

Neuroinflammation causes changes to the nodes of Ranvier in Multiple Sclerosis normal-appearing white matter.

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A thesis submitted for the degree of Doctor of Philosophy of Imperial College London.

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Statement of originality

I hereby declare that the work presented in this thesis is entirely my own, except otherwise acknowledged. This work has not been submitted for any other qualifications and at any other universities.

Patricia Gallego Delgado

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Contributions

In chapter 4: *Axo-glial pathology in a chronic model of meningeal inflammation*, the surgery of the rats was carried out by Dr. James and Dr. Browne, and the BSc student Joanna Meng contributed to the immunostaining and collection of data. In chapter 6: *Inducing paranodal axo-glial junction pathology in cerebellar slices*, MSc student Swetha Umanshankar contributed to the rat microglial culture studies.

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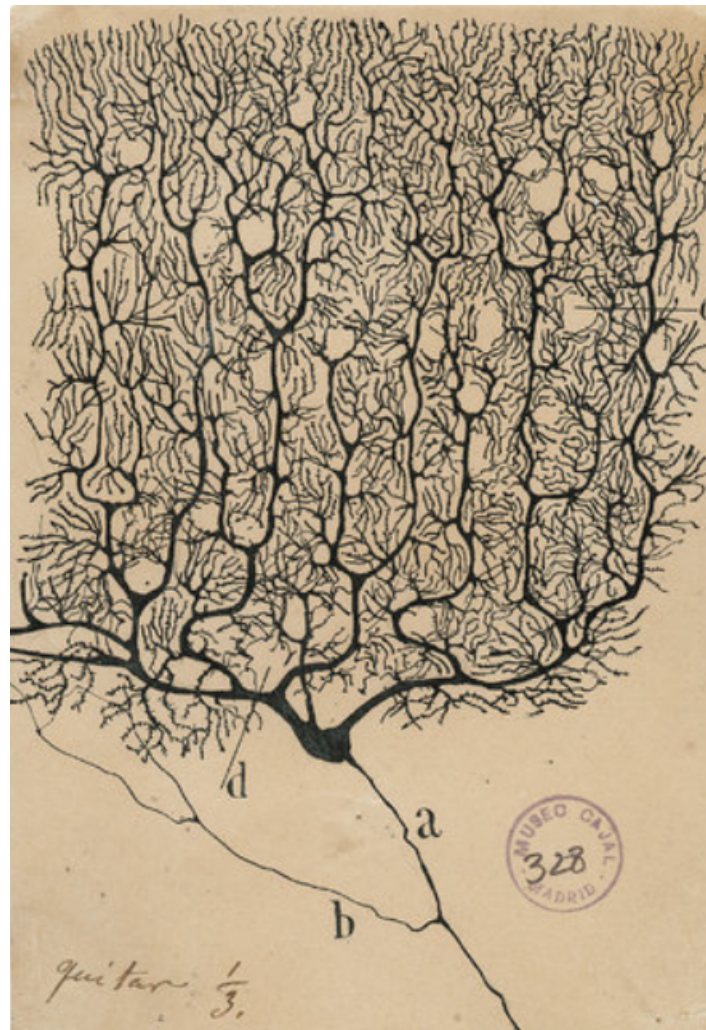
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Purkinje neuron drawing from a human cerebellum (Ramon y Cajal, 1899).



"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, gearings, even though such machinery also has its beauty. Neither do I believe that the spirit of adventure runs any risk of disappearing in our world. If I see anything vital around me, it is precisely that spirit of adventure, which seems indestructible and is akin to curiosity (Maria Skodowska-Curie, Nobel Prize winner in 1903 and 1911)"

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Abstract

Background: In addition to the focal demyelinating lesions in multiple sclerosis (MS), both imaging and neuropathological analyses have demonstrated the presence of diffuse pathology in both the white and grey matter, including changes to the structure of nodes of Ranvier in the normal-appearing white matter (NAWM). **Objective:** Study the structural and functional consequences of nodal pathology and the role of inflammation in them. **Methods:** We have examined the expression of the paranodal axonal protein Caspr1, the voltage-gated channels Na_v and $K_v1.2$ at nodes and juxtaparanodes respectively, and SMI32+ (dephosphorylated neurofilament) axons in NAWM areas from post-mortem progressive MS brains compared to controls. This axo-geometrical data on nodal changes was then integrated into a computational model of an axon developed with NEURON. To test our hypothesis, rats were injected into the cerebral subarachnoid space with lentiviral vectors for lymphotoxin- α and interferon- γ , and structural changes were examined 3 months later. Furthermore, a cerebellar tissue culture model was used to induce nodal pathology by the activation of microglia with TNF, interferon- γ , conditioned microglial medium and glutamate administration. **Results:** The paranodal domain in MS NAWM tissue was longer on average than in control and $K_v1.2$ channels appeared dislocated towards the paranode. These changes were associated with stressed axons and activation of microglia. When these changes were inserted into the computational model, a rapid decrease in velocity was observed as the paranodal peri-axonal space was increased, reaching conduction failure when the axons were less than $1\mu\text{m}$ of diameter. The same structural changes were observed in the corpus callosum of our rat model and were associated with microglia/astrocyte activation. TNF, interferon- γ , conditioned microglial medium and glutamate also generated paranodal elongation in the cerebellar cultures axons and was reversed by an NMDA blocker. **Conclusion:** Microglia

activated by pro-inflammatory cytokines may release high levels of glutamate, which triggers paranodal pathology in MS NAWM, contributing to axonal damage and subsequent conduction deficits.

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Abbreviations

AIS Axon Initial Segment

AMPA α -amino-3-hidroxi-5-metilo-4-isoxazolpropinico

AP Action Potential

APP Amyeloid Precursor Protein

ATP Adenosine Triphosphate

BBB Blood Brain Barrier

BMI Body Mass Index

C3 Complement 3

Ca²⁺ Calcium ions

CaMK Calmodulin dependent protein Kinase

CARS Coherent Anti-Stokes Raman Spectroscopy

Caspr1 Contactin Associated Protein

cDNA Complementary Deoxyribonucleic Acid

CFA Complete Freund's Adjuvant

CIPD Chronic Inflammatory Demyelinating Polyneuropathy

Cm Membrane capacitor

CMV Cytomegalovirus

CNS Central Nervous System

CSF Cerebrospinal Fluid

Cx Connexin

CyD Cyclophilin D

d_{core} Core Diameter

d_{fibre} Fibre Diameter

DA Dark Agouti

DIV Days In Vitro

DMEM Dulbecco's Modified Eagle

DMF Dimethylfumarate

DMT Disease Modifying Therapies

EAE Experimental Autoimmune Encephalomyelitis

EBV EpsteinBarr Virus

ECM Extracellular Matrix

EDSS Expanded Disability Status Scale

EEAT Excitatory Amino Acid Transporter

EM Electron Microscopy

FAD Focal Axonal Degeneration

FDA Food and Drug Administration

G Conductance

GBS Guillain-Barre syndrome

Gd Gadolinium

GFAP Glial Fibrillary Acidic Protein

GFP Green Fluorescent Protein

GLAST Glutamate Aspartate Transporter

GLT Glutamate Transporter

GM Grey Matter

GPI Glycan Phosphatidyl Inositol

GWAS Genome-Wide Association Studies

HBSS Hank's Balanced Salt Solution

HDAC1 Histone Deacetylase 1

HEK Human Embryonic Kidney

HH Hodgkin and Huxley

HLA Human Leukocyte Antigen

HPF High Pressure Freezing

HVEM Herpes Virus Entry Mediator

IFA Incomplete Freund's Adjuvant

IFN γ Interferon γ

Ig Immunoglobulin

IND Internode

JXP Juxtaparanode

K $_v$ Voltage-gated potassium channels

LT α Lymphotoxin α

LV Lenti-viral

MAG Myelin Associated Glycoprotein

MBP Myelin Basic Protein

MHCII Major Histocompatibility complex II

MK801 Dizocilpine

MLKL Mixed Lineage Kinase Domain Like protein

MNC Mononuclear Cells

MOG Myelin Oligodendrocyte Glycoprotein

MRI Magnetic Resonance Imaging

mRNA Messenger Ribonucleic Acid

MRS Magnetic Resonance Spectrometry

MS Multiple Sclerosis

MST Myelin Sheath Thickness

Na_v Voltage-gated sodium channels

NAA N-acetylaspartate

NAWM Normal Appearing White Matter

NBQX 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline

NF- κ B Nuclear Factor Kappa chain-enhancer of activated B cells

Nf155 Neurofascin 155

Nf186 Neurofascin 186

nl Number of Myelin Lamella

NMDA N-methyl-D-aspartate

NO Nitric Oxide

NOS Nitrogen Oxygen Species

NOs Nitric Oxide Synthase

OCT Optimal Cutting Temperature Compound

PBS Phosphate-Buffered Saline

PDL Poly-d-Lysine Hydrobromide

PFA Paraformaldehyde

PLP Proteolipid Protein

PMD Post-Mortem Delay

PML Progressive Multifocal Leukoencephalopathy

PNJ Paranodal Axoglial Junction

PNS Peripheral Nervous System

PPMS Primary Progressive Multiple Sclerosis

PRMS Progressive Relapsing Multiple Sclerosis

PTP Permeability Transition Pore

R Resistance

RGB Red Green Blue

RIPK Receptor-interacting serine/threonine-protein kinase

RNS Reactive Nitrogen Species

ROI Regions of Interest

ROS Reactive Oxygen Species

RRMS Relapsing-Remitting Multiple Sclerosis

SEM Standard Error of the Mean

SPMS Secondary Progressive Multiple Sclerosis

TLR Toll-like receptor

TNF Tumor Necrosis Factor

TNFR Tumor Necrosis Factor Receptor

TRADD Tumor Necrosis Factor Associated Death Domain

TRAF Tumor Necrosis Factor Receptor-Associated Factors

V_m Membrane potential

V_{max} Maximum velocity

WM White Matter

WT Wild Type

1

Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory, neurodegenerative, and demyelinating disease of the CNS (Dutta and Trapp, 2007). It is the most common neurological disability among young adults and it is estimated to affect around 2.2 million people worldwide (Wallin et al., 2019). Its main pathological features include demyelination, neuronal and axonal degeneration, focal and diffuse inflammation, gliosis and oligodendrocyte loss (Lassmann et al., 2007).

1.1.1 Epidemiology and Aetiology

The global prevalence of MS changes with latitude, sex and race/ethnicity. Prevalence of MS is significantly higher in countries of high latitude with an increase in up to 1.03 times per degree of latitude (Wallin et al., 2019). In children, the prevalence between boys and

1.1. Multiple Sclerosis

girls is similar, but by the fourth decade of life, the ratio is 2:1 in favour of women (Alonso and Hernán, 2008; Wallin et al., 2019). Although previous studies found that individuals of European descent have a higher probability of developing MS, compared to Asian, black, native Americans and Māori individuals (Rosati, 2001), later studies suggested that the black population had a larger incidence of MS, especially among women, compared to white, Hispanic and Asian population (Wallin et al., 2012; Langer-Gould et al., 2013). Initial studies about immigration suggested that immigrants who are under 15 years of age at migration from a low-risk country to a higher-risk were less likely to develop MS than those who migrate at an older age (Gale and Martyn, 1995; Hammond et al., 2000; McLeod et al., 2011). However, a recent study that followed a cohort of first and second-generation Danish individuals for 47 years demonstrated that first-generation immigrants who arrived in Denmark before the age of 15 had a higher likelihood of developing MS than their country of birth but lower than the ethnic Danes. Those who migrated after the age of 15 had a risk closer to their birth country (Munk Nielsen et al., 2019). These results emphasise the importance of environmental factors in childhood and adolescence, which seem linked to MS susceptibility.

The aetiology of MS is thought to involve a combination of genetic susceptibility and environmental exposure. The main environmental risk factors include exposure to EpsteinBarr virus (EBV), obesity during adolescence and childhood, vitamin D deficiency and cigarette smoking. Although more than 90% of the general population are EBV-positive, nearly 100% of MS patients had a past EBV infection (Lucas et al., 2011). Previous meta-analysis studies estimated that the probability of developing MS is ten times higher among EBV-positive than EBV-negative persons (Ascherio and Munch, 2000; Ascherio et al., 2001). Furthermore, infectious mononucleosis caused by EBV during early adulthood is one of the best-established risk factors for MS (Thacker et al., 2006; Handel et al., 2010; Guan et al., 2019). However, few individuals infected with EBV virus develop MS; therefore, other factors must intervene. Meta-analysis studies comparing the body mass index (BMI) between MS patients and controls have shown that childhood and adolescence obesity increased the risk of MS two-folds for females and males in their 20s with a BMI $\geq 27\text{-}30 \text{ kg}/\text{m}^2$ (Hedström et al., 2012; Gianfrancesco et al., 2014). Moreover, in-

dividuals with a BMI larger than 27 and EBV-positive had over a six-fold increased risk compared to people with a BMI smaller than 27 and EBV-negative (Hedström et al., 2015). Vitamin D deficiency is another well-established risk factor for MS (Goldberg, 1974; Ascherio et al., 2010, 2014). In fact, low 25(OH)D levels in early MS are a strong risk factor for long-term MS activity and progression. Values greater than or equal to 50 nmol/L (20 ng/mL) were associated with a lower disability during the subsequent four years, demonstrating the protective immunomodulatory effect of this vitamin (Ascherio et al., 2014). Lastly, cumulative exposure to cigarette smoking has been associated with a higher risk of MS (Hernan et al., 2001; Riise et al., 2003). Furthermore, some studies have associated disease progression to smoking (Hernan et al., 2005).

MS risk is proportional to the degree of relatedness to someone that has MS (Beecham et al., 2013). For example, while the risk of developing MS in the general population is 1 in 1000, it rises to 1 in 4 for identical twins where one twin is affected by (Boomsma et al., 2002; Fagnani et al., 2015). Further, it was estimated in genome-wide association studies (GWAS), that genetic variation might be responsible for toward half of the MS susceptibility between individuals (Boomsma et al., 2002; Beecham et al., 2013; Westerlind et al., 2014; Fagnani et al., 2015; Parnell and Booth, 2017). Variations in the HLA regions encoding glycoproteins for antigen presentation have been associated with MS susceptibility in multiple studies (Jersild et al., 1973, 1975; Moutsianas et al., 2015). In fact, over 200 gene variants have been detailed in a GWAS study using genotyping from more than 100,000 cases and controls (Patsopoulos, 2016). Most of these variants were associated with the immune system, including the HLA-DRB1*15:01 (Patsopoulos et al., 2013), IL2 and IL7R polymorphisms or genes that modulate tumour necrosis factor (TNF) (in this thesis the abbreviation TNF will be used instead of $\text{TNF-}\alpha$ since the term $\text{TNF-}\beta$ was dropped for $\text{LT-}\alpha$) (De Jager et al., 2009; Cotsapas and Mitrovic, 2018). Moreover, some polymorphisms can interact with the environmental risk factors mentioned above. For instance, the HLA-DRB1*15:01 allele confers a higher risk in smokers (Hedström et al., 2011), obese adolescents (Hedström et al., 2014) and EBV infected individuals (Sundqvist et al., 2012).

1.1.2 Clinical course and diagnosis

MS has a heterogeneous clinical course and diverse clinical manifestations. However, most patients (85-90%) experience reversible episodes of neurological deficits known as relapses followed by remission periods of clinical recovery that are variable and in many cases, incomplete (Brownlee et al., 2017). This form of MS is called relapsing-remitting (RRMS) (figure 1.1 A). Over time, the clinical improvement of the remission periods fades, disability becomes more prominent and relapses might be present or not; this form of MS is referred as secondary progressive multiple sclerosis (SPMS) (figure 1.1 A) (Lublin et al., 1996, 2014). Approximately 10-15% of patients have a progressive disease course in which they experience an accumulation of disability from the onset without relapses; these patients follow a primary progressive clinical course known as primary progressive multiple sclerosis (PPMS) (figure 1.1 B) (Lublin et al., 1996). Lastly, a minority of patients can experience progressive accumulation of disability from the onset with acute clinical relapses (Lublin et al., 1996). This form of MS is classified as progressive relapsing multiple sclerosis (PRMS) (figure 1.1 C). However, the last revisions suggest dropping the PRMS classification as it is considered vague and overlaps with other disease clinical course subtypes (Lublin et al., 2014). In order to quantify clinical neurological disability, clinicians use the expanded disability status scale (EDSS) (figure 1.1 D), which is a method widely used to rate MS disability at a specific disease duration (Kurtzke, 1983).

The median age of disease onset of RRMS ranges from 20-35 years old, whereas PPMS typically begins approximately at 40 years old, and there is no sex difference (Brownlee et al., 2017). A study of 431 cases at the UK MS Tissue Bank showed that the median age onset of RRMS was 31.5, the age at progression to SPMS was 44, the age of progression to a wheelchair was 50 and the age at death was 62 (Reynolds et al., 2011). Furthermore, the median time to death from the onset has been estimated to be approximately 30 years with a reduction of life expectancy of 5-10 years (Brønnum-Hansen et al., 2004). Therefore, MS can be a high economic burden for the national health systems. Previous analyses had estimated a cost of $\approx$ 14.6 billion euros in 2010 within Europe (Gustavsson et al., 2011) and 4.3 billion dollars in the United States in 2013 (Chen et al., 2017).

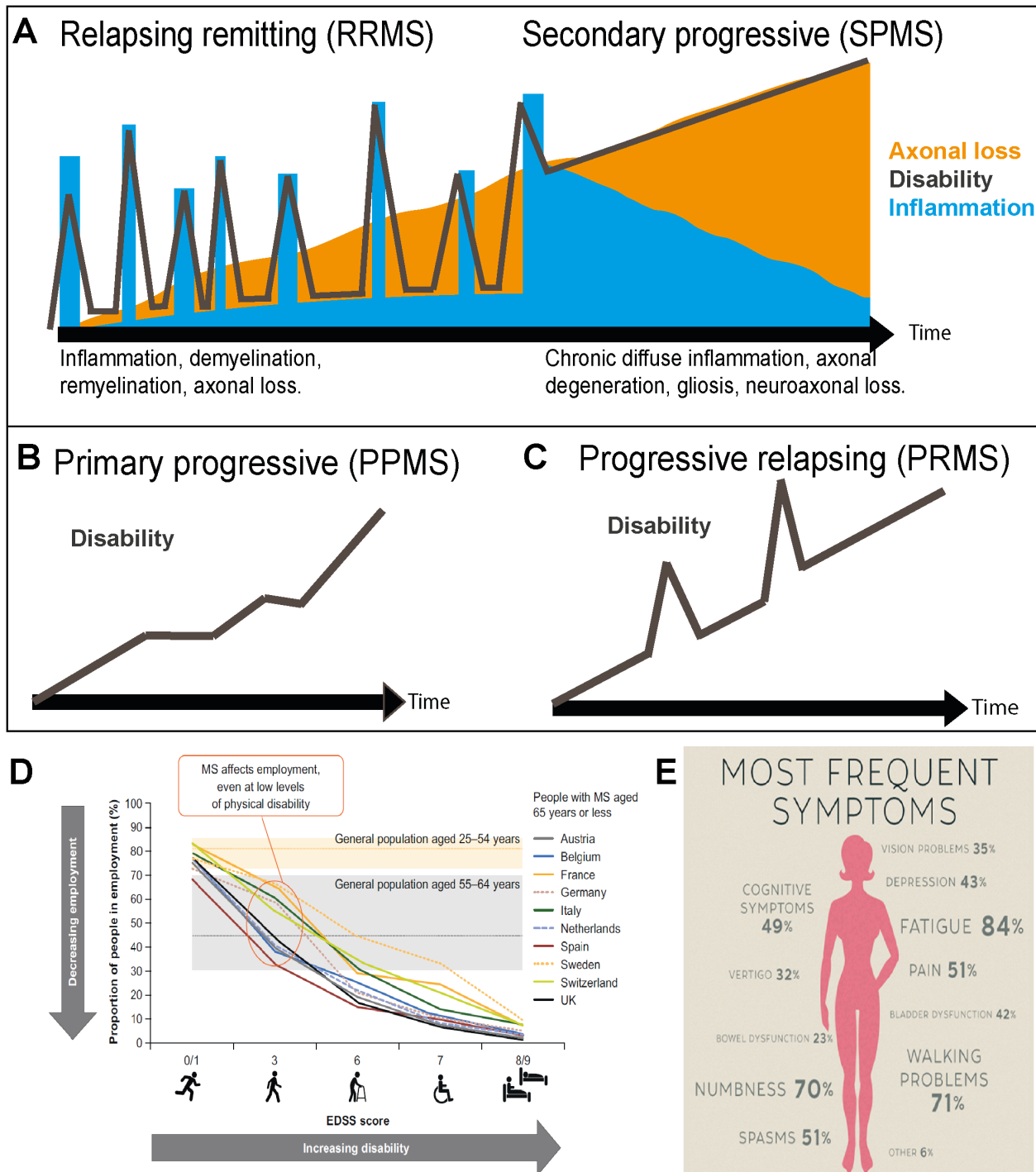


Figure 1.1: MS clinical description. (A) Clinical course diagram representing clinical disability (gray) over time for RRMS and SPMS. Axonal loss accumulation over time is represented in orange and inflammation profiles in blue. (B) Clinical course diagram representing clinical disability (gray) over time for PPMS. (C) Clinical course diagram representing clinical disability (gray) over time for PRMS. (D) Plot showing the relationship between increasing disability in MS measured by EDSS score (0-9) and the proportion of employment, modified from Giovannoni et al. (2016). (E) Image showing the most frequent clinical symptoms of MS, modified from Simmons (2015).

1.1. Multiple Sclerosis

Regarding MS diagnosis, magnetic resonance imaging (MRI) scans and cerebrospinal fluid (CSF) analysis are usually performed. MRI scan diagnosis follows McDonald criteria, in which at least one T2 bright lesion in at least two to four CNS locations are identified, and the appearance of a new lesion in a follow-up scan (Polman et al., 2011). CSF analysis is used to identify oligoclonal immunoglobulin bands via electrophoresis as 90 % of MS patients have an increase in immunoglobulin concentrations (Johnson et al., 1977). Lastly, evoked potential studies can be performed to detect the separation of lesions in space (Colombo et al., 2000).

Clinical manifestations in MS are very heterogeneous depending on the regions of the CNS affected (figure 1.1 E). Therefore, MS can result in motor, sensory, cognitive and psychiatric symptoms, including unilateral loss of vision, double vision, paralysis, spasms, bladder/bowel control problems, sexual dysfunction, numbness and paresthesia (Compton and Coles, 2008). Also, up to 65% of patients experience some degree of cognitive impairment (Winkelmann et al., 2007). For example, they can experience deficits in complex attention, efficiency of information processing, executive functioning, processing speed, and long-term memory (Chiaravalloti and DeLuca, 2008). A recent MRI study has observed that cognitively impaired MS patients have reduced network dynamics in default-mode, frontoparietal, and visual networks, but also show a loss of interplay between default-mode and visual networks (Eijlers et al., 2019). In addition, 70% – 90% of patients report fatigue as a symptom (Bakshi, 2003; Flachenecker and Meissner, 2008; Asano et al., 2015). Fatigue is described clinically as a subjective lack of physical and/or mental energy that is perceived by the individual or caregiver to interfere with the usual and desired activities (Christodoulou, 2005; Mills and Young, 2008). It is reported as the most common and invisible symptom of the disease (figure 1.1 E). Therefore, the consequences to the quality of life of patients are large, including unemployment (figure 1.1 D) and high risk of depression (Rao et al., 1991; Giovannoni et al., 2016). However, previous studies have not observed a correlation between fatigue and MRI or CSF parameters (Biberacher et al., 2018). Some of the working hypotheses to explain this symptom include mitochondrial dysfunction, in which axons are depleted of sufficient ATP, making conduction highly inefficient (Mao and Reddy, 2010). Other hypotheses point to axonal

degeneration pathways via glutamate toxicity or high release of superoxide species as the molecular mechanisms responsible for conduction deficiencies. Moreover, the fact that focal demyelinated plaques generate a degree of axonal transection (Ferguson et al., 1997; Trapp et al., 1998) forces the rest of the brain to compensate for the damage. Hence, it is considered another mechanism underlying MS cognitive symptoms such as fatigue. Although some pharmacological agents such as amantadine, modafinil or CNS stimulant pemoline have been demonstrated to reduce fatigue, the aetiology and physiology of fatigue remain unknown (Bakshi, 2003).

1.1.3 MS Treatment: Disease Modifying therapies

Although there is no cure for MS, there is an increasing number of disease-modifying therapies (DMTs) than can control the relapsing-remitting stage of MS. These therapies have been proven to decrease the frequency of relapses. However, their effectiveness in preventing the progressive neurological decline is minimal. The first treatments developed in the 1990s were injectables, consisting of the interferons IFN- β -1a, IFN- β -1b and glatiramer acetate. While the interferons are anti-inflammatory cytokines, glatiramer acetate is a mixture of amino acids that mimic the myelin basic protein (MBP), designed to compete and inhibit the interaction between myelin and immune cells. These therapies reduce the annual relapse rate by approximately one-third (Group et al., 1993; Johnson et al., 1995; Jacobs et al., 1996).

In 2004, natalizumab was developed as the first monoclonal antibody therapy (Miller et al., 2003; Polman et al., 2006). This treatment reduces lymphocyte migration across the blood-brain barrier (BBB) and acts by blocking α 4 β 1 integrin on the surface of the lymphocytes, which mediates attachment to the vascular cell adhesion molecule 1 on the vascular endothelial cells of the brain (Yednock et al., 1992). Natalizumab is an effective treatment that can reduce the relapse rate at one year up to 68%, but it has risks such as the development of progressive multifocal leukoencephalopathy (PML) (Kleinschmidt-DeMasters and Tyler, 2005). In 2010-2013, oral therapies that inhibit the immune activation in MS were developed: fingolimod, teriflunomide and dimethyl fumarate (DMF). Fingolimod

1.1. Multiple Sclerosis

is a fungal metabolite and an antagonist of S1P receptor present in cells including leukocytes. It causes T-cells to be sequestered in the lymph nodes, thereby reducing the number of circulating T-cells and the number of T-cells arriving into the CNS (Cohen et al., 2010; Kappos et al., 2010). Teriflunomide is a treatment used for rheumatoid arthritis that inhibits the proliferation of rapidly dividing cells such as T-cells by reducing their capacity to synthesize pyrimidine (O'Connor et al., 2011; Confavreux et al., 2014), and DMF reduces absolute lymphocyte counts although the complete mechanism is not fully characterised (Gold et al., 2012; Fox et al., 2012). In 2014-2016, the second generation of monoclonal antibodies were approved: alemtuzumab and daclizumab. Alemtuzumab depletes circulating T and B cells by binding to CD52 on mature lymphocytes (Cohen et al., 2012; Coles et al., 2012), whereas daclizumab targets the α subunit of the IL-2 receptor, reducing IL-2 signalling and increasing the intermediate affinity for the receptors (Gold et al., 2013; Giovannoni et al., 2014). Daclizumab was removed from the market as encephalitis and meningoencephalitis emerged in some patients. In 2017 the oral therapy cladribine was approved. Cladribine is a synthetic adenosine which becomes cytotoxic by disrupting DNA synthesis and repair when phosphorylated by a kinase. In the majority of cells, the drug remains inactivated, but it is thought to induce apoptosis in B-cells as their kinase: phosphatase ratio is higher (Leist et al., 2014; Giovannoni et al., 2018).

Lastly, the B cell-specific monoclonal antibodies rituximab and ocrelizumab have recently been tested for MS treatment. These antibodies deplete B cells by targeting the CD20 antigen, which is present in mature and developing B cells. Rituximab was the first B cell-specific monoclonal antibody developed for human use. A phase II placebo-controlled trial in MS proved that patients experienced fewer relapses and lesions with rituximab (Hauser et al., 2008). However, no phase III trials have been designed for it, and it is usually prescribed off-label as it has been approved for other conditions such as non-Hodgkin lymphoma. On the other hand, two phases III clinical trials comparing ocrelizumab to IFN- β -1a showed its efficacy and safety in RRMS (Hauser et al., 2017). Furthermore, another phase III clinical trial showed clinical benefits of ocrelizumab in PPMS (Montalban et al., 2016). Therefore, both the FDA and the European Medicines Agency approved ocrelizumab for the treatment of RRMS and PPMS in 2017.

The injectables are considered first-line therapies, whereas, natalizumab, alemtuzumab and fingolimod are second-line therapies, as they have in comparison higher adverse risks. Therefore, clinicians have the choice between starting treatment in an MS patient with a first-line therapy and then escalate to a second line, or start directly with second-line therapy. As previously mentioned, these treatments reduce the peripheral immune system response; but, there is a lack of neuroprotective treatments to inhibit neuro-axonal degeneration. Neuroprotective therapies could be used in combination with immunomodulatory treatments, thus promoting a higher degree of functional recovery as neuro-axonal degeneration is considered one of the main predictors of progressive neurological decline in MS.

1.2 MS Pathology

The main pathological features of MS include demyelinated lesions, focal and diffuse inflammation, neuronal and axonal degeneration, gliosis and oligodendrocyte loss. Demyelinating lesions were one of the first pathological features described in detail by Jean-Martin Charcot as *la sclérose en plaques* (Charcot, 1868, 1880). In his descriptions and illustrations, Charcot showed the expansion of lesions from the ventricles to the cerebral hemispheres, as well as axonal transection and remyelination within the plaques (Murray, 2009; Kumar et al., 2011). Demyelinating lesions can be observed in human post-mortem tissue, and MRI scans at any site of the CNS where myelin is present (figure 1.2). It is widely accepted that white matter (WM) plaques, as the hallmark of MS pathology, are sites of focal acute inflammatory demyelination sites associated with RRMS clinical acute relapses and changes in BBB permeability (Reynolds et al., 2011; Frischer et al., 2015). Furthermore, they are more likely to arise in high venous density areas (Charcot, 1880). However, there is high heterogeneity with regard to lesion load and lesion size among patients (figure 1.2 A) as any WM region within the CNS can be affected (Lucchinetti et al., 2000; Luchetti et al., 2018). Multiple MRI studies have demonstrated that the load and rate of appearance of new T2-weighted plaques correlate poorly with clinical disability measured by EDSS (figure 1.2 D) (Filippi et al., 1995; Losseff et al., 1996; Brex et al.,

1.2. MS Pathology

2002; Kappos et al., 1999; Bakshi et al., 2004). However, MRI studies are not often performed in the later stages of MS, and pathological features might differ from the early stages. A recent study in post-mortem tissue of progressive MS showed that patients with a severe disease course measured by EDSS had a substantial inflammatory lesion activity at time to death (Luchetti et al., 2018). Nonetheless, it is known that the presence of active lesions in progressive MS is not common. On the contrary, diffuse grey matter GM and WM pathology characterised by perivascular inflammatory infiltrates, diffuse microglial activation, diffuse axonal injury, neuronal loss, and astrocytic gliosis are more prominent in progressive MS and correlate with neurological decline (Kutzelnigg et al., 2005a; Reynolds et al., 2011; Lassmann et al., 2012).

