor neurons situated in the ventral horn.

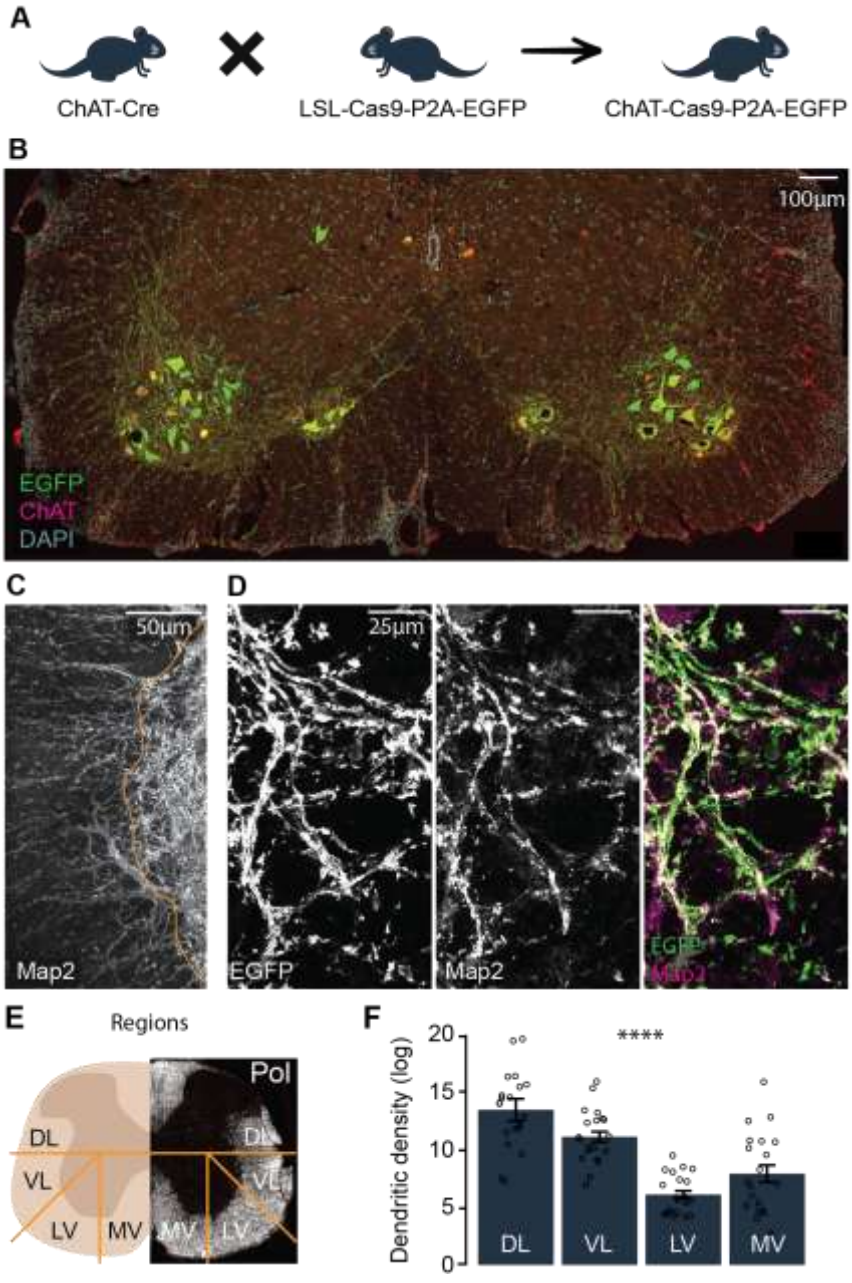



**Figure 12 | Dendrites in the white matter are coming from motor neurons.** **(A)** Breeding of ChAT-Cre mice together with floxed-Cas9-P2A-EGFP mice to obtain motor neuron specific expression of EGFP. **(B)** Confocal representative images of a lumbar spinal cord cross section. Motor neurons (ChAT+) are labelled with EGFP. EGFP+ neurites are seen to extend from the GM into WM. **(C)** Structures crossing the GM/WM border (yellow line) are dendrites (MAP2+). 5 µm Z-projection. **(D)** dendrites (MAP2) in the WM are coming from motor neurons (EGFP). 10 µm Z-projection. **(E)** Schematic of quantified areas within the white matter. **(F)** Quantification of dendritic density in the different regions of the healthy spinal cord (n = 20 sections in 5 mice, mean ± sem, ANOVA: p = 7.65e-12). Abbreviations: DL = dorsolateral, VL = ventrolateral, LV = lateroventral, MV = medioventral. Figure created by Adinda Wens, includes data from Marlena Helms (panel B) and Michael Keilholz (panel F).

Pilot studies performed by Michael Keilholz and me revealed that some areas contain more dendrites than others. Therefore, to account for variability in dendritic density across the white matter, I divided the white matter into 4 regions; dorsolateral (DL), ventrolateral (VL),

lateroventral (LV) and medioventral (MV) (Fig. 12, E). In healthy mice, the dendritic density is lowest within the LV region, likely due to the presence of the ventral root where many axons are exiting the spinal cord. The DL and VL areas have the most dendrites, possibly because of the proximity of the motor neuron somata in the ventral horns. (Fig. 12, F).

Further, I characterized how far these dendrites can extend into the white matter (Fig. 13, A). Therefore, as part of the characterization of dendritic features, I quantified the dendritic density in relation to the distance away from the grey matter border (Fig. 13, B).

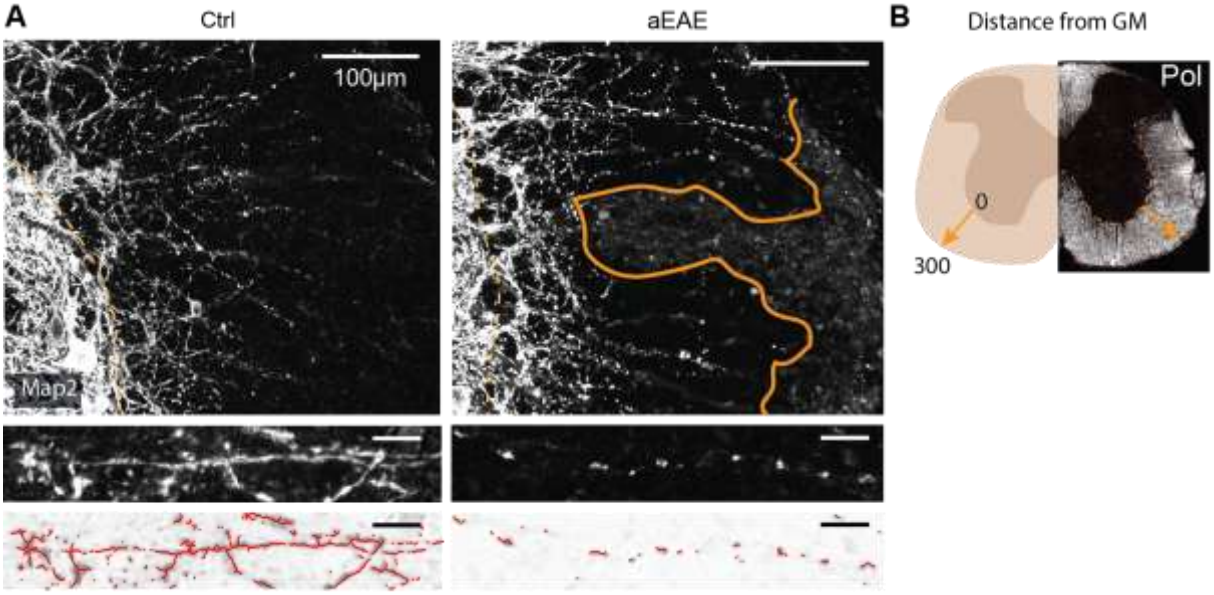



**Figure 13 | Dendritic degeneration within the spinal cord white matter. (A)** Confocal representative image of dendrites (MAP2) crossing from the grey matter into the white matter (dotted line) in healthy (left) and aEAE (right) animals. EAE lesion is outlined (yellow full line). Insets show closeups of dendrites, as well as an example of dendrite tracing for calculating dendritic density. **(B)** Schematic depiction of measurement of "Distance from GM".

As expected, the density is highest near the grey matter border (distance = 0 µm) and decreases towards the outer edge of the white matter (distance < 300µm) (Fig. 14, A and C, blue data points). Notably, some dendrites extend all the way to the outer edge of the white matter. As dendrites are found in the white matter, a compartment prone to developing inflammatory lesions in EAE, I wanted to investigate if they are affected by the condition. When comparing the dendritic density of aEAE mice to healthy mice, I observed that the dendritic density is significantly decreased (Fig. 14, A and B). The decrease can be observed in all regions. Interestingly, the dendritic loss was reversible and recovered spontaneously to healthy levels after the initial inflammation resolved in the chronic stage (Fig. 14, C and D).

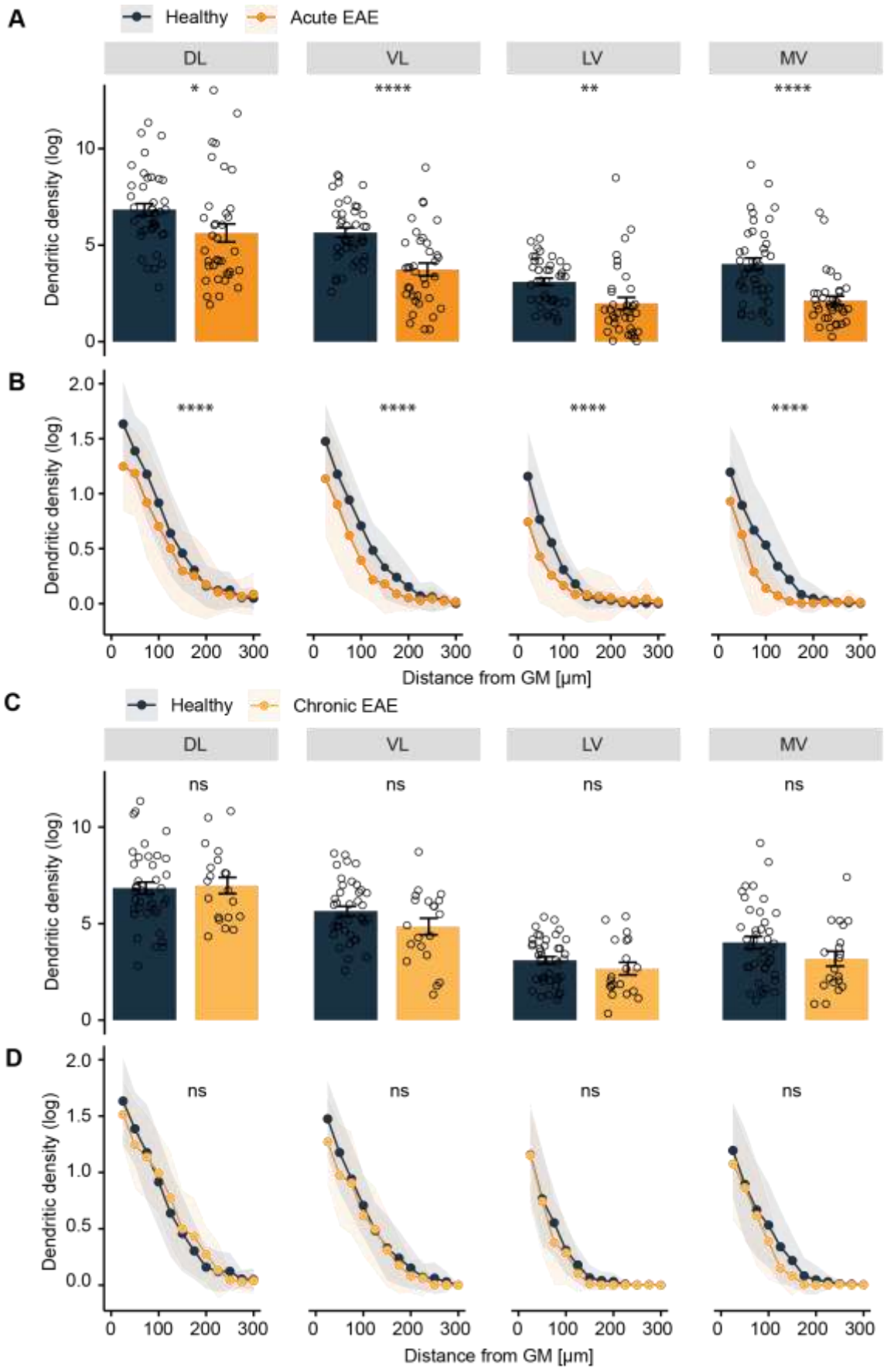

A
Healthy
Acute EAE
DL
VL
LV
MV
*
****
**
****
Dendritic density (log)
B
Distance from GM [µm]
C
Chronic EAE
ns
D

**Figure 14 | Dendritic degeneration within the spinal cord white matter. (A)** Dendritic density within different spinal cord areas for healthy and aEAE animals (healthy n = 40 areas in 5 animals, aEAE n = 36 areas in 5 animals, mean ± sem, normal distribution confirmed by the Shapiro–Wilk normality test, Student's T-test: P-values from left to right: p = 0,0353, 1.9e-05, 0,0031, 6,1e-06) **(B)** Dendritic density (log scale) of dendrites within the different regions in relation to the distance from the grey matter border in healthy and aEAE animals (n = 40 areas in 5 healthy mice, n = 36 areas in 5 aEAE animals, mean ± SD; normal distribution confirmed by the Shapiro–Wilk normality test, RM-ANOVA: P-values from left to right: p = 7.54e-10, 8.35e-11, 8.32e-08, 3.80e-11). **(C)** Dendritic density within different spinal cord areas for healthy and cEAE animals (healthy n = 40 areas in 5 animals, aEAE n = 20 areas in 5 animals, mean ± sem, normal distribution confirmed by the Shapiro–Wilk normality test, Student's T-test: ns.) **(D)** Dendritic density in relation to distance from the grey matter border for healthy and cEAE animals (n = 40 areas in 5 healthy mice, n = 20 areas in 5 cEAE animals, mean ± SD; normal distribution confirmed by the Shapiro–Wilk normality test, RM-ANOVA). Abbreviations: DL = dorsolateral, VL = ventrolateral, LV = lateroventral, MV = medioventral.

### IV.1.3. Dendritic Decline despite Neuronal Survival and Synaptic Connections

After the discovery of dendritic decline in the white matter, I was interested in determining whether the identified dendritic pathology is a primary or secondary event. In other words, to investigate if the damage occurs directly to the dendrites or if it is a consequence of preceding events affecting the neuron. This distinction is essential for understanding the underlying mechanisms of the pathology and for developing targeted therapeutic strategies.

In instances of neuronal decline, neurites are known to undergo swelling, fragmentation, and/or retraction (Takeuchi et al., 2005). To address this, I quantified the number of motor neurons situated in the ventral horn, as I had previously identified the dendrites in the white matter to originate from these cells. While some studies suggest a loss of motor neurons in MS and EAE, this claim remains a point of contention in the literature (Christopher P. Gilmore et al., 2009; Petrova et al., 2020; Vogt et al., 2009). Using the ChAT-Cre x LSL-Cas9-EGFP mice, I quantified the number of EGFP-positive cells located in the ventral horn in healthy, aEAE and cEAE mice (Fig. 12, B). There is no significant difference in the number of motor neurons across the conditions, meaning motor neurons are surviving throughout the course of EAE, at least up until 30 days after immunization (Fig. 15, C). These findings were further confirmed by Marlena Helms and me though an IHC staining with ChAT and NeuN antibodies (Supp. Fig. 1). These results eliminate the idea that a dendritic decline is merely the result of neuronal loss. Importantly, the observed dendritic decline also does not lead to or precede neuronal loss, as there is no evidence of motor neuron loss at the chronic stages of EAE (Fig. 15, C). This indicates that the dendritic pathology might be an independent phenomenon rather than a precursor or consequence of neuronal death.

Recognizing that neurons can exhibit neuritic beading prior to degeneration, I explored somatic volume changes as a label-free indicator of neuronal health (Dukkipati et al., 2018; Kiernan & Hudson, 1991). I measured the volume of motor neuron somata within the ventral horn of healthy and aEAE mice. Intriguingly, no significant difference in soma volume was observed (Fig. 15, D). Taking this into consideration, I assume dendritic beading to be an independent phenomenon which is not associated to neuronal death.