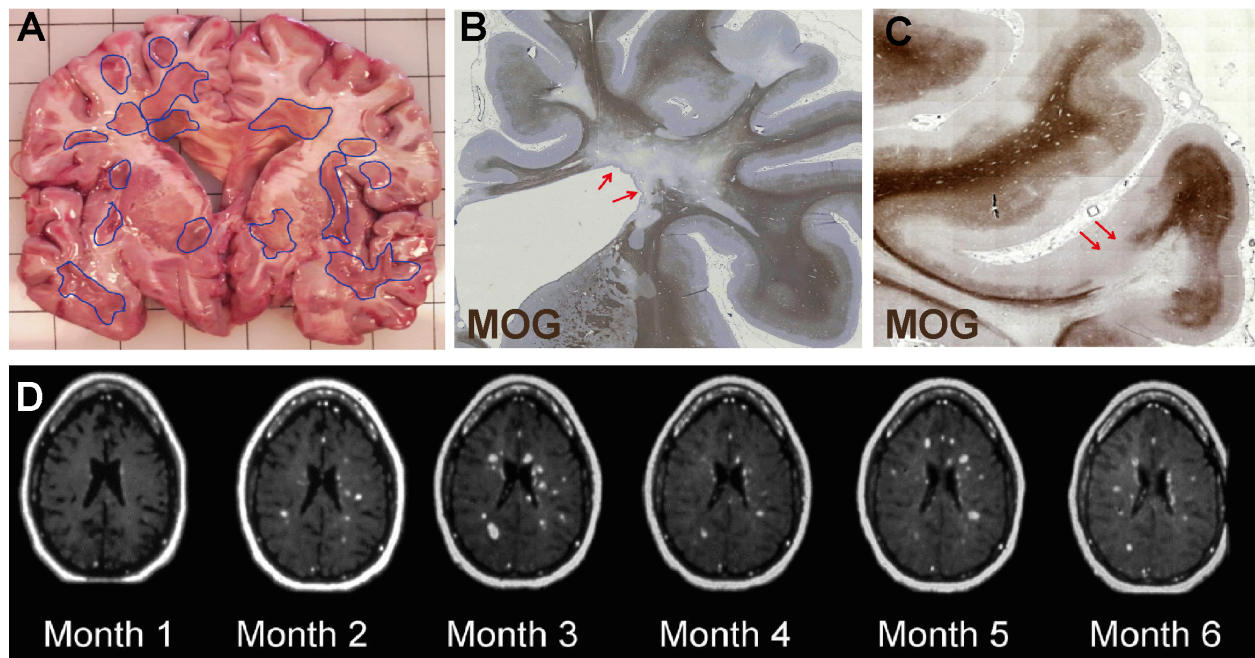


Figure 1.2: Focal demyelinating lesions. (A) Post-mortem brain tissue acquired from the UK MS Tissue Bank from a case of 44 years old with less than 20 years disease duration. Demyelinating lesions are circled in blue. (B) Peri-ventricular white matter lesions stained with MOG. (C) Cortical white matter lesions stained with MOG. (D) Gadolinium-enhanced MRI scans of a 25 years old female RRMS patient clinically silent throughout a 6 months course (Bakshi et al., 2004).

1.2.1 Inflammation and focal demyelination

Inflammation in the brain and spinal cord in MS involves adaptive immune cells (T-lymphocytes, B-lymphocytes, plasma cells and dendritic cells) and innate cells (macrophages) that infiltrate through a permeable BBB (figure 1.3 A-H), as well as the CNS resident innate cells (microglia) with inflammatory and phagocytic capacity (Dendrou et al., 2015). However, focal demyelination is suggested to be carried out by microglia and macrophages, which are stimulated by diffuse infiltration of lymphocytes (Barnett and Prineas, 2004; Reynolds et al., 2011). Infiltrated lymphocytes are mainly CD8⁺ T cells, whereas CD4⁺ T lymphocytes, B-lymphocytes and plasma cells usually represent a small component of the inflammatory infiltrate, rarely seen within the parenchyma (Magliozzi et al., 2007; Friese and Fugger, 2009; Reynolds et al., 2011).

Demyelinating plaques can be classified as active, chronic active, inactive and remyelinated (figure 1.3 I-O) depending on the presence of myelin debris in phagocytic cells and their distribution, which usually reveal a morphological phenotype of activated microglia and macrophages (Brück et al., 1995; Reynolds et al., 2011; Frischer et al., 2015). In the centre of active lesions (figure 1.3 I, L), the majority of phagocytic cells have a morphological phenotype of macrophages, while activated microglia predominate at the edge of the plaque, in the peri-plaque and even in the normal-appearing white matter (NAWM) (Lassmann, 2018a). Throughout the lesion, phagocytic cells might carry out myelin destruction with or without the deposition of immunoglobulins and complement components (Reynolds et al., 2011). Infiltrated T-lymphocytes, mainly CD8⁺, can also be found within the active lesion (Booss et al., 1983; Hayashi et al., 1988; Gay et al., 1997). Nonetheless, lymphocytes are predominantly located in the perivascular spaces and the subarachnoid space of the meninges (Babbe et al., 2000; Reynolds et al., 2011). This inflammatory process is usually associated with an increased BBB permeability, which has been quantified previously by changes in the expression of the protein dysferin, which is a marker of leaky endothelial cells (Hochmeister et al., 2006) or by markers of microvascular tight junctions such as zonula occludens-1 (Kirk et al., 2003). The expression of pro-inflammatory cytokines, chemokines and adhesion molecules have also been found

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in active lesions such as IL-1 β + and TNF+ microglia or VCAM-1+ blood vessels and astrocytes (Cannella and Raine, 1995; Huang et al., 2000). Focal demyelination at active lesions is also linked to a variable degree of acute axonal injury and axonal loss within the plaque and peri-plaque (Ferguson et al., 1997; Prineas et al., 2001; Trapp et al., 1998; Frischer et al., 2015; Lassmann et al., 2012).

In progressive MS, the majority of lesions can be classified as chronic active lesions (figure 1.3 J, M). These type of lesions are characterised by microglia and macrophages present at the lesion edge. In these lesions, a rim of activated microglia can be observed at the edge with some cells of a macrophage phenotype in between, which contain myelin degradation products, leaving the centre of the lesion with few cells (Prineas et al., 2001). In the peri-plaque, axonal injury has also been seen by disturbed fast axonal transport and the presence of axonal end bulbs (Frischer et al., 2015). Pro-inflammatory cytokines and adhesion molecules expression within these lesions has also been seen. For example, TNF was expressed at higher levels in microglia and macrophages and occasionally in astrocytes and endothelial cells at chronic active lesions (Cannella and Raine, 1995). Another type of lesions are inactive, which are the most abundant in the MS brain (Lassmann, 2018a) and are characterised by astrogliosis and lack of microglia and macrophages. In these lesions, astrocytes form a dense fibrillary scar that fills the demyelinated plaque (figure 1.3 K, N). Lastly, remyelinated plaques are characterised by axons with thin myelin (figure 1.3 O), which have been re-myelinated by new oligodendrocytes (Chang et al., 2002; Bramow et al., 2010). Remyelination varies significantly between different MS patients and might depend in part on lesion location (Lassmann, 2018a). For example, remyelinated lesions are more frequent in subcortical regions compared to the periventricular WM (Patrikios et al., 2006).

Active WM focal lesions are generally most numerous in the early phases of MS (RRMS) and become rare when patients have entered the progressive stage (Frischer et al., 2015; Lassmann, 2018a). On the contrary, diffuse atrophy in the WM demyelination, GM, GM demyelination and changes in the NAWM prevail in progression. Although atrophy (tissue loss) has been observed in all clinical stages of MS as the inflammatory lesions arise,

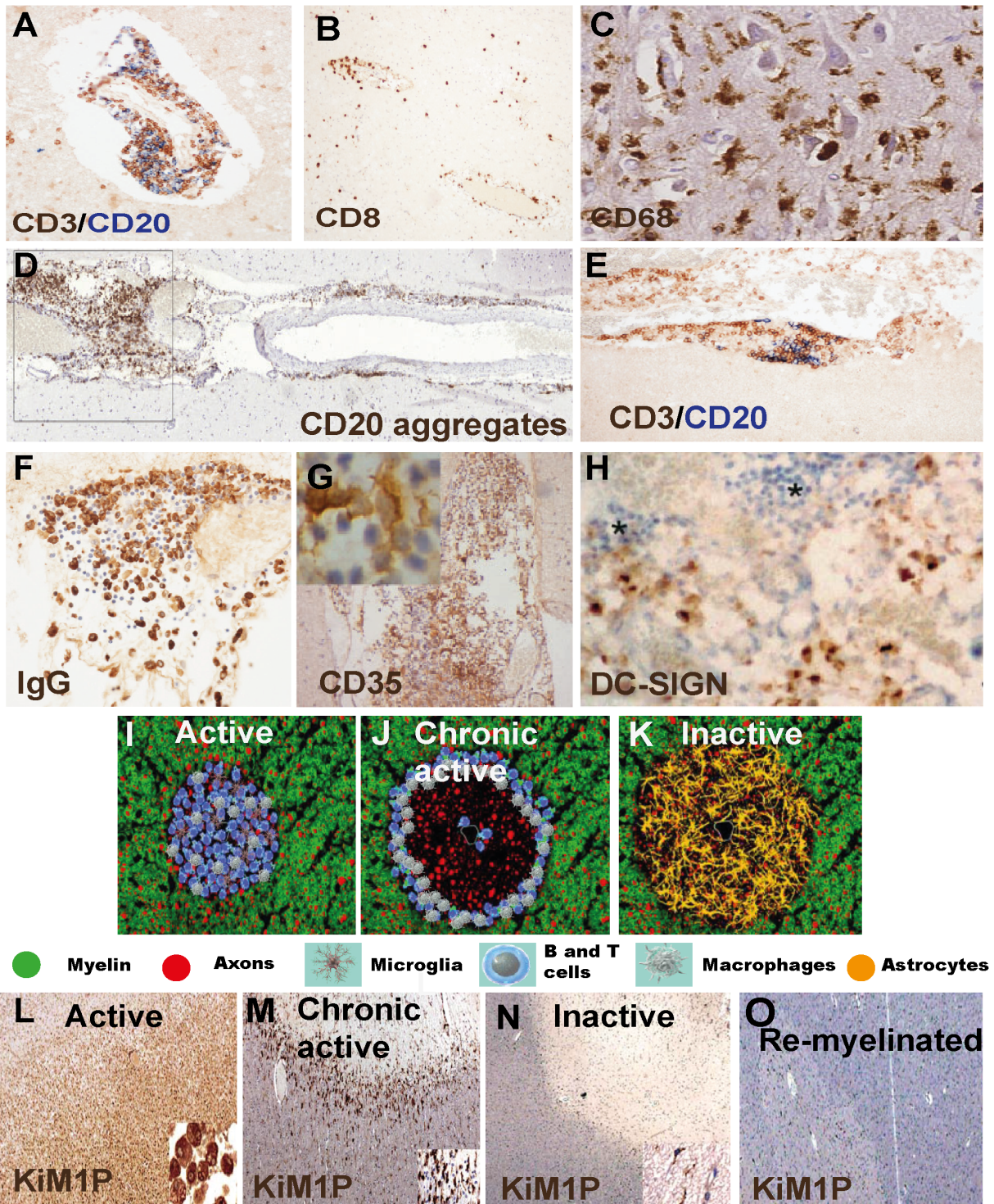


Figure 1.3: Immune infiltrates and lesion types. (A) CD3+ T cells and CD20+ B cells in lesioned tissue (Lassmann, 2018a). (B) CD8+ T lymphocytes in lesioned tissue (Lassmann, 2018a). (C) CD68+ microglia/macrophages in an active lesion (Bevan et al., 2018). (D) Meningeal CD20+ B cells (Howell et al., 2011). (E,F) Meningeal CD3/CD20 and IgG+ plasma cells (Lassmann, 2018a). (G) Meningeal CD35 B cells (Howell et al., 2011). (H) Meningeal DC-SIGN dendritic cells (Serafini et al., 2006). (I,J, K) Lesion types diagrams: active (I), chronic active (J) and inactive (K) (Goodin et al., 2016). (L, M, N, O) KiM1P+ microglia/macrophages in active (L), chronic active (M), inactive (N), re-myelinated (O) lesions (Frischer et al., 2015).

MRI studies have suggested that a component of atrophy is independent of focal inflammatory lesions due to the lack of gadolinium (Gd) contrast enhancement (Inglese et al., 2005; Miller et al., 2002). Furthermore, existing immunomodulatory DMTs have minimal effects on disease progression. Hence, this data suggests that in the early phases of MS, inflammation might be the driving force, whilst in the progressive phases, a chronic neurodegenerative process develops that is not dependent on the activity of the peripheral immune system (Reynolds et al., 2011; Lassmann et al., 2012; Lassmann, 2018a).

1.2.2 GM pathology

Extensive GM demyelination is associated with the progressive phases of disease (Bö et al., 2006). Cortical lesions contain very few CD3+ lymphocytes and CD68+ microglia and macrophages compared to WM lesions (Peterson et al., 2001; Lucchinetti et al., 2011). Transected axons and dendrites have been observed in active, chronic active and inactive cortical lesions, and the myelinated MS cortex (Peterson et al., 2001). The most common cortical lesions in the progressive phases are subpial as they are located in brain sulci and are closely associated with the inflammatory infiltrates found in the leptomeninges (Magliozzi et al., 2007; Lucchinetti et al., 2011; Bevan et al., 2018). Previous studies have also demonstrated the presence of ectopic lymphoid tissue in the SPMS meninges; containing B cells, T cells and plasma cells, and a network of follicular dendritic cells (figure 1.3 D-H) (Serafini et al., 2004; Franciotta et al., 2008; Pikor et al., 2016). Moreover, these meningeal lymphoid aggregates, which are considered important in sustaining MS immunopathology, have been associated with diffuse meningeal inflammation, cortical demyelinating lesions and a gradient of neuronal loss throughout the cortical layers (Magliozzi et al., 2010; Howell et al., 2011; Bevan et al., 2018). Similar aggregates had also been found within the large perivascular Virchow Robin spaces (fluid-filled spaces surrounding certain blood vessels) of the brain (Favaretto et al., 2017; Lassmann, 2018b).

1.2.3 Axonal loss and diffuse white matter pathology

A variable degree of axonal loss has been reported throughout the brain and the spinal cord of MS tissue (Ferguson et al., 1997; Trapp et al., 1998; Kornek and Lassmann, 1999; Evangelou et al., 2000; Lovas et al., 2000). Although axonal loss is thought to be more acute during the early phases of MS (Kuhlmann et al., 2002), axonal degeneration accumulates throughout the lifetime of a patient and correlates significantly with clinical disability (Narayanan et al., 1997; Bjartmar et al., 2000; Frischer et al., 2009; Haider et al., 2016). Axonal loss and injury can be identified by immunostaining for dephosphorylated neurofilament protein (SMI32) and amyloid precursor protein (APP), including the presence of axon swellings and ovoids (figure 1.4 A-E), within and around demyelinating plaques in WM (Ferguson et al., 1997; Trapp et al., 1998) and GM (Peterson et al., 2001; Bö et al., 2006; Lucchinetti et al., 2011).

Significant diffuse axonal injury has also been noted in NAWM of progressive MS tissue (figure 1.4 F), which may indicate an underlying disturbed axonal transport (Ferguson et al., 1997; Trapp et al., 1998; Kutzelnigg et al., 2005a; Frischer et al., 2009; Howell et al., 2010; Moll et al., 2011). In fact, NAWM tissue in the late stages of MS is highly abnormal; it is characterised by diffuse inflammation (CD3+ T lymphocytes have been observed within perivascular spaces (figure 1.4 I)) and profound activation of microglia and macrophages (figure 1.4 H) that are closely associated with the extent of degenerating axons in these regions (Allen and McKeown, 1979; Allen et al., 2001; Graumann et al., 2003; Kutzelnigg et al., 2005a; Moll et al., 2011). Axonal density in the NAWM of progressive MS spinal cord correlates with the density of MHCII+ microglia and meningeal CD3+ T cells (Androdias et al., 2010), and moderately with cortical GM demyelination (Kutzelnigg et al., 2005a). Although abnormalities in the NAWM become more prominent in progressive MS, axonal degeneration in NAWM of MS patients with short disease duration has also been seen (Bjartmar et al., 2001) (figure 1.4 G). Moreover, previous MRI studies have shown that the extent of NAWM atrophy (tissue damage) does not correlate with the load, location or size of focal demyelinating plaques (Evangelou et al., 2004; Kutzelnigg et al., 2005a). Therefore, although axonal injury can occur via anterograde

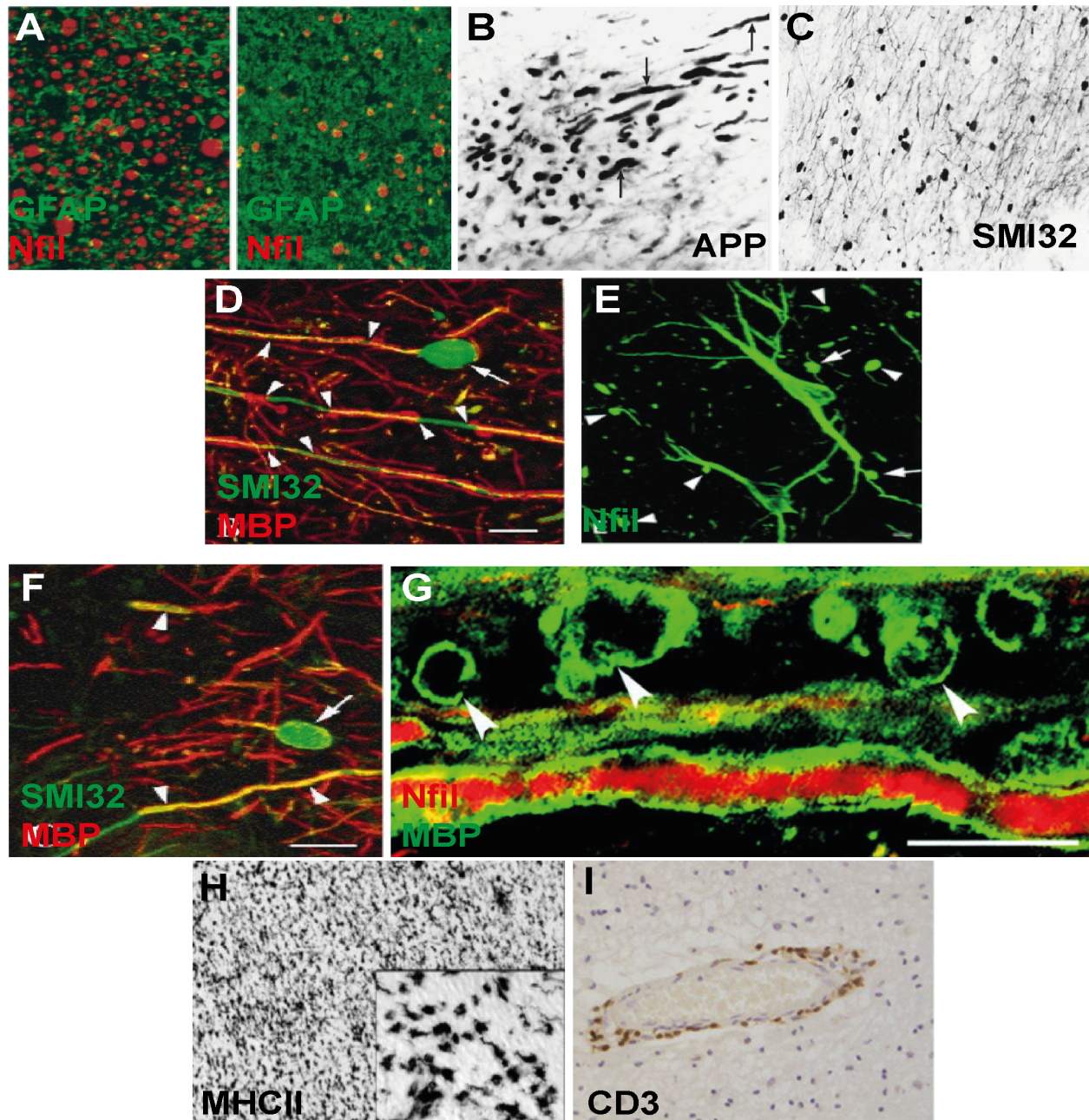


Figure 1.4: Axon injury in lesion and NAWM tissue. (A) Immunofluorescent images of Nfil+ axons and GFAP+ astrocytes in spinal cord of normal (left) and lesioned (right) tissue shows a reduction in axon density (Bjartmar et al., 2000). (B) Immunofluorescent image of APP+ axons in a brain lesion (Ferguson et al., 1997). (C, D) Immunofluorescent image of SMI32+ axons in lesioned tissue displays the presence of axonal bulbs (Trapp et al., 1998). (E) Immunofluorescent image of Nfil+ axons in a cortical lesion with swellings/bulbs (Peterson et al., 2001). (F) Immunofluorescent image of SMI32+ axons in MS NAWM tissue reveals the presence of swellings/ovoids (Trapp et al., 1998). (G) Immunofluorescent image of Nfil+ axons and MBP+ myelin showing neurodegeneration (Bjartmar et al., 2001). (H) Immunohistochemistry image of MHCII+ microglia in MS NAWM tissue of the spinal cord (Moll et al., 2011). (I) Immunohistochemistry image of CD3+ T-lymphocytes in MS NAWM tissue (Howell et al., 2010).

(Wallerian) and/or retrograde axonal degeneration from demyelinating lesions (Evangelou et al., 2000, 2004; Dziedzic et al., 2010; Haider et al., 2016), diffuse inflammation and activation of microglia are considered the driving forces of diffuse axonal injury (Lassmann et al., 2007). Multiple molecular mechanisms to explain diffuse axon injury have been proposed. Some of them include mitochondrial function insufficiency and ATP reduction, increased Na⁺ ion loading, the reversal of Na⁺/K⁺ ATPase, glutamate toxicity and subsequent calcium loading within axons (Frieze et al., 2014).

1.3 Studying MS: animal models, in-vitro and in-silico systems

1.3.1 MS animal models

The choice of an animal model to study MS varies depending on the pathological features under investigation. Models range from myelin mutants and chemically-induced lesions used to study demyelination and remyelination, viral models to study aetiology, and autoimmune models to study immune-mediated pathologies (Baker et al., 2011). Myelin mutants such as *shiverer* (myelin basic protein (MBP) mutant), *rumpshaker* and *jimpy* (proteolipid protein (PLP) mutants) mice, as well as gene knockout animals such as the myelin-associated glycoprotein (MAG) knockout (Inuzuka et al., 1985), show dysmyelination, altered neurotransmission and in some instances clinical disease.

However, the experimental allergic encephalomyelitis (EAE) animal model is the one that has been most widely used to study MS pathogenesis. The EAE model was first discovered when immune brain lesions were observed after the injection of attenuated rabies virus grown in rabbit brains to model post rabies vaccine encephalomyelitis (Rivers et al., 1933). It was found that the lesions were a direct consequence of the immune response against antigens present in the rabbit brain, and their development was inconsistent. Subsequent studies showed that if heat-killed bacteria was added as an adjuvant to emulsify the brain tissue, the immunisation was reproducible. Therefore, in the EAE models, complete Freund's adjuvant (CFA) is administered to stimulate phagocytic up-

1.3. Studying MS: animal models, in-vitro and in-silico systems

take and presentation of antigens, and therefore subsequent activation of auto-reactive CD4⁺ T-cells. The EAE model strengthened the idea that MS is a CNS disease driven by activated T-cell destruction of myelin. Initially, it was shown that Biozzi ABH and SJL mice, dark agouti (DA) rats and strain 13 guinea pigs develop relapsing EAE after active sensitisation with whole myelin, while C57BL/6 inbred mouse strain was relatively resistant (Furlan et al., 2009; Baker et al., 2011). Later, the discovery that myelin oligodendrocyte glycoprotein (*MOG*_{35–55}) could induce reproducible EAE (Mendel et al., 1995), which was effective in C57BL/6 mice, led the generation of numerous gene-knockout mice models (Fazekas and Tabira, 2000). Currently, the most common form to induce EAE in C57BL/6 mice is by injecting the antigen, mainly *MOG*_{35–55} or *PLP*_{139–151}, emulsified in CFA containing mineral oil and mycobacterium tuberculosis, followed by intraperitoneal injections of pertussis toxin (Mix et al., 2010; Baker et al., 2011). The clinical course of EAE models includes monophasic clinical episodes of paralysis (female Lewis rats sensitised with MBP), chronic relapsing (SJL mice sensitised with *PLP*_{139–151} and DA rats with *MOG*_{35–55}) and chronic progressive (C57BL/6 mice immunised with *MOG*_{35–55}) (Gold et al., 2006b). However, these models have disadvantages including that lesions develop mainly at the spinal cord, and that the induced inflammation is mainly mediated by auto-reactive MHC Class II CD4⁺ T-cells (Lassmann and Bradl, 2017).

Although these models are useful, MS is a highly heterogeneous disease with different neuropathological and immunopathological features. Therefore, in order to discover underlying molecular mechanisms, other experimental systems apart from animal models are usually used.

1.3.2 Other experimental systems

Post-mortem human MS tissue is one of the experimental platforms that can be used to study MS. For instance, meningeal inflammation and subsequent neuronal loss in MS was only observed after a systematic study in post-mortem MS tissue (Magliozzi et al., 2010; Howell et al., 2011). Immunofluorescence studies, as well as, DNA, RNA and protein analysis of this tissue can provide sufficient data to develop rationale hypotheses regard-

ing molecular mechanisms that can then be tested in animal models, in-vitro and in-silico systems. Although animal models might be the best approach to analyse the complexity of the nervous system, these raise problems of ethics and accessibility (Doussau et al., 2017) and are not always comparable with human diseases. Organotypic brain slices are the best in-vitro alternative to study brain function in physiological or pathological conditions as they preserve the cytoarchitecture during weeks to months, with the possibility to easily perform pharmacological experiments. Slices are grown at an interface between air and culture medium in which culture inserts composed of semipermeable membranes are placed in 6-well plates containing culture medium in a regular $\text{CO}_2 - 37^\circ\text{C}$ incubator (Stoppini et al., 1991). The semipermeable membranes enable the diffusion of metabolic substrates, hormones and neurotrophic factors from the culture medium. These inserts cause less tissue thinning than the initial roller-tube technique (Gähwiler, 1981), and they do not auto-fluoresce, allowing immunofluorescence experiments. Furthermore, they can be kept in vitro for several weeks without significant disruption to the cellular architecture. However, one of the limitations is that they are usually derived from embryonic or neonatal tissue ($< P12$), and the resulting neural network is still immature. For demyelinating diseases such as MS, the viability of cerebellar slices in long-term cultures (Zhang et al., 2011) have made them an excellent model system.

Regarding in-silico systems, computational models can be developed. For instance, action potential (AP) propagation along axons can be modelled with software simulators such as the widely used NEURON (Hines and Carnevale, 2006) or Brian (Goodman and Brette, 2009). These simulators are based on the mathematical equations derived by Hodgkin and Huxley (HH) from experimental data obtained in a squid axon to explain AP propagation in biophysical terms (Hodgkin and Huxley, 1952). The main advantages of using these systems are that they can serve as a platform to narrow the number of hypotheses before pursuing lengthy and costly experimental approaches. Nonetheless, modelling has disadvantages, such as issues with not having enough data or computational power to model complex systems or poor understanding of the underlying dynamics.

Therefore using a combination of in-vitro, in-vivo and in-silico systems would be the best

approach to solve experimental questions of complex multi-factorial neurodegenerative diseases such as MS.

1.4 CNS myelinating cell: The oligodendrocyte

Oligodendrocytes are a type of glial cell within the CNS that arise during development from the oligodendrocyte precursor cells. The formation of the myelin sheath has been considered the main function of the oligodendrocytes. Most oligodendrocytes generate between 20 and 60 myelinating plasma membrane processes which wrap multiple CNS axons concentrically, and myelin compaction occurs subsequently (Snaidero et al., 2014; Simons and Nave, 2016). Furthermore, oligodendrocytes are metabolically active cells (Fünfschilling et al., 2012; Saab and Nave, 2017) connected to their myelinated axons via cytoplasmic-rich myelinic channels in which the movement of macromolecules can reach the internodal peri-axonal spaces. Therefore, the myelinating oligodendrocytes not only enable the axon to conduct AP conduction in a fast and reliable manner due to the high resistance and low capacitance of the myelin sheath, they also can support axons metabolically by providing metabolites such as lactate through these cytoplasmic-rich myelinic channels (Fünfschilling et al., 2012; Saab and Nave, 2017).

1.5 Structure of a CNS myelinated axon

Myelinated axons are the neural processes that enable the transmission of information in a fast, reliable and cost-efficient manner across long distances in the form of electrical impulses. Previous studies have estimated that the individual arbours from cholinergic neurons in the basal forebrain can have > 1000 branch points and total axon lengths of up to 50 cm (Wu et al., 2014). Therefore, bi-directional axonal transport is important to communicate on between the axon and the soma as the latter can be cm away. In the CNS, axons larger than $0.2\ \mu\text{m}$ in diameter surpass the threshold for myelination as estimated by previous studies (Goebbels et al., 2017). Stochastic computational models estimated that axons smaller than $0.1\ \mu\text{m}$ diameter become functionally inoperable because of the generation of spontaneous AP, which disrupts efficient electrical transmission (Faisal et al.,

2005). Therefore, most of the CNS axons are wrapped concentrically by an electrically insulating membrane, called the myelin sheath, produced by oligodendrocytes (Nave and Werner, 2014).

Separating adjacent lengths of the myelin sheath are non-myelinated regions: the nodes of Ranvier (Ranvier, 1874). High-pressure EM studies have shown that the lateral edges of the outermost myelin layer loop define the dimensions of the node, and consecutively the inner myelin layer loops terminate increasingly distally from the nodes, comprising the paranodes (Snaidero et al., 2014). The paranodes or paranodal axo-glial junctions (PNJ) are the largest vertebrate intercellular junction known that connects the axolemma and the myelin sheath (figure 1.5 C). Moreover, the myelinating lateral edges consist of a series of cytoplasmic loops separated by 2.5-3 nm, attached to each other by tight junctions of claudin-11 (Morita et al., 1999; Gow et al., 1999; Rasband and Peles, 2016) (figure 1.5 C). In addition to the PNJ, there is another axolemma domain referred to as the juxtaparanode (JXP), which lies underneath the myelin sheath and it is rich in voltage-gated K_v1 channels. Lastly, the juxtaparanode connects with the long axon membrane domain underneath myelin called the internode (IND) (figure 1.5 A, B).

1.5.1 Structure of the myelin sheath

Myelin is crucial for optimal neuro-axon physiology since it provides metabolic and structural support from the oligodendrocyte to the neuron. Traditionally, myelin was considered a passive component of the CNS with high electrical resistance and low capacitance to improve electrical impulse propagation. These characteristics describe the compacted component of the myelin sheath. EM images have illustrated the ultra-structural appearance of the compacted myelin sheath (figure 1.5 E), characterised by electron-dense and electron-light layers, called the major dense lines and intraperiod spaces, respectively (Peters, 1960; Simons and Nave, 2016). The intraperiod space exists between the extracellular surfaces of the myelin layers while the intracellular surfaces fuse, forming the dense lines (figure 1.5 E). The latter are considered adhesion zones and previous studies have determined that they follow a period of approximately ≈ 15.6 nm (Hildebrand et al., 1993;

1.5. Structure of a CNS myelinated axon

Agrawal et al., 2009).

Furthermore, high pressure freezing EM was used to better show that myelin also contains non-compacted cytoplasmic regions (Peters, 1960; Möbius et al., 2008; Edgar and Griffiths, 2009; Snaidero et al., 2014), including the end loops of myelin adjacent to the nodes, the paranodes, and a single channel around the perimeter of the glial cell process (Stassart et al., 2018). These cytoplasmic spaces are called the myelinic channel system, and they provide a connection between the oligodendrocyte soma and the peri-axonal space, allowing the distribution of nutrient and metabolites (Nave, 2010). They can contain septin filaments, microtubules, vesicles and multivesicular bodies (Buser et al., 2009; Patzig et al., 2016) (figure 1.5 C). The adaxonal cytoplasmic space contains ion channels, glutamate receptors and metabolic transporters in the glial membrane (figure 1.5 E) (Rinholm et al., 2011; Lee et al., 2012; Nijland et al., 2014; Domenech-Estévez et al., 2015; Saab et al., 2016). This region is also called the axo-myelinic synapse since it faces the peri-axonal space along the axon (Micu et al., 2016). EM images of transverse CNS axons reveal the presence of the peri-axonal space between the myelin sheath and the axolemma in the paranodal, juxtaparanodal and internodal compartments (figure 1.5 A-E). Studies with fluorescent dextrans (figure 1.5 G) have measured a paranodal cross-sectional area of 170 nm^2 and an internodal width between 2-4 nm (Mierzwa et al., 2010). Therefore, the myelin sheath is more than an inert insulating membrane (Simons and Nave, 2016) and the axo-myelin unit interactions at the PNJ are crucial for understanding potential axon injury and spread.

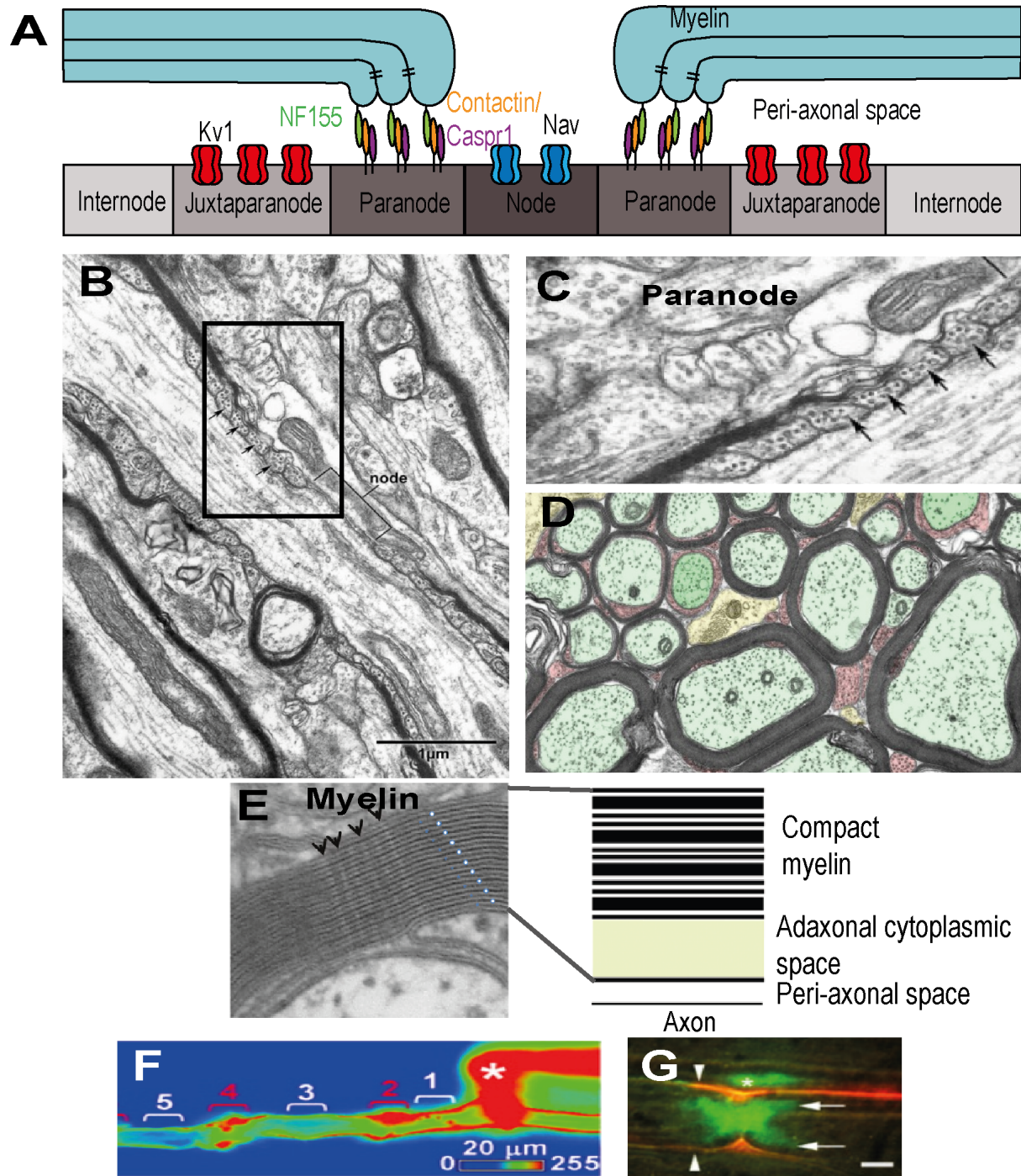


Figure 1.5: Myelinated axon. (A) Diagram representing the distinct axon domains: nodes, paranodes, juxtaparanodes and internodes. (B, C) Longitudinal EM image of a node from the primary visual cortex of a 27 year old monkey and (C) an amplified image of the PNJ (images extracted from the collection of Dr. Alan Peters). (D) Transverse EM image of the corpus callosum of a 6 year old monkey (image extracted from the collection of Dr. Alan Peters). (E) EM image of compacted myelin (Edgar and Griffiths, 2009) and a diagram representing the different layers (Simons and Nave, 2016). (F) Live image of the lucifer yellow tracer filling oligodendrocytes in ex-vivo spinal cord slices demonstrating the existence of the myelinic channel system (Velumian et al., 2011). (G) Image showing that fluorescent tracers are able to flow underneath the PNJ and JXP demonstrating the existence of the peri-axonal space (Mierzwa et al., 2010).

1.5.2 The Nodes of Ranvier

The nodes of Ranvier are the only myelin-free gaps along the axon between two contiguous myelin sheaths. Therefore, they are crucial for saltatory AP propagation (Brill et al., 1977; Bostock and Sears, 1978). At the nodes, axoplasm is rich in microtubules and neurofilaments that are less highly phosphorylated and more closely spaced than in the internode (Stassart et al., 2018). Nodes contain a high density of clustered voltage-gated Na_v channels ($Na_v1.1$, $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$, $Na_v1.9$) (Fjell et al., 2000; Boiko et al., 2001; Henry et al., 2005; Duflocq et al., 2008; Black et al., 2012). In addition, voltage-gated K_v channels ($KCNQ2$, $KCNQ3$ and $K_v3.1b$) (Kole and Cooper, 2014; Devaux et al., 2003, 2004) to regulate neuronal excitability are also present in the nodal compartment (Battefeld et al., 2014; King et al., 2014). In order to anchor and stabilise voltage-gated channels, nodes are enriched with cytoskeletal and scaffolding proteins (figure 1.6 A), in particular Ankyrin G (Kordeli et al., 1995) and βIV spectrin in the axoplasm (Berghs et al., 2000). In the extracellular matrix (ECM), the channels bind to neurofascin186 (Nf186) and NrCAM (Davis et al., 1996) and, VcanV2 and Bcan interact with Bral1, hyaluronan and NF186 (Susuki, 2013). Apart from the nodal scaffolding proteins, paranodal axo-glial junctions are fundamental to nodal assembly and voltage-gated channel clustering during development (Amor et al., 2017; Thaxton et al., 2011).

1.5.3 Paranodal Axo-glial junctions

The paranodes (PNJ) are the regions of interaction between the cytoplasmic myelin loops and the axolemma. They constitute the main site of attachment between the oligodendrocyte and the axon (Wiley and Ellisman, 1980; Rosenbluth, 1984), and they are part of the myelinic channel system (Rosenbluth, 1995; Rasband and Peles, 2016; Stassart et al., 2018). The interaction between myelin and the axolemma is formed by the binding between neurofascin155 (Nf155) on the glial side (Davis et al., 1996; Tait et al., 2000) and contactin1-associated1 protein (Caspr1) on the axonal side (Einheber et al., 1997; Peles et al., 1997; Menegoz et al., 1997; Rios et al., 2000; Charles et al., 2002) and then with 4.1B, αII and βII spectrins in the axoplasm (Ogawa et al., 2006; Simons et al., 2014) (figure 1.6

B). These intercellular connections form septate-like adhesive junctions, which are critical for tethering the myelin sheath to the axon (Salzer, 1997). Specifically, the myelin protein Nf155 forms trans non-covalent immunoglobulin interactions (Ig5-Ig6) with the axonal cis heterocomplex of contactin 1 and Caspr1 (Rios et al., 2000; Tait et al., 2000; Bhat et al., 2001; Boyle et al., 2001; Charles et al., 2002). Moreover, the oligomannose type sugars of contactin1 are required for association with its glial partner Nf155 (Labasque et al., 2014). On the other hand, the interaction between contactin1 and Caspr1 occurs in the endoplasmic reticulum and is required for the insertion of Caspr1 into the axolemma (Faivre-Sarrailh et al., 2000; Boyle et al., 2001).

The correct organisation of these septate-like domains is crucial for rapid and efficient AP propagation in myelinated axons throughout the CNS. Paranodes play multiple functions in the axon, and the correct integrity of the nodes of Ranvier depends greatly on them. They function as physical barriers by enabling the differential clustering of voltage-gated Na_v and K_v channels at the node and juxtaparanode (Chiu and Ritchie, 1980; Barrett and Barrett, 1982; Rosenbluth, 1995), and preventing the lateral movement of these ion channels to preserve the nodal dimensions (Rosenbluth, 1995, 2009). Paranodes are also considered electrical barriers as they prevent electrical shunting underneath the myelin sheath. They also enable nutrients, metabolites and ions to diffuse through the periaxonal space between the node and juxtaparanode (Trapp and Kidd, 2000; Mierzwa et al., 2010). Thus, the integrity of these junctions is fundamental for maintaining the stability of the different axolemma compartments (Rosenbluth, 2009). In fact, a mutation in the gene CNTNAP1, which encodes for the paranodal Caspr1 protein was found in patients with severe non-syndromic arthrogryposis multiplex congenita. These patients showed a significant reduction in motor nerve conduction velocity (<10 m/s) and severe structural abnormalities at the nodes of Ranvier such as nodal length elongation (Laquerriere et al., 2013).

1.5.4 Juxtaparanodes and Internodes

Juxtaparanodes (JXP) are compartments underneath the myelin sheath with a high density of voltage-gated K_v channels ($K_v1.1$, $K_v1.2$, $K_v1.4$ and $K_v\beta2$) that lie in adjacent to the PNJ (Wang et al., 1993; Mi et al., 1995; Rasband et al., 2001; Kew and Davies, 2010). Voltage-gated K_v channels are clustered in the juxtaparanode by the complex formed by contactin-2 (TAG1) and Caspr2 in the membrane, and 4.1B, α II and β II spectrins in the axoplasm (Poliak and Peles, 2003). Moreover, K_v channels also bind to ADAM22 and PSD95/93 (Ogawa et al., 2010) (figure 1.6 C). However, the interaction between K_v channels and Caspr2, TAG-1 and ADAM22 remains unknown (Rasband and Peles, 2016).

The internodes (IND) are the domains underneath the myelin sheath that connect to the next JXP along the axon. They can contain proteins such as the immunoglobulin cell adhesion molecules nectins (NECL1, NECL4) in the axon membrane (Park et al., 2008; Liu et al., 2019) and myelin-associated glycoprotein (MAG) in the myelin membrane (Trapp et al., 1989) (figure 1.6 D). The length of the myelinated internodes can affect conduction speed as it influences the spacing between the nodes of Ranvier (Bechler et al., 2015). A recent study has shown that the length of internodes generated by the myelinating oligodendrocytes can be influenced by the micro-environment of normal versus input-deprived axons (Osanai et al., 2018).

1.5.5 Mitochondria distribution and axoplasm structure

The most encountered cellular machinery within the axoplasm is mitochondria, the power energy sites of the neuron. However, EM studies have shown that the distribution of mitochondria in CNS and PNS axons is fundamentally different. While in the PNS mitochondria are mainly clustered at the node of Ranvier, in the CNS they are located predominantly in the internodal and juxtaparanodal compartments (figure 1.6 C, D, F) (Edgar et al., 2008; Perge et al., 2009; Chiu, 2011; Ohno et al., 2011). Previous studies estimated in the optic nerve that the volume occupied by mitochondria rises linearly for small-diameter axons ($\approx 0.5 \mu m$), whereas for larger diameters the relationship is quadratic ($>0.7 \mu m$) (Perge et al., 2009). This result suggests that the energy budget

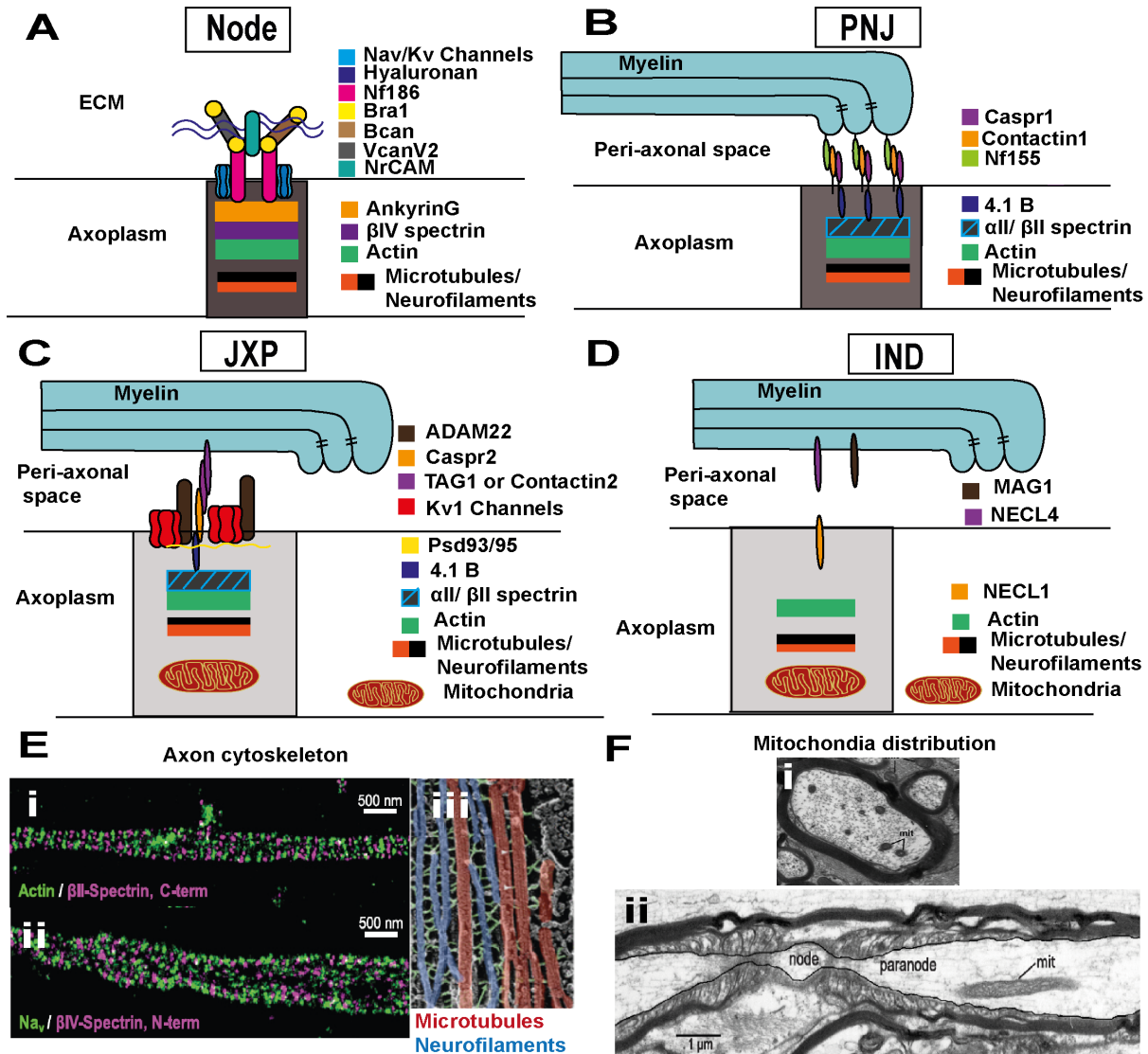


Figure 1.6: Molecular organisation of a CNS myelinated axon. (A) Molecular structure of the node characterised by the clustering of high density of Na_v and K_v voltage gated channels (blue). These channels are anchored by their linkage to Ankyrin G and β IV spectrin in the axoplasm, and to Nf186 and NRCAM in the ECM. In addition, Nf186 interacts with VcanV2, Bcan, Bral1 and hyaluronan. (B) Molecular structure of the paranode comprised by the interaction between Nf155 in the glial side, contactin1-Caspr1 in the axonal side and 4.1B, α II and β II spectrins in the axoplasm. (C) Molecular structure of the juxtaparanode characterised by the clustering of K_v voltage gated channels linked to ADAM22. In addition, the linkage of TAG1 and Caspr2 binds the myelin sheath to the axolemma, which is strengthened in the axoplasm by 4.1B, α II and β II spectrins. (D) Molecular structure of the internode which includes adhesion proteins such as NECL1-4 and MAG. (E) (i,ii) Images built with stochastic optical reconstruction microscopy to show the periodic cytoskeletal structure in axons formed by actin and spectrins (Xu et al., 2013); (iii) Ultrastructural representation of the core cytoskeleton of axons comprised by parallel arrays of neurofilaments and microtubules interconnected by cross-linker (green) (Hirokawa, 1982; Yuan et al., 2017). (F) (i) Transverse EM image of an axon shows the presence of mitochondria. (ii) Longitudinal EM image shows that mitochondria in CNS axons are located in the JXP and IND (Perge et al., 2009).

correlates with fibre diameter, which is also proportional to conduction speed (Rushton, 1951). Regarding morphology, mitochondria are thin and elongated in healthy axons. However, previous studies in EAE models have shown mitochondrial swelling before axonal transection (Nikić et al., 2011). Further, mitochondria can travel long distances on the axon's microtubules (figure 1.6 E) by two main motor protein families, the dyneins and kinesins (Melkov and Abdu, 2018).

As we can see in figure 1.6, the axoplasm is rich in actin filaments, neurofilaments, microtubules and spectrins (Peters and Vaughn, 1967). While microtubules serve as scaffolds for axon transport, neurofilaments extensively cross-bridge and interconnect with other components of the cytoskeleton, and serve as docking sites for vesicular organelles (Yuan et al., 2017). The periodic actin-spectrin based structure provides elastic and stable support to the axon (figure 1.6 E). Moreover, spectrin specificity could influence the molecular organisation of the plasma membrane. In the PNJ, JXP and IND, α II and β II spectrins dominate while the nodes contain β IV spectrin (figure 1.6 A-D). This regional specialisation can restrict the movement of channels (Xu et al., 2013). In fact, previous studies in a β II spectrin knock-out mice model showed that the juxtaparanodal K_v channels dislocated towards the PNJ (Zhang et al., 2013). However, the maintenance of voltage-gated Na_v and K_v clusters in the node and JXP might also depend on the trophic support from the oligodendrocyte. Previous studies in mutant mice unable to synthesise sulfatide had decreased numbers of channel clusters, altered nodal length and dislocation of K_v channels (Ishibashi et al., 2002).

1.6 Diffuse axon injury mechanisms in NAWM

There are different possible neuro-axon injury mechanisms in MS. Axonal loss can result from anterograde and/or retrograde degenerative processes coming from demyelinating focal plaques (Friese et al., 2014). Anterograde degeneration, also known as Wallerian degeneration (Waller, 1850) consists of the degeneration of the sections of the axon distal to the injury and the subsequent clearance of the axonal debris by microglia and macrophages. On the contrary, retrograde degeneration, also known as dying back, as-

sumes that the degeneration starts at the distal ends of axons and spreads retrogradely (Coleman, 2005). In MS, retrograde axonal degeneration could be triggered in one or more focal lesions, which would lead to degeneration of the entire axon distal to these lesioned sites. Nonetheless, in both anterograde and retrograde degenerative mechanisms, the extent of axonal injury is determined by the dissemination of focal demyelinating plaques (DeLuca et al., 2006; Haider et al., 2016). On the contrary, axonal injury in MS NAWM tissue, as we have mentioned in previous sections, correlates with diffuse inflammation driven by locally activated microglia/macrophages, and therefore can be driven by a mechanism independent from focal inflammatory demyelination (Lassmann, 2018a). Hence, multiple molecular mechanisms could be underlying NAWM diffuse axon injury, including oxidative damage, mitochondria dysfunction and glutamate excitotoxicity (Frieze et al., 2014).

1.6.1 Oxidative damage and mitochondrial dysfunction

Chronically activated microglia and macrophages can release high amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The overproduction of ROS and RNS (oxidative stress), as potent intracellular oxidants, can lead to damage to nucleic acids, proteins, and lipids, and lead to cellular death (Khansari et al., 2009; Su et al., 2013). Previous literature has shown that the extensive oxidative damage in MS demyelinating lesions can lead to intra-axonal mitochondrial dysfunction (Lu et al., 2000; Van Horssen et al., 2008; Haider et al., 2011). If mitochondria are damaged, superoxide production can increase significantly within the axoplasm (Dutta et al., 2006; Mahad et al., 2009). Mitochondria synthesise ATP by oxidative phosphorylation for adequate AP conduction, thereby also produce ROS. Thus, in environments where ROS levels are increased, mitochondrial DNA mutations can accumulate and compromise the oxidative phosphorylation efficiency. For example, high levels of NO can inhibit mitochondrial respiration and reduce ATP synthesis (Su et al., 2009). The inhibition of the mitochondrial respiratory chain by NO could involve the failure of the energy-dependent Na^+/K^+ -ATPase which leads to an increase in the intra-axonal Na^+ levels and therefore in the intra-axonal Ca^{2+} by reversing the function of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Stys et al., 1992). Moreover, ROS

1.6. Diffuse axon injury mechanisms in NAWM

and NOS can activate enzymes including myeloperoxidase, xanthine and NADPH oxidases that can drive structural axonal degeneration (Gray et al., 2008; Fischer et al., 2012).

Some of the features that demonstrate mitochondrial dysfunction association with oxidative stress in MS demyelinating plaques include impaired NADH dehydrogenase (DH) activity (Lu et al., 2000), NADPH oxidase expression (Fischer et al., 2013), increased mitochondrial density in both axons and astrocytes expressing mtHSP70 (Witte et al., 2009), decreased activity of the mitochondrial complex IV (Mahad et al., 2008), decreased levels of the metabolite N-acetyl aspartate (NAA) (Benarroch, 2008; Mao and Reddy, 2010; Su et al., 2013) and reduced mitochondrial gene expression (Dutta et al., 2006). Further, another study has observed that the presence of the nuclear enzyme histone deacetylase 1 (HDAC1) in damaged axons impair mitochondrial movement by forming complexes with the motor proteins kinesins (KIF2A and KIF5), which are involved in anterograde transport of protein complexes, mRNA and membranous organelles in MS tissue and in a cuprizone-induced demyelination mouse model (Kim et al., 2010). Moreover, they found that the interaction of HDAC1 with the kinesins could happen in cultured neurons after the exposure of glutamate and TNF. This impairment in mitochondrial transport along axons has also been observed in an EAE mouse model (Sorbara et al., 2014). Therefore, from these studies, we can conclude that the impairment in anterograde transport of mitochondria compromises the ability of neurons to replenish mitochondria in long axons.

Regarding non-lesioned tissue, the decrease in nuclear DNA encoded mitochondrial transcripts has been related to the deficiency of the respiratory chain complex I and complex III in neurons located in non-demyelinated regions of the motor cortex (Dutta et al., 2006; Broadwater et al., 2011; Witte et al., 2013), cingulate gyrus and frontal cortex (Witte et al., 2013). Moreover, another study in SPMS tissue found respiratory deficient neurons with mitochondrial DNA deletions encoding complex IV in the cortical layer IV and the immediate subcortical WM (Campbell et al., 2011). Also, magnetic resonance spectrometry (MRS) studies, which calculate the chemical composition of brain tissue by the analysis of certain peaks in the resonance spectra, have found decreased NAA levels in NAWM tissue (Fu et al., 1998).

Mitochondrial damage can significantly affect neuronal physiology as they are highly dependent on oxidative metabolism for efficient and fast AP propagation. One of the suggested mechanisms by which oxidative stress can lead to mitochondrial dysfunction, apart from the accumulation of mutations in mitochondria DNA and disrupted mitochondrial transport, is the opening of the permeability transition pore (PTP). It is a transient pore in the inner mitochondrial membrane that opens in response to stimuli such as ROS, NOS and calcium and depends on the matrix protein cyclophilin D (CyPD) (Su et al., 2013; Friese et al., 2014). Previous studies have shown that the opening of this pore is mediated by p66ShcA (p66) protein (Su et al., 2013). p66 diffuses towards the mitochondrial intermembrane space, behaves as a redox enzyme by oxidizing cytochrome c, reducing oxygen into superoxide and hydrogen peroxide, which mediates the opening of the PTP (Giorgio et al., 2005). An EAE mouse model with a knock out of the p66 protein showed a consistent trend of reduced clinical impairment and significant neuropathology, compared to wild type (WT) mice, despite unaffected immune cell infiltration (Su et al., 2012). Additionally, a study in an EAE mouse model showed that in demyelinating lesions, the earliest ultra-structural sign of axonal injury before demyelination is mitochondrial pathology. They reported that it precedes axon fragmentation, co-localises with axonal swelling, usually occurs around the nodes of Ranvier, correlates with local microglia and macrophage activation and can be reversed with nitric oxide (NO) scavengers (Nikić et al., 2011). An earlier study showed that NO donors could cause reversible conduction block in axons and drive degeneration (Smith et al., 2001). Moreover, experimentally blocking action potential in CNS axons results in a decrease in the movement of mitochondria, whereas enhancing neuronal firing increases the mitochondrial movement and the size of mitochondrial stationary sites in the nodal/paranodal axoplasm (Ohno et al., 2011).

However, it is also essential to notice that myelinated axons have a compensation system since oligodendrocytes and astrocytes can support axons in case of an energy-deprived environment by transferring lactate via monocarboxylic acid transporters (MCT1, MCT2) to perform oxidative phosphorylation and produce ATP (Tekkök et al., 2005; Saab and Nave, 2017). Furthermore, previous studies with conditional Cox10 knockout mice in

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which oligodendrocytes and Schwann cells fail to assemble mitochondrial cytochrome c oxidase (COX) showed no signs of demyelination or axonal pathology in the CNS, suggesting that oligodendrocytes can survive via glycolysis and provide lactate to myelinated axons (Fünfschilling et al., 2012).

In conclusion, the elevated release of ROS, RNS species, glutamate and cytokines such as TNF from activated microglia and macrophages in MS NAWM tissue can damage mitochondria located in the juxtaparanodal/internodal compartments and compromise significantly the integrity of myelinated axons by impairing mitochondrial anterograde transport, damaging mitochondrial DNA or promoting the opening of the PTP.

1.6.2 Glutamate excitotoxicity

Glutamate is the major excitatory neurotransmitter in the CNS, and under physiological conditions, glutamate levels are highly regulated. The concentration of glutamate inside the axon is approximately 10 mM, whereas in the extracellular space it is low, around 0.5-4 μ M. Moreover, glutamate cannot penetrate the BBB, so it has to be synthesised from glucose or glutamine (Malekzadeh Shafaroudi et al., 2018). However, in neurodegenerative diseases such as MS, glutamate synthesis and release and re-uptake can be disrupted, leading to increased concentrations of extracellular glutamate, which can be neurotoxic (Miladinovic et al., 2015; Macrez et al., 2016). Multiple MRS studies have demonstrated elevated glutamate levels in acute MS lesions and NAWM tissue compared to non-neurological controls (Srinivasan et al., 2005; Tisell et al., 2013; Azevedo et al., 2014). Increased glutamate levels have also been found in the CSF of MS patients (Sarchielli et al., 2003). Furthermore, genome-wide association analysis of 382 MS patients showed that patients had a higher number of associated alleles from genes in glutamatergic pathways, which were also associated with decreased levels of NAA (Baranzini et al., 2010).

In MS, chronically activated microglia, macrophages, neutrophils and dendritic cells are considered the main sources of glutamate (Piani et al., 1991; Parpura et al., 2004; Pacheco et al., 2006; Takeuchi et al., 2006; Evonuk et al., 2015). Dendritic cells can release glutamate

when in contact with T-cells (Pacheco et al., 2006). Moreover, T-cells can induce glutamate release from these antigen-presenting cells (Evonuk et al., 2015). The activation of microglia and macrophages can promote the release of high amounts of glutamate through the cysteine/glutamate antiporter Xc⁻ (Pampliega et al., 2011; Evonuk et al., 2015) or via the connexins 32 (cx32) or connexins 43 (cx43), which are subunits part of the microglial gap junctions (Takeuchi et al., 2006; Eugénin et al., 2001). Gap junctions allow intracellular propagation of second messengers (Ca^{2+}), metabolites (glucose and glutamate), and nucleotides (ATP and RNA) between adjacent cells (Goldberg et al., 1999, 2002; Harris, 2001, 2007; Sáez et al., 2003; Valiunas et al., 2005). Also, they can facilitate molecular transfer between the cytoplasm and extracellular milieu (De Vuyst et al., 2007; Laird, 2010; Takeuchi and Suzumura, 2014).

In terms of glutamate synthesis, microglia and macrophages produce glutamate mainly by two mechanisms. Firstly, via glutaminase hydrogenase which converts α -ketoglutarate to glutamate. Secondly, via glutaminase from extracellular glutamine brought into the cell via glutamine transporters (Newsholme and Calder, 1997; Nissim, 1999; Takeuchi and Suzumura, 2014). However, pro-inflammatory cytokines such as TNF, IFN γ and LT α enhance specifically the expression of glutaminase and cell-surface hemichannels, such as Cx32 and Cx43 in activated microglia and macrophages (Eugénin et al., 2001; Takeuchi et al., 2006). Moreover, abnormal expression of glial connexins, such as astrocytic Cx43 and oligodendrocytic Cx32 and Cx47, has been observed in human MS lesioned tissue and in EAE mouse models both in lesions and in NAWM tissue (Brand-Schieber et al., 2005; Markoullis et al., 2012).

Glutamate signalling is mediated by ionotropic receptors (NMDA and AMPA), and glial glutamate transporters (EAAT1 and EAAT2, also known as SLC1-A3 or GLAST and SLC1-A2 or GLT-1, respectively) are expressed in astrocytes, microglia, and oligodendrocytes. Mature myelinating oligodendrocytes express AMPA/kainate receptors on their soma while their processes are enriched in NMDA receptors (Salter and Fern, 2005). Specifically, NMDA receptors are located in the cytoplasm filled spaces of the oligodendrocytes, which include the adaxonal space and the PNJ (Piña-Crespo et al., 2010; Micu

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et al., 2016; Saab and Nave, 2017). AMPA receptors are Na^+ permeable and their activation leads to depolarization, which in turn enables the removal of the NMDA receptor Mg^{2+} block (Nowak et al., 1984) and Ca^{2+} ions to flow in (Dingledine et al., 1999). High intracellular Ca^{2+} could be buffered in the neuronal soma by the endoplasmic reticulum (Subramanian and Meyer, 1997). NMDA receptors are also Ca^{2+} permeable and an increment in extracellular glutamate could overstimulate them, leading to calcium overload within the axoplasm. Given the presence of Ca^{2+} sensitive enzymes such as calpain-1 and phospholipase C in central myelin, Ca^{2+} accumulation could promote degradation of a number of cell adhesion protein such as Nf155 or cytoskeletal proteins involved in membrane trafficking such as tubulins, septins, ERM or Rab3 (Jahn et al., 2009). The consequences of these effects are greater in oligodendrocytes as myelin proteins have a half-life of the order of several weeks to months. For instance, the half-life of the bulk of cholesterol in the CNS has been estimated to be at least five years (Björkhem et al., 1998). Thus, myelin protein degradation could be very severe and trigger axonal degeneration.

Glutamate could also activate calmodulin-dependent protein kinases (CaMK), which activates nitric oxide synthase (NOs) and increases the intracellular NO (Takeuchi and Suzumura, 2014). The increment of NO and ROS (Loikkanen et al., 1998), as we have mentioned in the previous section, can lead to mitochondrial dysfunction. Impaired mitochondrial activity linked to Ca^{2+} accumulation due to glutamate excitotoxicity has been shown by NAD(P)H fluorescence in brain slices (Rosenstock et al., 2010) and hippocampal neurons (Stanika et al., 2010; Kambe et al., 2011). Further, glutamate could modulate the opening of the mitochondrial PTP (Gincel and Shoshan-Barmatz, 2004) and impair anterograde mitochondrial transport leading to neuritic beading (Kim et al., 2010). Furthermore, direct administration of glutamate has also been associated with paranodal splitting in the spinal cords of myelinated axons in an EAE mouse model (Fu et al., 2011) and in the spinal cord of adult guinea pigs (Fu et al., 2009). Therefore, multiple studies in EAE models have shown that blockers of NMDA and AMPA receptors improve the neurological dysfunction of the animals and limit degeneration (Pitt et al., 2000; Smith et al., 2000; Kanwar et al., 2004; Centonze et al., 2009). NMDA and AMPA channels can also be found clustered at the synaptic cleft, and therefore any modification in their function can

lead to changes in AP generation and excessive synaptic excitation (Romanos et al., 2019).