Dendrites, as critical postsynaptic receivers, integrate signals coming from other presynaptic neurons. These signals are transmitted through synapses either located on specialized structures, spines, or directly on the dendritic shaft. Notably, our lab has previously identified a reduction in spines on cortical layer III and IV neurons a cortical EAE model of MS (Jafari et al., 2021). Building upon these findings, Michael Keilholz visualized the synapses within the spinal cord white matter using the synaptic marker protein Synapsin-1. He observed that the synapses within this compartment do not receive inputs on spines, like in the cortex, but rather directly on the dendritic shaft (Fig. 15, B). To explore the potential link between a loss of synaptic activity and dendritic loss, Michael Keilholz quantified the synaptic density on dendrites in healthy animals and on remaining dendrites both within and outside of lesions in aEAE animals. I processed his raw data and performed the statistical analysis. Our findings suggest that we can discount a loss of synapses as the causative factor for dendritic loss as the synaptic density on dendrites remains consistent across the various conditions examined (Fig. 15, A).

As previously published by our lab, nanoruptures in the plasma membrane drive axonal degeneration in EAE through calcium influx (Witte et al., 2019). As axons are neurites as well, dendrites might undergo a similar form of degeneration. To explore this possibility, Michael Keilholz conducted an experiment analogous to the one performed by Witte et al. He gauged the passive uptake of a Texas Red-labelled 3kDa dextran administered to the subdural space of the lumbar spinal cord, into dendrites. I processed his quantification and performed the statistical analyses. In contrast to the axonal uptake of the dextran, the dye failed to enter into dendrites (Fig 15, E). This observation indicates that dendrites, unlike axons, lack nanoruptures and thus do not receive a calcium influx through said ruptures.

In light of the collective findings above, I rule out motor neuron loss, synaptic loss, and membrane damage in dendrites as potential causes for the observed dendritic degeneration. Consequently, I pivot my focus towards alternative mechanisms that could lead to these dendritic changes. Particularly dendrites are specialized cellular structures for receiving extracellular inputs. Such signalling molecules have the potential to initiate cascades within the neuron, ultimately contributing to the degeneration of dendrites.

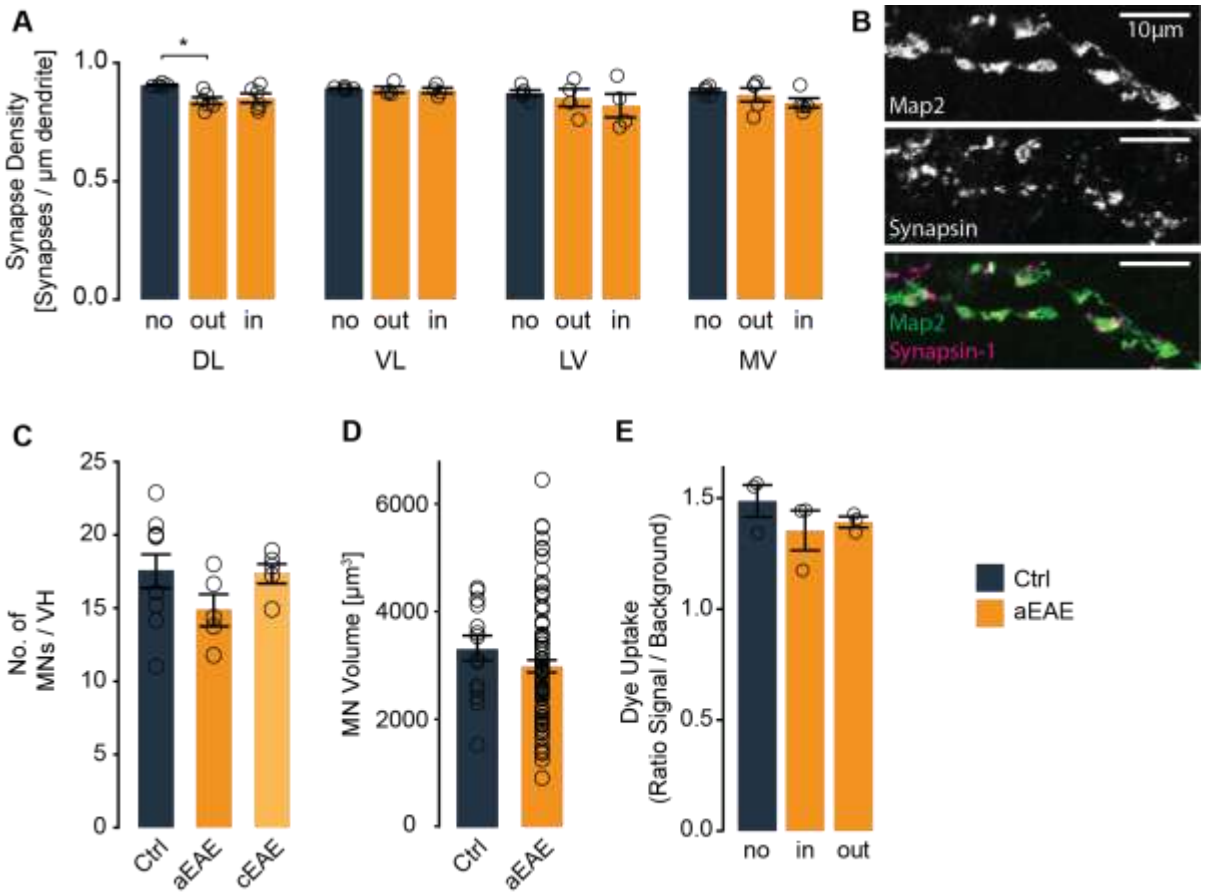



**Figure 15 | Dendritic loss is independent of motor neuron loss. (A)** Synaptic density as number of synapsin-1 puncta per µm dendrite. (Healthy n = 5 mice, aEAE n = 6 mice, mean ± sem, ANOVA: p=0,022, post-hoc t-test: 0,018). **(B)** Confocal representative image of MAP2 and Synapsin-1 IHC staining. **(C)** Number of motor neurons per ventral horn of ChAT-Cre x LSL-Cas9-EGFP animals in healthy, aEAE and cEAE conditions (Healthy n=10 mice, aEAE n=5 mice, cEAE n=5 mice, mean ± sem, normal distribution confirmed by the Shapiro–Wilk normality test, ANOVA: ns). **(D)** Motor neuron volume compared between healthy, aEAE and cEAE conditions in slices of 5 µm thickness. (Healthy n = 241 neurons in 2 mice, aEAE n= 1593 neurons in 3 mice, mean ± sem, Wilcoxon signed-rank test: ns). **(E)** Measurement of Texas Red 3kDa dextran uptake in dendrites located within (in) or outside (out) a lesion in aEAE, and in healthy animals (no lesion). Dye was measured inside and outside of dendrites to calculate the signal ratio between dendrite and background. (Healthy n = 3 mice, aEAE n = 3 mice, mean ± sem, Kruskal-Wallis test: ns). Figure created by Adinda Wens, includes data from Michael Keilholz (panel A, B and E) and Marlena Helms (panel C).

## IV.2. Genetic Editing of Motor Neurons

To investigate whether secreted effector molecules are influencing the dendritic degeneration observed above, I looked for a way to remove their respective receptors in motor neurons. Given that many signalling molecules can exert effects on a plethora of different cell types, it is important to develop a method that will enable a focused examination of their impact on motor neurons specifically. To achieve the editing of a number of candidate receptors in motor neurons, I turned to the promising CRISPR/Cas9 gene-editing technology. CRIPSR/Cas9 engineering provides the advantage of rapid in-house generation of KO mouse lines, preventing the need for purchasing and importing genetically-modified mouse lines for each candidate. It also allows for the generation of non-commercially available KO-lines, taking away the limitations as to which receptors can be investigated. While the CRISPR/Cas9-method has demonstrated remarkable efficacy in manipulating protein expression across diverse cell types, it is acknowledged that *in vivo* neuronal editing presents unique challenges, underscoring the need for careful consideration and refinement in its application.

The goal is to efficiently deplete a range of membrane receptors commonly found on dendrites, and to do this selectively in motor neurons. To achieve this, I designed a workflow consisting of an *in vitro* and *in vivo* pipeline, in close collaboration with Emily Melisa Ullrich Gavilanes, as well as with the help of Arek Kendirli, Clara de la Rosa del Val and Almir Aljovic in our lab.

### IV.2.1. Step 1: *In vitro* CRISPR Editing Pipeline

As described previously in the introduction, the target gene for Cas9-cleavage and subsequent KO can be specified by modifying the sequence of the gRNA to match the desired gene sequence to be cleaved. In spite of this apparent simplicity, the editing efficiency by Cas9 greatly varies depending on the exact location within the target gene sequence it binds to. To determine which gRNA's result in effective cleavage of the genome, I adapted a workflow established in our lab by Arek Kendirli and Clara de la Rosa del Val. The system uses haematopoietic progenitor cells from the bone marrow of transgenic Cas9-expressing mice, which are conditionally immortalized through the insertion of an oestrogen-regulated form of the transcription factor Hoxb8 (Cas9-HoxB8 cells) (Redecke et al., 2013). For each target gene, at least 3 gRNAs were tested through retroviral transfection of Cas9-HoxB8 cells. With the help of a fluorescent marker, successfully infected cells were isolated by FACS. After DNA extraction, the cleaving efficiency was determined through frameshift analysis (Fig. 16, A). As a proof-of-concept experiment,

Emily Melisa Ullrich Gavilanes and I targeted the Ifngr1 gene. Using "Tracking of Indels by Decomposition" (TIDE) analysis of the genomic DNA, we found a 69% editing efficiency of the *Ifngr1* gene (Fig. 16, C and D), which lead to a decrease of IFNGR1 on a protein level measured through FACS (Fig. 16, E).

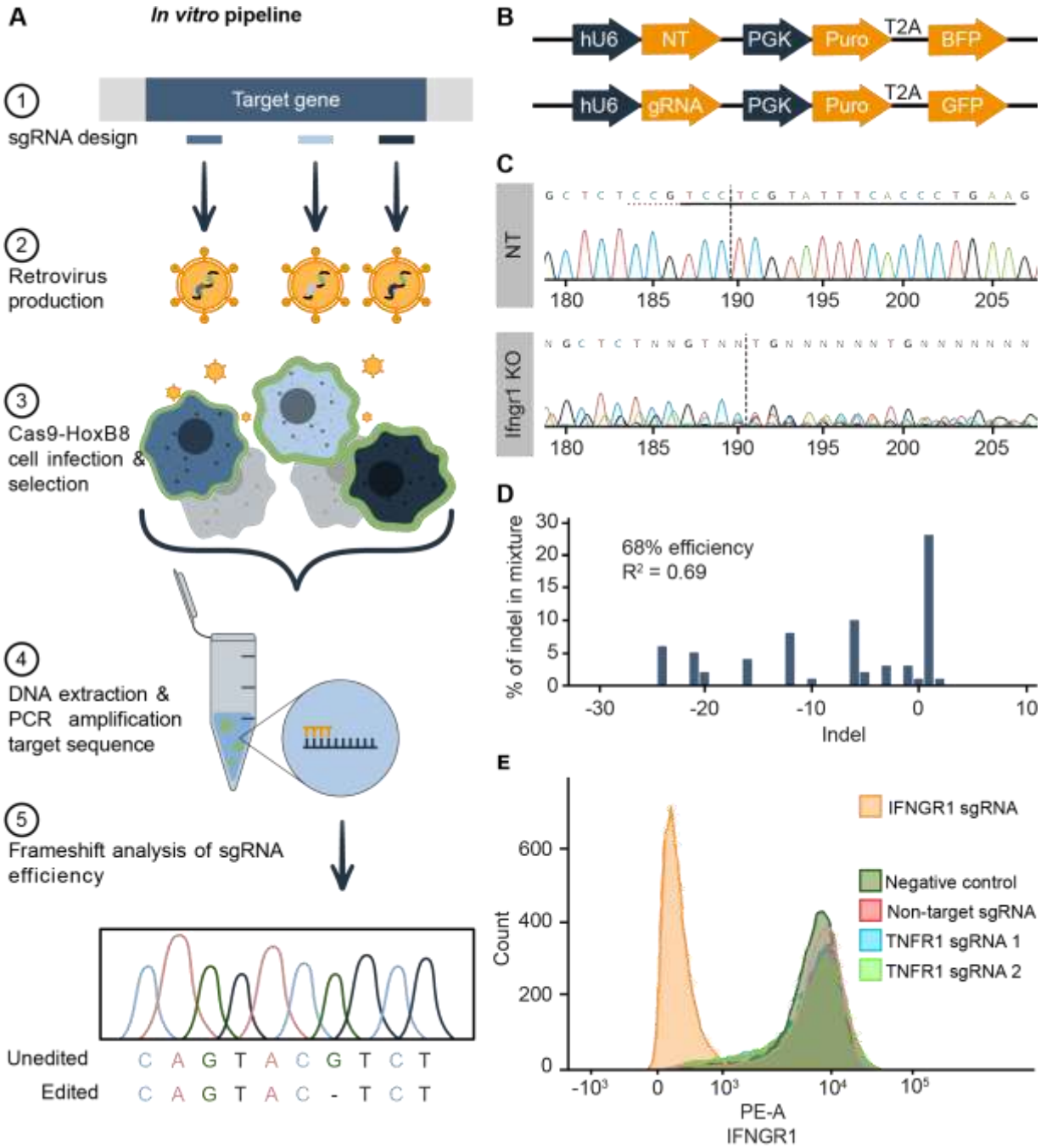



**Figure 16 | *In vitro* validation of efficient gRNAs. (A)** *In vitro* pipeline for screening of gRNA's leading to efficient CRISPR-editing. Several gRNAs are designed for each target gene and transfected into Cas9-HoxB8 cells using retroviral vectors. Cells containing the fluorescent markers, indicating successful uptake of the viral DNA, are selected via FACS. Their DNA is extracted and the target region is amplified through PCR. The DNA is sequenced and analysed using TIDE for frameshift detection. **(B)** Retroviral expression cassette for non-targeted (NT) or targeted gRNA(gRNA) delivery. NT plasmids contain a BFP fluorescent marker, plasmids used with gRNAs for target

proteins carry a GFP sequence. **(C)** Frameshift analysis of genomic DNA from Cas9-HoxB8 cells exposed to retroviruses containing NT or Ifngr1 gRNAs. **(D)** TIDE analysis results comparing NT and Ifngr1-edited DNA. Cells treated with Ifngr1 gRNA retroviruses show a 68% editing efficiency. **(E)** FACS histogram of IFNGR1 protein expression level of cells transduced with Ifngr1 gRNA (orange) compared to NT gRNA (red), or gRNAs targeted towards a different gene ( *Tnfra* gene: blue and light green), as well as a negative control (WT mice, dark green). Figure created by Adinda Wens, all data collected in colaboration with Emily Melisa Ullrich Gavilanes.

### IV.2.2. Step 2: *In vivo* CRISPR Editing Pipeline

In order to insert the selected gRNAs from the previous step selectively into neurons in vivo, Emily Ullrich and I leveraged a tool brought to the introduced at our institute by Almir Aljovic: AAV-PHP.eB viral vectors (Chan et al., 2017; Deverman et al., 2016). These viruses are known to effectively cross the BBB upon intravenous systemic injection, in contrast to many other AAV serotypes which have to be administered intraparenchymally. They also exhibit a strong neuronal tropism, outperforming other AAV serotypes. Indeed, when the AAV-PHP.eB was administered via tail vain injection, infection of neurons within both the brain and spinal cord was obtained (spinal cord: Fig. 17, C, brain: data not shown).

To demonstrate genome editing *in vivo* in adult mice, we infected LSL-Cas9-EGFP mice with AAV-PHP.eB viruses expressing Cre, the selected *Ifngr1* gRNA and nuclear targeted EGFP (Fig 17, B). The nuclear localization of EGFP allows for selection of infected neurons by FACS and determination of editing efficiency (Fig. 17, C, right panel). After incubation of 3 weeks, we performed TIDE analysis on the DNA of infected neuronal nuclei. This analysis revealed an editing efficiency of 83% in the brain and 87% in the spinal cord (Fig. 17, D), superseding *in vitro* efficiency (Fig. 16, D). This approach offers a technique for manipulating neurons in adult mice without the need for local administration, ensuring that the observed effects result from protein knockout in the EAE environment rather than inflammation due to surgical interventions.

So far, we have measured CRISPR editing efficiency on the genomic level. In further experiments using the principles of this workflow, we further validated the extent of the *in vivo* editing at different expression levels. In an experiment attempting to knock out the lysosomal pre-protein Prosaposin (PSAP), we obtained a KO of 95% efficiency in the brain on the genomic level (Fig. 18, B). When isolating the mRNA and running bulk RNA sequencing on the samples, she also found a significant reduction of PSAP on the mRNA level (Fig. 18, C). Subsequently, as another proof-of-concept experiment, we knocked out the neuronal nuclear protein, NeuN. We applied AAV-PHP.eB viruses via intracortical injections containing a NeuN-specific gRNA and an mCherry fluorescent marker (Fig. 18, D). As before, we observed adequate editing efficiency of 86,5% in the brain DNA (Fig. 18, E). After sorting

of mCherry positive nuclei from these mice, we demonstrated a reduction of NeuN on the protein level via FACS (Fig. 18, F). Another part of the tissue was prepared for IHC staining with NeuN antibodies, which also showed a lack of NeuN in the mCherry-positive cells (Fig. 18, G). Lastly, using the same Ifngr1-gRNA as before, we performed an experiment to gauge the effect of IFNGR1 KO on spine loss within the cortical MS model (c-MS). Upon juxtaposed injection of NT- and Ifngr1-gRNA, we observed that animals with Ifngr1 KO neurons had more spines than unedited neurons in the c-MS model, suggesting a protective effect of Ifngr1 KO in synaptic loss (Fig. 18, J).

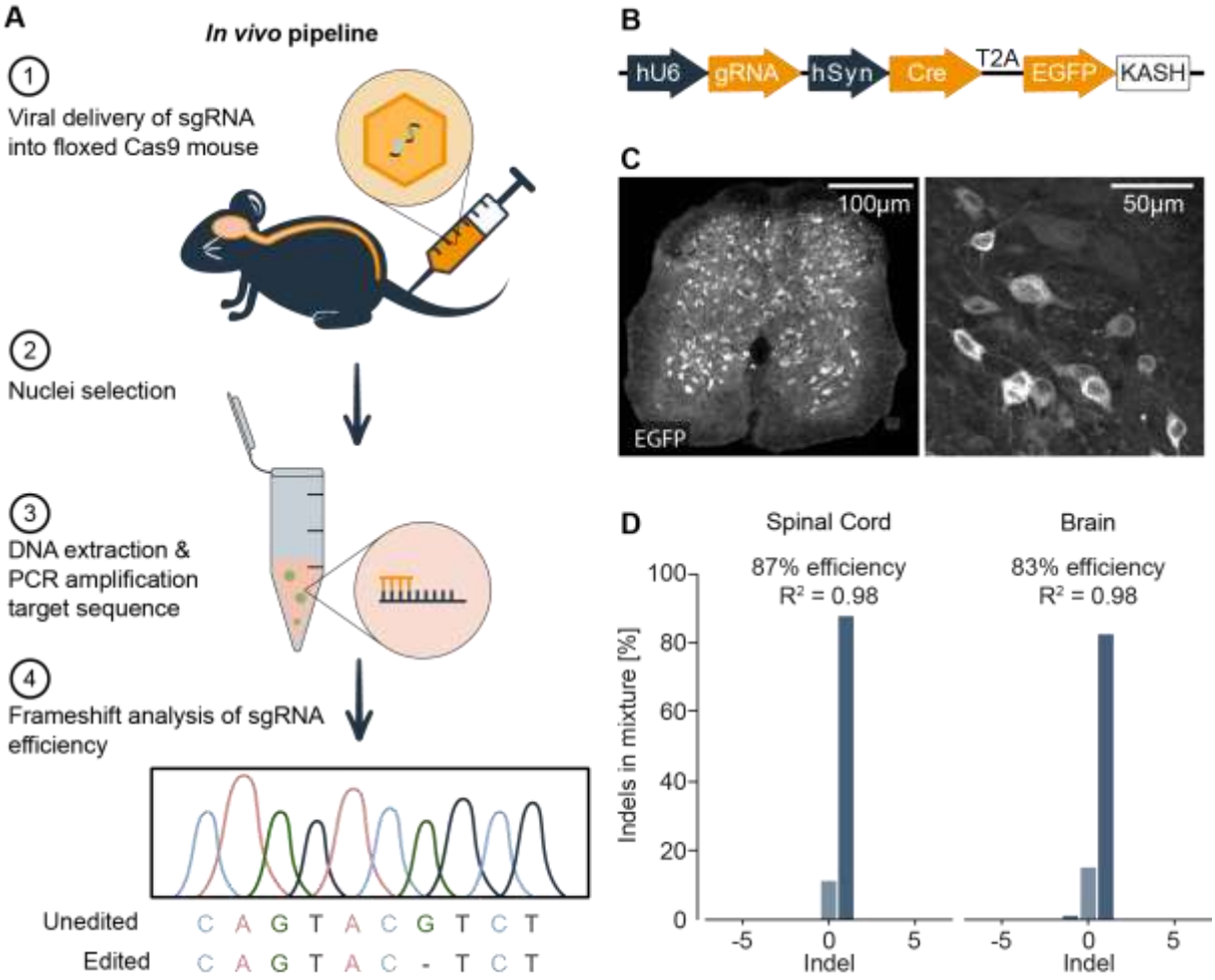
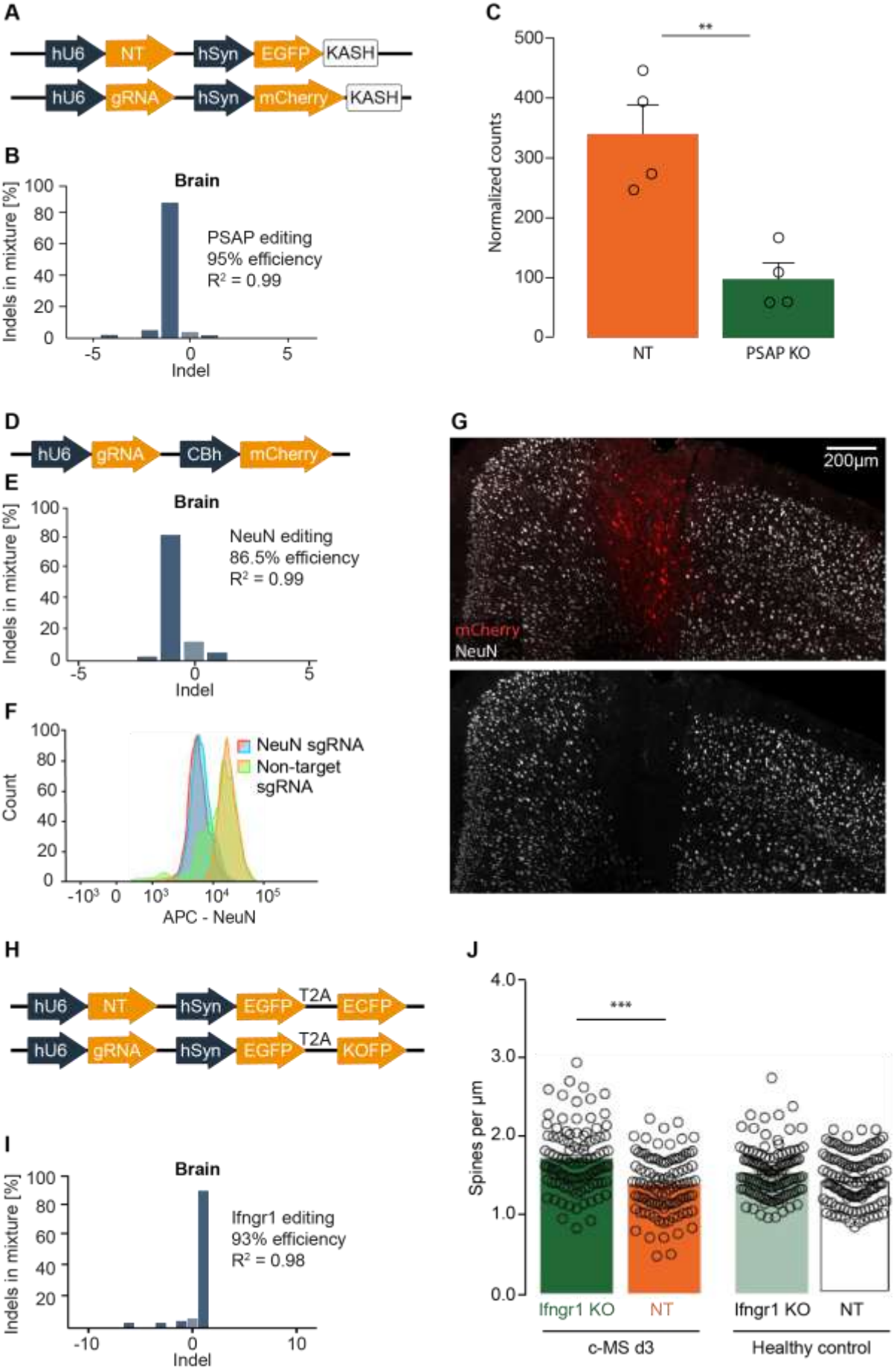



**Figure 17 | *In vivo* workflow for editing of adult neurons using CRISPR/Cas9. (A)** *In vivo* pipeline for CRISPR-editing of neurons in adult mice. Selected gRNAs identified *in vitro* are inserted in AAV-PHP.eB viral vectors and administered via tail vein injection. Cells expressing EGFP on the nuclear membrane are selected via FACS. Their DNA is extracted and sequenced for TIDE frameshift analysis. **(B)** AAV-PHP.eB expression cassette for gRNA delivery in floxed Cas9 mice containing a Cre sequence under the neuronal hSyn promoter, together with a nuclear EGFP. **(C)** EGFP expression in the lumbar spinal cord. Close-up of neurons in the spinal cord showing cytoplasmic- and nuclear membrane-specific EGFP expression after viral infection (right). **(D)** Editing efficiency of Ifngr1-gRNA in the spinal cord and brain after systemic application. Figure created by Adinda Wens, all data collected in collaboration with Emily Melisa Ullrich Gavilanes.

Altogether, these results provide evidence that (i) gRNAs can be delivered to neurons via non-invasive intravenous injections of AAV-PHP.eB virus, (ii) that Cas9 is capable of creating targeted indels in adult neurons and (iii) these indels result in decreased RNA and protein levels of targeted proteins and have structural effects. Thus, our pipeline is validated as capable of rapid and simple generation of knockouts in adult mice.

∇ **Figure 18 | Validation of *in vivo* CRISPR/Cas9 editing of adult neurons on the RNA, protein and structural level. (A, D and H)** AAV-PHP.eB expression cassettes used for KO experiments. All cassettes contain a gRNA placeholder sequence following the transcriptional hU6 promoter. Several fluorescent marker proteins are added to the constructs, depending on the needs of the experiments. **(B, E, I)** Indel efficiency analysis (TIDE) of the different knockouts. In all experiments, an editing efficiency of over 85% is achieved. **(C)** Measurement of PSAP mRNA levels from bulk RNA sequencing. (mRNA normalized count quantification, NT n = 4, PSAP KO n = 4; mean ± sem; unpaired Students t-test, $p < 0{,}01$). **(F)** FACS plot of nuclei transduced with NeuN gRNA (red and blue) and NT gRNA (orange and green) based on APC-NeuN expression (NeuN KO n = 2, NT n = 2). **(G)** Confocal representative image of NeuN gRNA infected cortical neurons (mCherry). NeuN staining (white) and mCherry infected neurons (red, upper panel). Apparent lack of NeuN signal in the area of mCherry positive neurons (lower panel). **(J)** Quantification of spine density of cortical neurons in c-MS d3 (left) and healthy control (right) mice. Neurons infected with Ifngr1 gRNA retain more spines during the acute phase of c-MS (Ifngr1 gRNA n = 100 dendrites, NT gRNA n = 100 dendrites in n = 10 c-MS d3 mice; Ifngr1 gRNA n = 120 dendrites, NT gRNA n = 120 dendrites in n = 12 healthy control mice; mean ± sem; Kruskal-Wallis test followed by Dunn's multiple comparisons test, $p < 0.001$). Figure created by Adinda Wens, includes data from Emily Melisa Ullrich Gavilanes (panel B and J) and Veronika Pfaffenstaller (panel C).


A
hU6
NT
hSyn
EGFP
KASH
hU6
gRNA
hSyn
mCherry
KASH
B
Brain
Indels in mixture [%]
PSAP editing
95% efficiency
R² = 0.99
Indel
C
Normalized counts
**
NT
PSAP KO
D
hU6
gRNA
CBh
mCherry
E
Brain
Indels in mixture [%]
NeuN editing
86.5% efficiency
R² = 0.99
Indel
F
Count
NeuN sgRNA
Non-target sgRNA
APC - NeuN
G
200µm
mCherry
NeuN
H
hU6
NT
hSyn
EGFP
T2A
ECFP
hU6
gRNA
hSyn
EGFP
T2A
KOFP
I
Brain
Indels in mixture [%]
Ifngr1 editing
93% efficiency
R² = 0.98
Indel
J
Spines per µm
***
Ifngr1 KO
NT
Ifngr1 KO
NT
c-MS d3
Healthy control

## IV.3. Role of Candidate Mediators in Dendritic Pathology

The final aim of this project was to investigate whether extrinsic signals within the EAE tissue can lead to dendritic damage via receptors present on the neuronal membrane.

In order to screen the effects of several receptors which might be implicated in causing the degeneration of motor neuron dendrites, I knocked out candidate receptors using the CRISPR editing pipeline described in the previous chapter. To obtain motor neuron specificity, I used the ChAT-Cre x LSL-Cas9-EGFP line (Fig 12, A). This way, only motor neurons, will express Cas9, rendering them susceptible to CRISPR editing. The gRNAs specific to DNA sequences within candidate genes are delivered via AAV-PHP.eB vectors, previously shown to be effective in infecting spinal cord motor neurons (Fig. 17, C). A Cre-dependent nuclear kusabira orange fluorescent protein (KOFP) sequence within the viral construct enables tracking of infection efficiency of motor neurons by the viral vector (Fig. 19, B). The EGFP and KOFP reporters serve as an indication which neurons produce Cas9 and contain gRNA's, respectively, both components necessary for successful CRISPR-editing.

As introduced in chapter I.1.4.2, due to the inflammatory reaction in MS and EAE, immune cells present within lesions can release various pro- and anti-inflammatory cytokines (Galli et al., 2019). Certain cytokines can alter glutamatergic transmission, hereby causing synaptic hyperexcitability (Stampanoni Bassi et al., 2017). As it is known that an imbalance or abundance of certain cytokines and glutamate can have neurotoxic effects, e.g. dendritic beading, I decided to knock-out the cytokine receptor IFNGR1, as well as manipulate the AMPA and NMDA glutamate receptors to assess their role in dendritic degeneration in the context of EAE lesions found in the murine spinal cord.

### IV.3.1. Interfering with the Interferon-Gamma Pathway in Motor Neurons does not improve Dendritic Integrity in aEAE

The interferon-gamma receptor is a multimer consisting of two IFNGR1 and two IFNGR2 chains, triggering intracellular responses via the Jak/STAT signalling pathway. To test the influence of IFN-γ on spinal cord motor neurons in EAE, I targeted the ligand-binding IFNGR1 chain of the receptor for CRISPR-editing to interfere with the signalling cascade.

A custom plasmid was designed and cloned in the lab for tracking motor neuron infection. A nuclear KOFP in between 2 lox sites (double-floxed inverted open-reading frame, DiO) enables expression of a fluorescent marker selectively in infected motor neurons after recombination by Cre, which itself is expressed selectively under the motor neuron specific ChAT-promoter. After recombination of the DNA, KOFP will be expressed under the hSyn

promotor, adding another layer of neuron-specificity for marker expression (Fig. 19, B). This system enables visualization of infection efficiency of the viral vector and delivery of the gRNA to the motor neurons. The most efficient IFNGR1-targeting gRNA that came out of the CRISPR-pipeline was cloned into the gRNA-backbone within the plasmid. A plasmid containing an gRNA sequence which was not complementary to any part in the DNA was used as a non-targeted control (NT).

The viral vectors containing either the IFNGR1-specific or NT gRNA-plasmid were administered systemically via the tail vein. This Ifngr1-gRNA has shown an 87% efficiency in inducing indels in spinal cord neurons (Fig. 17, D). A period of 3 weeks was allowed for IFNGR1 turnover, leading to a knock out of the protein, as well as expression of the KOFP reporter. Afterwards, mice were immunized and sacrificed at the peak of disease (Fig. 19, A).