In conclusion, elevated glutamate levels could be one of the main contributors to diffuse axonal injury in MS NAWM tissue as previous studies have demonstrated the presence of high levels of this molecule at these regions and due to the presence of chronically activated microglia and macrophages as the primary sources of glutamate in the inflamed WM.

1.7 The action potential (AP)

The AP is the fundamental signal of the nervous system. Each AP signal is a rapid and transient change in the electrical potential across the plasma membrane, which propagates from the soma of the neuron along the axon and it is transmitted between cells through synapses.

1.7.1 AP recording

In order to measure AP conduction experimentally, patch clamping technique was developed by the Nobel Prize winners Sakmann and Neher (Sakmann and Neher, 1984). This technique allowed the recording of voltage membrane and ionic currents over time. Moreover, it allowed the resolving of single-channel currents through cell-attached patches. Sakmann and Neher (1984) showed that the behaviour of voltage-gated channels in the axolemma was stochastic and digital (open/close), due to the thermodynamic fluctuations they are subjected to. Thus, they can be modelled mathematically as transistors that follow a binomial distribution. The discovery of the patch clamping technique supported the results obtained by the first biophysical model of the AP conduction developed by the Nobel Prize winners Hodgkin and Huxley (HH) (Hodgkin and Huxley, 1952). This model was derived from their experimental observations in a giant squid axon to determine the dynamics of channels and explain the generation of each AP. From their experiments, they arrived at the development of an electrical circuit model, which simulates how AP is conducted. They showed that current propagating through the cellular membrane could be modelled as the charging and discharging of a capacitor (C_m)

1.7. The action potential (AP)

or via time and voltage-dependent resistances in parallel with the capacitor (figure 1.7 B). Each of the resistances corresponds to the Na^+ , K^+ and leakage currents present in the membrane flowing through voltage-gated ion channels ($g=1/R$). Further, each current can be determined by the driving force generated by the difference between the membrane potential and the reversal potential (equilibrium) of each ion (E_l , E_{Na} and E_K), and the maximum open probability related to each ion channel (figure 1.7 B).

1.7.2 AP conduction

Physiologically, there is an electrochemical gradient across the membrane that determines the resting membrane potential of the neuron ($V_m = -90$ mV). The concentration or diffusion gradient is created because K^+ permeability is higher than Na^+ . Therefore, following this gradient, K^+ ions tend to flow from inside to outside, whereas the Na^+ ions do it in the opposite direction. This creates an electric field, which makes the inside of the membrane more negative than the outside. Therefore, the electric drift forces K^+ ions to stay in the axoplasm, while the Na^+ would be prone to flow inside. The resting membrane potential or Nerst equilibrium is achieved when the diffusion force equals the electric drift.

The injection of positive current depolarises the membrane. If the voltage surpasses a certain threshold, an AP is fired; otherwise, the signal is passive (figure 1.7 A). The AP signal is a regenerative and an all-or-nothing event. This term means that no matter how strong the stimulating current is if it does not reach the firing threshold, no AP can be generated. In fact, the amplitude of the AP does not depend on the strength of the stimulus, it is encoded in the frequency of the stimulation, and if the stimulus persists, multiple APs would be generated.

The AP propagates along the axon via the opening and closing of the voltage-gated channels of the axonal membrane. Hodgkin and Huxley showed that Na_v channels contain four gates, three of them control the opening of the channel (m), while one controls the closing of the channel (h). Meanwhile, K_v channels have four opening gates (n). However, m, n and h have different kinetics that depend on voltage and time. During an AP

signal, the axonal membrane is depolarised by the entrance of Na^+ ions inside the cell through the voltage-gated Na_v channels (figure 1.7 A). In this phase, the m and h gates are open, while the n gates remain closed. At the peak of the AP, the h gates start to close, while the n gates start to open. Therefore, at the falling phase, the K^+ ions flow outside the membrane repolarising the electrical potential (figure 1.7 A). Lastly, during the hyperpolarising phase, the m gates close, while the n gate stays open and hence K^+ ions can keep flowing towards the extracellular space (figure 1.7 A). Therefore, for the recovery and maintenance of the resting membrane potential, the active metabolic function of the pump Na_v/K_v ATPase transports three Na^+ to the outside of the cell and two K^+ ions to the inside.

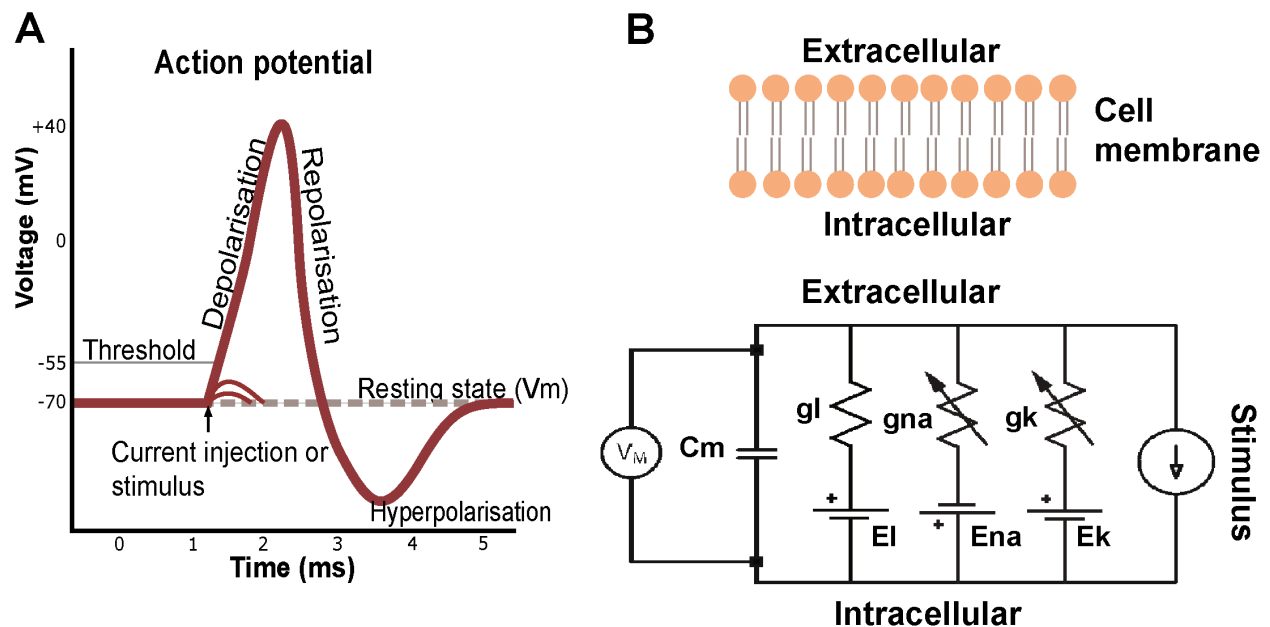


Figure 1.7: Action Potential. (A) Voltage change (mV) versus time (ms) plot during the generation of an AP. This signal is only generated when the membrane voltage surpasses the firing threshold. In the diagram, depolarisation, repolarisation and hyperpolarisation phases are represented. (B) The dynamics of the AP across the membrane can be represented in the form of an electrical circuit. In this image, the electrical circuit corresponds to the one in the HH model (Hodgkin and Huxley, 1952).

In myelinated axons, the AP is only generated at the nodes of Ranvier, as the only non-myelinated sites along the axonal length with a high density of clustered voltage-gated Na_v channels. The high resistance and low capacitance properties of myelin insulate the

1.7. The action potential (AP)

rest of the axolemma electrically, enabling passive current to flow longitudinally inside the axon towards the next node (Huxley and Stämpeli, 1949). Since the AP is only measured at the nodes, this type of conduction is widely referred to as saltatory conduction.

1.7.3 AP simulation

Since the Hodgkin and Huxley biophysical model of the AP (figure 1.7 B), multiple computational simulations have increased the complexity of these models. Currently, some of the widely used electrical models include the integrate and fire model or the leaky integrate and fire model (Laing and Lord, 2010). Furthermore, regarding the propagation of the AP, most models describe neuronal geometry (axon and dendrites) as cylindrical structures modelled following the classical cable theory. However, in order to avoid simplifications of the neuro-axonal structure using this theory, compartment models have been created to establish different electrical properties for different regions of the neuron and axon. For instance, in the case of a myelinated axon, with compartmental models, the node, paranode, juxtaparanode and internode could have distinct biophysical features, as well as the axoplasm, peri-axonal space and myelin sheath spaces (Cohen et al., 2020). Deterministic simulators such as NEURON (Hines and Carnevale, 1997) and stochastic simulations such as Modigliani (Faisal et al., 2005) have been used to model the morphology of axons and neurons compartmentally and capture the average and stochastic behaviour over many repeated events.

1.7.4 AP disruption in MS

AP conduction can be highly disrupted in MS by demyelinating lesions and changes in the proteins that preserve the dimensions of the nodes of Ranvier. It has been shown that when an axon is demyelinated in an MS lesion, the propagation becomes passive and therefore the conduction speed is significantly reduced, leading to a conduction block, which is one of the major causes of devastating MS symptoms such as paralysis and blindness (Smith, 1994). In fact, the conduction block in the first stages of demyelination will depend on the proportion of current available to depolarise the node versus the

needed current (Smith, 1994). However, the conduction block can be reserved in demyelinated axons when the expression of voltage-gated Na_v channels is increased (Craner et al., 2004). Nonetheless, the speed of this conduction would remain inefficient and slow as it would require the activation of voltage-gated Na_v channels along the entire length of the axolemma.

The disruption of the paranodes could disrupt the dimensions of the nodes in MS and widen up the peri-axonal space connecting the node with the juxtaparanode. In turn, this will enable current shunting beneath the myelin sheath, reducing the proportion of passive current flowing to the next node and eventually slowing down the AP conduction. Furthermore, the disruption of the paranode could enable K_v channels to dislocate toward the PNJ, making them more exposed to the extracellular space. The increment in the concentration of K^+ in the extracellular domain would increase the risk of K^+ induced depolarisation and the generation of spontaneous activity that can provoke epileptic seizures (Rosenbluth, 2009). In fact, MS patients have a higher probability of epileptic seizures than the general population (Kelley and Rodriguez, 2009). Also, more energy is required to pump K^+ back again inside the cell to recover the resting membrane potential. Concerning the voltage-gated Na_v channels clustered at the nodes of Ranvier, any decrease in their density could affect the efficiency of the propagation as a minimum density of Na_v channels is needed to surpass the depolarising firing threshold of an AP signal. For instance, disruption in paranodal axo-glial junctions could enable the dislocation of Na_v channels underneath the myelin sheath, and less number of these channels would experience the change in voltage. Moreover, in MS, axonal swellings could alter the axonal diameter. The capacitance and resistivity of the cellular membrane are proportional to its surface area and determines the rate at which Na_v channels open (Babbs and Shi, 2013). Hence, any increase in surface area due to inflammatory swellings would decrease AP conduction speed. Changes in nodal length could also affect AP conduction as more area of the membrane would be needed to be depolarised (Arancibia-Carcamo and Attwell, 2014).

In summary, the proper structure of the myelin sheath and the different domains at the

nodes of Ranvier, are crucial for efficient and fast AP conduction in myelinated axons.

1.8 Hypothesis

The disruption of the structure of paranodal axo-glial junctions structure by microglial and macrophages activation in MS NAWM leads to an elongation of the non-compacted myelin loops from the axolemma, which in turn produces defects in AP conduction.

1.9 Aims

The general aims of this thesis include:

- To characterise paranodal axo-glial pathology present in human post-mortem MS NAWM tissue and correlate it to local microglial and macrophage activation and axonal cytoskeleton disruption (**Chapter 3**);
- To quantify and characterise paranodal axo-glial pathology in the NAWM regions in a chronic model of meningeal inflammation induced by lentiviral vectors containing $LT-\alpha$ / $IFN-\gamma$ (**Chapter 4**);
- To simulate in-silico paranodal axo-glial disruption and estimate changes in conduction velocity, amplitude and width of the AP propagation under different structural disruptions at the nodes of Ranvier (**Chapter 5**);
- To investigate the best strategy to trigger glutamate release in primary microglial cultures by different combinations of pro-inflammatory cytokines including TNF, $IFN\gamma$ and $LT\alpha$, as well as, inducing and quantifying paranodal axo-glial pathology in organotypic cerebellar tissue slices by direct exposure to pro-inflammatory cytokines, high glutamate microglial-conditioned medium and direct glutamate administration (**Chapter 6**);

2

Methods

2.1 Human post-mortem tissue selection and processing

Tissue blocks for this study were provided by the UK Multiple Sclerosis Society Tissue Bank at Imperial College London. Brains were collected following fully informed consent by the donors via a prospective donor scheme following ethical approval by the National Multicentre Research Ethics Committee (MREC 02/2/39). Thirty-four blocks from twenty cases of neuropathologically confirmed SPMS (Table 2.1; 11 females, mean age of 54.3 years (range 38-76 yrs), post-mortem delay (PMD) mean of 22.03 hours (range 12-48 hrs), and a mean disease duration of 28.35 years (range 12-42 yrs)) and sixteen blocks of nine non-neurological controls were selected (Table 2.2; 3 females, mean age of 73.67 years (range 50-88 yrs) and post-mortem delay (PMD) mean of 23.12 hours (range 8-48 hrs)). Tissue blocks (2x2x1 cm) were chosen from the pre-central gyrus, which contains the primary motor cortex and the cerebral peduncle located in the midbrain. These two brain

2.2. Primary and secondary antibodies

regions were chosen due to the presence of white matter (WM) tracts that contain highly longitudinally aligned axons that would make quantitative analysis easier. The blocks were snap-frozen in isopentane on dry ice, cryoprotected in 30% sucrose, stored at -75°C in a ultra-low temperature freezer (New Brunswick Scientific, Labx) and cryosectioned at a thickness of $10\mu\text{m}$ with the cryostat CM1850 (Leica).

2.2 Primary and secondary antibodies

The primary antibodies used in this project were: mouse anti-MOG (clone Y10, Prof Reynolds, Imperial College London, UK); rabbit anti-myelin basic protein (MBP) (Polyclonal, Merck, Darmstadt, Germany); mouse anti-neurofilament-H protein (clone NE14; Merck, Darmstadt, Germany); mouse anti-dephosphorylated neurofilament protein (clone smi32; Biolegend, San Diego, CA, USA); rabbit anti-neurofascin 155 (NFF3: provided by P.J.B (Tait et al., 2000)); rabbit anti-caspr (clone EPR7828, Abcam, Cambridge, UK); mouse anti-caspr (clone K65/35; Neuromab, Davis, CA, USA); mouse anti-pan- Na_v channels (clone K58/35, Neuromab, Davis, CA, USA); mouse anti- K_v 1.2 channels (clone K14/16, Neuromab, Davis, CA, USA); mouse anti-HLA-DR (clone TAL.1B5, Dako Agilent, Santa Clara, CA, USA); rabbit anti-IBA1 (Polyclonal IgG, Wako Pure Chemical Corporation, USA); rabbit anti-Glial Fibrillary Acidic Protein (GFAP) (Polyclonal, Dako Agilent, Santa Clara, CA, USA); mouse anti-Calbindin1 (clone CB-955, Merck, Darmstadt, Germany); mouse anti-NeuN (clone A60, Merck, Darmstadt, Germany); and mouse anti-porin (clone 20B12AF2, Abcam, Cambridge, UK) (Table 2.3).

All the secondary antibodies used for immunofluorescence were purchased from ThermoFisher Scientific (USA): Alexa Fluor 546 Goat Anti-Mouse IgG (H+L), Alexa Fluor 546 Goat Anti-Rabbit IgG(H+L), Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) Fluor 488 Goat Anti-Rabbit IgG (H+L), Alexa Fluor 488 Goat Anti-Mouse IgG1, Alexa Fluor 546 Goat Anti-Mouse IgG1, Alexa Fluor 647 Goat Anti-Mouse IgG1, Alexa Fluor 488 Goat Anti-Mouse IgG2b, Alexa Fluor 647 Goat Anti-Mouse IgG2b, Alexa Fluor 488 Streptavidin, Alexa Fluor 546 Streptavidin, Alexa Fluor 647 Streptavidin. For immunohistochemistry and to couple with Alexa Fluor Streptavidins, the following biotinylated antibodies were

used from Vector Laboratories (UK) : biotinylated anti-rabbit IgG (H+L) made in goat, biotinylated anti-mouse IgG (H+L) made in horse and biotinylated anti-mouse IgG2a made in goat (Life Technologies, ThermoFisher Scientific).

2.3 Immunohistochemistry of post-mortem human tissue

For immunofluorescence, sections were fixed with 4 % paraformaldehyde (PFA, Sigma-Aldrich) for 30 min for all the antigens (Table 2.3), except for the Pan-Na antibody for which fixation was not longer than 10 min. After fixation, sections were post-fixed with 100 % methanol at -20 ° C (Sigma) for 10 min and washed in 0.1M PBS-0.3 % Triton X-100 (Sigma-Aldrich) three times, five minutes each. After post-fixing, sections were blocked and permeabilized with 10 % normal horse/goat serum (Sigma-Aldrich) in PBS-0.3 % Triton X-100 for 1 hr at room temperature. Finally, sections were incubated overnight at 4 ° C in a humid chamber with primary antibodies (Table 2.3) containing 10 % normal horse/goat serum and PBS-0.3 % Triton X-100 (Sigma-Aldrich). After primary antibody incubation, sections were thoroughly rinsed in 0.1M PBS at least three times, five minutes each. After rinsing, sections were incubated with the appropriate secondary antibody conjugated to biotin for 1 hr for MOG, SMI32 and Pan-Nav antigens, and rinsed in 0.1M PBS. After rinsing, sections were incubated with Alexa Fluor streptavidin or the appropriate species-specific secondary antibody conjugated to the proper fluorochrome, for 2 hrs at room temperature. Finally, the tissue was rinsed in 0.1M PBS and dH_2O , nuclear counterstained with DAPI (diluted in 1:2000, Sigma-Aldrich) and mounted with Vectashield Antifade Mounting Media (Vector Laboratories). The coverslips (24x50 mm, VWR, International) were fixed to the slides (SuperFrost Plus, VWR, International, USA) with nail-polish.

Immunohistochemistry was performed for HLA-DR antigen with the ImmPRESS HRP Anti-Mouse IgG (Peroxidase) Polymer Detection Kit, made in horse (Vector Laboratories). Using this kit, the tissue endogenous peroxidase activity was blocked with the BLOXALL Blocking Solution (SP-6000) for 10 min and for unspecific staining with 2.5 % horse serum for 20 min. After blocking and washing with 0.1M PBS, the primary antibody was

2.3. Immunohistochemistry of post-mortem human tissue

	Blocks	Age(yrs)	PMD(hrs)	Gender	Duration(yrs)	Cause of death
1	MS461-A1B4	43	13	Male	21	Bronchopneumonia
2	MS461-A13D	43	13	Male	21	Bronchopneumonia
3	MS500-A15B	50	32	Male	29	Urinary Sepsis
4	MS500-A13D	50	32	Male	29	Urinary Sepsis
5	MS510-A15B	38	19	Female	22	Pneumonia
6	MS510-A1C5	38	19	Female	22	Pneumonia
7	MS444-A1B6	49	18	Male	20	Renal Failure
8	MS478-A13C	63	24	Female	39	Metastatic bowel cancer, MS
9	MS478-A1B5	63	24	Female	39	Metastatic bowel cancer, MS
10	MS535-A1C4	65	12	Female	40	MS
11	MS535-A1B5	65	12	Female	40	MS
12	MS404-A13D	55	17	Female	34	Septicaemic shock, Pneumonia
13	MS404-A15B	55	17	Female	34	Septicaemic shock, Pneumonia
14	MS584-A1B4	42	26	Female	12	MS
15	MS584-A1D3	42	26	Female	12	MS
16	MS466-A1B6	65	25	Female	36	MS, Pneumonia lung cancer
17	MS466-A15D	65	25	Female	36	MS, Pneumonia lung cancer
18	MS549-A14D	50	8	Male	29	MS
19	MS422-A14D	58	25	Male	25	Chest Infection, MS
20	MS411-A12C	61	24	Male	29	Pneumonia, MS
21	MS587-A1B5	58	36	Female	20	Cardiac arrest, MS
22	MS567-A15B	45	48	Female	23	MS
23	MS567-A12C	45	48	Female	23	MS
24	MS542-A1B4	76	12	Female	35	Pneumonia, MS
25	MS517-A13D	48	12	Female	25	Chest Sepsis MS
26	MS517-A16B	48	12	Female	25	Chest Sepsis MS
27	MS406-A16B	62	23	Male	42	Chest Infection Pneumonia, MS
28	MS406-A13D	62	23	Male	42	Chest Infection Pneumonia, MS
29	MS523-A15C	63	20	Female	32	Bronchopneumonia, MS
30	MS523-A1B5	63	20	Female	32	Bronchopneumonia, MS
31	MS530-A13C	42	15	Male	21	MS
32	MS530-A16B	42	15	Male	21	MS
33	MS585-A15C	53	27	Female	27	Bronchopneumonia, MS
34	MS585-A15B	53	27	Female	27	Bronchopneumonia, MS
	34 BLOCKS	54.30	22.03	22F: 12M	28.35	

Table 2.1: Clinical data of post-mortem MS tissue. Table showing the age, gender, disease duration, and cause of death of the MS patients, as well as the block name and the post-mortem delay (PMD) in hours of the blocks.

	Blocks	Age(yrs)	PMD(hrs)	Gender	Cause of death
1	C72-A1D3	77	26	Male	Pneumonia
2	C72-A1C4	77	26	Male	Pneumonia
3	C48-A16B	68	10	Male	Colon cancer
4	C54-A1D2	66	16	Male	NA
5	C54-A15B	66	16	Male	NA
6	C75-A1B1	88	8	Male	COPD
7	C75-A1C2	88	8	Male	COPD
8	C76-A13D	87	31	Male	Pneumonia
9	C76-A11C	87	31	Male	Pneumonia
10	PDC29-P11B	82	48	Male	Liver and lung cancer
11	PDC29-P12D	82	48	Male	Liver and lung cancer
12	PDC39-A21C	50	28	Female	Renal cancer
13	PDC40-A12B	61	15	Female	Ovarian Cancer
14	PDC40-A12C	61	15	Female	Ovarian Cancer
15	C74-A11D	84	22	Female	Old age
16	C74-A13B	84	22	Female	Old age
	16 BLOCKS	73.67	23.12	5F: 11M	

Table 2.2: Clinical data of post-mortem non-neurological control tissue. Table showing the age, gender, the cause of death of the non-neurological control donor cases, as well as the block name and the post-mortem delay (PMD) in hours of the tissue blocks.

incubated overnight at 4 ° C in a humid chamber. Then the ImmPRESS Polymer Reagent was incubated at room temperature for 30 min and the signal was developed with the peroxidase substrate solution (ImmPact DAB, SK-4105, Vector Laboratories). The tissue was then rinsed with tap water for 5 min to stop the reaction. Lastly, it was counter-stained with hematoxylin (Sigma-Aldrich) to see the nuclei for 5 min, washed with tap and distilled water, dehydrated with washes of 70 %, 90 % and 100 % of ethanol (2 min each), cleared with xylene for 10min and mounted with DPX mounting medium (Sigma-Aldrich).

2.4. Lentiviral vectors

	Antigen	Specificity	Concentration	Clone	Source
1	MOG	Myelin oligodendrocyte protein	1:50	Mouse Y10	Prof Richard Reynolds
2	MBP	Myelin Basic Protein	1:500	Rabbit Polyclonal	Merck
3	Nfil	Neurofilament-H protein	1:500	Mouse NE14	Merck
4	SMI32	De-phosphorylated neurofilament	1:500	Mouse SMI32 (IgG1)	Biolegend
5	Nf155	Paranodal protein	1:500	Rabbit NFF3	Provided by P.J.B
6	Caspr1	Paranodal protein	1:300	Rabbit EPR7828	Abcam
7	Caspr1	Paranodal protein	1:500	Mouse K65/35 (IgG1)	Neuromab
8	Pan- Na_v	Voltage-gated sodium channels	1:100	Mouse K58/35 (IgG2a)	Neuromab
9	$K_v1.2$	Voltage-gated potassium channels	1:100	Mouse K14/16 (IgG2a)	Neuromab
10	HLA-DR+	Human Leukocyte Antigen	1:200	Mouse TAL.1B5	Dako Agilent
11	IBA1	Calcium-binding protein	1:500	Rabbit Polyclonal	Wako
12	GFAP	Glial Fibrillary Acidic Protein	1:500	Rabbit Polyclonal	Dako Agilent
13	Calbindin1	Calbindin1 (Purkinje neurons)	1:500	Mouse CB-955 (IgG1)	Merck
14	NeuN	Neuron-Specific Nuclear Protein	1:500	Mouse A60	Merck
15	Porin	VDAC1 (Mitochondria)	1:500	Mouse 20B12AF2	Abcam

Table 2.3: Primary antibodies details. Table showing the antigen, specificity, concentration, clone and source of the primary antibodies used in this project.

2.4 Lentiviral vectors

Lentiviral (LV) vectors expressing human $LT\alpha$ (LVLT α), human IFN γ (LVIFN γ) or enhanced green fluorescent protein (LVGFP) genes were produced by Dr. Rachel James. In order to produce these vectors, human immunodeficiency virus type 1 (HIV-1) transfer vector (326-pRRL-sincppt-CMV-eGFP-WPRE genome plasmid), which carries the human cytomegalovirus promoter (CMV) promoter was used. Complementary DNA sequence

(cDNA) for human LT α or IFN γ were codon designed for rat including a 5 Kozak sequence and synthesised by Gene Art (Life Sciences, Paisley, UK). Moreover, the human transgene DNA fragments were excised by restriction digest with Xba1 and Sal1 prior to purification by preparative agarose gel electrophoresis. Gene specific primers were used to amplify the cytokine transgene with the addition of Xba1 and Sal1 restriction sites for directional cloning into the transfer vector. The GFP fragment was removed from the transfer plasmid by digestion with Xba1 and Sal1 and the transgene DNA fragments ligated in-frame using the same restriction sites.

To produce the lentiviral vectors, HEK 293T cells were transfected with vector plasmid (pRRLsincppt-CMV-TNF-WPRE, pRRLsincppt-CMV-IFN -WPRE or pRRLsincppt-CMV-GFP-WPRE), packaging vector plasmids expressing the HIV-1 gag/pol gene (pMD2-LgRRE), HIV-1 Rev (pRSV-Rev) and VSV-G envelope plasmids (pMD2-VSV-G) following the addition of 2 mol/L CaCl₂. 16 hrs later the virus production was induced by sodium butyrate (Sigma-Aldrich). The viral supernatant was collected 36 hrs post sodium butyrate and filtered through a 0.45 μ m pore size filter. Preparations were concentrated by centrifugation at 6000 rpm for 16 hrs at 4 °C and the pellet resuspended in ice-cold phosphate-buffered saline (PBS) and was further concentrated by ultracentrifugation for 90 min at 20,000 rpm, 10 °C. The pellet was resuspended in 100 μ l ice-cold TSSM formulation buffer (20 mmol/L Tromoethamine, 100 mmol/L NaCl, 10 mg/mL sucrose and 10 mg/mL mannitol). The biological and physical titres were calculated as described previously. The lentiviral genome copy number was calculated using the Clontech Lenti- X qRT-PCR Titration kit (Takara).

2.5 Stereotaxic surgery and tissue processing

Surgeries were carried out by Dr. Browne and Dr. James. All animal experiments were carried out under the regulations of the UK Home Office. Eleven eight-ten week old female Dark Agouti (DA) rats (140-160 g) were obtained from Janvier (France) and kept in groups of four in a 12 hrs light/dark cycle with food and water provided ad libitum. Stereotaxic injections of lentiviral vectors into the subarachnoid space were carried out

2.5. Stereotaxic surgery and tissue processing

using at 0.9 mm caudal to bregma, on the midline, following previously published methods (Gardner et al., 2013). Rats were either naïve with no treatment, immunised with MOG in incomplete Freund's Adjuvant (IFA) or IFA alone. The induction of sub-clinical experimental autoimmune encephalomyelitis (EAE) was induced as described previously (Gardner et al., 2013). Rats were anaesthetised with isoflurane and immunised intradermally at the base of the tail (dorsal aspect) with 5 μ g of recombinant mouse MOG (amino acids 1-119, corresponding to the external Ig-like domain) diluted in sterile PBS and emulsified in an equal volume of IFA (Sigma-Aldrich, 100 μ l total volume) or with PBS alone emulsified in IFA. However, in this study, only animals immunised with IFA were analysed (n=3 naïve rats, n=3 rats injected with LV expressing GFP genes, and n=5 rats injected with LV expressing LT α /IFN γ genes). The stereotactic surgery was performed at 20-23 days post-immunisation.

Rats were fully anaesthetised (2 % isoflurane; Abbott Laboratories, Berkshire, UK and oxygen 2 l/min), their scalps were shaved and disinfected with Videne antiseptic solution (Ecolab). Subcutaneous injections of 0.9 % saline (Sigma) and 0.01 mg/kg buprenorphine (Vetergesic; Alstoe Animal Health, North Yorkshire, UK) were performed to provide post-surgery rehydration. Rats were positioned on the stereotaxic frame (Stoelting, Dublin, Ireland) and an incision was made through the scalp to visualise the skull bregma. A power drill (RS Components, Northamptonshire, UK) was used to make a hole at 0.9 mm (figure 2.1 A, B) caudal to bregma, at the motor cortex. Injections were performed with a finely calibrated glass capillary attached to a 26 gauge needle of a 10 μ l Hamilton syringe was made (Hamilton, Graubünden, Switzerland). The needle was inserted to a depth of 2.4 mm below the dural membrane. At this point, using an automated infuser (KD Scientific, USA), 4 μ l of lentiviral vectors (2 μ l of each vector) diluted in TSSM with 0.5 mM monastral blue tracer were introduced at a rate of 0.2 μ l/min. Viruses were injected at a total of 1×10^{12} genomic copies/ μ l (GC/ μ l) for LT α and 1×10^{10} GC/ μ l for GFP and IFN γ . The needle was left in place for 5 min to allow diffusion of the sample from the area of injection, then withdrawn and the incision was sutured (Mersilk; Covidien, Ireland). The sutures were removed after 7-10 days and the animals were monitored daily.

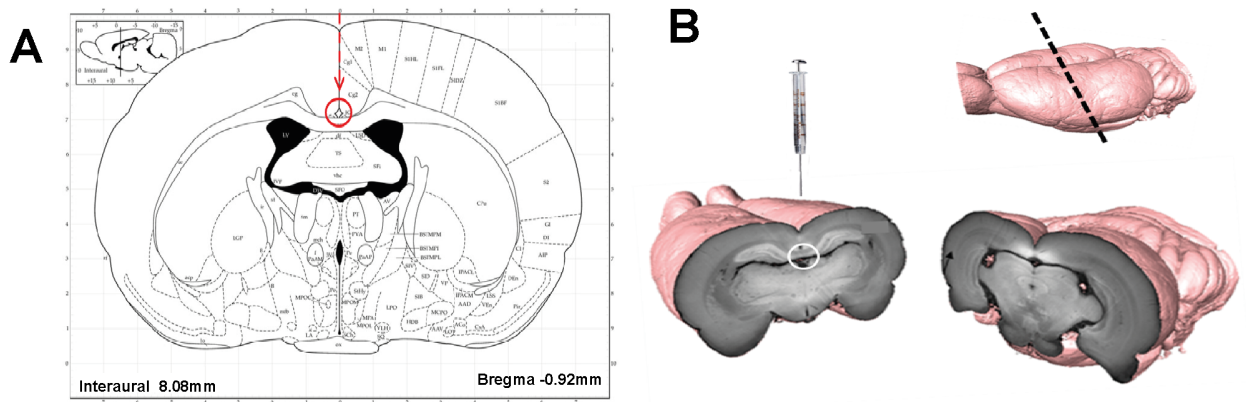


Figure 2.1: Stereotaxic surgery. (A) Diagram of the rat brain at -0.92 mm bregma. (B) Digital reconstruction of a rat brain (Browne, 2015).