Our experiment showed an infection efficiency of 81 ± 0.01 % of motor neurons in the control group (NT) and 87± 0.01% in the IFNGR1 KO group (Fig. 19, E). A high infection efficiency allows for the assumption that most motor neurons are edited and an effect of the KO should be apparent when quantifying the dendrites. The infection by neither of the viral vectors affected the neuronal numbers within the lumbar spinal cord (Fig. 19, D). This confirms that neither the infection by the virus, nor the KO of IFNGR1 leads to neurotoxicity.

To assess if the knock out of the IFNGR1 protein could prevent the dendritic damage observed in aEAE, Diego Ruiz and I quantified the dendritic density across the white matter of the upper and lower lumbar spinal cord based on MAP2 signal, similar to the previous experiments. Interestingly, the dendritic density showed no significant difference between the NT and INFGR1 KO mice at aEAE in any of the regions (Fig. 20, A and B). Because the MAP2 signal represents dendrites coming from all possible neuronal populations, and only motor neurons were edited, we also quantified the dendritic density based on the EGFP signal. This quantification exclusively shows dendrites originating from motor neurons. To our surprise, we also did not reveal any significant difference between the two conditions (Fig. 20, C and D). From this experiment, we assume there is no effect of IFN-γ on motor neuron dendrites via the IFNGR1 at the acute stages of EAE.

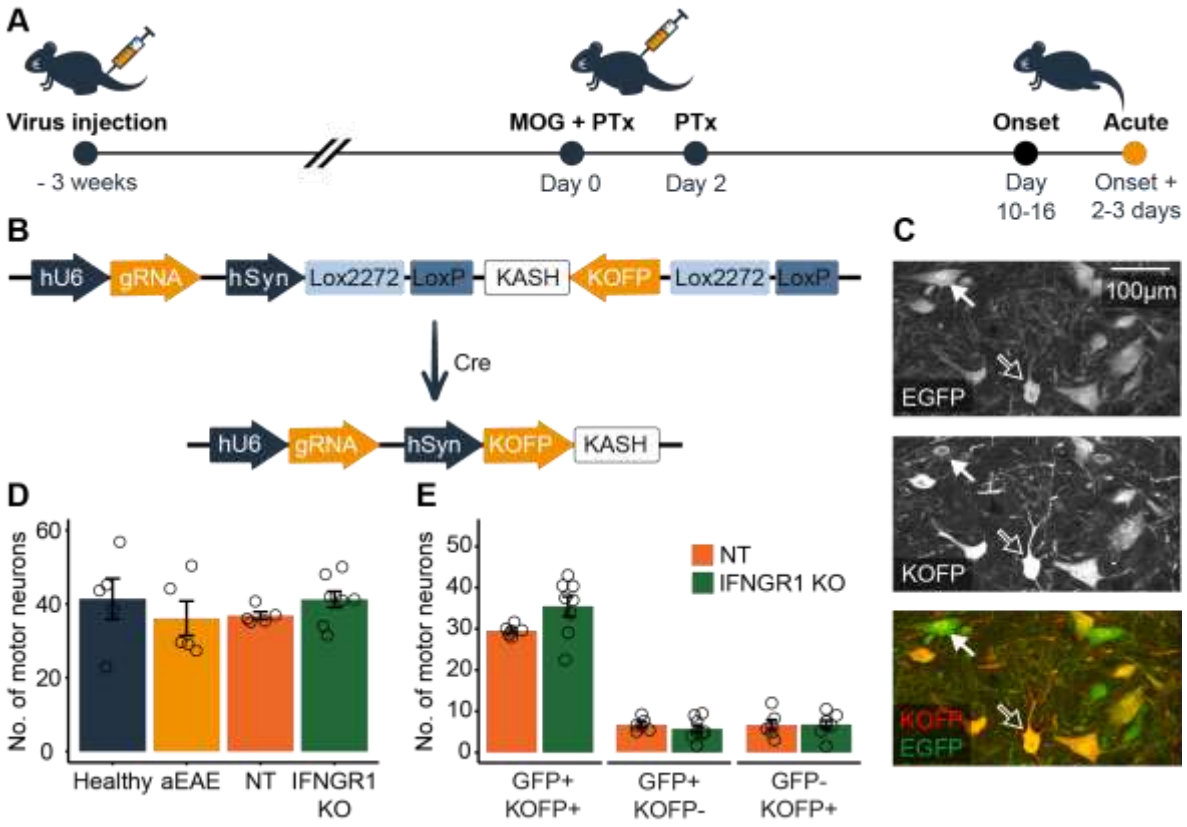
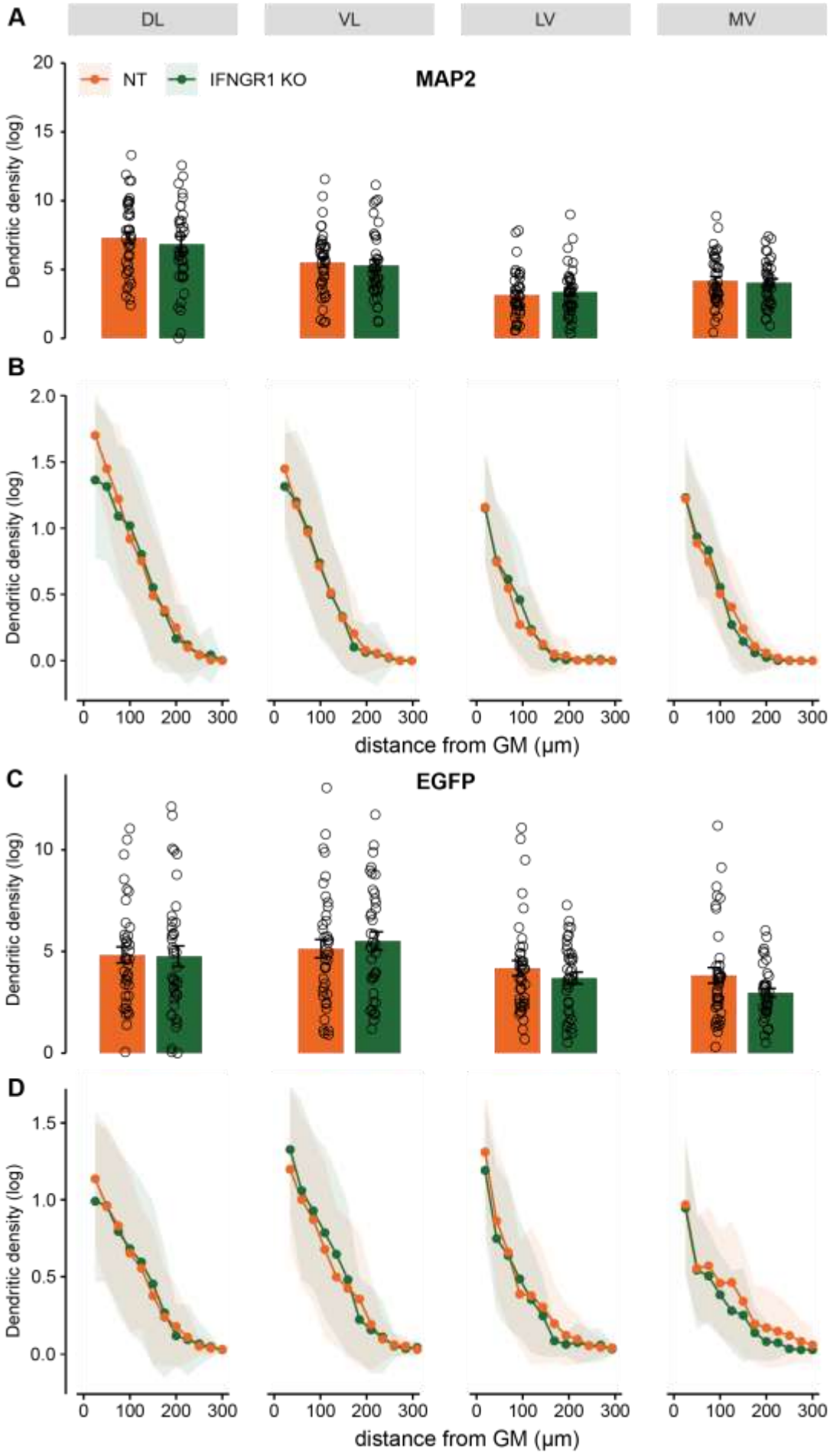



**Figure 19 | Validation of infection efficiency for KO of IFNGR1. (A)** Timeline of the *in vivo* CRISPR-editing in mice with EAE induction. **(B)** Viral construct for the delivery of gRNAs and Cre-dependent expression of nuclear KOFP. **(C)** Confocal representative image of EGFP positive motor neurons and KOFP positive infected cells. Full arrow: neuron with clear nuclear localization of KOFP, empty arrow: neuron where KOFP has leaked into the cytoplasm. **(D)** Mean number of motor neurons is not different between healthy, aEAE and CRISPR KO animals (Mean of 8 slices per mouse, healthy n = 5 mice, aEAE n = 5 mice, NT n = 5 mice, IFNGR1 KO n = 8 mice, mean ± sem, normality confirmed with Shapiro-Wilk test, ANOVA: ns. **(E)** Quantification of double and single positive neurons to assess infection efficiency using AAV-PHP.eB viral vectors (Mean of 8 slices per mouse, NT n = 5 mice, IFNGR1 KO n = 8 mice, mean ± sem, normality confirmed using Shapiro-Wilk test, Student's T-tests: ns). Figure created by Adinda Wens, includes data from Diego Ruiz (panel D and E).

∇ **Figure 20 | IFNGR1 KO does not rescue dendritic degeneration. (A, C)** Total dendritic density (log) per quantified area within different regions of the spinal cord based on MAP2 **(A)** and EGFP **(C)** signal (mean ± sem, normality checked with Shapiro-Wilk test, Student's T-Tests: ns). **(B, D)** Mean dendritic densities (log) in relation to the distance from the GM border based on MAP2 **(B)** and EGFP **(D)** signal (mean ± SD, normality checked with Shapiro-Wilk test, RM-ANOVA: ns). (A-D: NT n = 40 areas in 6 animals, IFNGR1 KO n = 38 areas in 7 animals). Abbreviations: DL = dorsolateral, VL = ventrolateral, LV = lateroventral, MV = medioventral. Figure created by Adinda Wens, all data was collected in collaboration with Diego Ruiz.


A
DL
VL
LV
MV
NT
IFNGR1 KO
MAP2
Dendritic density (log)
B
distance from GM (µm)
C
EGFP
D

### IV.3.2. Preventing NMDAR-dependent Calcium-Influx does not ameliorate the Dendritic Phenotype in aEAE

The N-methyl-D-aspartate receptor (NMDAR) is a heterotetramer, consisting of 2 mandatory GluN1 subunits, as well as additional GluN2 and/or GluN3 subunits, derived from distinct gene families *Grin1-Grin3*, respectively. The NMDAR is a glutamate-gated cation channel, predominantly permeable for $Ca^{2+}$. The receptor is known to regulate functional and structural synaptic plasticity as well as play an essential role in the pathophysiology of a variety of neurological diseases, including MS, via excitotoxicity. An overabundance of calcium-influx through the receptor could lead to dendritic beading and degeneration (Mandolesi et al., 2015).

In a similar fashion as the experiment described above, I wanted to assess the effects of NMDAR-dependant $Ca^{2+}$-influx on dendritic pathology in aEAE. Since aberrant calcium-influx has been shown to be harmful in axons, but no pores have been observed in dendrites (Fig. 15, E), I want to investigate whether different possible sources of calcium are implicated in the degeneration of dendrites.

As a further development to the system established before, I exchanged the hSyn promotor with a CMV promoter. The CMV promotor increases the expression level of the KOFP reporter, making quantification of double positive neurons more reliable (Fig. 21 A). The motor neuron specificity is furthermore warranted through the ChAT-dependent Cas9 and Cre expression within the transgenic mice, as well as the strong neuronal tropism of AAV-PHP.eB vectors. When checking the in vivo editing efficiency of the NR1-gRNA, I observed that 96% of the DNA isolated from infected spinal cord neurons contained indels (Fig, 21, B). The viral infection efficiency of motor neurons in the lumber spinal cord was 84,14 ± 0,04% and 90,0 ± 0,02% for mice receiving AAV-PHP.eB virus containing NR1- and NT-gRNA's, respectively (Fig. 21, C). Also here, because of the high infection efficiency, any effect of the KO should be observable in the dendritic quantification. When looking at overall motor neuron numbers between the conditions, it appears that the KO of NR1 could affect the survival of motor neurons in aEAE (Fig. 21, D). Importantly, it should be noted that the sample size of the group is too small to draw conclusions and more samples should be added to the experiment to make a conclusive statement.

When quantifying the dendritic density based on MAP2 (dendrites from all neurons) or EGFP (dendrites from motor neurons) signal, I did not observe a difference in dendritic density between the two conditions in most of the regions (Fig. 22, A-D). Significant changes are shown in the dorsolateral area, more specifically, a further loss of dendrites is observed in NR1 KO mice. This decline is probably attributable to the loss of motor neurons in these

animals. Although it seems that the prevention of NMDAR-dependent Ca2+-influx does not have an influence on the dendritic density or even aggravates the condition, it is important to increase the sample size of the experiment to increase the power of the study.

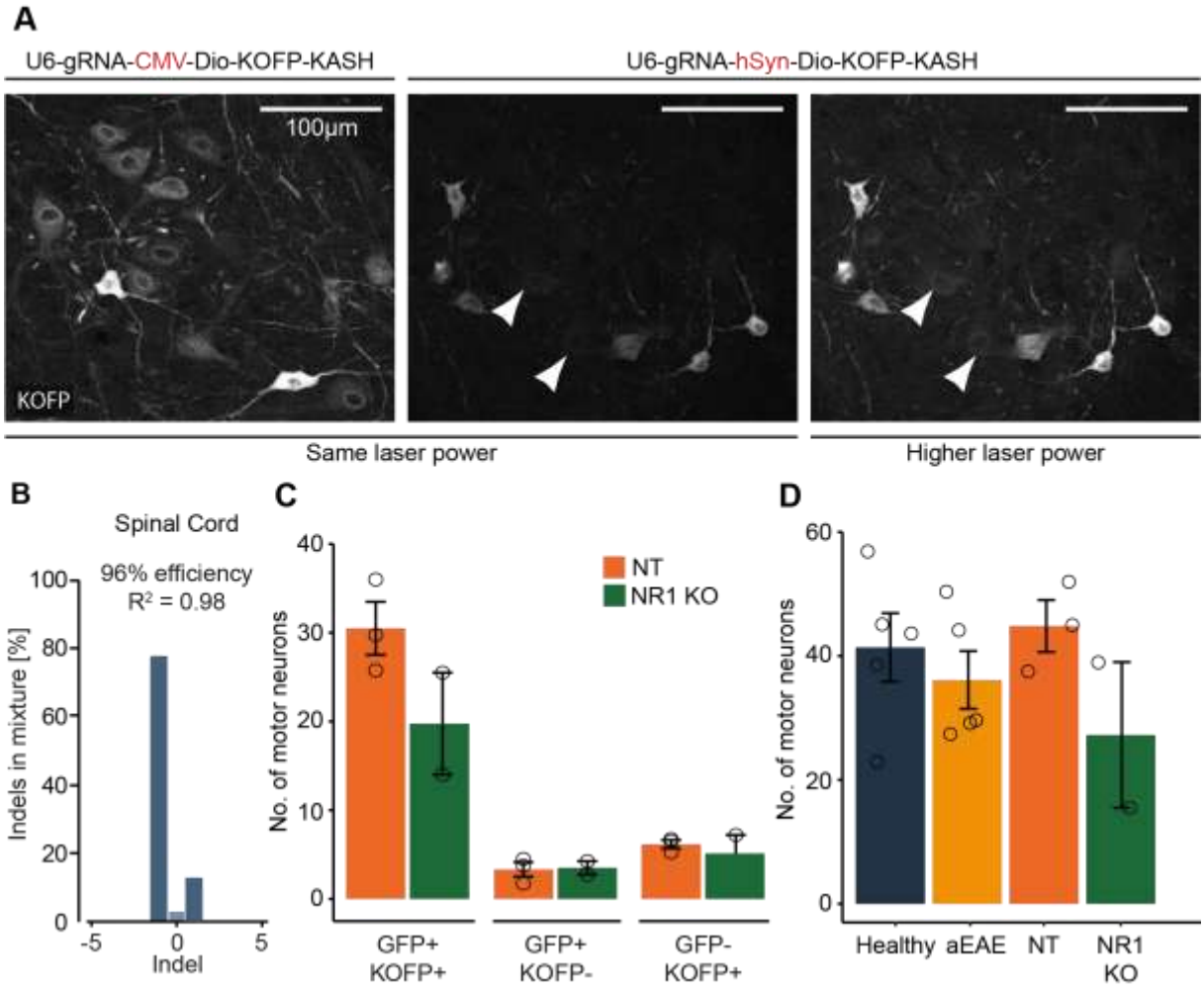
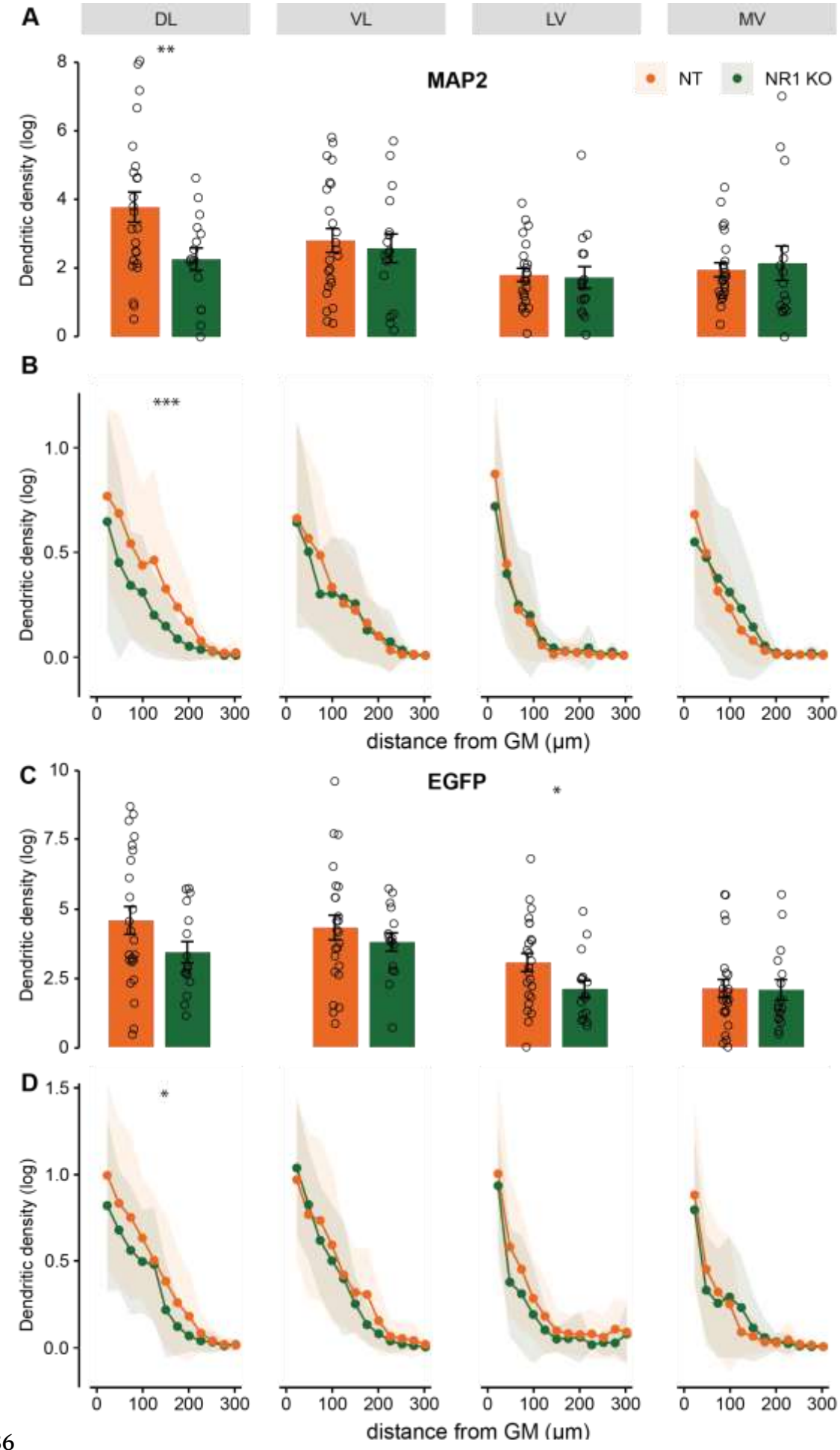



**Figure 21 | Validation of indel and infection efficiency for the manipulation of NR1. (A)** Changing the hSyn (right panels) for a CMV promotor (left panel) improves the expression of the KOFP reporter protein to facilitate identification of infected neurons. **(B)** Editing efficiency of the NR1-gRNA within neurons in the spinal cord using TIDE analysis. **(C)** Number of double and single positive motor neurons to assess infection efficiency of the virus (mean of 8 sections per animal, NT n = 3, NR1 KO n = 2, mean ± sem, normality checked with Shapiro-Wilk test, Student's T-tests: ns). **(D)** Amount of motor neurons per coronal section (10µm thickness) (Healthy n = 5, aEAE n = 5, NT n = 3, NR1 KO n= 2, mean ± sem, normality checked with Shapiro-Wilk test, ANOVA: ns). Figure created by Adinda Wens.


A
DL
VL
LV
MV
MAP2
NT
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**
Dendritic density (log)
B
***
distance from GM (µm)
C
EGFP
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D
*
distance from GM (µm)

**Figure 22 | NR1 KO may aggravate dendritic pathology. (A, C)** Total dendritic density (log) per quantified area within different regions of the spinal cord based on MAP2 **(A)** and EGFP **(C)** signal (mean ± sem, normality checked with Shapiro-Wilk test, Student's T-Tests: **p = 0,0083, *p = 0,038). **(B, D)** Mean dendritic densities (log) in relation to the distance from the GM border based on MAP2 **(B)** and EGFP **(D)** (mean ± SD, normality checked with Shapiro-Wilk test, RM-ANOVA: ***p = 0.0009, *p = 0.039). (A-D: NT n = 24 areas in 3 animals, NR1 KO n = 16 areas in 2 animals). Abbreviations: DL = dorsolateral, VL = ventrolateral, LV = lateroventral, MV = medioventral. Figure created by Adinda Wens.

### IV.3.3. Blocking $Ca^{2+}$ Influx via AMPAR does not prevent Dendritic Fragmentation

Another type of ionotropic glutamate channel that has been implicated in excitotoxicity in EAE is the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR). The AMPAR is a tetramer, consisting of different combinations of GluA1-4 proteins. The permeability of the channel is governed by the GluA2 subunit. Most of the GluA2 subunits undergo a posttranscriptional modification of glutamine (Q) to arginine (R). If the GluA2 subunit remains unedited (GluA2(Q)), the channel is permeable for $Na^+$, $Ca^{2+}$ and $K^+$. After editing of the mRNA, the GluA2 subunit will contain a positively charged arginine (GluA2(R)), which renders most of the AMPARs $Ca^{2+}$ impermeable.

In order to find out whether AMPAR-dependent $Ca^{2+}$-influx plays a role in dendritic pathology, I made use of the EGFP-dnGluA2 mouse line, established in the lab of Dwight E. Bergles (Khawaja et al., 2021). Through Cre-inducible overexpression of the edited GluA2, cell-selective calcium-impermeability can be achieved. By using the AAV-PHP.eB virus containing Cre recombinase under the hSyn promotor (Fig. 23, A), I can overexpress the GluA2(R)-subunit in this mouse line, thereby increasing their integration into the neuronal AMPA receptors in adult animals. The GluA2(R) subunit is fused to an extracellular EGFP, allowing for verification of expression (Fig. 23, E). Upon systemic tail vein injection of the virus, I observed that after 3 weeks, 99% of the motor neurons in the lumbar spinal cord were infected and expressed the GluA2(R) subunit (Fig. 23, C (right) and D). Upon injection of a virus lacking Cre, no EGFP signal was present (Fig 23, C (left)). From this, we can assume that an increased amount of AMPA receptors in virtually all motor neurons are now calcium impermeable. The mice were sacrificed at the peak of disease and dendrites were quantified as specified above (Fig. 19, A). The increase of GluA2(R) subunits into AMPARs seemingly did not change the number of motor neurons in the spinal cord ventral horns, again confirming the non-neurotoxicity nature of the intervention (Fig. 23, B). Surprisingly, interfering with the calcium influx through the AMPARs via GluA2 did not prevent the dendritic decay observed in aEAE in any of the regions (Fig. 23, F and G).

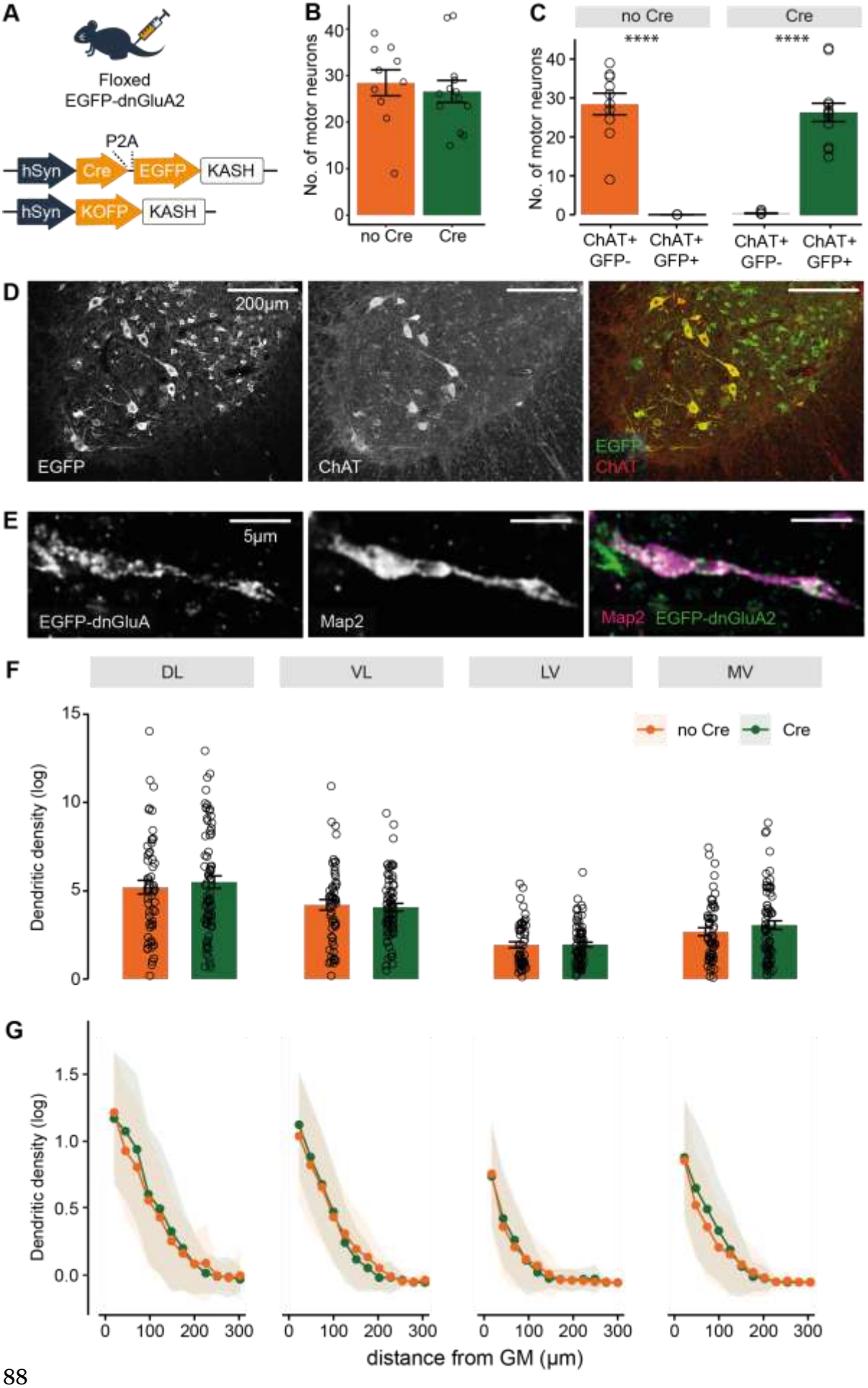
A
Floxed
EGFP-dnGluA2
P2A
hSyn
Cre
EGFP
KASH
hSyn
KOFP
KASH
B
No. of motor neurons
no Cre
Cre
C
no Cre
Cre
****
****
No. of motor neurons
ChAT+ GFP-
ChAT+ GFP+
ChAT+ GFP-
ChAT+ GFP+
D
200µm
EGFP
ChAT
EGFP
ChAT
E
5µm
EGFP-dnGluA
Map2
Map2
EGFP-dnGluA2
F
DL
VL
LV
MV
no Cre
Cre
Dendritic density (log)
G
Dendritic density (log)
distance from GM (µm)

**Figure 23 | Interfering with AMPA-dependent Ca2+-influx does not change dendritic damage. (A)** IV Injection of AAV-PHP.eB virus into the tail vein of floxed EGFP-dnGluA2 animals. Constructs used for the experiments contained (Cre) or lacked (no Cre) a Cre sequence, together with nuclear-localizing reporter proteins. **(B)** Comparison of number of motor neurons between animals that received Cre or no Cre viruses (mean ± sem, student's t-test: ns). **(C)** Quantification of infection efficiency of motor neurons by the viral vectors based on expressed EGFP and ChAT staining (mean ± sem). (B-C Mean of 8 sections per animal, no Cre n = 10, Cre n = 13). **(D)** Confocal representative image of neurons within the ventral horn of the lumbar spinal cord in a Cre-injected animal. **(E)** Confocal representative image of the expression of EGFP-tagged GluA2(R) subunits (green) on MAP2 dendrites (magenta). **(F)** Total dendritic density (log) per quantified area within different regions of the spinal cord based on MAP2 signal (mean ± sem, normality checked with Shapiro-Wilk test, Student's T-Tests: ns). **(G)** Mean dendritic densities (log) in relation to the distance from the GM border based on MAP2 signal (mean ± SD, normality checked with Shapiro-Wilk test, RM-ANOVA: ns). (F-G: no Cre n = 59 areas in 10 animals, Cre = 72 areas in 13 animals). Abbreviations: DL = dorsolateral, VL = ventrolateral, LV = lateroventral, MV = medioventral. Figure created by Adinda Wens.

# Chapter V - Discussion

MS is a multifaceted autoimmune and neurodegenerative disease characterized by inflammation, demyelination, and neurodegeneration within the CNS. While considerable research has focused on the white matter lesions of MS, the involvement of grey matter pathology has historically been understudied. This gap in research is significant, given that grey matter pathology has emerged as a crucial predictor of disease progression and severity (Fisniku et al., 2008). Damage to grey matter structures has been increasingly recognized as a determinant of the irreversible disability associated with MS (Schlaeger et al., 2014). Unlike white matter lesions, which are primarily composed of activated immune cells and demyelinated axons, grey matter lesions can form away from inflammatory hubs and directly affect neuronal integrity (Lassmann, 2018b). Therefore, understanding the mechanisms driving grey matter pathology is imperative for the development of effective therapies.

In the exploration of grey matter pathology in the spinal cord, I found that dendrites can extend into the white matter and that these dendrites appear beaded and fragmented. The primary objective of my study was to characterize this phenomenon and to elucidate the molecular mechanisms underlying this dendritic pathology within the grey matter of the spinal cord in the EAE model of MS. By focusing on dendrites, I aimed to uncover potential pathogenic pathways affecting neurons directly and find therapeutic targets for the direct treatment of neurons.

My research was driven by the hypothesis that specific extrinsic signals within the inflamed and demyelinated environment of the EAE spinal cord might lead to dendritic degeneration, as the dendrites within the grey matter appeared to be unaffected. This situation showcases an unusual mix of white and grey matter pathology, where neuronal components typically belonging to the grey matter are exposed to disease mechanisms characteristic of the white matter. By investigating these diffuse signals and their receptors on neurons, I aimed to identify pathways that could be targeted to preserve dendritic integrity and, by extension, neuronal function and health. This approach is particularly relevant as current MS therapies primarily target the immune system to reduce inflammation but fall short in addressing the neurodegenerative component of the disease, especially in its progressive stages.

In this discussion, I will interpret the findings of my study, considering the implications of dendritic pathology in grey matter for disease progression in MS. I will also explore the shortcomings of the current study and suggest potential improvements and further approaches to better understand and treat this aspect of the disease.