2.5.1 Tissue processing and immunohistochemistry

At the termination of the study, rats received an overdose of sodium pentobarbital (200 mg/ml Euthatal; Merial Animal Health, Essex, UK) by intraperitoneal injection. Then, rats were perfused with 50 ml PBS followed by 100 ml 4 % PFA in PBS through the left ventricle at 90 days post viral injection. Brains were removed and post-fixed in 4 % PFA (4 hrs, room temp), prior to cryo-protection in 30 % sucrose solution in PBS (48 hrs or until equilibrated). Then, brains were embedded in optimal cutting temperature compound (OCT; Tissue-Tek; Sakura, The Netherlands), frozen in isopentane on dry ice and cut at 10 μ m in the coronal plane throughout the brain.

The rat tissue sections were stained following the same protocol as the human tissue except the initial 4 % PFA fixation step.

2.6 Primary rat microglial culture

P0-P2 Sprague Dawley rats (Charles River Laboratories, USA) were decapitated following the UK Home Office regulations under the project licence PPL/70/8916. Every step of the procedure was carried out in a laminar flow hood (Scanlaf, Fortuna, LaboGene, Denmark). The brains were isolated, the cerebral cortices were dissected with sterile autoclaved tweezers and dissecting scissors and freed of meninges to avoid any fibroblast

2.6. Primary rat microglial culture

contamination. The cortices of three pups were transferred to a 50 ml sterile polypropylene conical tube (Falcon, A Corning Brand) with dissection medium, which contained Hank's Balanced Salt Solution (HBSS 1X, Gibco, ThermoFisher Scientific). The supernatant was removed and replaced by digestion mix. The digestion mix contained Minimum Essential Media (MEM with 4 mM glutamine, Gibco, ThermoFisher Scientific), 2.5 mg/ml Papain (14 units/mg, Sigma-Aldrich, Merck), 40 μ g/ml DNase II (980 Units/mg, Sigma-Aldrich, Merck) and 240 μ g/ml L-Cysteine (Sigma-Aldrich, Merck). The 50 ml falcons were placed in a water bath (Fisher Scientific) at 37 °C for one hour; every 15 min the tissue was dissociated through gentle pipetting. After 1 hr the cortices were re-suspended in 30 mL warm dissection medium, filtered through a 70 μ m nylon cell strainer (Corning Incorporated, USA) to remove any non-dissociated fragments and centrifuged at 500 rpm for 5 min (Heraus Multifuge X3R, Thermo Scientific). The supernatant was discarded and the pellet was re-suspended in 15ml of culture medium. The culture medium was Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM F12, Gibco, ThermoFisher Scientific) supplemented with 5ml streptomycin-penicillin (10,000 U/mL, Gibco, ThermoFisher Scientific), 5 ml L-glutamine (200 mM, Gibco, ThermoFisher Scientific) and 50 ml heat-inactivated fetal bovine serum (Sigma, Merck). The culture medium was filtered through a 0.2 μ m filter unit (Nalgene Rapid-Flow Sterile Disposable Filter Unit, Thermo Scientific). The mixture of dissociated cells was plated in a T75 culture flask (Nunc EasYFlask 75 cm² cell culture flasks, Thermo Scientific), previously coated with poly-D-lysine hydrobromide (PDL, Sigma, Merck) for 2 hrs and washed with sterile water 3 times. The mixed glial cultures (figure 2.2 A) were maintained for one week in a humidified incubator (HeraCell Vios 160i, ThermoFisher) at 37 °C and 5 % CO₂ for one week and half medium was changed every 2 days to replace growth factors. After a week, astrocytes formed a connected confluent dense monolayer in the bottom of the plate, whereas the majority of the microglia were floating. Primary microglial cells were isolated from the astroglial cell bed by mechanical agitation by vigorously tapping the flasks. Subsequently, the cells were plated in a 24-well plate (Nucleon Delta Surface, Thermo Scientific) at a cell density of 5×10^4 cells/well (figure 2.2 B). After 24 hrs, microglia were attached to the bottom of the plate. Therefore, they were ready to be treated with

pro-inflammatory cytokines. In order to check the integrity of the primary microglial cultures, they were imaged with an inverted microscope (Olympus CKX53, Tokyo, Japan).

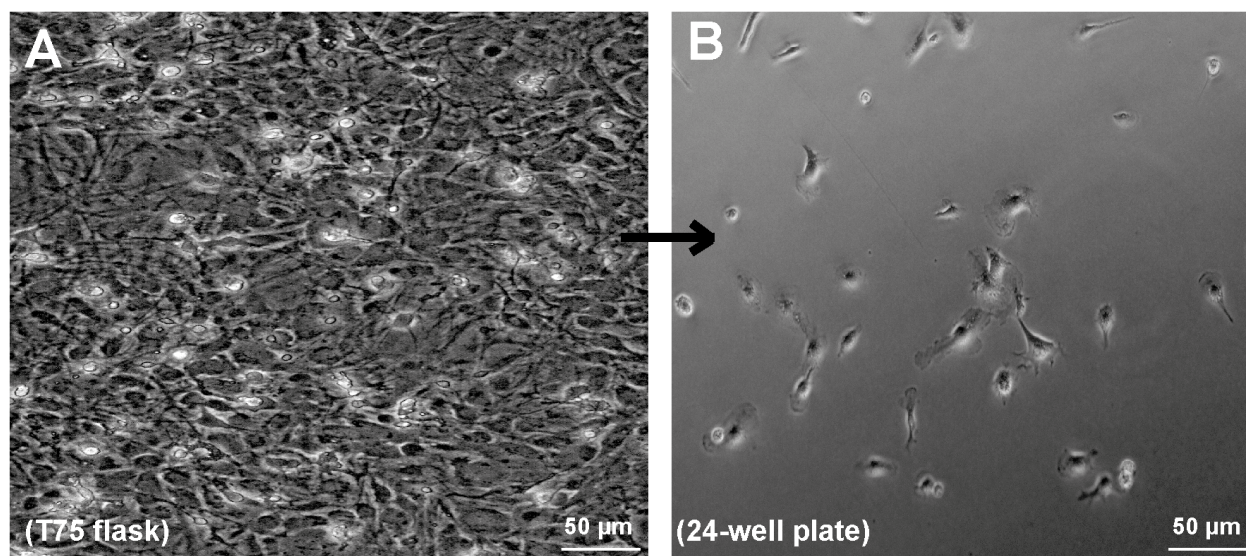


Figure 2.2: Primary glial cultures. (A) Live-image of astrocytes and microglia cultures; astrocytes formed a dense connective monolayer in bottom of the plate, whereas microglia were floating. (B) Live-image of primary microglia in a 24-well plate at a cell density of 5×10^4 .

2.7 Cerebellar organotypic tissue cultures

Organotypic cerebellar slices were chosen as they are widely used to study myelination processes in physiological and pathological conditions such as MS (Marzban et al., 2015). Further, only a single type of myelinated axon is maintained in cerebellar slices: the axons of the Purkinje cells, whereas the axons of afferent fibres (mossy fibres and climbing fibres) or efferent fibres (deep cerebellar nuclei) degenerate after a few days in vitro (Zhang et al., 2011; Doussau et al., 2017).

P8/P9 Sprague Dawley rats (Charles River Laboratories) were decapitated following the UK Home Office regulations under the project licence PPL/70/8916 (figure 2.3 A). Every step of the procedure was carried out in a laminar flow hood (Scanlaf, Fortuna). Brains were dissected from caudal to rostral by removing the cranium to expose the brain with sterile autoclaved tweezers and dissecting scissors (during the procedure tools were also sterilised using the dry sterilizer Germinator 500, Braintree Scientific, inc.). The brains

2.7. Cerebellar organotypic tissue cultures

were cut in half, separating both hemispheres, and plated in a petri dish (60 mm, Corning tissue-culture treated culture dishes) containing a sterile filter paper and cold dissection medium (Dulbecco's Modified Eagle Medium (DMEM), Gibco, supplemented with 5 ml of streptomycin-penicillin (10,000 U/mL, Gibco, ThermoFisher Scientific) (figure 2.3 B). One of the hemispheres was mounted with superglue (Loctite, superglue gel) onto the slicing platform of the Leica VT1200s vibratome positioned in the parasagittal plane. In order to set the correct parameters on the vibratome and for proper positioning of the brain in the platform, a rectangular piece of sterile solid agar (4 g of agar (Sigma) in 200 ml of distilled water) was also attached to the platform (figure 2.3 C). The brains were sliced sagittally at 400 μm thickness in dissection medium using the maximum vibration (around 1.2 mm) and the minimum speed (0.9 mm/s) of the vibratome, with a sharp edge sterilised blade (Gillette).

The cerebellar slices were captured from the dissection medium using autoclaved glass pasteur pipettes (Fisher Scientific) and transfer into a 60 mm petri dish containing cold medium until the whole hemisphere was sliced. Later, they were plated on sterile Bio-pore PTFE membrane of 0.4 μm pore size (Millicell-CM culture inserts, Merck) in a sterile six well plate (Costar, Corning Incorporated) with 1 ml of nutrient medium underneath every culture insert (figure 2.3 D). The nutrient medium contained 200 ml Neurobasal-A-medium (Gibco, ThermoFisher Scientific) and 100 ml Hank's Balanced Salt Solution (HBSS 1X, Gibco, ThermoFisher Scientific) supplemented with 5 ml streptomycin-penicillin (10,000 U/mL, Gibco, ThermoFisher Scientific), 5 ml L-glutamine (200 mM, Gibco, ThermoFisher Scientific), 4.4 ml D-glucose (200 g/L, Gibco, ThermoFisher Scientific) and 2 ml vitamin B27 Plus Supplement (50X, Gibco, ThermoFisher Scientific). The culture medium was filtered through a 0.2 μm filter unit (Nalgene Rapid-Flow Sterile Disposable Filter Unit, Thermo Scientific). One-two cerebellar slices were plated per insert and maintained in an incubator (HeraCell Vios 160i, ThermoFisher) at 37 °C , 5 % CO₂ for 9-10 days, replacing half the medium every other day to replace the growth factors. In order to check the integrity of the slices, the macroscopic structure was checked with the inverted microscope (Olympus CKX53). They were also stained with the apoptotic marker propidium iodide (PI, Molecular Probes). PI was added at a concentration

of 5 $\mu\text{g}/\text{ml}$, incubated for 60 min in the culture medium and imaged with the inverted microscope (figure 2.3 G, H). After 9-10 days in vitro (DIV) healthy slices flattened to approximately 100 μm . The slices that looked flattened and transparent were selected for further experiments (figure 2.3 F, H).

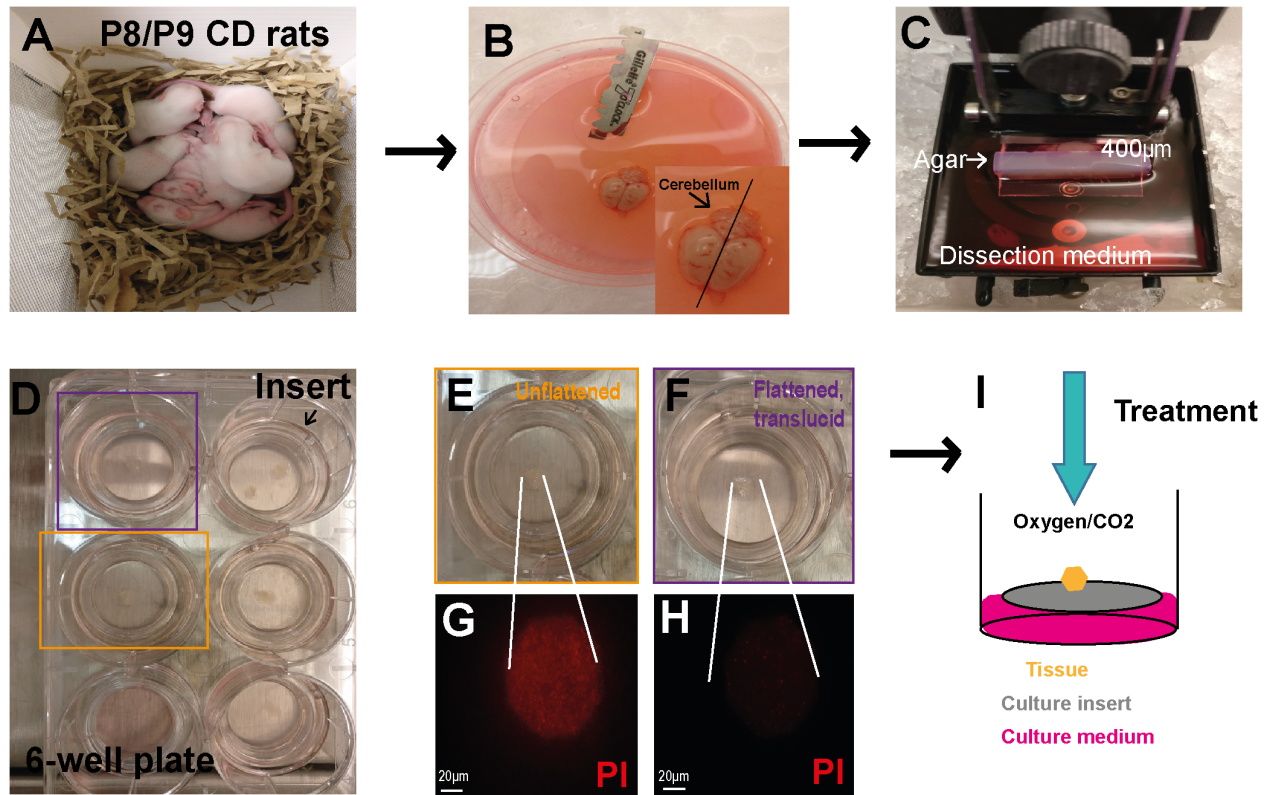


Figure 2.3: Diagram of the organotypic cerebellar tissue cultures protocol. (A) Image of P8/P9 CD Sprague Dawley rats. (B) Image showing how the dissected brains were cut by plating them in a petri dish containing a sterile filter paper and cold dissection medium. (C) Image of the vibratome showing the rectangular piece of agar that allowed the proper position of the brain hemisphere in the parasagittal position. Slices were cut at 400 μm thickness. (D) Image of a 6 well plate showing the cerebellar slices on top of the culture inserts. The purple rectangular shows a flattened/healthy slice (F), while the orange rectangle (E) points to an unflattened/unhealthy slice. (G, H) PI staining images of a healthy (H) and an unhealthy (G) slice. (I) Diagram of the different elements of this culture system: culture medium, culture insert and tissue slice; all placed in an incubator at 37 $^{\circ}\text{C}$, 5 % CO_2 for 9-10 days.

2.8 Cerebellar and microglial culture treatment

The cerebellar slices and the microglial cultures were treated with the pro-inflammatory cytokines TNF (recombinant rat TNF, Biolegend, San Diego, CA, USA) and $\text{IFN}\gamma$ (re-

2.8. Cerebellar and microglial culture treatment

combinant rat IFN γ , Biolegend, San Diego, CA, USA). The microglial cultures were also treated with the pro-inflammatory cytokine LT- α (recombinant human LT- α , Abcam). The cerebellar slices were treated either 3 times 24 hrs apart with TNF and IFN γ at 50 ng/ml, or twice 24 hrs apart at a concentration of 100 ng/ml (figure 2.4 B). The microglial cultures were treated either once or twice with doses of 100 ng/ml or 200 ng/ml of TNF, IFN γ , LT α , TNF+IFN γ and LT α +IFN γ (each dose was administered every 24 hrs) (figure 2.4 A (purple)). Moreover, the cerebellar cultures were also treated with conditioned microglial medium after two 100 ng/ml doses of TNF+IFN γ (in this case, each well contained 800 μ l of conditioned medium and 200 μ l of nutrient medium) (figure 2.4 B).

Furthermore, the cerebellar slices were treated directly with glutamate (L-Glutamic acid, Sigma-Aldrich, Merck). The glutamate was dissolved in 1M HCl and sterile water at a stock concentration of 25 mM. Specifically, the cerebellar slices were treated with glutamate twice every 24 hrs at a concentration of 75 μ M and 100 μ M (figure 2.4 B). Furthermore, the slices treated with 100 ng/ml of TNF+IFN γ were also incubated at the second dose with 0.6 mM (Fu et al., 2009) of MK-801 maleate, which is a non-competitive NMDA antagonist (ab120028, Abcam, UK).

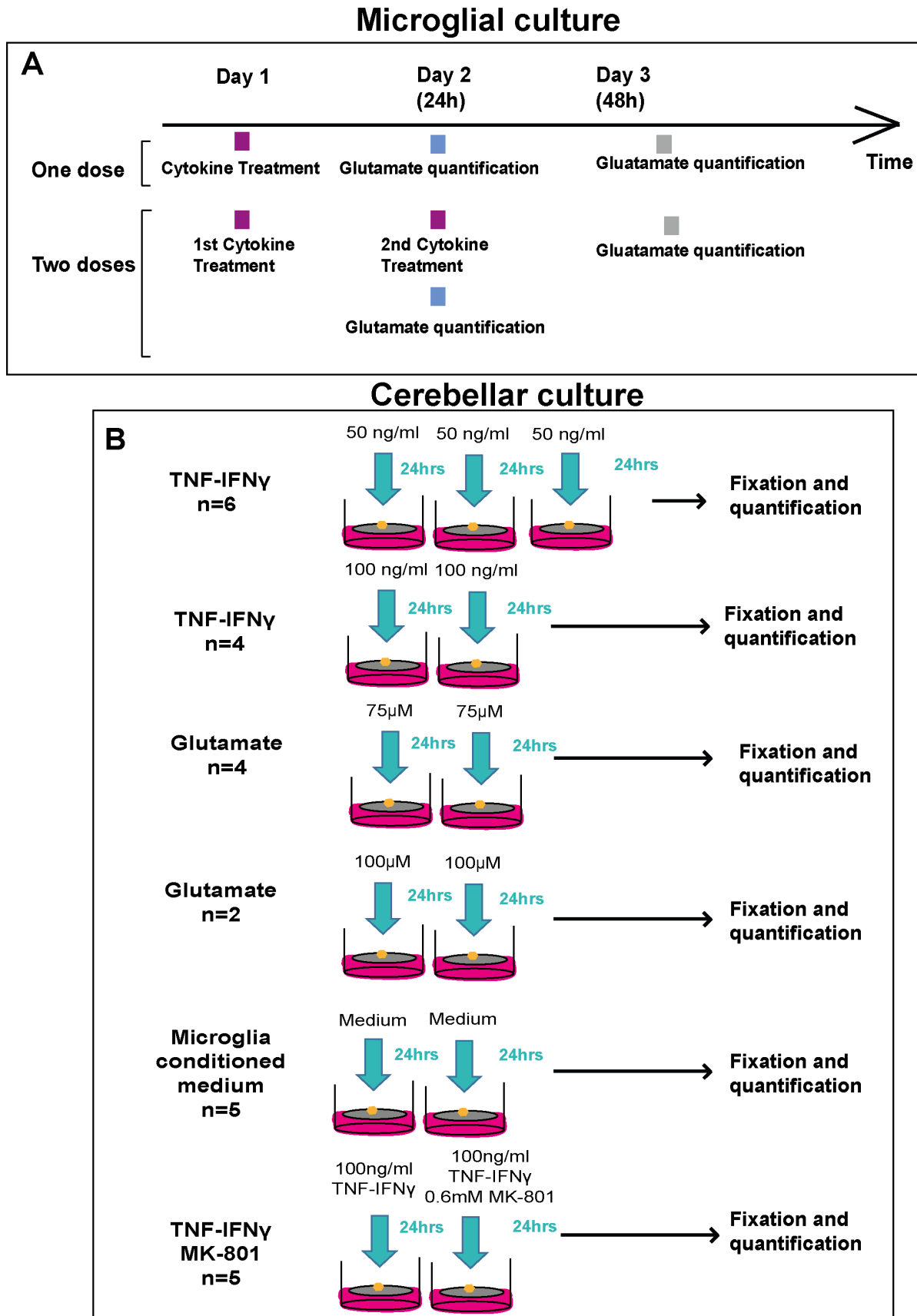


Figure 2.4: Diagrams of the cerebellar and microglial culture treatments.

2.9 Immunofluorescence of the cerebellar culture slices

For immunofluorescence of the cerebellar slices the protocol was acquired from previous studies (de Mendoza et al., 2011). The cultures were fixed with cold 4 % PFA (1 ml added on top of the filter and 1 ml underneath) for 1 hr at room temperature. After fixation, sections were post-fixed with 20 % methanol and washed in 0.1M PBS-0.3 % Triton X-100. For further permeabilization, slices were incubated with 0.1M PBS-0.3 % Triton X-100 overnight. After permeabilization, slices were blocked with 20 % bovine serum albumin (BSA, Sigma, Merck) and PBS-0.3 % Triton X-100 for a minimum of 4 hr at room temperature. Later, slices were removed from the insert by cutting around the tissue carefully using scissors to then transfer the slices to a 24-well plate (Costar, Corning Incorporated). Primary antibodies were added at their corresponding working concentration (Table 2.3) and incubated overnight at 4 ° C in a humid chamber. After primary antibody incubation, sections were thoroughly rinsed in 0.1M PBS, three times 10 min each. After rinsing, sections were incubated with the appropriate secondary antibody conjugated to the appropriate fluorochrome for 4 hrs at room temperature. The tissue was rinsed in 0.1M PBS (three times, 10 min each) and dH_2O , nuclear counterstained with DAPI (diluted in 1:2000) and transferred to the slides (SuperFrost Plus, VWR, International) with tweezers. Lastly, the tissues were mounted with Vectashield Antifade Mounting, and the coverslips (24x50 mm, VWR, International) were fixed to the slides with nail-polish.

Some of the microglial cultures were also fixed with cold 4 % PFA for 10 min and stained with IBA1 following the same protocol as the human tissue.

2.10 Glutamate colorimetric assay

The glutamate concentration measured in from the primary microglial medium with the glutamate colorimetric assay kit from Abcam (no. 83389). The kit comprised the glutamate standards, an assay buffer, a developer solution and an enzyme mix solution. The glutamate standard was prepared by diluting 5 μl of the glutamate standard solution in 495 μl assay buffer. The diluted standard was used to prepare the six standards which

correspond to a glutamate concentration in the range of 0-10 nmol. A reaction mix was prepared with 90 % of assay buffer, 8 % of developer and 2 % of mix, and a background mix was prepared with 92 % of glutamate assay buffer and 8 % of developer solution. The microglial medium samples were aliquoted in a 96-well plate (Costar, Corning Incorporated) and diluted 1:2 with glutamate assay buffer to keep the glutamate concentrations within the standard curve values of the kit. For the samples and standard wells, 100 μ l reaction mix was added. The background mix was only added to the background samples containing just medium to account for the endogenous glutamate. Then, the 96-well plate was incubated at 37 °C for 30 min and scanned at a wavelength of 450 nm to quantify the samples absorbances with a SPARKS multimode microplate reader (Tecan, Switzerland). The glutamate concentration from the sample readings was extrapolated from the standard curve regression equation, and the absorbances were corrected for the background values. Results were expressed in μ M and represented by the mean $\pm$ standard error of the mean (SEM) of 3-4 duplicates per condition (Control, TNF, IFN γ , LT α , TNF+IFN γ and LT α +IFN γ).

2.11 Image acquisition and analysis

Immunostained sections from the post-mortem human tissue and the rat tissue were imaged with both an epifluorescence Olympus BX63 microscope and the SP8 Leica confocal (Germany). The cerebellar organotypic cultures were imaged exclusively with the confocal microscope as they were approximately 100 μ m thick. For the latter, a range of 4-6 z-stacks were taken with an average thickness of 15 μ m with a step-size of 0.3 μ m. Sequential line scanning was performed when more than two channels were acquired. All images were analysed using Fiji (Image J, NIH, USA) and prepared in Illustrator (Adobe Systems). The quantification of all the cases/samples was performed with the observer blinded to case identification. The regions of interest (ROI) were areas defined as NAWM in the post-mortem human tissue, and the corpus callosum, cingulate gyrus and external capsule in the chronic meningeal inflammation rat tissue. Specifically, in human MS tissue, NAWM regions were defined as being at least 2 mm away from a focal demyelinating

2.11. Image acquisition and analysis

lesion. MOG immunofluorescence images were taken at 4x to get an overall scan area of the whole block to select NAWM regions, as MOG shows myelin integrity.

In the post-mortem human tissue, 10 images of HLA-DR+ staining from NAWM regions were taken at 20x. Microglial activation was analysed by quantifying the area occupied by HLA-DR+ microglial labelling microglia by thresholding the images to a specific intensity per image acquired and dividing them by the total area of each image. In the rat tissue, the local number of IBA1+ microglia and GFAP+ astrocytes were counted at 60x. Also, the area occupied by microglia and astrocytes was measured by thresholding from 10 ROIs at 20x.

PNJ disruption was analysed by measuring the axonal length of paranodal protein Caspr1 in the post-mortem human tissue, rat tissue and cerebellar organotypic tissue slices. Caspr1, Pan- Na_v , K_v 1.2 and Caspr1-SMI32 triple and double immunofluorescent images were captured with a 60x or 63x oil immersion objective for higher magnification. Only Caspr1 positive axons that were in focus were measured in 10 ROIs in the human and rat tissue. In the cerebellar organotypic slices the ROIs corresponded to a range of 4-6 z-stacks located in the regions with a high density of Purkinje cell axons. Per tissue block, rat section and cerebellar organotypic tissue slice, 200 focused Caspr1-stained paranodes were measured.

2.11.1 Quantifying disruption of the paranodal axo-glial junction

For quantification of voltage-gated K_v 1.2 and Na_v channel dislocation, Caspr1- K_v 1.2 and Caspr1- Na_v double fluorescent images were taken with the 60x oil-immersion objective for each human and rat sample. Only positive Caspr1- K_v 1.2 and Caspr1-Pan Na_v axons were studied, in 10 ROIs, with a minimum of 50 axons per tissue block. RGB line intensity profiles of Caspr1, K_v 1.2 and Pan Na_v were obtained per axon along length to assess potassium and sodium channel dislocation. An overlapping region between two signals was defined as the axonal area where K_v or Na_v channels were located in the paranodal axolemma, which meant that a certain degree of K_v or Na_v channel dislocation had occurred. Using RGB intensity line profiles acquired with Fiji, intensity signals of K_v 1.2

or Na_v and Caspr1 were subtracted from one another per axon. If the difference between both signals was smaller than a variable threshold at the same point in distance, an overlapping region was confirmed. Moreover, the total proportion of axons with overlapping regions was measured in MS and non-neurological human tissue, and in the GFP, LT α -IFN γ and naïve rat tissue. Mean $\pm$ SEM of all line profiles were calculated respectively per case and across groups (the code used to calculate channel dislocation can be found at AppendixA.1).

2.12 Computational model

The computational model of a myelinated axon composed of 21 nodes generated with the simulator NEURON (Hines and Carnevale, 2006). A double cable core model was solved with the backward Euler integration method. Simulations across seven different diameters were initiated and allowed to reach the resting potential for 1 ms before a current of 2 nA was injected in the mid-point of node 1 as a square pulse of 0.1 ms duration. The propagation of the AP along the axon was measured up to 10 ms (2000 points plotted/ms). Measurements were recorded at node 4 and 16 of the 20 nodes created. AP amplitude, width and conduction velocity were measured. The anatomical and biophysical parameters introduced in the model are specified in Chapter 5 and the code used for the creation of the model can be found at the Appendix A.2.

2.13 Data analysis

All the data was processed in R (R Project for Statistical Computing) and plotted with the package ggplot2. For the human tissue data in Chapter 3, the rat tissue data in Chapter 4 and the organotypic tissue cultures in Chapter 6, the non-parametric Mann-Whitney test was used to compare groups and non-parametric Spearman rank correlation test for correlation between variables. Moreover, in the rat tissue data, the Kruskal-Wallis test was also used to compare across groups. For the microglial culture studies in Chapter 6, the non-parametric Friedman rank sum test was used to compared across treatment groups and timings, and Wilcoxon pairwise comparison with a Bonferroni correction was used to

2.13. Data analysis

compare between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Mean $\pm$ SEM per group was calculated in all the cases and box plots (showing the median, the 25-75 % quartiles, the minimum and the maximum), bar graphs or point graphs were plotted.

Axo-glial pathology in MS

3.1 Introduction

Pathological changes in the white matter of the progressive MS brain include focal demyelinating lesions, focal and diffuse neuro-axonal loss, perivascular immune cell infiltration, within a global inflammatory response in the whole brain and meninges (Schumacher et al., 2017). In NAWM, neuropathological studies have shown that it contains diffuse inflammation (Allen et al., 2001), degenerating axons labeled by dephosphorylated neurofilament (SMI32) or amyloid precursor protein (APP) (Trapp et al., 1998; Fu et al., 1998; Bjartmar et al., 2000, 2001), chronically activated microglia (Trapp et al., 1998; Graumann et al., 2003; Kutzelnigg et al., 2005b; Moll et al., 2011) and a compromised BBB (Kirk et al., 2003). Furthermore, neuro-axonal injury is recognised as an early and persistent factor in MS pathology and may be one of the best predictors of long-term irreversible neurological decline (Lassmann et al., 2012; Simons et al., 2014), that continues

into the late stages of progressive MS and is linked to the profound and chronic activation of microglia and macrophages. Therefore, the molecular mechanisms responsible for progressive axonal injury throughout NAWM might be other than the classical dying-back pattern propagating out of demyelinating lesions. Previous studies in EAE mice suggest that the earliest ultra-structural sign of axonal damage in myelinated axons in acute lesions, is mitochondrial pathology, which precedes axon fragmentation, co-localises with axonal swelling, emerges at the nodes of Ranvier, and is surrounded by activated microglia and macrophages (Nikić et al., 2011). This type of non-classical axon loss mechanism could indicate that the paranodal axo-glial junctions, as regions with a very high concentration of proteins, could be considered important targets of focal inflammatory attacks, that could lead to the initiation of the diffuse axonal injury, as described in the NAWM MS tissue (Simons et al., 2014).

3.1.1 Paranodal axo-glial junctions disruption

Previous studies have shown that the fine structures of the PNJs may be disrupted in MS lesions (Wolswijk and Balesar, 2003), in their surroundings (Howell et al., 2006), in the NAWM, and the EAE animal model of MS (Howell et al., 2010). Furthermore, animal model studies performed with conditional knockouts of the paranodal proteins Caspr1 (Bhat et al., 2001), GPI-linked contactin (Boyle et al., 2001) and Nf155 (Pillai et al., 2009) showed that they fail to form paranodal septate-like junctions and present physiological symptoms such as tremor, motor paresis, ataxia and a significant reduction in conduction velocity. In these cases, the disruption of the paranodal junctions implied a widening of the space between the paranodal loops and the axolemma, and complete dislocation of the juxtaparanodal voltage-gated channels K_v1 towards the paranodes. Regarding the voltage-gated Na_v channels, they remained clustered at the nodes in most of the studies. Although paranodal junctions, as physical barriers, are crucial for Na_v channels clustering, it has been shown that the clustering depends on two glial-dependent mechanisms: their attachment to the extracellular matrix (ECM) proteins working through Nf186, and the assembly of the paranodal junctions. When both Nf186 and paranodal junctions are lost, Na_v channels cannot be assembled at the nodes (Amor et al., 2017). However, Nf186

is key for the maintenance of Na_v channel clusters so that Nf186-deficient mice will have a significant reduction of Na_v channels over time (Thaxton et al., 2011). Moreover, myelin-deficient rats (Arroyo et al., 2002), hypo-myelinated shiverer mice lacking MBP (Rasband et al., 1999), Jimpy mice lacking PLP (Jenkins and Bennett, 2002), as well as mice lacking certain galactolipids (Dupree et al., 1999; Poliak et al., 2001), sulfatides (Ishibashi et al., 2002), and gangliosides (Susuki et al., 2007) show aberrant axo-glial junctions. Mice with conditional null mutations in the genes for the paranodal cytoskeletal proteins β II spectrin (Zhang et al., 2013), α II spectrin (Huang et al., 2017) and 4.1.B (Cifuentes-Diaz et al., 2011; Buttermore et al., 2011) displayed disrupted paranodal junctions and voltage-gated channels K_v1 dislocation, suggesting that the paranodal diffusion barrier also depends on the integrity of the actin-spectrin axon cytoskeleton (Xu et al., 2013). All of these studies point to the fact that paranodal axo-glial junctions are an intricate connection between the oligodendrocyte and the axon, the structural integrity of which is maintained by the respective cytoplasmic cell-adhesion proteins. Thus, it is the structural arrangement of proteins that makes them effective electrical barriers to avoid current shunting underneath the myelin sheath, and effective physical barriers to maintain the correct location of voltage-gated channels and the dimensions of the nodal, paranodal and juxtaparanodal compartments, to ensure fast and reliable AP conduction.

It is essential to characterise the extent of paranodal axo-glial disruption, and its relationship to chronically activated microglia and macrophages located in the surroundings of the axons. In MS, this characterisation is relevant to understand the mechanisms underlying the significant detrimental consequences to AP conduction and the loss of trophic support from the oligodendrocyte leading to diffuse axonal injury. Its characterisation could contribute to the development of neuroprotective therapies by hindering axonal injury progression and help elucidate the molecular mechanisms for cognitive deficit treatments, such as fatigue.

3.1.2 Aims

The following experiments aimed to characterise the pathology of the paranodal axo-glial junctions and the expression of Caspr1/*Na_v*/*K_v* proteins in the NAWM of 20 SPMS and 10 non-neurological control cases, using snap-frozen post-mortem tissue blocks from the cerebral peduncles and the pre-central gyrus. The specific aims of the study were:

- To characterise the myelin integrity and inflammation present in NAWM regions in all the cases by immunohistochemical analysis;
- To quantify and characterise paranodal axo-glial pathology by elucidating the expression of the paranodal Caspr1, the nodal *Na_v*, and the juxtaparanodal *K_v*1.2 proteins;
- To characterise the relationship of axo-glial pathology to local microglial and macrophage activation, and axonal cytoskeleton disruption.
- To characterise the relationship of axo-glial pathology to its corresponding clinical data.

3.2 Results

In order to study the myelin integrity and diffuse inflammation of the pre-central gyrus, and the cerebral peduncles, of the selected post-mortem human tissue blocks, myelin oligodendrocyte glycoprotein (MOG), human leukocyte antigen DR (HLA-DR+) and SMI32 immunostaining was performed. Specifically, 34 blocks of 10 pathologically confirmed MS cases (17 from the pre-central gyrus and 17 from the cerebral-peduncles), and 16 blocks of 10 non-neurological control cases (7 from the pre-central gyrus and 9 from the cerebral-peduncles) were chosen for the study. From the MOG-images, we chose 8-10 NAWM areas as regions of interest (ROIs), with the condition that they have to lay at least 2 mm away from focal demyelinating lesions (figure 3.1 A). The area occupied by microglia/macrophages was quantified with the marker HLA-DR+, which labels MHC class II cell surface receptors, as a measurement of innate inflammation (figure 3.1 B). SMI32,

which labels de-phosphorylated neurofilament, was used to indicate axonal stress (figure 3.1 C). Furthermore, axonal pathology was analysed at the nodes of Ranvier: using Caspr1 as a marker of the paranode, voltage-gated Na_v for the node and voltage-gated $K_v1.2$ for the juxtaparanode (figure 3.1 D).

3.2.1 Caspr1 stained paranodal disruption

In order to determine if an increment in paranodal length was present in the selected ROIs of MS NAWM tissue, the paranodal length of Caspr1 stained structures (figure 3.2 A) was measured. In total, 6800 Caspr1 (200 paranodes per block) stained paranodes from 34 blocks of 10 pathologically confirmed MS (figure 3.2 C) and 3200 Caspr1 stained paranodes from 16 blocks (200 paranodes per block) of 10 control cases (figure 3.2 B) were analysed. Caspr1-stained paranodal length was 21.7 % longer on average in the MS NAWM tissue ($2.8 \pm 1.1 \mu\text{m}$; figure 3.2, blue box-plot) than non-neurological control tissue ($2.3 \pm 0.8 \mu\text{m}$; **** $p < 0.0001$; figure 3.2 D, gray box-plot). However, the average increment in paranodal length did not provide much information; what is essential is to notice is that the paranodal distributions between the two groups were significantly different. In fact, 43 % of the paranodes in the MS tissue were longer than the 75 % percentile of the control group ($2.7 \mu\text{m}$). Furthermore, to get more information about these distributions, we separated the measured paranodal data by length ranges for each group (figure 3.2 E). 11.14 % of paranodes in the MS group were longer than $4 \mu\text{m}$ compared to the 4.1 % in the non-neurological control group (figure 3.2 E (pink and purple)). Regarding the variability of paranodal length between the two brain areas examined, paranodes in the cerebral peduncles of the mesencephalon were $2.81 \pm 0.019 \mu\text{m}$ long and 12% of them were longer than $4 \mu\text{m}$ (box plots labelled with "cbp", figure 3.2 F). On the other hand, in the precentral gyrus blocks, paranodes were $2.79 \pm 0.017 \mu\text{m}$ long and 10.38 % of them were longer than $4 \mu\text{m}$ (box plots labelled with "pcg", figure 3.2 F). Overall, our data demonstrates that the length of the paranodes was highly disrupted in a significant proportion of MS brains when compared to the non-neurological controls (figure 3.2 F).

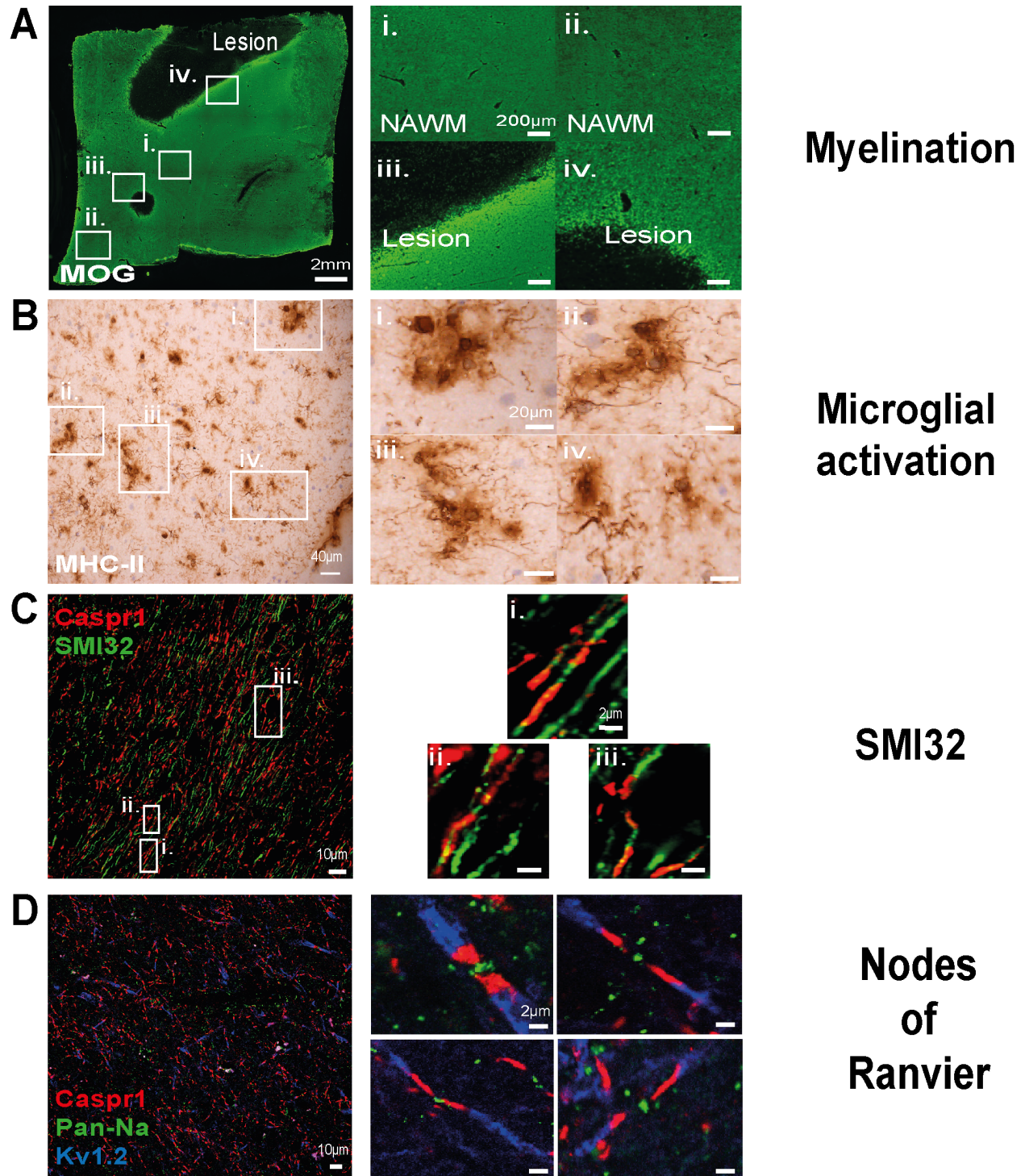


Figure 3.1: MS NAWM regions were characterised by immunostaining with MOG, MHC-II, Caspr1, SMI32, Pan-Na and K_v 1.2. (A) MOG-myelin immunostaining image. NAWM regions of interest (ROIs) were selected (i, ii) from demyelinating lesions (iii, iv). (B) Image of HLA-DR+ stained microglia. An activated amoeboid microglial morphology was found throughout the ROIs (i, ii, iii, iv). (C) Confocal image of SMI32+ axons (green) labelling dephosphorylated neurofilaments and Caspr1-stained paranodes (red) (i, ii, iii). (D) Confocal image of Caspr1-stained axo-glial paranodal junctions (red), K_v 1.2-stained juxtaparanodes (blue) and Na_v stained nodes (green) from selected ROIs.

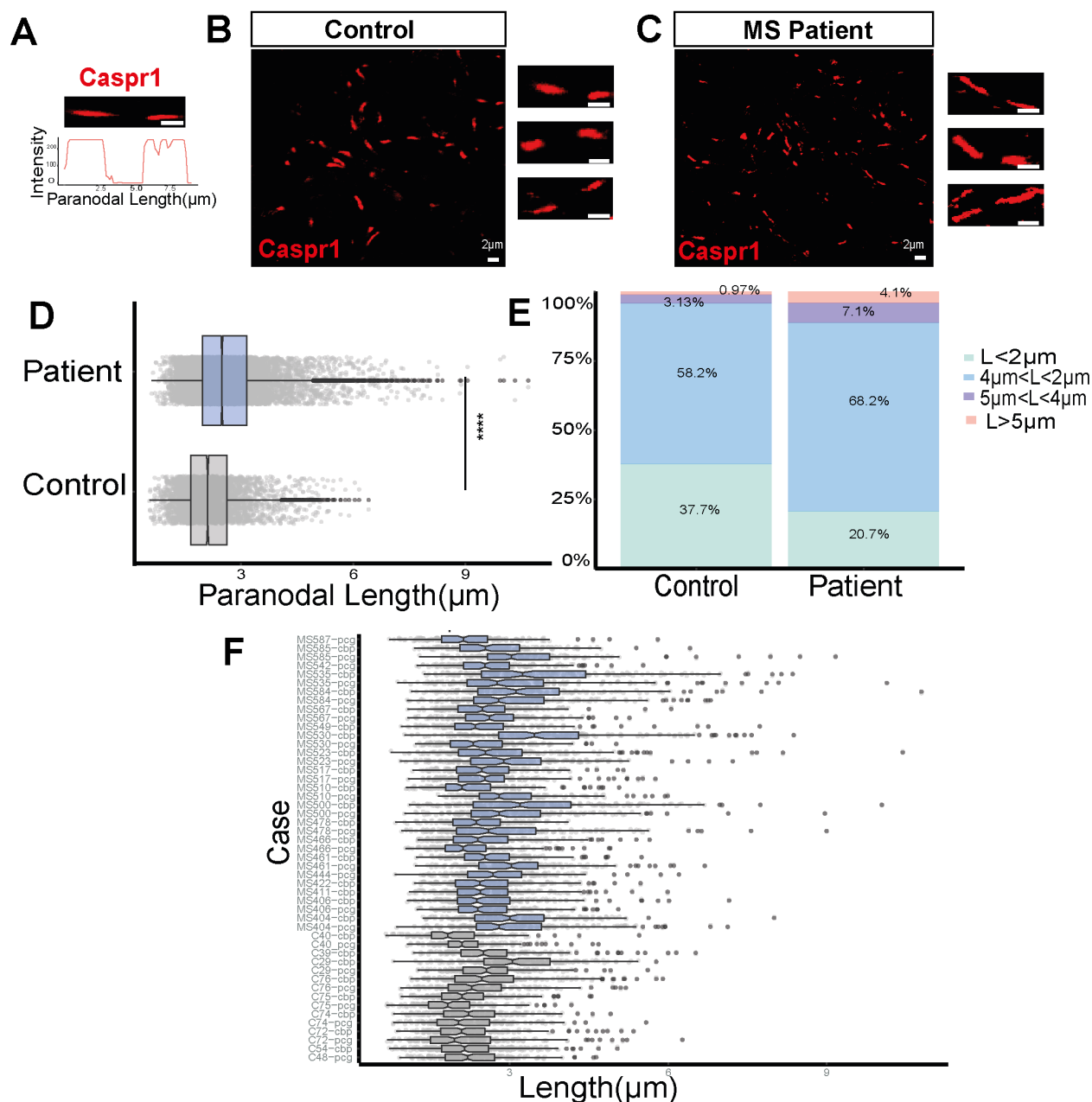


Figure 3.2: MS NAWM tissue contained a larger proportion of elongated Caspr1-paranodes compared to non-neurological control tissue. (A) Confocal image of a single Caspr1-stained paranode, and its intensity profile of Caspr1. The paranodal length corresponds to the distance between the half maximum intensity of each side of the profile. (B) Confocal images of paranodes from human post-mortem NAWM MS were longer than the ones from non-neurological control (C) tissue. (D) Significant different distributions of paranodal length between Caspr1-measured paranodes in NAWM MS and non-neurological control tissue (**** $p < 0.0001$, Mann-Whitney test). (E) The paranodal length distributions were separated by length ranges and represented in a bar plot. NAWM MS tissue contained a larger proportion of paranodes longer than 4 μm (purple) and 5 μm (pink) than control tissue. (F) Distributions of paranodal length from NAWM MS and non-neurological control tissue per case. In the y axis the indexes *cbp* correspond to the cerebral peduncle blocks, while indexes *pcg* correspond to pre-central gyrus blocks).

3.2.2 Paranodal disruption associated with microglial activation

Previous studies from our laboratory have suggested that an increment in paranodal length was associated with nearby microglial and macrophage activation in the MS brain (Howell et al., 2010). In order to quantify microglial activation, (figure 3.3 A, B), the area occupied by microglia and macrophages was quantified from MHC class II immunohistochemistry images (figure 3.3 B) from 10 NAWM regions of $679.99 \mu\text{m} \times 511.99 \mu\text{m}$. We found statistically significant correlations between the mean paranodal length of all measured axons per block and the mean microglial area (figure 3.3 C , $r=0.43$, $*p < 0.05$). Furthermore, the relationship was stronger when the area occupied by microglia was correlated to the proportion of paranodes longer than $4\mu\text{m}$ (figure 3.3 D, $r=0.46$, $**p < 0.01$). Also, rounded microglia characterised by thicker and shorter processes in nodules were observed in some of the cases (figure 3.3 B).

3.2.3 Paranodal disruption of stressed-damaged SMI32 axons

In order to determine if the axons with longer paranodes in MS NAWM tissue corresponded to stressed/damaged axons, immunostaining for de-phosphorylated neurofilaments (SMI32) was performed (figure 3.4). Neurofilaments are heavily phosphorylated in healthy myelinated axons and not stained by SMI32 antibodies (Trapp et al., 1998). Thus, SMI32 staining can indicate if the disrupted elongated paranodes in MS NAWM tissue correspond to an underlying axonal pathology (figure 3.4). Caspr1-stained paranodal was measured for SMI32+ or SMI32- axons (figure 3.4 A). Caspr1 paranodes were elongated when associated with damaged/stressed SMI32+ axons ($3.89 \pm 0.1 \mu\text{m}$) compared to SMI32- ($2.49 \pm 0.07 \mu\text{m}$) or non-neurological controls ($2.33 \pm 0.01 \mu\text{m}$) (figure 3.4B, $**** p < 0.0001$). Furthermore, average paranodal length for SMI-32+ was 66.9 % longer than control paranodal length and 56.2 % longer than SMI32- average paranodal length. In addition, axonal swellings (figure 3.4 C, i.) and transections near the node of Ranvier were observed (figure 3.4 C, ii). No correlations were found between mean/median paranodal length and clinical outcomes measures such as age, post-mortem delay (PMD) or duration of the disease.

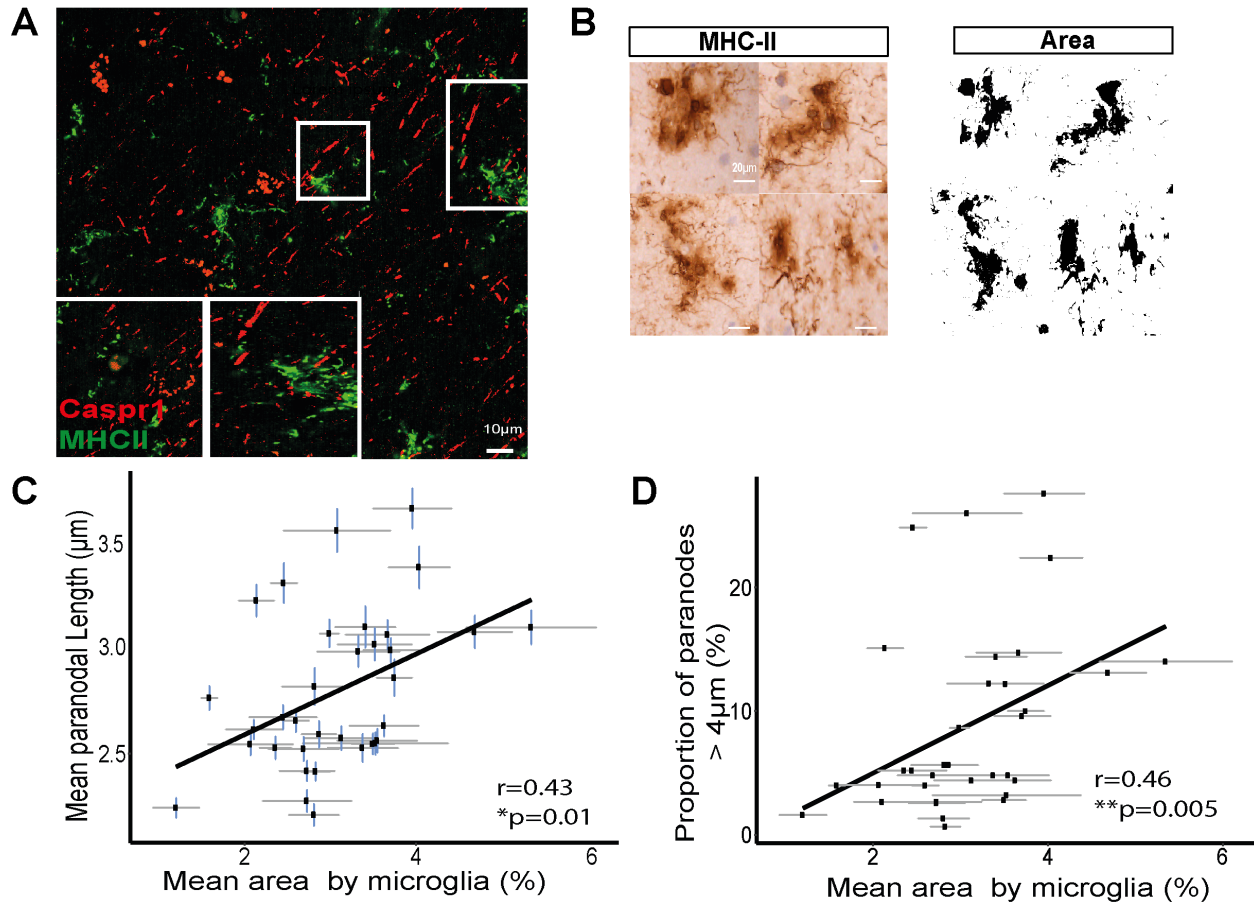


Figure 3.3: Paranodal elongation was associated to microglial activation in MS NAWM tissue. (A) Confocal image of Caspr1 stained paranodes with HLA-DR+ (green) stained microglia and macrophages. (B) Activated microglia were measured as the mean area occupied by HLA-DR+ microglia and macrophages applying a threshold mask in 10 ROIs. (C) The mean paranodal length per block correlated with the mean area occupied by activated microglia ($r = 0.43$, $*p < 0.05$, Spearman's rank correlation test). (D, E) The mean area occupied by activated microglia per block correlated significantly with the proportion of paranodes longer than $4\mu\text{m}$ ($r = 0.46$, $**p < 0.01$) and $5\mu\text{m}$ ($r = 0.51$, $**p < 0.01$). The horizontal arrows in the graphs denote the standard error of the paranodal mean, and microglial area (grey).

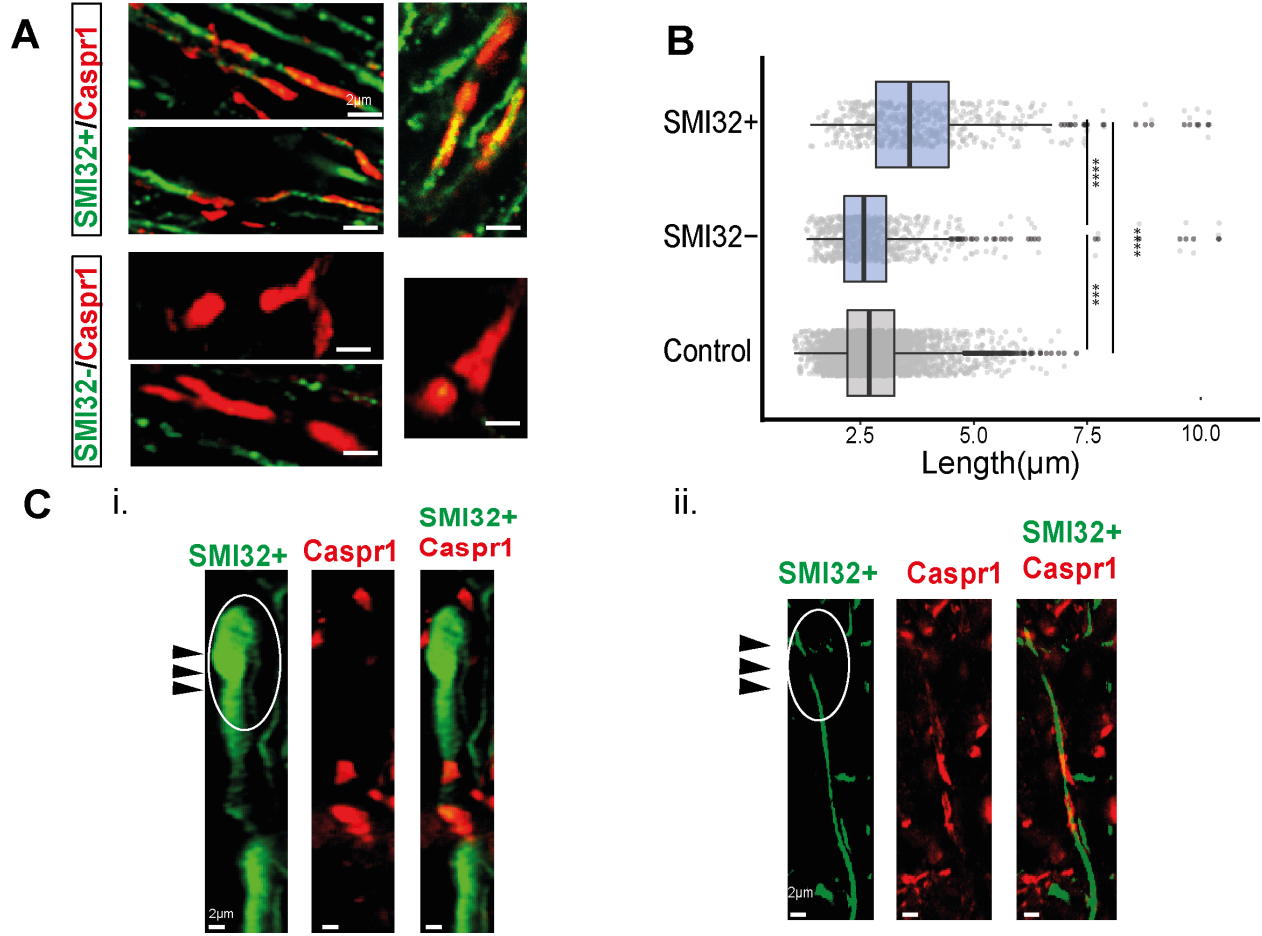


Figure 3.4: SMI32+ axons characterised by dephosphorylated neurofilaments had elongated paranodes. (A) Confocal images of long Caspr1 paranodes co-stained with SMI32+ and SMI32- axons. (B) Paranodal length distributions of SMI32+ and SMI32- from MS NAWM tissue and from non-neurological control tissue (*** $p < 0.0001$, Mann-Whitney test). The box-plot shows that the paranodal length of SMI32+ axons is longer than SMI32-. (C) Some of the axons have swellings (i) and/or are transected (ii) very close to the node of Ranvier.

3.2.4 Dislocation of voltage-gated channels

Dislocation of voltage-gated K_v channels

To assess the dislocation of $K_v1.2$ channels and their relationship to paranodal disruption, RGB intensity profiles were acquired for the paranodal Caspr1 and juxtaparanodal $K_v1.2$ signals of all the axons quantified (figure 3.5).

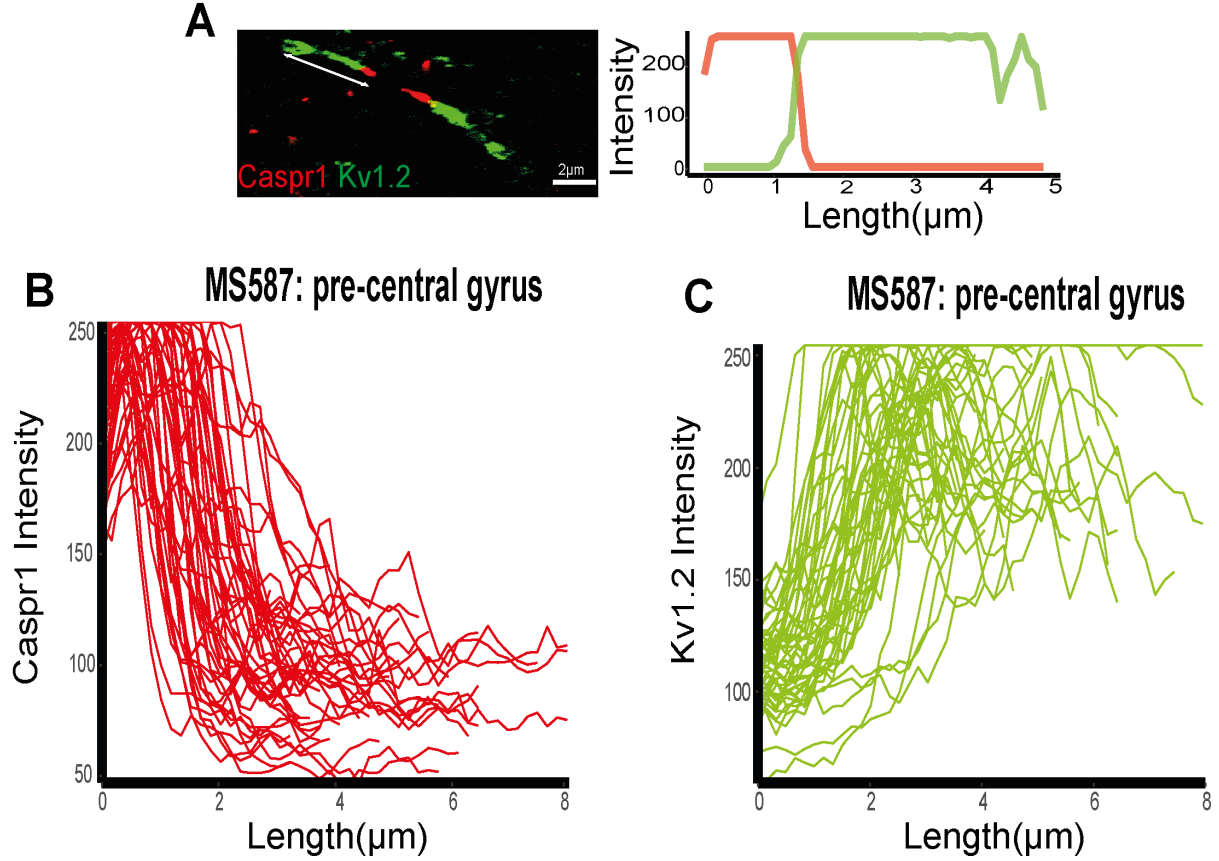


Figure 3.5: Caspr1 and $K_v1.2$ RGB profiles of NAWM region in a MS case. (A) Confocal image of a double immunofluorescence of Caspr1-stained paranode and juxtaparanodal voltage-gated $K_v1.2$. (B) Caspr1 Intensity profiles of all the axons measured in the pre-central gyrus block from the post-mortem tissue case MS587. (C) $K_v1.2$ Intensity profiles of all the axons measured in the pre-central gyrus block from the post-mortem tissue case MS587.

In order to quantify co-localisation between the fluorescent signals, we subtracted one signal (Caspr1, red) from the other ($K_v1.2$, green) for every single axon to analyse the proportion of overlapping regions (figure 3.6 B). Ideally, if both signals are at the same location, the difference would be 0 (figure 3.6 A). However, RGB intensity signals for Caspr1 and $K_v1.2$ signals contained intrinsic noise as figure 3.6 A shows. Therefore, we defined that when the difference between the two signals was lower than the variable threshold θ , that value was an overlapping region $S(\theta)$. Furthermore, the proportion of overlap between Caspr1 and $K_v1.2$ signals per group was calculated, by dividing the total number of overlapping regions of the control and MS NAWM groups, by the total number

3.2. Results

of axons studied in each group (the algorithm used can be found in Appendix A1).

$$S(\theta) = \{axon | overlap < \theta\} \quad (3.1)$$

$$overlap(axon) = \sum_{i=1}^n |Caspr1(x_i) - K_v1.2(x_i)| \quad (3.2)$$

$$overlap_{K_v} = \frac{|S(\theta)|}{axons} \quad (3.3)$$

Varying the threshold θ , we observed that MS NAWM tissue contained a larger proportion of axons with overlapping regions at every threshold θ analysed compared to the non-neurological control group (thresholds analysed from 0 to the maximum of the intensity signal) (figure 3.6 B). If we set the intensity threshold (θ) to 50, the proportion of overlapping regions between MS and control groups were statistically different (**** $p < 0.0001$, Mann-Whitney test). Moreover, the proportion of overlapping regions per block at this threshold correlated significantly with the mean paranodal length ($r = 0.49$, ** $p < 0.01$, figure 3.7 A, B), and with the proportion of paranodes longer than $4 \mu\text{m}$ ($r = 0.57$, *** $p < 0.001$, figure 3.7). Therefore, we can conclude that MS NAWM tissue had more damaged axons with dislocated voltage-gated $K_v1.2$ channels (figure 3.6 B, blue), and therefore disrupted/elongated PNJs than non-neurological NAWM control tissue (figure 3.6 B, grey), as we have demonstrated in the immunofluorescence images of figure 3.6 B.