## V.1. Motor Neuron Survival in Spinal Cord Grey Matter

I concentrated on characterizing and editing motor neurons of the spinal cord because immunohistochemistry showed that most dendrites within the white matter are ChAT positive. This observation is consistent with findings in other species, including rats and cats, where retrograde labelling techniques were used (Rose & Richmond, 1981; Zhu et al., 2003). The literature on lower motor neuron loss in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) is contentious. Some studies have reported significant neuronal loss in MS patients, while others have found no such evidence. For instance, Petrova et al. reported widespread anterior horn neuron loss throughout the spinal cord in MS patients (Petrova et al., 2020). Vogt et al. provided further evidence, noting lumbar motor neuron loss in both MS patients and various EAE mice models (Vogt et al., 2009). In contrast, Gilmore and colleagues observed motor neuron loss in the cervical and thoracic regions of the spinal cord but did not find a similar loss in the lumbar region of MS patients (Christopher P. Gilmore et al., 2009). Adding to this, a study using THY1-YFP transgenic mice and TUNEL histochemistry reported no motor neuron loss in the lumbar spinal cord of EAE animals, despite the matching genetic background and EAE induction method as the aforementioned study (Bannerman et al., 2005). These conflicting results underscore the ongoing debate and highlight the necessity for further investigation to better understand the extent and nature of motor neuron loss in MS and EAE.

In this dissertation, my findings also indicate the absence of significant motor neuron loss in the lumbar spinal cord of EAE animals. These results have been further confirmed by additional immunohistochemical analysis, performed in our lab together with my colleague Marlena Helms. Following the hypothesis that the inflammation can disrupt certain staining methods, we explored if different results would be obtained by immunohistochemical analysis. So, additional to quantifying the motor neurons based on the EGFP signal coming from our ChAT-Cre x LSL-Cas9-EGFP animals, we stained the sections from aEAE animals with ChAT and NeuN antibodies. We observed that only 52% of $EGFP^+$ motor neurons were positive for ChAT. This was surprising as the ChAT-promoter was the driver of the EGFP-expression in the transgenic mouse line. When we compared ChAT or NeuN positive neurons between control and aEAE animals, we did not see a difference in motor neuron numbers (Supp. Fig. 1). These observations are consistent with the results from the EGFP-based quantification and some of the previous literature above, but stands in contrast to other reports. Notably, it has been suggested that motor neuron loss may be closely linked to the presence of local grey matter lesions (Christopher P. Gilmore et al., 2009). This could explain the discrepancies observed in various studies, as not all reports examine the

presence or extend of grey matter lesions. This hypothesis aligns with my findings, as the grey matter of the lumbar spinal cord in our EAE model is largely spared of lesions (93% only in white matter; unpublished, see also dissertation Stefan Syma) potentially accounting for the absence of motor neuron loss.

Apart from motor neuron numbers, I also assessed other variables such as motor neuron volume and synaptic density. Our results show that there is no difference in motor neuron volume between healthy and EAE mice, in alignment with what was previously observed in rats (Marques et al., 2006). While changes in motor neuron size can be used as an indicator of neuronal health, they are particularly challenging to interpret in the context of neuronal loss. Some evidence suggests that motor neuron size is increased in MS cases compared to controls, potentially due to pathological swelling of motor neurons (Christopher P. Gilmore et al., 2009). Conversely, Bannerman et al. found smaller motor neuron perikarya in the lumbar spinal cord at 98 days post-immunization, a much later time point than my own quantifications, which were conducted during the acute stages of EAE (Bannerman et al., 2005). They proposed that motor neuron perikaryal atrophy is unlikely to result from motor neuron axotomy but rather from a loss of necessary trophic input due to EAE-induced motor neuron dendritic pathology or chronic exposure to pro-inflammatory cytokines. This supports a similar hypothesis proposed in this thesis; that dendritic pathology might be a cause rather than a consequence of neuronal atrophy. Synaptic density was assessed via immunohistochemistry using a synapsin-1 antibody, a well-established presynaptic marker. revealed no loss of synapsin-1 puncta on the remaining dendrites, which is consistent with previous findings (Bellizzi et al., 2016). However, it has also been noted that in the EAE hippocampus, postsynaptic structures can be diminished while presynaptic structures remain intact (Bellizzi et al., 2016). Thus, in the context of this study, it would be valuable to also investigate postsynaptic markers. Although motor neuron dendrites in the white matter do not receive inputs on spines but rather directly on the dendritic shaft, it would be intriguing to explore whether dendritic degeneration begins at the sites of synaptic input.

Even though the chronic time point in this thesis did not reveal motor neuron loss, it was only 30 days post-immunization, which is short compared to the 98 days in Bannerman's study and may have been too brief to observe significant effects. Furthermore, even in studies where neuronal loss was observed, the magnitude of detected neuronal loss is proportionally lower than that of synaptic loss (Petrova et al., 2020). This suggests that dendritic pathology could indeed be a phenomenon that precedes neurodegeneration. Additionally, the transient nature of dendritic disruption might allow neurons to recover fully after replenishment, preventing cell death altogether.

## V.2. Transient Dendritic Pathology in the White Matter

In this study, I observed that there is no dendritic loss in the grey matter. This contrasts with the findings of Bannerman et al., who reported strong beading in the grey matter, albeit based solely on visual pattern classification without quantitative evaluation (Bannerman et al., 2005). My results align more closely with those of Zhu et al., who observed only a moderate dendritic loss in the grey matter of rats with EAE, but mainly sees a decline of dendrites in the white matter (Zhu et al., 2003).

The selective vulnerability of dendrites in white matter can be attributed to several factors. Research has shown that immune cell infiltration, marked by $CD45^+$ leukocytes, begins in the meninges and then progresses to the parenchyma (Shrestha et al., 2017). This "outside-in" pattern of infiltration suggests that inflammatory cells and soluble mediators are initially concentrated in the meninges and white matter of the spinal cord. Therefore, the dendrites in these regions are likely exposed to higher levels of inflammatory factors for a longer duration, making them more susceptible to damage. Since my analysis focused on dendrites in grey matter during the acute phase, it would be valuable to examine later time points to see if dendritic loss occurs as inflammation spreads further inward. Alternatively, the conductance on distal dendrites could be higher in white matter, making them more susceptible to excitotoxicity. This means that similar levels of excitotoxicity might cause more damage to dendrites within white matter compared to grey matter. Additionally, the grey matter may have a higher capacity to clear excess glutamate due to the presence of more synapses. Lastly, the distribution of receptors along dendrites varies depending on their proximity to the cell body. This spatial distribution of receptors could lead to selective injury depending on the insulting agent (Dodt et al., 1998).

A significant observation from this study is that despite the profound structural dendritic pathology found in the white matter of the spinal cord, these deficits seem largely reversible once the initial acute inflammation alleviates. During symptomatic recovery, dendritic density returns to healthy levels, indicating a capacity for endogenous neuronal repair, and in line with previous research performed with rats (Zhu et al., 2003). All animals used for dendritic analysis at chronic stages reached a motoric score ≥2,5 at peak of disease, suggesting that the chronic mice experienced at least the same disease severity as the mice analyzed at the acute timepoint. Another study in rats has revealed that synaptic covering on motor neurons is reduced during EAE exacerbation, while non-autoimmune inflammation and non-immune demyelination do not induce synaptic stripping, emphasizing the specificity of this phenomenon to EAE (Marques et al., 2006). They hypothesize it might not be a coincidence that the recovery of synapses and motor

symptoms coincide, as the process of remyelination takes much longer and would not be a sufficient explanation. We could broaden this hypothesis to encompass the recovery of dendrites, given that the majority of synaptic inputs are situated on these structures. Unlike the irreversible event in which an axon is transected, dendritic damage in white matter appears reversible to the degree of reaching healthy levels again (Kerschensteiner et al., 2004). Of course, this transiency stands in stark contrast to the persistent damage seen in human MS lesions. The discrepancy may be attributed to differences in duration of the inflammatory process. In the EAE model, acute inflammation peaks a few days post-immunization, followed by partial recovery, with most animals not relapsing within the 30-day experimental period. MS patients, however, endure multiple relapses over many years. The cumulative effect of age and subtle damage that exhausts repair or compensation mechanisms after each relapse might contribute to the permanent damage observed in MS. Therefore, it might be worthwhile to investigate dendritic damage in a mouse model with a relapsing-remitting disease course (e.g. EAE in SJL/J mice with PLP immunization)(Mattner et al., 2013). This underscores the importance of early intervention to prevent neurodegeneration and allow endogenous recovery mechanisms to counteract MS progression. Paralleling the dendritic pathology observed in the spinal cord, our lab has documented transient synaptic loss in a cortical EAE model in 8 - 14 week old mice (Jafari et al., 2021). When the cortical model was applied to middle-aged mice (10-12 months old), they exhibited a phenotype showing sustained spine loss for up to 84 days (unpublished, PhD thesis Emily Melisa Ullrich Gavilanes). It would be interesting to investigate whether the dendritic pathology in the spinal cords of middle-aged EAE mice exhibits similar persistence, thereby more closely mirroring the progressive pathology seen in MS patients.

The phenomenon of dendritic recovery itself presents an intriguing avenue for further study. While our current research focuses on preventing dendritic loss, investigating the mechanisms of dendritic repair could provide new therapeutic targets, particularly for the progressive stages of MS. Potentially, one could collect neuronal repair signatures through transcriptome sequencing of motor neurons in the spinal cord, akin to the work of Schattling et al. (Schattling et al., 2019). By comparing RNA profiles from healthy mice, mice at the peak of disease, and mice in remission, it would be possible to identify potential repair pathways. These pathways could be related to cytoskeletal remodelling or neurite growth, which are implicated in regrowth mechanisms observed in Drosophila (Thompson-Peer et al., 2016). By focusing on possible repair mechanisms, one could develop strategies to trigger repair processes even in progressive stages of MS. Identifying these pathways would not only enhance our understanding of neuronal recovery but also provide a foundation for developing treatments that promote repair and regeneration in MS patients.

## V.3. Advances of CRISPR Editing in (Motor)Neurons

Since its discovery, CRISPR/Cas9 technology has revolutionized genome editing, providing a quick and efficient means of modifying genetic sequences. In this thesis, we have leveraged this powerful tool to explore a role of specific proteins in dendritic pathology. Our CRISPR workflow comprises the following *in vitro* and *in vivo* steps:

In the *in vitro* phase, we employed Cas9-expressing HoxB8 cells to screen various gRNAs targeting different loci within the genes of interest. This step was crucial for identifying the most effective gRNA for inducing frameshift mutations. The choice of HoxB8 cells, derived from mice, offered several advantages. First, it ensured genomic compatibility with our mouse models. Furthermore, these cells are easy to maintain and manipulate in culture, providing a robust system for initial CRISPR screening. While one might argue that primary cultured neurons would be more relevant for our ultimate goal of neuronal CRISPR editing, HoxB8 cells presented fewer challenges in transfection and survival during spin infections. Additionally, neurons are non-proliferative, requiring frequent isolations from pups, whereas HoxB8 cells proliferate rapidly, ensuring a high turnover rate and reliable expression of gene knockouts across generations.

The *in vivo* component of our pipeline involved Cas9-expressing mice and the use of AAV-PHP.eB viral vectors for gRNA delivery. This approach circumvented the need for generating and / or acquiring knockout lines for each candidate gene. The use of AAV-PHP.eB virus (Chan et al., 2017; Deverman et al., 2016), with its strong neuronal tropism and efficient systemic delivery has eliminated the need for invasive surgeries, simplifying the procedure and minimizing confounding local inflammation or damage, thus ensuring that any observed effects were due to EAE rather than procedural artefacts. Additionally, the timing of knockout induction could be precisely controlled, as we performed the knockouts right before immunization, minimizing the impact of gene disruption during earlier stages of life.

To validate our system, we first performed a knockout of NeuN, a well-established target, confirming our methodology on the protein level with reliable antibodies (Platt et al., 2014). This project represents one of the first efforts to target genes beyond NeuN in adult motor neurons, an important step forward given the complexities of neuronal editing. Unlike other CRISPR studies that manipulate cell types *ex vivo* before reintroducing them into animals, work with animals at prenatal stages or manipulate cells other than neurons, our approach involved direct *in vivo* editing of adult motor neurons, a method that has proven particularly challenging (Gaj et al., 2017; Straub et al., 2014; Wheeler et al., 2023).

Our results demonstrated a high infection and editing rate of motor neurons, surpassing infection rates observed in other studies targeting the spinal cord (Chen et al., 2023). Recent advancements include the insertion of two or more gRNA sites into a single vector, which could further enhance the efficiency of gene disruption (Yin et al., 2023). The nuclear targeting of the reporter fluorescent protein (KOFP), was key for identifying infected neurons. I attempted to express a fluorescent protein within the cytoplasm to facilitate dendrite-specific quantification, but encountered challenges possibly due to the large size of motor neurons and insufficient fluorophore expression because of limited expression time. A significant limitation of our study is the lack of reliable antibodies for validating the knockout of the candidates at the protein level, which would provide the ultimate confirmation of our editing success. As an alternative, we relied on genomic indel tracking (TIDE) and complemented our findings with observed shifts in RNA expression from transcriptomic data, as well as IHC staining of alternative proteins and structural analysis (Fig. 18). The knockout of other proteins for which reliable antibodies exist could provide protein-level proof. Analogous to NeuN, targeting of an alternative nuclear protein could be checked by isolating neuronal nuclei and FACS analysis, which could provide a relatively fast and easy read-out. As a proof-of-concept, using conditional knockout mice for individual candidates could offer validation of the findings, although this would counter the advantages of the CRISPR technology.

Due to the time-consuming nature of developing the method and quantification strategies, only two candidates using the CRISPR technology were included in the thesis. Testing candidates individually necessitates numerous animals and prolonged time for tissue processing and quantification. Thus, streamlining the screening process to achieve higher throughput of candidate validation may expedite candidate identification. One potential alternative could involve assessing motor function, as we hypothesize that motor scores may correlate more closely with synaptic and dendritic damage, as well as recovery, rather than with de- and remyelination. However, even this approach remains a one-by-one candidate screening. It may be worthwhile to explore strategies that allow for multiple candidates to be tested simultaneously. While CRISPR screens in the CNS are emerging, they are primarily conducted in prenatal animals (Zheng et al., 2024). Future studies could leverage our systemic AAV-PHP.eB approach to deliver multiple viruses carrying gRNAs targeting different genes in adult mice. By adjusting the library titres, the risk of multiple gene perturbations within individual cells could be minimized. Nevertheless, identifying an appropriate readout for dendritic phenotypes remains a significant challenge. Motor score assessments would not be suitable in this approach, as the effect of neurons with each edit would probably be too diluted to detect. Additionally, tracing dendrites back to their

originating neurons and linking them to specific DNA edits is not feasible when the dendrites are fragmented. One could think of a viral fluorescent cell barcoding system to link neurons and their (fragmented) structures. Although virally labelling neurons with fluorescent proteins was attempted during the time of this thesis, it was unsuccessful – possibly because the time post-infection was insufficient to fully fill the large motor neurons with fluorescent proteins.

## V.4. Candidate Mechanisms mediating Dendritic Pathology

As shown in this thesis, the dendritic loss occurs at the peak of disease, and resolves during remission (Fig. 14). Other studies have shown at later time points that during relapse and recovery, MAP2 levels similarly drop and come back, respectively (Zhu et al., 2003). We established there was no loss of motor neurons, nor were there any somatic volume changes. We also observed that the spine density on remaining dendrites was unchanged, hinting that synaptic loss and deprivation of inputs would not precede or be the cause of dendritic loss. Lastly, we also did not observe an uptake of a 3kDa dextran, eliminating the hypothesis of uptake of extracellular calcium via nanoruptures, as seen as an important mechanism in axonal pathology (Witte et al., 2019).

Taking into account the neuroinflammatory pathology of MS and its models, the initial dendritic damage is possibly linked to inflammatory process or processes that are elicited through inflammation. Most of the white matter lesions observed in our EAE model develop subpially, corroborating with the pattern of outside-in immune cell infiltration from the meninges into the parenchyma (Shrestha et al., 2017), and paralleling the formation of cortical lesions in MS. In the cortex, meningeal inflammation results in diffuse neuronal and axonal loss, extending beyond the subpial lesions into the NAGM. As neuronal decline is also observed in the myelinated NAGM, the diffuse neuronal disease pattern is unlikely to be solely due to demyelination, highlighting the importance of soluble factors (Klaver et al., 2015). It has also been proposed that meningeal inflammation and the diffuse activation of $CD68^+$ macrophages/microglia are associated with diffuse axon loss in the spinal cord of MS patients (Androdias et al., 2010). This diffuse activation of microglia was replicated in the characterization of Iba1+ cells in the EAE spinal cord within this thesis (Fig. 10), possibly impacting not only axons but also dendrites. Furthermore, the work of Stefan Syma in our lab indicated an increased number of lymphocytes and macrophages in the EAE lesions during the acute disease phase (unpublished, see dissertation Stefan Syma). The temporal correlation of these immune cells with the attack and recovery phases aligns with the observed dendritic degeneration. In addition to immune-mediated damage, dendritic loss may also be driven by excitotoxicity (Greenwood et al., 2007). Elevated glutamate levels

have been detected in the CSF of MS patients, particularly during relapses when immune cell infiltration peaks (Sarchielli et al., 2003). Cytokines released by immune cells can provoke glutamate release from glia, impair its reuptake, and even regulate the expression of glutamate receptors (Bezzi et al., 2001; Viviani et al., 2003; Yu et al., 2002). This interaction illustrates how inflammation and excitotoxicity are closely interlinked, with their combined effects likely contributing to dendritic damage. Based on this interplay, I hypothesize that immune cell infiltration during active disease stages provides a source of cytokines and glutamate within the inflamed white matter, and that these soluble factors act on neuronal receptors, driving dendritic pathology. Given the plausibility of these neuroinflammatory mechanisms, I aimed to manipulate the receptors for these candidate mediators specifically within neurons to determine if blocking them could prevent dendritic beading in our model.

The selection of IFN-γ as a candidate was based on its prominent role in MS pathology and its well-documented effects in EAE. IFN-γ is particularly noteworthy due to its discrepant effects in MS and EAE, which have been a major point of contention and critique of the EAE model. Extensive evidence underscores the central role of $CD4^+$ T-helper cells in MS pathogenesis, particularly IFN-γ-producing TH1 cells and IL-17-producing TH17 cells, which are prevalent in active MS lesions within the CNS and are crucial for the development of EAE (Androdias et al., 2010). In MS, intravenous IFN-γ treatment in a clinical trial triggered relapses in a substantial number of participants (Panitch et al., 1987). Conversely, a non-placebo-controlled trial of an anti-IFN-γ antibody showed it suppressed MS, unlike an anti-TNF antibody, which did not (Skurkovich et al., 2001). In EAE, IFN-γ exhibits disease stage-specific effects. Treatment of mice with IFN-γ during the initiation phase of EAE exacerbates disease severity, while treatment during the effector phase attenuates the disease (Naves et al., 2013). These findings highlight the immunological role of IFN-γ in the relapsing-remitting stages of MS. However, they do not necessarily reflect the long-term effects on neurons, which might only become apparent during the more progressive stages of MS. This gap suggests that the role of IFN-γ in neuronal pathology and its potential contribution to dendritic changes in the chronic stages of MS warrants further investigation.

After exposing neurons in culture or cortical slice cultures to IFN-γ, researchers have observed specific dendritic degeneration without accompanying neuronal death. Notably, axons remained unaffected. Intriguingly, even when IFN-γ was applied solely to distal axons, retrograde dendritic degeneration was still observed (Kim et al., 2002). This suggests that the exposure causing dendritic degeneration does not need to be localized at the dendrite itself but could occur anywhere along the axon. However, this observation contradicts my findings in two significant ways. Firstly, I observed selective degeneration of dendrites in

the white matter, while dendrites from the same neurons in the grey matter appeared unaffected, making a retrograde degeneration unlikely (Fig. 11 and 14). Secondly, our CRISPR approach, designed to knock out IFNGR1 throughout the entire neuron – including axons – did not prevent dendritic degeneration, making retrograde degeneration via axonal pathway activation unlikely (Fig. 20). It is important to note that, without validation of the knockout at the protein level, we cannot be certain that enough IFNGR1 subunits were removed by the time of EAE onset. While we confirmed IFNGR1 knockout in HoxB8 cells at the protein level using FACS (Fig. 16), these cells are rapidly proliferating. Therefore, the progeny of edited cells would lack the receptor from the outset. In neurons, the turnover rate of membrane receptor proteins is presumed to be shorter than our incubation period, suggesting that the receptors should theoretically be absent as well (Sun & Schuman, 2022). However, due to the unsuitability of the FACS antibody for IHC staining of EAE tissue, we could not confirm the knockout in situ.

Returning to the link between inflammation and excitotoxicity, it has been proposed that IFN-γ-dependent dendritic beading operates through a complex involving the IFNGR and AMPAR. This interaction is associated with an increased $Ca^{2+}$-influx following IFN-γ exposure. Importantly, studies have shown that $Ca^{2+}$ chelators, as well as AMPAR antagonists such as NBQX and JSTx-3, can prevent the IFN-γ-induced dendritic beading (Mizuno et al., 2008). To assess the AMPA-dependent $Ca^{2+}$-influx in EAE, I performed an experiment using a different approach. Instead of blocking the AMPAR using antagonists, I used a transgenic mouse line together with AAV-PHP.eB viruses to achieve pan-neuronal overexpression of the GluA2(R) subunit, which renders the AMPAR $Ca^{2+}$ impermeable. Despite achieving overexpression in nearly all motor neurons, no change in the dendritic damage was observed at aEAE (Fig. 23). This was rather surprising, given that motor neurons are known for their low calcium buffering capacity and high levels of AMPARs on their membranes (Van Den Bosch et al., 2006). This makes motor neurons particularly vulnerable to AMPA-mediated excitotoxicity, whereas other neurons are thought to be more resistant to AMPAR overstimulation. The intentional proteolysis of glutamate receptors to prevent calcium-mediated damage is proposed to be a natural defence mechanism (Armada-Moreira et al., 2020). The reduction of glutamate receptors by motor neurons is suggested as a strategy to avoid excitotoxicity in EAE, allowing the cell to focus resources on survival and axonal recovery (Marques et al., 2006). Despite these expectations, my results suggest that dendritic beading might be driven by mechanisms other than AMPA-mediated $Ca^{2+}$-influx.

While there is substantial evidence pointing towards the involvement of AMPA receptors as the main glutamate receptor in EAE motor neuron pathology, evidence also suggests possible NMDA receptor involvement. Activated microglia-conditioned medium rapidly induced numerous beads in most neurites *in vitro*. Blocking NMDA receptors nearly completely abrogated mitochondrial dysfunction and neurotoxicity in these cultures (Takeuchi et al., 2005). This suggests that neuritic beading can be induced by activated microglia, which are present in MS and EAE lesions, and that it could occur through NMDA receptor signalling. Excitotoxic but sub-lethal NMDAR stimulation of neurons can cause spine loss, dendrite branch swelling, and dendrite shrinkage, in a calcium-dependent manner (Hoskison et al., 2007; Zhu et al., 2003). From cultured hippocampal neurons, we learned that NMDAR-dependent changes are closely related to F-actin and microtubule restructuring in spines and dendrites, respectively, while pre- and postsynaptic marker proteins are retained (Halpain et al., 1998). This suggests that the restructuring of the cytoskeleton due to NMDAR stimulation, rather than synapse loss, leads to dendritic disruption. This aligns with my findings in which the synaptic density on remaining dendrites in aEAE was not reduced (Fig. 15). However, despite these indications, the depletion of the NR1 subunit of the NMDAR via CRISPR-editing in my experiments did not prevent dendritic beading. As previously stated, verification of CRISPR editing on the protein level would be needed to confirm the (extend of) absence of NR1 subunits, however, I encountered challenges in identifying a suitable antibody for immunohistochemical staining of fixed EAE tissue.

Unfortunately, despite manipulating these individual receptors in this thesis, I did not observe any prevention of dendritic loss during the acute stage of EAE. This result is particularly surprising, considering the stress conditions that dendrites experience in EAE. Previous studies have demonstrated that dendrites can degenerate even under physiological levels of glutamate when neurons are under stress during chronic neuroinflammation (Bellizzi et al., 2016; Bellizzi et al., 2005). Perhaps manipulating other receptors could yield different results. IFN-γ, TNF, IL-17, and GM-CSF are among the prominent cytokine candidates released within the inflamed CNS of MS patients that cause tissue injury (Attfield et al., 2022). TNF-α induces rapid exocytosis of AMPA receptors in hippocampal cultures via TNFR1 receptors, particularly GluR2-lacking AMPA receptors, which are permeable to calcium (Stellwagen et al., 2005) and potentiates AMPA-induced excitotoxicity (Bernardino et al., 2005). Sequestering TNF-α after spinal cord injury using a soluble TNF-α receptor significantly reduced AMPAR trafficking and neuronal excitotoxicity in animal models (Ferguson et al., 2008). Additionally, the presence of TNF-α can stimulate glutamate release from microglia, increasing local glutamate concentrations

(Takeuchi et al., 2006). Unlike IFN-γ, TNF-α does not induce dendritic beading when administered in pure neuron cultures (Kim et al., 2002), indicating its direct neurotoxicity is relatively weak. Although TNF-α itself does not directly cause dendritic beading, these findings suggest it may regulate neuronal membrane protein composition and circuit homeostasis in ways that exacerbate excitotoxic damage. Thus, manipulating TNF-α receptors could provide interesting insights. The direct effects of other cytokines on neurons are less well-studied. It is known that certain neuronal populations in the brain and spinal cord express IL-17 and GM-CSF receptors (Luo et al., 2019; Reed et al., 2020; Schäbitz et al., 2008). However, conflicting results exist regarding their direct action on neurons (Donatien et al., 2018). IL-17 has been shown to potentiate NMDA-mediated EPSCs in spinal cord slices, although its direct effect on neurons may be mediated through interactions with MOG-specific Th17 cells rather than direct receptor activation (Luo et al., 2019). In demyelinating EAE lesions, this interaction has been observed to cause extensive axonal damage and fluctuations in neuronal intracellular $Ca^{2+}$ concentration (Siffrin et al., 2010). Finally, the activation of microglial cells via GM-CSF production by encephalitogenic T cells has been shown to be crucial for the induction of EAE (Ponomarev et al., 2007). In MS patients, GM-CSF is also known to exert pro-inflammatory effects on monocytes (Vogel et al., 2015). While its role in promoting inflammation in both MS and EAE is well-established, GM-CSF appears to have a neuroprotective effect on neurons, such as by countering programmed cell death (Schäbitz et al., 2008). Therefore, neuronal IL-17 and GM-CSF receptors do not currently appear to be promising targets.

Another consideration could be that manipulating single receptors may not be sufficient to produce a discernible result. For instance, recent studies with human neuronal stem cells derived from embryonic stem cells have highlighted synergistic effects of multiple cytokines such as IL-17, TNF, and IFN, which exacerbate disruption of neuritic integrity (Meyer-Arndt et al., 2023). Similarly, in motor neurons, AMPA-induced $Ca^{2+}$ influx appears to occur not only through $Ca^{2+}$-permeable AMPA receptors but also via voltage-gated $Ca^{2+}$ channels (Joshi et al., 2011). This suggests that calcium influx may not be completely halted by my current experimental approach. Therefore, it is plausible that multiple receptors are involved in causing the dendritic pathology, and that functional redundancies in these pathways may compensate for the effects of targeting single receptors, masking any observable outcomes.

To elucidate the role of calcium in the dendritic degeneration observed in motor neurons during EAE, it would be crucial to assess calcium levels within these neurons. Evaluating calcium involvement will clarify its role in dendritic beading during neuroinflammation and

assess the effectiveness of the receptor manipulations conducted in this thesis. However, conducting *in vivo* calcium imaging poses challenges, particularly in determining calcium fluxes in motor neurons due to their ventral location and limited accessibility for imaging.

Lastly, it is plausible that dendritic beading in EAE stems from mechanisms unrelated to direct ligand-receptor effects on neurons. This hypothesis will be further explored in the final discussion section.

## V.5. Mechanisms of Dendritic Damage independent of Ligand-Receptor Interactions

It is plausible that the loss of dendrites in the EAE spinal cord is not (solely) attributable to direct ligand/receptor effects on neurons, as hypothesized in this thesis. Therefore, I will explore alternative mechanisms that could potentially contribute to dendritic beading and degeneration. In the following discussion, I will critically examine these alternative mechanisms, evaluating the evidence both supporting and challenging their involvement in the specific dendritic pathology observed in this study.

It is well established that activated microglia play a crucial role in the pathological environment of MS and EAE. While my focus has been primarily on their capacity to release glutamate and secrete cytokines, another pivotal function of microglia is phagocytosis. To explore potential mechanisms underlying the primary spine loss observed in the cortex of MS patients, Jafari et al. employed the cortical EAE model and utilized *in vivo* imaging to demonstrate that microglia are responsible for synaptic removal (Jafari et al., 2021; Jürgens et al., 2015). Importantly, they found that microglia selectively target synapses exhibiting increased intracellular calcium levels. Given that I did not directly measure calcium levels following my receptor manipulations, it remains plausible that calcium levels were elevated during EAE in NMDAR- and AMPAR-manipulated neurons, thereby triggering microglial phagocytosis of dendritic structures. Although activated microglia were also detected in the spinal cord grey matter – where no loss of dendritic density was observed (Fig. 10) – I previously hypothesized that the spinal cord grey matter might possess greater buffering capacities than the white matter due to its higher synaptic density. It could allow for more efficient clearance of excessive glutamate and thus prevent calcium influx and microglial engulfment. Notably, while visual examination did not reveal MAP2 inclusions in microglia, proper investigation and quantification would be necessary to further affirm or rule out this possibility. In addition to MAP2, it would be worthwhile to investigate whether postsynaptic proteins, such as PSD-95, are present within microglial lysosomes.

Another mechanism through which neuronal structures may be tagged for elimination is via the complement system. Complement components have been found around white matter lesions and intracortical lesions in MS patients, and elevated levels have also been detected in normal-appearing grey and white matter compared to control tissue (Ingram et al., 2014). Specifically, C1qA RNA expression in neurons and C3a-R-bearing microglia have been identified in close proximity to cortical neurons (Watkins et al., 2016). Notably, neurons associated with these microglia exhibit altered nuclear morphology, which could indicate cellular stress or damage. The complement system can mediate synapse removal in the CNS, particularly via the C1q-C3 axis (Stevens et al., 2007). Pharmacological inhibition of C3 using Rosmarinic acid, as well as C3 knockout models, have been shown to prevent early dendritic loss, microglia-mediated synaptic phagocytosis in the dentate gyrus, and memory impairment in EAE mice (Bourel et al., 2021).

Lastly, the accumulation of toxic protein aggregates is a well-known factor in neuronal degeneration, as seen in classical neurodegenerative diseases, e.g. amyloid-β protein and tau in Alzheimer's disease, α-synuclein in Parkinson's disease, and huntingtin in Huntington's disease. Recent studies have indicated similar aberrant protein accumulation in MS and EAE, suggesting a disturbance in the protein homeostasis of neurons. For instance, protein aggregates composed of the presynaptic protein bassoon have been shown to directly contribute to neuronal injury in MS (Schattling et al., 2019). Similarly, the axon-specific mitochondrial anchor protein Syntaphilin (SNPH) accumulates in the soma and dendrites in the *shiverer* mouse model, which mimics progressive MS. This accumulation of SNPH in dendrites increases their vulnerability to excitotoxic insults and impairs mitochondrial recycling, making neurons more prone to dendritic shrinkage and eventual death (Joshi et al., 2019). These findings highlight the importance of protein homeostasis in maintaining neuronal integrity and suggest that protein aggregation may be a contributing factor to dendritic damage in EAE and MS.

## V.6. Outlook and Perspectives

In this thesis, I conducted a detailed morphological characterization of dendritic pathology in EAE, with a primary focus on the lumbar spinal cord. The findings highlight the acute vulnerability of dendrites, particularly within the white matter, where degeneration was observed, while dendrites in the grey matter remained unaffected. This raises intriguing questions about the selective susceptibility of dendrites in different regions. Could the proximity of white matter dendrites to inflammatory mediators, intrinsic properties of their structure or local buffering capacity of the environment explain this vulnerability? It

would be interesting to explore whether grey matter dendrites might also degenerate at later stages when inflammation has had more time to infiltrate the parenchyma.

No evidence of neuronal loss, reduced synaptic density, or dendritic nanoruptures was observed, suggesting that the decline in dendritic density is independent of such processes and may be driven by soluble mediators present in the inflamed environment. A particularly surprising finding was the transient nature of dendritic damage, with dendritic density recovering to baseline levels following the resolution of inflammation. It remains to be seen whether this regenerative capability persists through multiple relapses, or whether dendritic recovery becomes compromised over time. Therefore, assessing dendrites in middle-aged animals with EAE or an animal model which exhibits a more pronounced relapsing-remitting disease course(Mattner et al., 2013). could be used to investigate this further.