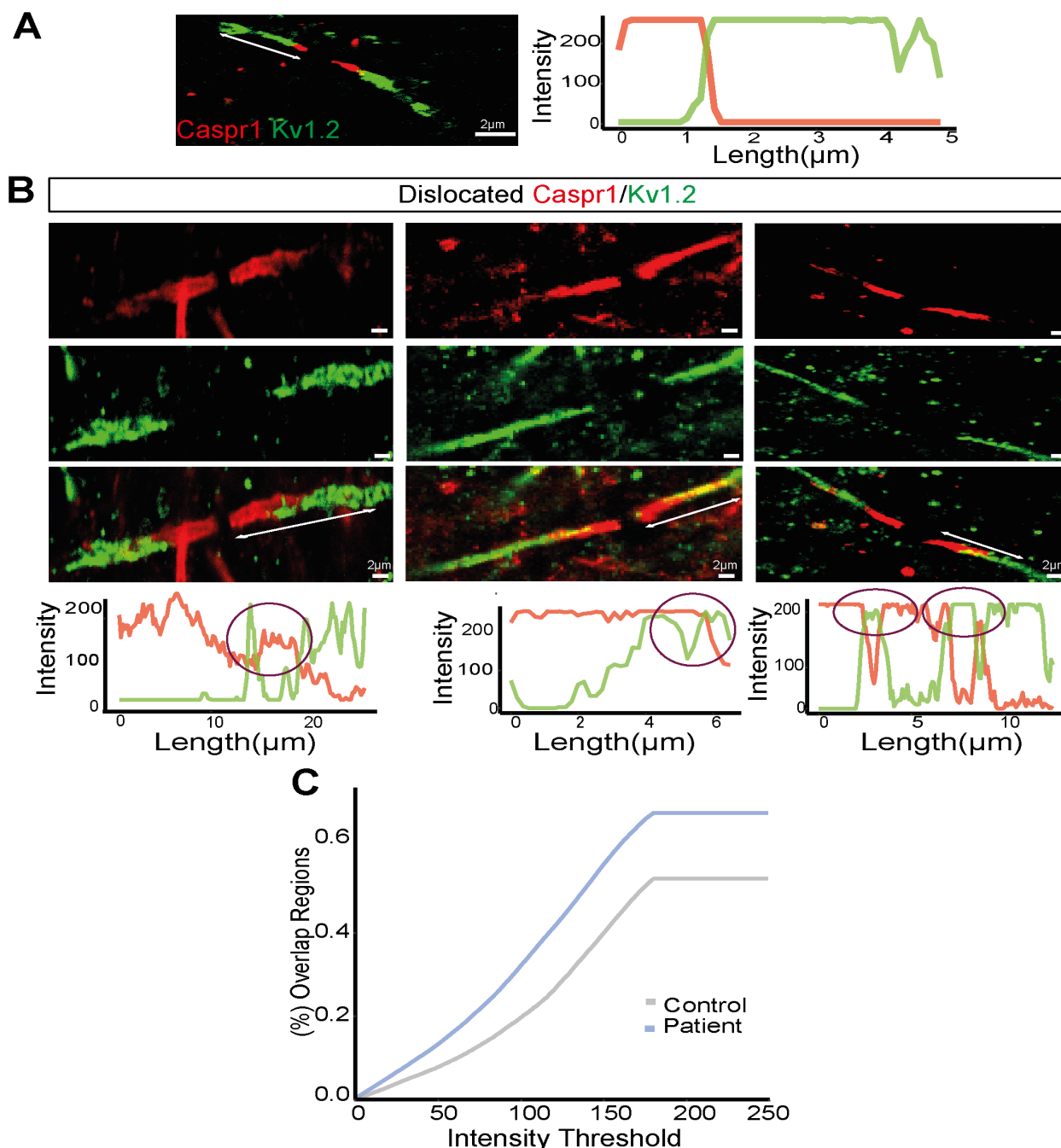


Figure 3.6: MS NAWM tissue contained a larger proportion of regions where Caspr1 and Kv1.2 channels were overlapping. (A) Confocal image from a node showing the expression of Caspr1 in the paranode (red) and Kv1.2 in the juxtaparanode (green) and its respective RGB intensity profile. (B) Confocal images from nodes where Caspr1 in the paranode (red) and Kv1.2 in the juxtaparanode (green) are co-localising, and their respective intensity RGB profiles. The purple circles denote regions where both signals are co-localising: overlapping regions. We continued the analysis by subtracting Caspr1 signals from Kv1.2 signals and when the difference between them was smaller than a variable Intensity Threshold, we considered that point as an overlapping region. For every threshold calculated, the proportion of overlapping regions was larger in MS NAWM tissue (blue) than in non-neurological control tissue (grey, C).

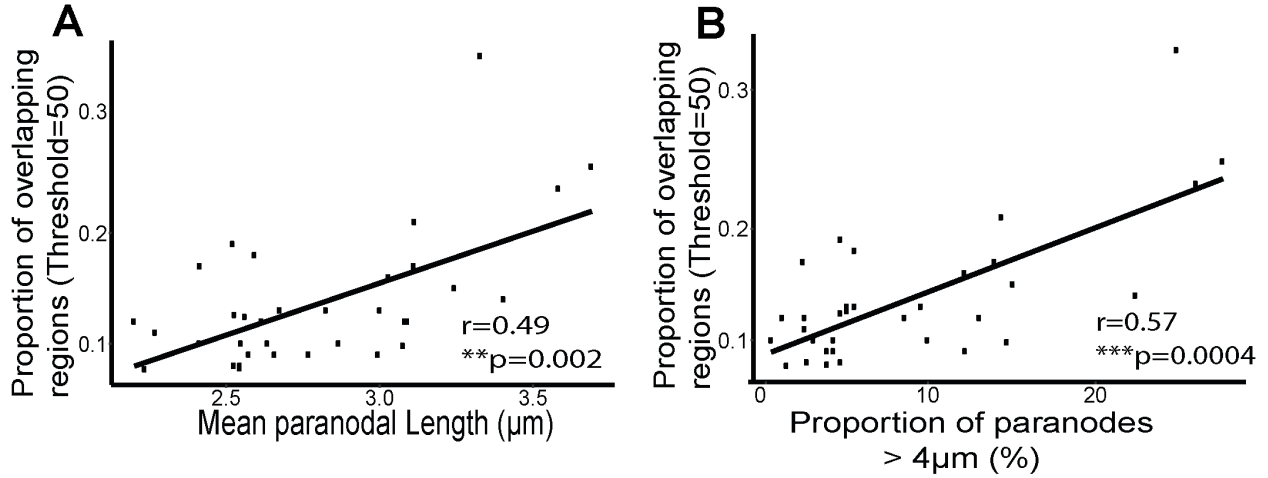


Figure 3.7: Paranodal elongation was associated with Caspr1- K_v overlapping in MS NAWM tissue. (A) The mean paranodal length per block correlated with the proportion of overlapping regions at an intensity threshold of 50 ($r = 0.49$, $**p < 0.01$, Spearman's rank correlation test). (B) The proportion of paranodes longer than $4 \mu\text{m}$ per block correlated with the proportion of overlapping regions at an intensity threshold of 50 ($r = 0.57$, $***p < 0.001$, Spearman's rank correlation test)

Dislocation of voltage-gated Na_v channels

To assess the dislocation of Na_v channels and PNJ disruption, intensity profiles of Na_v (green) and Caspr1 (red) immunostaining images of all the axons imaged were acquired (figure 3.8 A), and followed the same process as with the $K_v1.2$ signals. However, no significant overlap was found between voltage-gated Na_v channels and the paranodal axoglial junctions (figure 3.8 A, B). Although the integrity of the paranodal junctions could be compromised in our ROIs, the clustering of voltage-gated Na_v channels is maintained through their attachment to ECM proteins such as Nf186.

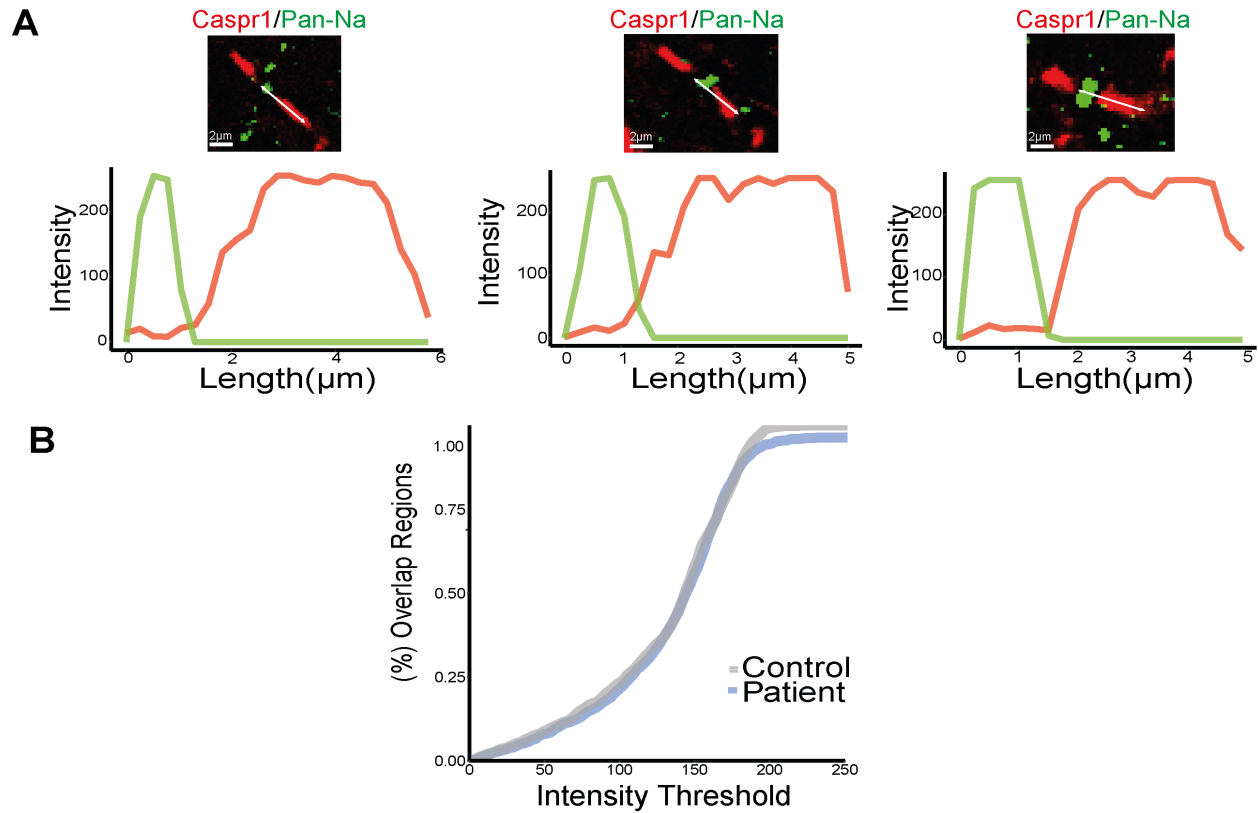


Figure 3.8: The location of nodal Na_v channels was not disrupted in MS NAWM tissue. (A) Confocal images of a double immunofluorescence of Caspr1-stained paranode and nodal voltage-gated Na_v channels with the RGB Intensity profile of both immunofluorescence signals across the nodal and paranodal compartments. (B) Caspr1 signal was subtracted from Na_v , and when the difference between them was smaller than a variable Intensity Threshold, that point was considered an overlapping region. For every threshold calculated, the proportion of overlapping regions was very similar in both groups.

3.2.5 Further analysis of the Caspr1, $K_v1.2$, and Na_v RGB Intensity Profiles

In order to compare the average intensity distribution between the MS group and the non-neurological control group (figure 3.9 A,C,E), the overall mean and standard error of all the RGB intensity profiles for Caspr1, $K_v1.2$ and Na_v signals along the nodal, paranodal and juxtaparanodal length were computed. The Caspr1, $K_v1.2$ and Na_v axonal profiles were not defined at the same points in distance at every RGB intensity profile measured. Therefore, in order to calculate the average of all of the profiles along the same length occupied in the axonal membrane, length bins were created from which the midpoint of each one was taken to calculate the overall mean and standard error per bin. Also, the intensity of each profile was scaled by the maximum length to observe the intensity / density distribution of Caspr1, $K_v1.2$ and Na_v independently of length (figure 3.9 B,D).

Assuming that the intensity of the signal is proportional to the density of the protein, then the average Caspr1 intensity profile of the MS group was higher at every length bin compared to the control group, especially at longer paranodal lengths (figure 3.9 A). However, $K_v1.2$ intensity was larger at first, and then became lower than the control group along the juxtaparanodal length (figure 3.9 C). The Na_v mean intensities between both groups were similar (figure 3.9 E). When the variable length from the distributions was removed, there was much more noise (the standard error per bin was higher since each RGB profile had a different length) (figure 3.9 B,D,F). The overall mean intensities of Caspr1 were slightly larger in MS compared to the non-neurological control group (figure 3.9 B). The mean intensities of $K_v1.2$ between the control and the MS group followed a different pattern suggesting a different channel distribution (figure 3.9 D). The average Na_v intensity profile of the MS group was slightly smaller than the control group (figure 3.9 A,B).

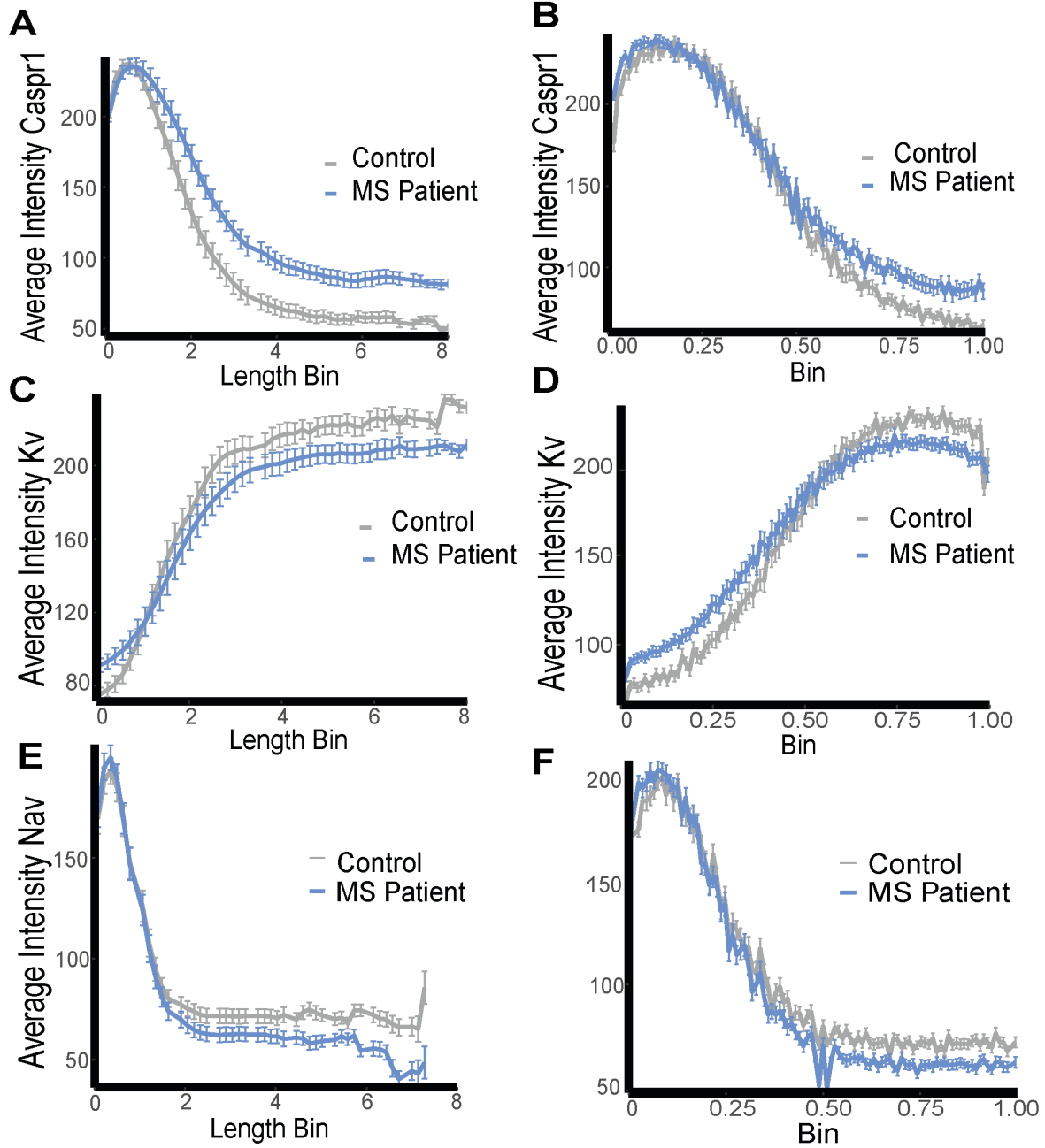


Figure 3.9: The average intensity profile of the Caspr1, $K_v1.2$ and Na_v RGB signals suggested that in MS NAWM tissue Caspr1 and $K_v1.2$ could have diffused along the axolemma. (A) Average intensity profile $\pm$ standard error of all the Caspr1-stained paranodes along length. (B) Average intensity profile $\pm$ standard error of Caspr1-stained paranodes scaled by the maximum length per profile. (C) Average intensity profile $\pm$ standard error of all the $K_v1.2$ -stained juxtaparanode along length. (D) Average intensity profile $\pm$ standard error of all the $K_v1.2$ -stained scaled by the maximum length per profile. (E) Average intensity profile $\pm$ standard error of all the Na_v -stained nodes along length, (F) Average intensity profile $\pm$ standard error of all the Na_v -stained nodes scaled by the maximum length per profile. The post-mortem MS tissue profiles were plotted in blue and non-neurological control tissue in grey.

3.3 Discussion

3.3.1 Axonal degeneration in MS

Many histopathological studies have characterised and quantified axonal degeneration in MS post-mortem tissue in focal demyelinating lesions in both white matter and grey matter (Trapp et al., 1998; Kutzelnigg et al., 2005b) and in NAWM (Bjartmar et al., 2001; Kutzelnigg et al., 2005b; Moll et al., 2011). However, characterisation studies of the pathology at the intersection between the axon and the oligodendrocyte, at the axo-glial junction, are scarce. In MS, the intertwined nature of axon and myelin pathology is especially relevant. Although demyelination is one of the main histopathological features of the disease, axonal injury is observed from the earliest stages, and it is one of the main predictors of long-term outcome and the transition from RRMS to SPMS (Trapp and Nave, 2008; De Stefano et al., 2001; Simons et al., 2014). Furthermore, axonal transection has also been observed in myelinated axons (Nikić et al., 2011). Therefore, in order to understand the molecular mechanisms behind axon and oligodendrocyte injury, we cannot study neuronal and glial pathology as separate entities.

The nodes of Ranvier represent the only points of direct contact between myelin and the axon, specifically at the paranodal axo-glial junctions, also known as "axon-myelin units" (Simons et al., 2014; Stassart et al., 2018), and it is suggested that pathology at these sites could be one of the targets of the inflammatory mediated attacks in MS. However, paranodal and juxtaparanodal pathology has been described in only a few studies in human MS tissue (Howell et al., 2006, 2010; Kastrioti et al., 2015), while its effects and causes remained unknown. Nonetheless, there are several studies in chronic inflammatory demyelinating polyneuropathy (CIPD) pointing to IgG4 autoantibodies against nodal and paranodal proteins such as Caspr1 (Doppler et al., 2016), Nf155 (Devaux et al., 2016) and Contactin-1 (Doppler et al., 2015) that lead to paranodal disruption and comprise 10 % of patients with CIPD (Bunschoten et al., 2019). These autoantibodies are also reported in some patients with Guillain-Barre Syndrome (GBS) (Delmont et al., 2017). However, the prevalence of these IgG4 autoantibodies against Nf155 was not significant in PMS or

RRMS compared to controls (Stich et al., 2016). Therefore, although these autoantibodies against paranodes can dismantle the axo-glial junctions, and cause AP conduction deficits in some neuropathies such as CIPD and GBS (Manso et al., 2016), they might not play a major role in MS paranodal axo-glial pathology.

In this chapter, we were interested in the analysis of paranodal axo-glial junctions in non-lesioned post-mortem MS tissue from the pre-central gyrus and cerebral peduncle, which were selected by immunofluorescence staining with MOG antibodies (figure 3.1 A). Several studies have associated chronically activated microglia and macrophages to diffuse axonal pathology throughout the NAWM (Kutzelnigg et al., 2005b; Moll et al., 2011; Singh et al., 2013). In our study, activated microglia/macrophages characterised by their more amoeboid morphology (thicker and shorter processes) were visualised by immunofluorescence staining with HLA-DR+ and were found in higher numbers in all the MS tissue blocks compared to the non-neurological control tissue (figure 3.1 B). This data is consistent with previous studies in NAWM post-mortem tissue in which in situ hybridisation analysis revealed the expression of mRNA HLA-DR+, known to be generally expressed by microglia, throughout the NAWM of the majority of MS cases, whereas in control white matter was not detectable (Zeis et al., 2007). Furthermore, pathological studies have pointed to the CNS innate immune system as one of the main drivers of axonal degeneration and damage (Barnett and Prineas, 2004; Marik et al., 2007). Pro-inflammatory microglia could produce diffuse axonal damage in myelinated axons by secreting cytotoxic cytokines (Hanisch, 2002), free radicals, (Boje and Arora, 1992; Haider et al., 2011; Fischer et al., 2012) and glutamate (Takeuchi et al., 2006; Barger et al., 2007), which could contribute to mitochondrial pathology and energy deprivation (Mahad et al., 2009).

3.3.2 PNJ disruption

PNJs serve multiple functions for fast and reliable saltatory AP conduction as they are effective electrical and physical barriers (Rosenbluth, 2009). Thus, the correct molecular architecture across the axonal membrane and oligodendrocyte must be kept intact. The observed longitudinal elongation of the Caspr1-stained paranodal axo-glial junctions in-

indicates an evident PNJ disruption in a population of paranodes in MS NAWM compared to non-neurological control tissue (figure 3.2D). 11.14 % of the paranodes in the MS group were longer than 4 μm (figure 3.2E), which is more than 50 % longer than the average paranodal length in the brain ($\approx 2 \mu\text{m}$). This data is similar to the elongation of Nf155-stained paranodes noted previously (Howell et al., 2010). Previous literature showed that paranodal disruption could be linked to ageing (Hinman et al., 2006). In fact, a recent deep learning analysis of MRI scans from MS patients suggests that the process of ageing in the brain is faster in MS patients (Herent et al., 2018). However, in our data, the average age of the MS cases is lower than the non-neurological controls (see Chapter 2). Thus, we suggest that the neurodegenerative pathological features of MS, such as chronic inflammation, might be driving the paranodal disruption as well as the ageing and not the other way around.

The paranodal axo-glial junction elongation, measured either by the paranodal axonal protein Caspr1 or, by its myelin counterpart, Nf155, might indicate that they could have detached one from each other. Therefore, it could represent a progressive detachment of the myelin end loops from the axolemma, and between the loops themselves, that would result in a complete detachment of the myelin tongues. When we calculated the overall mean of the Caspr1 RGB profiles between the MS and the non-neurological control groups (figure 3.9A), the Caspr1 mean intensity was larger compared to the control group at every length, specifically at longer ones. If we assume that the mean intensity of Caspr1 is proportional to its concentration/density, this distribution suggests that some Caspr1 proteins could have lost their anchorage, and could have diffused along the axonal membrane. When we scaled the data by distance (figure 3.9 B), the average intensity/density of Caspr1 between groups was very similar, suggesting that there was not a difference in protein concentration, but a structural disruption. The progressive detachment of the myelin loops from the axolemma could increase the width of the peri-axonal space at the paranode and the distance between the myelin loops (figure 3.10 A). Moreover, it could also lead to a progressive increment of the peri-axonal space at the juxtaparanodal compartment (figure 3.10 B), as observed in Nf155 null mice that have a lack of septate junctions at the paranode (Pillai et al., 2009).

Myelinating oligodendrocytes do not only isolate electrically the axons for saltatory propagation, they also provide trophic support to their function and integrity. In fact, previous studies with PLP-null mice showed that oligodendrocyte pathology can perturb anterograde and retrograde axonal transport (Edgar et al., 2004). Further, a high-pressure freezing (HPF) electron microscopy study (Snaidero et al., 2014) showed that mature myelin contains non-compacted cytosolic channels called myelinic channels. These channels serve as a route for the oligodendrocyte to transport proteins and axo-protective molecules to the paranodal axo-glial junctions (Simons and Nave, 2016). These non-compacted myelin regions that form the myelinic channels include the outer and inner peri-axonal tongues of myelin membranes and the PNJs (Nave, 2010; Velumian et al., 2011). Moreover, normal myelinic transport is considered a requirement for healthy axonal function and structure. Thus, any disruption in the axo-glial transport along the myelinic channels of proteins that maintain the adequate structure and function of the axo-glial junctions could damage them.

Chronic inflammation in MS could perturb axo-glial transport, and one of the main access routes for damage is through the peri-axonal space where the axo-myelinic synapse is present (Stys, 2011). Further, through this system the axon can inform the oligodendrocyte of its metabolic needs. In fact, recent studies have shown that the oligodendrocytes can sense changes in the electrical activity by the internodal/paranodal membrane NMDA receptors. The larger release of glutamate in the peri-axonal space could be used as a marker of increased axonal electrical activity and energy needs. In this case, the oligodendrocyte increases expression of glucose transporters to perform glycolysis and provide lactate to the axon for aerobic ATP production and lipid synthesis. This way, oligodendrocytes can modulate the metabolic support to the axon (Fünfschilling et al., 2012; Saab et al., 2016; Saab and Nave, 2017). Thus, an increment in the peri-axonal space could change signalling to oligodendroglial receptors and disrupt adequate metabolic support to the axon.

In addition, an increment in the peri-axonal space could lead to direct detrimental effects to AP conduction. The resistance of the paranodes and juxtaparanodes would be

reduced, whereas the capacitance would increase. The PNJs would then be electrically leaky and allow passive nodal current shunting underneath the myelin sheath, slowing down the conduction progressively. If all the paranodes throughout the brain surrounded by chronically activated microglia/macrophages were disrupted, a percentage of nodes within an axon would be damaged. Depending on the proportion of nodes affected, the conduction velocity would be proportionally reduced or even cancelled. This physiological imbalance in conduction throughout the brain could translate into some of the cognitive symptoms that MS patients suffer such as fatigue (Van Kessel and Moss-Morris, 2006). Fatigue is one of the most debilitating and common symptoms in patients, and refers to exhaustion of a mental state, a physical state or both (Krupp et al., 1988; for Clinical Practice Guidelines et al., 1998). Thus, it could be suggested that the severity of this symptom could be associated to the proportionality of disrupted nodes within axons. Furthermore, the common therapeutic drugs to treat fatigue (4-AP, amantidine) have demonstrated efficiency in only some patient subgroups (Bowman et al., 1997; Rossini et al., 2001). Although, these results could be explained by the heterogeneity of MS, neuroprotective treatments targeted to protect the integrity of the axo-glial junctions could be a possible new therapeutic approach.

3.3.3 PNJ disruption associated with chronic microglial activation

Although it is hard to follow axons longitudinally through immunofluorescence images, we observed that not all the paranodes of an axon are sequentially disrupted. A patch of paranodes surrounded by activated microglia/macrophages are more likely to be damaged (figure 3.3A). We found a correlation between the area occupied by HLA-DR+ stained microglia/macrophages, and the mean length of the paranodes (figure 3.3 B, C), and the proportion of paranodes longer than 4 μm (figure 3.3 D). Assuming that microglia and macrophages acquire a more amoeboid morphology and increase in number when they are activated, and thus the overall area occupied by microglia is larger, our data suggests that they could be secreting toxic molecules that could disrupt the structure of the paranodes. In fact, the production of ROS, NOS, glutamate, cytokines and chemokines by microglia, astrocytes and other immune cells are suggested to promote oxidative stress,

mitochondrial damage and dysfunction, demyelination and Ca^{2+} influx (Filosto et al., 2011; Friese et al., 2014; Dendrou et al., 2015).

Since the PNJs are composed of non-compacted myelin (Snaidero and Simons, 2014) and NMDA receptors are located in the processes of the oligodendrocytes, specifically at the non-compacted myelin (Salter and Fern, 2005), glutamate can induce an increase in Ca^{2+} levels inside the myelin (Micu et al., 2016). In MS, elevated glutamate levels have been demonstrated in both lesions and NAWM tissue (Srinivasan et al., 2005; Baranzini et al., 2010) and in the CSF (Sarchielli et al., 2003), compared to non-neurological controls. Thus, proteases such as Ca^{2+} dependent cysteine calpain1 could be activated, and subsequently, proteolyse cytoskeletal proteins that would disrupt the intricate protein connection that forms paranodal junctions (Siman et al., 1989; Schafer et al., 2009). The latter mechanism could explain the results of a study in which coherent anti-Stokes Raman scattering in-vivo imaging of myelin was performed in the spinal cord of a murine model, and paranodal splitting and juxtaparanodal K_v1 channels dislocation was observed after direct administration of glutamate (Fu et al., 2011).

3.3.4 PNJ disruption is associated with axonal stress

Persistent high levels of glutamate can also cause intracellular Ca^{2+} overload in the axon, activating degradative enzymes that trigger axonal injury and mitochondrial dysfunction (Grasselli et al., 2013). Therefore, the disruption of paranodal junctions could also come from within the axon. In our data, most of the axons with dephosphorylated neurofilaments (SMI32+) had longer paranodes than SMI32- axons (figure 3.4 A, B), which suggests that both phenomena are related, and when a paranode is elongated, there is an underlying axonal pathology as well. Phosphorylation of neurofilament proteins in axons is considered important to regulate axonal transport rate and provide stability (Shea et al., 2003). Thus, SMI32- axons will be more prone to contain swellings due to a possible disruption in their transport, as we have seen in our tissue (figure 3.4 B), and in other studies (Trapp et al., 1998). Previous literature has shown that mitochondrial transport is compromised in MS, and might be mediated by the translocation of the nuclear histone

deacetylase 1 (HDAC1) to the axonal membrane and subsequent local axonal beading (Kim et al., 2010). Another possible mechanism that could contribute to degeneration in the axon is oxidative stress caused by superoxide species (ROS and NOS) released by microglia and macrophages. Superoxide production can increase inside the axon significantly, especially if the mitochondria are damaged, as in MS (Dutta et al., 2006; Mahad et al., 2009). Mitochondria synthesise ATP by oxidative phosphorylation for adequate AP conduction, thereby also producing ROS. In environments where ROS levels are increased, mitochondrial DNA mutations can accumulate and compromise the oxidative phosphorylation efficiency. For example, high levels of NO can inhibit mitochondrial respiration and reduce ATP synthesis (Su et al., 2009). Therefore, axonal transport and conduction could be disrupted in MS, as they are highly energy-demanding processes. Moreover, ROS and NOS can activate enzymes including myeloperoxidase, xanthine and NADPH oxidases (NOX) that can drive structural axonal degeneration (Gray et al., 2008; Fischer et al., 2012). For instance, paranodal proteins at the axonal cytoskeleton such as 4.1B and β IV spectrin and α II spectrin could be degraded. Moreover, a previous study showed that all of these events occur near the nodes in a murine EAE model, and can be reversed by NO scavengers (Nikić et al., 2011). This data corroborates what we have observed in MS tissue, in which SMI32+ axonal transections are located near the nodes of Ranvier (figure 3.4 C (i, ii)).

Therefore, we can suggest that microglial/macrophage secretion of glutamate, ROS and NOS might trigger multiple molecular mechanisms in either the oligodendrocyte and/or the axon, that will contribute to axon degeneration, and consequently to paranodal axo-glial disruption.