To investigate specific molecular pathways involved in dendritic degeneration, I developed an *in vivo* AAV-PHP.eB CRISPR/Cas9-based approach to target receptors of key inflammatory and excitotoxic mediators in neurons – the primary source of dendrites within the white matter of the lumbar spinal cord. While our gene-editing technique allowed for CRISPR-based knockouts at the DNA level, protein validation was limited due to the absence of suitable antibodies. This is a critical caveat, as we were unable to confirm (the extent of) the receptor knockouts. Using this CRISPR/Cas9 pipeline, I targeted key receptors such as the inflammatory cytokine receptor IFNGR and the glutamate receptor NMDAR. Despite these efforts, no significant protective effect on dendritic structure was observed, and the pathology remained consistent with that seen in unedited wild-type neurons. Also the blocking of AMPA-mediated calcium influx did not produce a change in dendritic density. This outcome raises further questions about the complexity of immune signalling in neuroinflammation. It is possible that blocking single cytokine or glutamate receptors may not be sufficient to observe a protective effect, as multiple pathways and redundant signalling mechanisms may obscure the impact of individual receptor manipulations. Additionally, it remains a possibility that dendritic degeneration is not mediated directly by cytokines or glutamate but rather through alternative mechanisms, such as the complement system, calcium dysregulation, or microglial engulfment. Future studies could investigate dendritic engulfment by assessing whether microglia phagocytose dendrites, for instance by measuring MAP2 engulfment. Next to classical histological assessment, techniques like the recently developed FEAST, which allows for the dissection of the lumbar grey matter and the isolation of microglia for FACS screening, could prove valuable in such an analysis (Dissing-Olesen et al., 2023). Additionally, calcium

imaging studies would be interesting to confirm the findings from the AMPA-dependent $Ca^{2+}$-blocking experiment and to further elucidate a role of calcium influx in the observed dendritic beading and degeneration.

This thesis lays the groundwork for a versatile gene-editing tool that could be applied to investigate a variety of neuronal pathologies in the CNS. Although the method was specifically developed to study mediators of dendritic pathology, it can also be adapted to explore other aspects of neuronal dysfunction, such as axonal or mitochondrial pathology. While I focused on motor neurons by using the ChAT-Cre line, this approach can be easily modified to target other neuronal populations by employing alternative cell-type-specific Cre lines. For instance, using the DAT-Cre line could specifically target dopaminergic neurons, which are particularly vulnerable in Parkinson's disease (Bäckman et al., 2006). The systemic administration and high infection efficiency of AAV-PHP.eB make this an especially powerful tool for studying neuronal populations without disturbing the local environment—an important consideration in neuroinflammatory diseases. If CRISPR editing can be confirmed at the protein level, the combination of CRISPR/Cas9 technology together with AAV-PHP.eB could provide an effective means of probing the genetic mechanisms underlying neuronal pathology in models of various neurological diseases.

A key question that remains is how dendritic pathology influences the onset and progression of disease in the EAE model. I hypothesized that dendritic damage might contribute to motor dysfunction and that the recovery of motor symptoms could be associated with dendritic restoration at the chronic stages. However, due to the lack of significant effects following receptor manipulations, I was unable to fully explore this relationship. It would be interesting to investigate whether protecting dendrites during the acute phase of EAE could potentially prevent the onset of paralysis.

In conclusion, the findings from this thesis highlight the complexity of dendritic degeneration in the EAE model of MS. Despite efforts to target individual cytokine and glutamate receptors, the expected prevention of dendritic loss was not realized. Future research should aim to explore a wider array of cytokines, the combined effects of multiple inflammatory mediators, calcium dysregulation, and the intricate interactions between neurons and immune cells. Such studies will be crucial for building a more comprehensive understanding of dendritic pathology. The technical advancements and insights presented in this work provide a valuable foundation for future molecular investigations into various aspects of neuronal pathology across a range of neurological diseases.

# Supplementary Figures

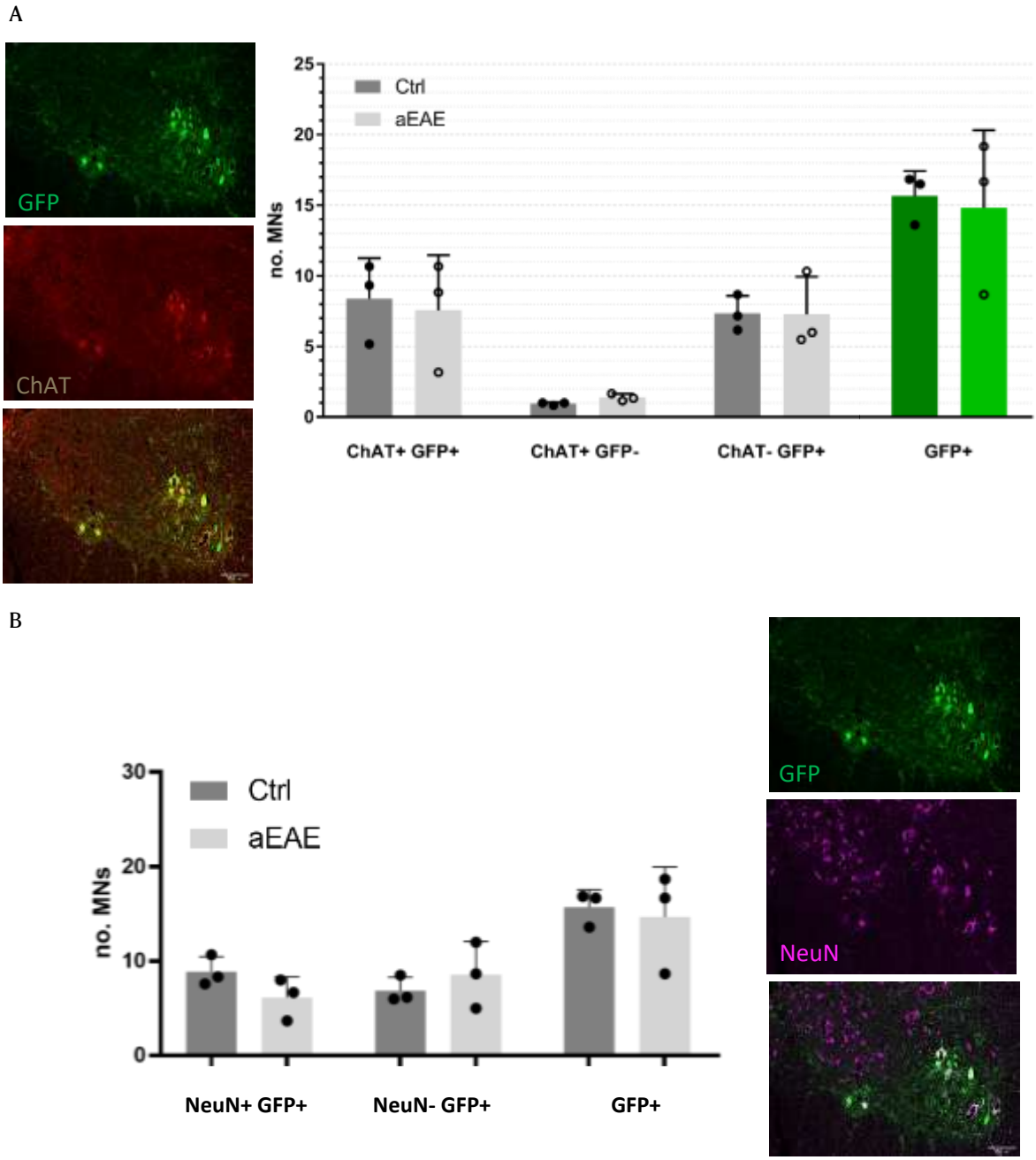


**Figure S1 | Validation of motor neuron numbers in healthy and aEAE animals using different staining methods.** **(A)** Quantification of motor neurons based in ChAT IHC staining. Confocal representative image of ChAT staining in ChAT-Cre x LSL-Cas9-EGFP animals (left). Number of motor neurons per ventral horn (right) **(B)** Quantification of motor neurons based on NeuN staining and morphology (left), with representative confocal images (right). (A-B) Healthy n = 3 mice, aEAE n = 3, mean ± sem, normality tested with Kruskal-Wallis test, Student's T-tests: ns. Figure created by Adinda Wens, all data was collected in collaboration with Marlena Helms.

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# List of Publications

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2. Mezydlo, A., Treiber, N., Ullrich Gavilanes, E. M., Eichenseer, K., Ancău, M., **Wens, A.**, Ares Carral, C., Schifferer, M., Snaidero, N., Misgeld, T., & Kerschensteiner, M. (2023). Remyelination by surviving oligodendrocytes is inefficient in the inflamed mammalian cortex. Neuron, 111(11), 1748–1759.e8. https://doi.org/10.1016/j.neuron.2023.03.031

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- Figure 7 | Dendrite and spine changes across various neurological disorders. (140-149)

**Figure 2 | The use of different MRI sequences to visualize MS lesions.**

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**Figure 5 | Variety in the architecture of neuronal dendritic trees.**

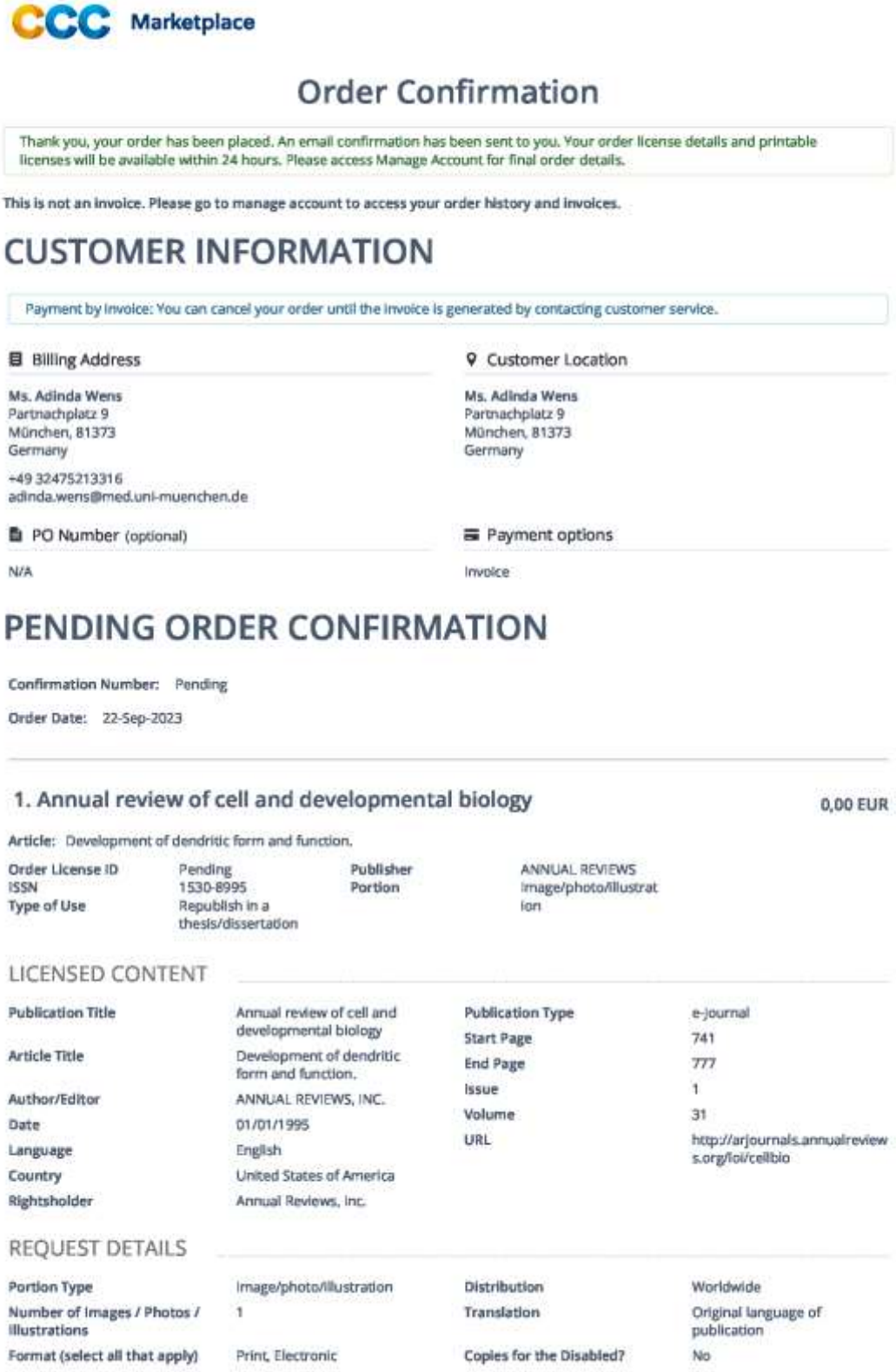

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**Figure 7 | Dendrite and spine changes across various neurological disorders.**

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# Author Contributions

Pilot studies for the histological characterization of dendritic degeneration were performed by Michael Keilholz and optimized by Adinda Wens.

- Michael Keilholz established the staining and imaging methods.
- Adinda Wens improved the staining protocols, automized quantification methods and established the analysis pipelines in R.

The quantification of motor neurons was done in part by Marlena Helms and Diego Ruiz, as part of their internships for the master programme, under supervision of Adinda Wens.

- Marlena Helms quantified the motor neurons in chronic EAE and control mice. She performed tissue processing, staining and imaging of relevant tissue.
- Diego Ruiz performed the quantification of motor neurons in acute EAE mice for the IFNGR1 CRISPR KO experiment. He performed tissue processing, staining and imaging of relevant tissue.
- Adinda Wens quantified motor neurons in all remaining experiments as well as tissue processing, staining and imaging, as well as EAE induction of all animals in these experiments.

Staining of (motor)neurons using different histological markers in chronic EAE tissue were part of the work for the internship in our lab by Marlena Helms.

- Marlena Helms performed the tissue processing, staining and imaging.
- Adinda Wens performed the immunization and provided supervision.

The quantification of dendritic density in the lumbar spinal cord is as well performed by Marlena Helms, Diego Ruiz and Adinda Wens.

- Marlena Helms quantified the dendrites in chronic EAE tissue.
- Diego Ruiz quantified the dendrites in acute EAE within the IFNGR1 CRISPR KO mice.
- Adinda Wens provided supervision, and performed quantification in all remaining experiments.

The experiments for measuring the uptake of cadaverin into dendrites were mainly performed by Michael Keilholz, under supervision of Dr. Paula Sanchez. They did all animal work, as well as post-processing of the tissue and imaging. Michael Keilholz performed all the quantification. Adinda Wens proofed the data and performed the statistical analyses.

The animal work, tissue processing and quantification of synapses on remaining dendrites were carried out by Michael Keilholz. Some of the data was later reanalysed by Adinda Wens and she performed all statistical analyses.

## Author Contributions

The establishment of the CRISPR/Cas9 pipeline was possible through the collaboration between Dr. Arek Kendirli, Dr. Almir Aljovic, Dr. Emily Melisa Ullrich Gavilanes, Clara de la Rosa del Val, Veronika Pfaffenstaller and Adinda Wens.

- Dr. Arek Kendirli assisted in the design and cloning of AAV-vectors used for *in vivo* CRISPR experiments. He performed and/or supervised the FACS sorting of gene edited nuclei. He introduced the use of Cas9-expressing HoxB8 cells for CRISPR editing in the lab and established the cell culture methods. Together with Clara de la Rosa, they pioneered the *in vitro* CRISPR pipeline using retroviral vectors.
- Dr. Almir Aljovic brought the materials and knowledge for generation of PHP.eB viruses to the lab. He generated the viruses necessary for the NeuN CRISPR/Cas9 KO.
- Dr. Emily Melisa Ullrich Gavilanes performed the surgeries and post-operational care for the generation of the NeuN, and PSAP CRISPR/Cas9 KO. She stained and imaged the brain tissue for verification of KO of NeuN on the protein level. Together with Adinda Wens, she established the CRISPR pipeline for genomic engineering of neurons in the CNS. They both shared the work for the TIDE analysis done in this thesis.
- Adinda Wens established the *in vitro* and *in vivo* CRISPR pipeline for the genomic engineering in motor neurons. She also aided in the generation of the NeuN CRISPR/Cas9 KO used for the verification of editing in neurons. Together with Diego Ruiz and Marlena Helms, she cloned all necessary plasmids for the KO of the target genes.
- Veronika Pfaffenstaller performed analysis on the RNA sequencing data of the PSAP knock out.

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