3.3.5 PNJ disruption and the subsequent dislocation of voltage-gated K_v1 channels

The structural disruption of the axo-glial junctions does not only have electrical consequences; their function as physical barriers will also be compromised, leading to the dislocation of voltage-gated channels such as K_v1 and Na_v . Our data shows that the MS

NAWM tissue had a higher proportion of regions where $K_v1.2$ and Caspr1 intensities were co-localising, compared to the non-neurological control tissue (figure 3.6 B, C). This result points again to the disruption of the paranodal axo-glial junctions; the proportion of overlapping regions at specific thresholds was correlated significantly with the paranodal mean and the proportion of paranodes per block longer than $4\ \mu\text{m}$ (figure 3.7). Therefore, this means that a proportion of the voltage-gated juxtaparanodal K_v1 channels could have been dislocated towards the paranode. When we calculated the overall intensity mean of all the RGB profiles between the MS and non-neurological control group, we found that the intensity of K_v1 channels was higher at the beginning compared to the control group, and was lower than the control as the length is larger (starting from the paranode to the juxtaparanode), (figure 3.9B). This result points to the fact that the density of voltage-gated K_v1 in the MS group changed in the juxtaparanode, towards the paranode. Moreover, previous studies in MS tissue showed that the juxtaparanodal proteins TAG1 and Caspr2, which anchor K_v1 channels to the axolemma appeared reduced in the NAWM MS tissue (Kastriti et al., 2015). Thus, the partial disruption of the paranode, as well as, the possible dissociation of the tripartite juxtaparanodal complex of TAG-1/Caspr2/ K_v1 , could explain voltage-gated K_v1 channels diffusion along the membrane.

Further, this pathological feature could also trigger an increment in the width of the juxtaparanodal peri-axonal space (figure 3.10 B, D). The dislocation of K_v1 channels could cause some detrimental effects in AP conduction. For instance, the higher rate of epileptic seizures in MS (Kelley and Rodriguez, 2009) could be related to elevated K^+ ion concentration in the extracellular space (Smart et al., 1998; Du et al., 2016), which could stem from the increment in peri-axonal space that makes voltage-gated K_v1 channels more exposed. Although experimental studies in the 1980s did not find significant changes in AP propagation after blocking these channels with 4-aminopyridine (4-AP) in the PNS (Smart et al., 1998) or with K_v1 knock-outs in large dorsal columns axons (Kocsis and Waxman, 1980), studies in small-calibre axons such as the optic nerves found a broadening of the AP and changes in afterpotential depolarisations (Devaux et al., 2002). These channels contribute to the re-polarisation of the AP, differently depending on the type of axons, mainly because in the node there are other K_v channels such as $K_v3.1b$ (Devaux

et al., 2003) and the slowly activating KCNQ, which can also re-polarise the AP.

Regarding the nodal voltage-gated Na_v channels, we did not observe any dislocation of these channels from the node (figure 3.8). The tight clustering of Na_v channels is regulated by the function of the paranodal junctions as diffusion barriers, but also by their anchorage to the extracellular matrix proteins through Nf186 (Thaxton et al., 2011; Amor et al., 2017). Previous studies in MS tissue showed that Nf186 appeared disrupted in demyelinating lesions (Howell et al., 2006). However, in our data, although a percentage of paranodes were disrupted, voltage-gated Na_v channels remained clustered via Nf186. However, when we plotted the overall mean of all the RBG profiles of Na_v between the MS and the non-neurological control group, the intensity/density of Na_v channels was slightly lower in the MS group. Nonetheless, only a significant reduction in the density of voltage-gated Na_v channels would be expected to modify AP conduction in the case that there is not enough of them to reach the depolarising threshold.

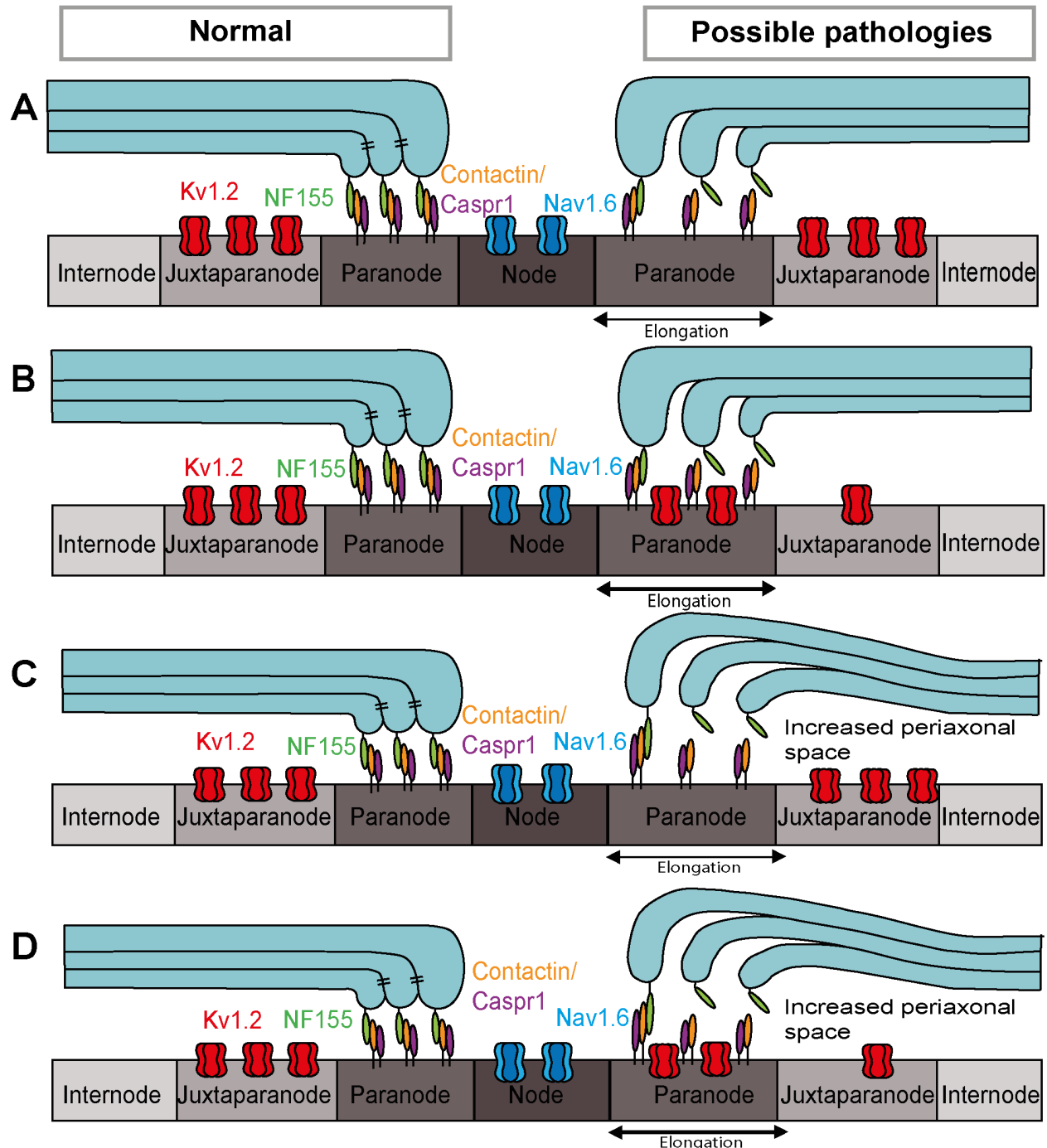


Figure 3.10: Diagrams of possible paranodal axo-glial junction disruptions. (A) Paranodal disruption caused by the detachment of the myelin loops from the axolemma increases the periaxonal space of the paranodal compartment. (B) Paranodal disruption caused by the detachment of the myelin loops from the axolemma increases the periaxonal space of the paranodal and juxta- and paranodal compartments. (C) Paranodal disruption caused by the detachment of the myelin loops from the axolemma generates an increment of the peri-axonal space of the paranodal junction compartment and the dislocation of $K_v1.2$ from the juxta- and paranode. (D) Paranodal disruption caused by the detachment of the myelin loops from the axolemma generates an increment of the peri-axonal space of the paranodal and juxta- and paranodal compartments, and the dislocation of $K_v1.2$ from the juxta- and paranode.

3.4 Conclusions

In conclusion, our histopathological data have shown that MS NAWM tissue contained a proportion of significant elongated Caspr1-stained paranodes compared to non-neurological control tissue, indicating an apparent disruption of these junctions. Further, paranodal elongation was positively correlated with activated microglia and, associated with axonal stress labelled by SMI32, indicating an underlying axonal pathology. Furthermore, the paranodal elongation was accompanied by a dislocation of juxtaparanodal voltage-gated K_v1 channels towards the paranodes, whereas the voltage-gated channels Na_v remained clustered at the node. Therefore, these results suggest that diffuse activation of microglia in MS could be associated with paranodal axo-glial junction pathology in myelinated axons. These structural changes at the node are likely to have functional consequences for AP conduction including slowing down propagation.

The following chapters in the current thesis stem from this chapter's results. In order to study the direct relationship between pro-inflammatory microglia and paranodal axo-glial disruption in NAWM, we performed the same histopathological analysis in a chronic rat model of meningeal inflammation generated by the chronic exposure to the pro-inflammatory cytokines $LT\alpha$ and $IFN\gamma$. This model was characterised by chronically activated microglia and the absence of white matter demyelination (Chapter 4). Regarding the potential AP deficits that these paranodal changes could provoke, we developed a computational model of an axon composed of 21 nodes with the simulator NEURON to predict conduction defects (Chapter 5). Lastly, in order to elucidate some of the molecular mechanisms underlying the pathology, we set up a cerebellar organotypic tissue culture platform (Chapter 6).

4

Axo-glial pathology in a chronic model of meningeal inflammation

4.1 Introduction

Paranodal axo-glial disruption has previously been shown in a rodent EAE model immunised with *MOG*_{35–55} peptide in complete freunds adjuvant (CFA) (Howell et al., 2010; Fu et al., 2011). In this model, lesions develop at the pial margins of the spinal cord mediated by auto-reactive MHC Class II CD4⁺ T-cells, leading to both demyelination and significant axonopathy (Lassmann and Bradl, 2017). However, this model is a relatively poor reflection of the pathology seen in the MS brain. In contrast, in this chapter, paranodal axo-glial pathology was examined in a rat model of chronic meningeal inflammation. In this model, inflammation was initiated by chronic production of pro-inflammatory cytokines within the meninges, and therefore, it was confined to the brain. Furthermore, it is characterised by extensive activation of microglia and macrophages, and the absence

of focal demyelinating lesions in the white matter. It does, however, feature subpial demyelination and neuronal loss in the cerebral cortex, similar to that seen in the MS brain. The substantial microglial activation that is seen throughout the cortex in this model means that it can be used as a model of chronic CNS inflammation. Thus, we tested if chronic inflammation throughout the brain driven by activated microglia/macrophages was sufficient to generate disruption at the nodes of Ranvier.

4.2 Chronic exposure to $LT\alpha$ and $IFN\gamma$ induces meningeal inflammation

Extensive subpial cortical pathology, neuronal loss and demyelination has been observed in the pre-central gyrus of human SPMS cases containing ectopic B-cell follicle-like structures in the meninges (Magliozzi et al., 2010; Howell et al., 2011). These findings are a key feature of progressive MS pathology, not seen in any other brain inflammatory condition. Furthermore, elevated numbers of TNF and $LT\alpha$ mRNA-expressing mononuclear cells have been observed in the CSF and blood from MS patients (Rieckmann et al., 1994; Navikas et al., 1996). In order to understand the underlying mechanisms that drive pro-inflammatory cytokines to diffuse through the BBB and generate cortical pathology, a chronic model of meningeal inflammation was developed. In this model, lentiviral vectors encoding for the $LT\alpha$, $IFN\gamma$ and TNF genes were injected into the subarachnoid space of rat brains to transfect the fibroblasts and endothelial cells of the meningeal subpial layer to then induce cortical pathology (the HIV-1 derived lentiviral vectors contained the general CMV promoter). Chronic expression of the up-regulated pro-inflammatory cytokines in the CSF of rats led to neuronal loss in the cortical layers and chronic inflammation throughout the brain (James et al, submitted to *Acta Neuropathologica*).

During the development of this chronic model of meningeal inflammation, a group of rats were immunised with MOG emulsified in the adjuvant IFA. After 28 days, they were injected with the lentiviral vectors (figure 4.1). Some animals were sensitised with the myelin protein MOG in order to verify if circulating anti-MOG antibodies were necessary

to generate similar pathology. The two control groups were rats injected with green fluorescent protein (GFP) gene and naïve rats. In the current chapter, a subgroup of rats from this study was analysed: the rats that were immunised with IFA, injected with lentiviral vectors encoding the $LT\alpha$, $IFN\gamma$ and GFP genes, and sacrificed 90 days post-injection (figure 4.1, yellow box). This subgroup of animals was selected to avoid MOG-induced demyelinating lesions to study paranodal axo-glial pathology at NAWM regions.

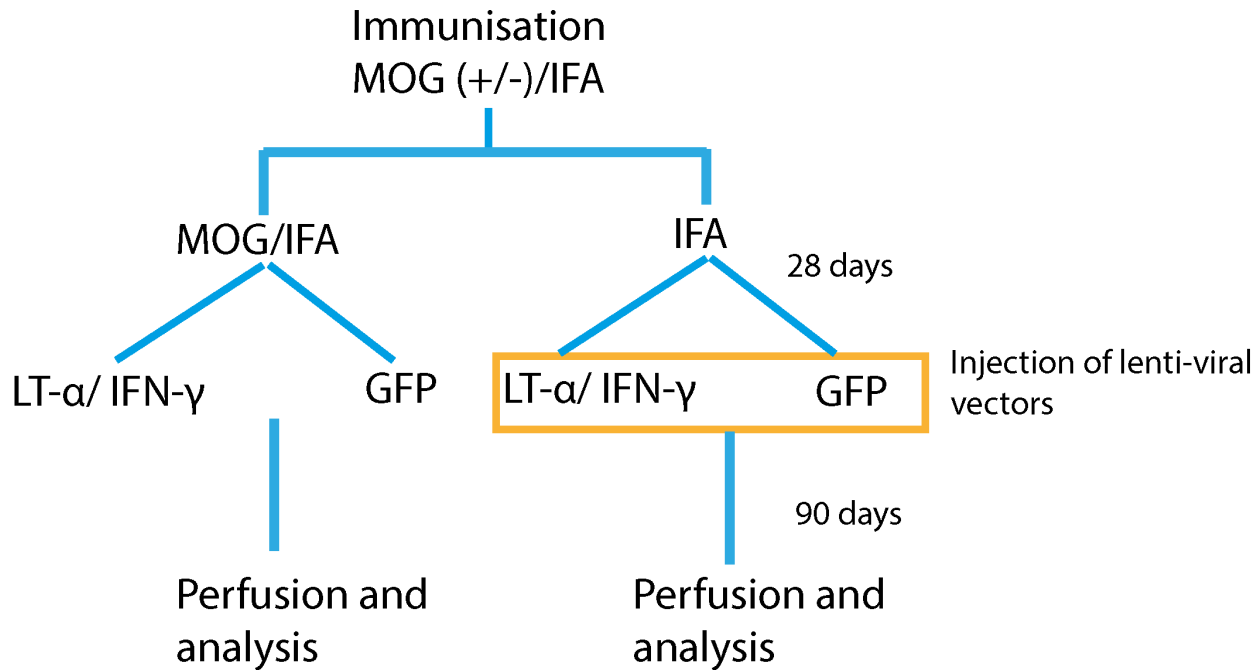


Figure 4.1: Experimental Diagram. Diagram of the study carried out by Dr. James to induce chronic meningeal inflammation. The rats were sensitized with IFA or MOG/IFA for 28 days. Subsequently they were injected with lentiviral vectors encoding $LT\alpha$ and $IFN\gamma$ genes for 90 days. The yellow box denotes the subgroup of rats that was analysed in the current chapter.

4.2.1 Lymphotoxin- α ($LT\alpha$)

Tumor necrosis factor superfamily molecules, such as $LT\alpha$ (also known as $TNF-\beta$ and $TNFSF1$), play a role in many physiological functions including inflammation, proliferation, differentiation, and cell death (Locksley et al., 2001). There are two types of lymphotoxins: $LT\alpha$ and $LT\beta$. $LT\alpha$ is secreted by lymphocytes as a homotrimeric molecule ($LT\alpha_3$). $LT\beta$ is a type II transmembrane protein that can bind to soluble $LT\alpha$ to form the heterotrimers: $LT\alpha_1\beta_2$ and $LT\alpha_2\beta_1$. While $LT\alpha_1\beta_2$ binds $LT\beta$ receptors ($LT\beta R$), $LT\alpha_2\beta_1$ and soluble $LT-$

4.2. Chronic exposure to $LT\alpha$ and $IFN\gamma$ induces meningeal inflammation

$\alpha 3$ homotrimers bind to TNF receptors 1 and 2 (TNFR1 and TNFR2), and herpes virus entry mediator (HEVM) (Sonar and Lal, 2015). LIGHT (CD258, also known as TNFSF14) is expressed on T cells and can interact with both $LT\beta R$ and HEVM (herpes virus entry mediator). Interaction of LIGHT with HEVM has a co-stimulatory function, whereas with $LT\beta R$ induces apoptosis. In fact, LIGHT-deficient mice develop more severe MOG_{35-55} EAE due to increased activation of microglia and apoptosis within the CNS (Maña et al., 2013).

Both soluble TNF and $LT\alpha$ homotrimeric molecules can activate TNFR1 and TNFR2. TNFR1 contains TRADD (tumor receptor associated death domain) that can initiate multiple downstream signaling pathways. It can initiate the classical nuclear factor kappa-light-chain-enhancer (NF- κ B) pathway that stimulates inflammation and cell survival (Hsu et al., 1995), the caspase-8 driven pathway for apoptosis, and RIPK3-MLKL pathway for the formation of a necrosome to induce necroptosis (Remijssen et al., 2014). On the other hand, activation of TNFR2 or $LT\beta R$, can initiate the recruitment of TRAFs (tumor receptor associated factors) and lead to the NF- κ B canonical pathway that mediates inflammatory responses or the NF- κ B non-canonical pathways involved in immune cell differentiation and maturation and secondary lymphoid organogenesis (Shih et al., 2011). In fact, $LT\alpha$ knockout mice have developmental defects in the lymph node formation, and lack of Peyer's patches (De Togni et al., 1994). Moreover, $LT\alpha$ null MOG_{35-55} immunised EAE mice showed reduced demyelination and CNS inflammation (Suen et al., 1997), and in a cuprizone-induced demyelination model showed a decreased number of microglia at lesions, and not effect in the rate of re-myelination (Plant et al., 2005). TNFR1 and TNFR2 are expressed in oligodendrocytes, neurons, astrocytes (Raine et al., 1998; Brambilla et al., 2011), and microglia (Takeuchi et al., 2006; Veroni et al., 2010; Han et al., 2015).

$LT\alpha$ in MS

Both TNF and $LT\alpha$ have been detected in acute and chronic MS brain lesions (Selmaj et al., 1991). Also, elevated numbers TNF and $LT\alpha$ mRNA-expressing mononuclear cells (MNC) have been observed in blood and CSF from MS patients (Rieckmann et al., 1994;

Navikas et al., 1996), in particular when examined during exacerbation (Rieckmann et al., 1995; Matusevicius et al., 1996). Further, a more recent study showed that B cells from progressive MS produced significantly more TNF and $LT\alpha$ than the ones from RRMS patients (Stein et al., 2018). They are also upregulated in the meninges and CSF of post-mortem MS cases with high levels of meningeal inflammation and cortical pathology (Magliozzi et al., 2018). Thus, these cytokines could play a major role in formation of the ectopic B-cell follicle-like structures in the meninges leading to neuronal pathology (Magliozzi et al., 2010).

4.2.2 Cortical characterisation of the meningeal inflammatory model induced with $LT\alpha/IFN\gamma$

In order to amplify chronic inflammation throughout the brain, $LT\alpha$ was coupled with $IFN\gamma$ in this model. $IFN\gamma$ is a pro-inflammatory cytokine secreted by T-cells that can prime microglia allowing an exaggerated microglial response to a secondary inflammatory stimulus such as TNF or $LT\alpha$. A cortical characterization analysis carried out by the master student Li Yi showed that the animals injected with lentiviral vectors for $LT\alpha/IFN\gamma$ presented a decrease in NeuN+ cell density in cortical layer I, a significant recruitment of microglia, and the absence of significant demyelination, compared to naïve and GFP rats (figure 4.2).

4.2. Chronic exposure to $LT\alpha$ and $IFN\gamma$ induces meningeal inflammation

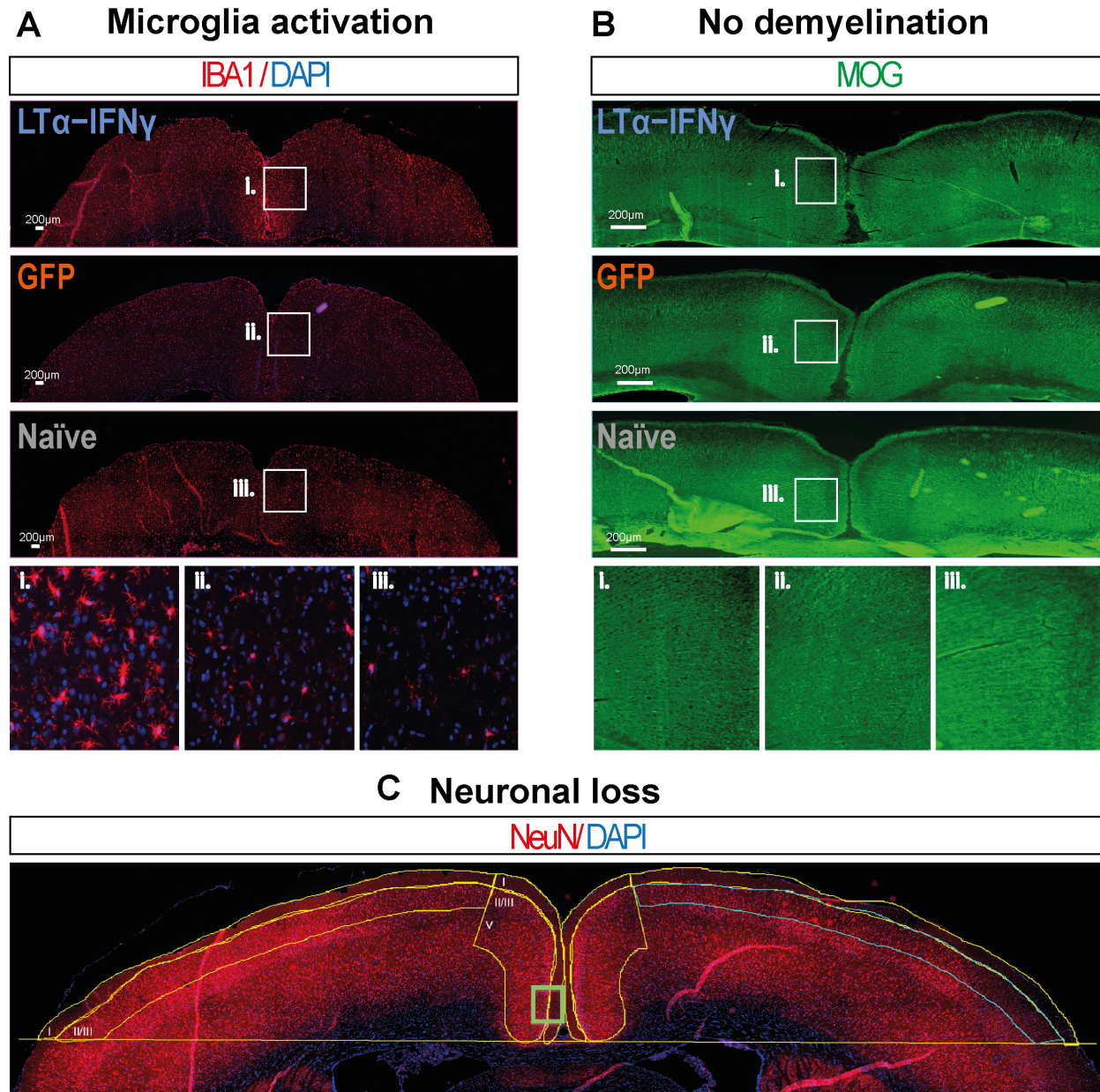


Figure 4.2: Cortical characterisation of the $LT\alpha/IFN\gamma$ rat tissue. (A) Images of IBA1-stained microglia in $LT\alpha/IFN\gamma$ (i), GFP (ii) and naïve (iii) rats. (B) Images of MOG stained myelin stained in $LT\alpha/IFN\gamma$ (i), GFP (ii) and naïve (iii) rats. (C) Image of NeuN+ stained neurons in the cortical layers I, II, III, IV and V of a $LT\alpha/IFN\gamma$ rat.

4.2.3 Aims

In order to determine whether the persistent presence of pro-inflammatory cytokines in the CSF could lead to paranodal axo-glial pathology in an experimental setting, the fol-

lowing experiments aimed to characterise and quantify this pathology in a chronic model of meningeal inflammation by the expression of Caspr1/ K_v proteins and its relationship to chronic inflammation. Therefore, the specific aims of the study were:

- To quantify and characterise paranodal axo-glial pathology by elucidating the expression of paranodal Caspr1 and juxtaparanodal K_v 1.2 in the NAWM regions of the corpus callosum, cingulate and external capsule in LT α /IFN γ , GFP and naïve rats. These WM regions were selected in order to observe the nodes of Ranvier of longitudinal axons in coronal sections at NAWM regions, and due to their proximity to the site of injection of the lentiviral vectors;
- To quantify the local number microglia and astrocytes in the corpus callosum, cingulate and external capsule in LT α /IFN γ , GFP and naïve rats and correlate them to paranodal axo-glial pathology parameters.

4.3 Results

In order to characterise the myelin integrity, microglial and astroglial recruitment at the corpus callosum, cingulate and external capsule of the rat sections, stainings for MOG (figure 4.3 C, D, E), IBA1 (figure 4.3 F, G, H) and GFAP (figure 4.3 I, J, K) were performed in the three groups of rats studied (LT α /IFN γ (n=5), GFP (n=3) and naïves (n=3), figure 4.3 A, B). From the MOG-images, 10 NAWM areas were chosen as regions of interest (ROIs) (figure 4.3 A).

4.3.1 Caspr1 stained paranodal disruption

Caspr1 and voltage-gated K_v 1.2 channels were immunostained to quantify paranodal and juxtaparanodal disruption in the murine model (figure 4.4 A, B, C, D). In total, 1000 Caspr1-stained paranodes (200 paranodes per rat) from the LT α /IFN- γ group, 600 from the GFP group, and 600 from the naïve were analysed (figure 4.4 E). Caspr1-stained paranodal length was 36.4 % longer on average in LT α /IFN γ ($2.4 \pm 0.5 \mu\text{m}$; figure 4.4 E, blue box-plot) than in the naïve ($1.8 \pm 0.4 \mu\text{m}$; figure 4.4 E, gray box-plot) and 15.9 % than in

4.3. Results

the GFP ($2.1 \pm 0.5 \mu\text{m}$; figure 4.4 E, orange box-plot). Moreover, 82 % of the paranodes in the $\text{LT}\alpha/\text{IFN}\gamma$ group were longer than the 75 % percentile of the control group ($1.98 \mu\text{m}$), and 49 % longer than the 75 % percentile of the GFP group ($2.37 \mu\text{m}$) (figure 4.4 E). Paranodal length distributions of each group showed that 9.7 % of the paranodes in the $\text{LT}\alpha/\text{IFN}\gamma$ group were longer than $3 \mu\text{m}$ compared to the 4.8 % in the GFP group, and 0 % in the naïve group (figure 4.4 F (purple and pink)). In fact, paranodal length distributions were significantly different: whereas 77.5 % of the paranodes in the naïves were shorter than $1 \mu\text{m}$, in the GFP group 46.7 %, and in the $\text{LT}\alpha/\text{IFN}\gamma$ group, the value decreased to 19.3 % (figure 4.5 F). Thus, we can conclude that the paranodal axo-glial junctions in the $\text{LT}\alpha/\text{IFN}\gamma$ group were highly disrupted regarding their length, compared to the GFP and naïve groups.

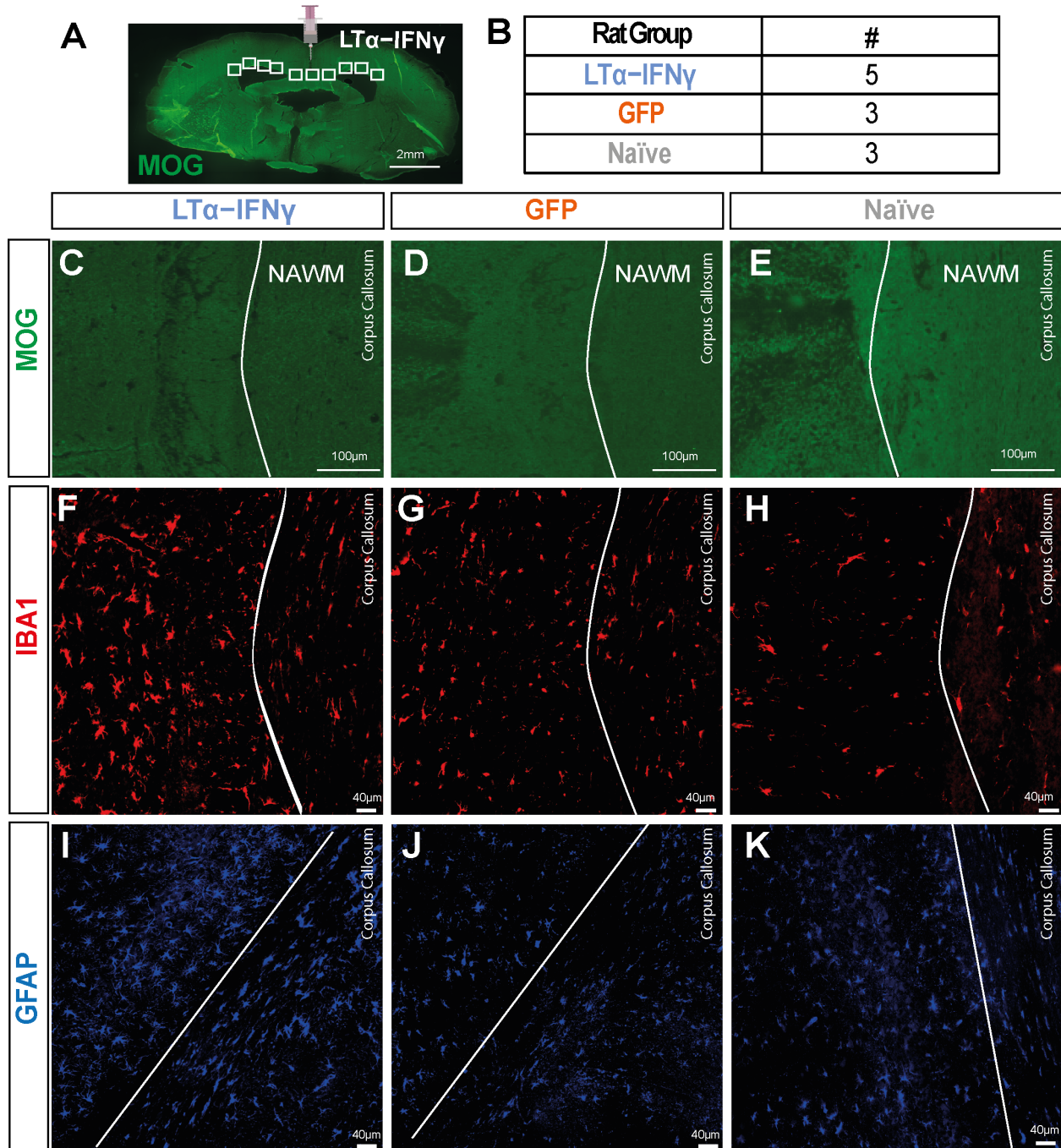


Figure 4.3: Characterisation of the NAWM regions of LT α /IFN γ , GFP and naïve rats at the corpus callosum, cingulate and external capsule with MOG, IBA1 and GFAP. (A) Immunofluorescent image of a coronal rat section stained with MOG. Lentiviral vectors encoding LT α and IFN γ genes were injected in the midline of the brain. The white rectangles are a representation of the 10 selected ROIs at the corpus callosum, cingulate and external capsule. (B) Table of the number of animals used: 5 rats were injected with LT α /IFN γ , 3 rats with GFP and 3 naïves. (C,D,E) Immunofluorescent images of myelin stained with MOG of the LT α /IFN γ , GFP and naïve rats. (F,G,H) Immunofluorescent images of microglia stained with IBA1 of the LT α /IFN γ , GFP and naïve rats. (I,J,K) Immunofluorescent images of astrocytes stained with GFAP of the LT α /IFN γ , GFP and naïve rats.

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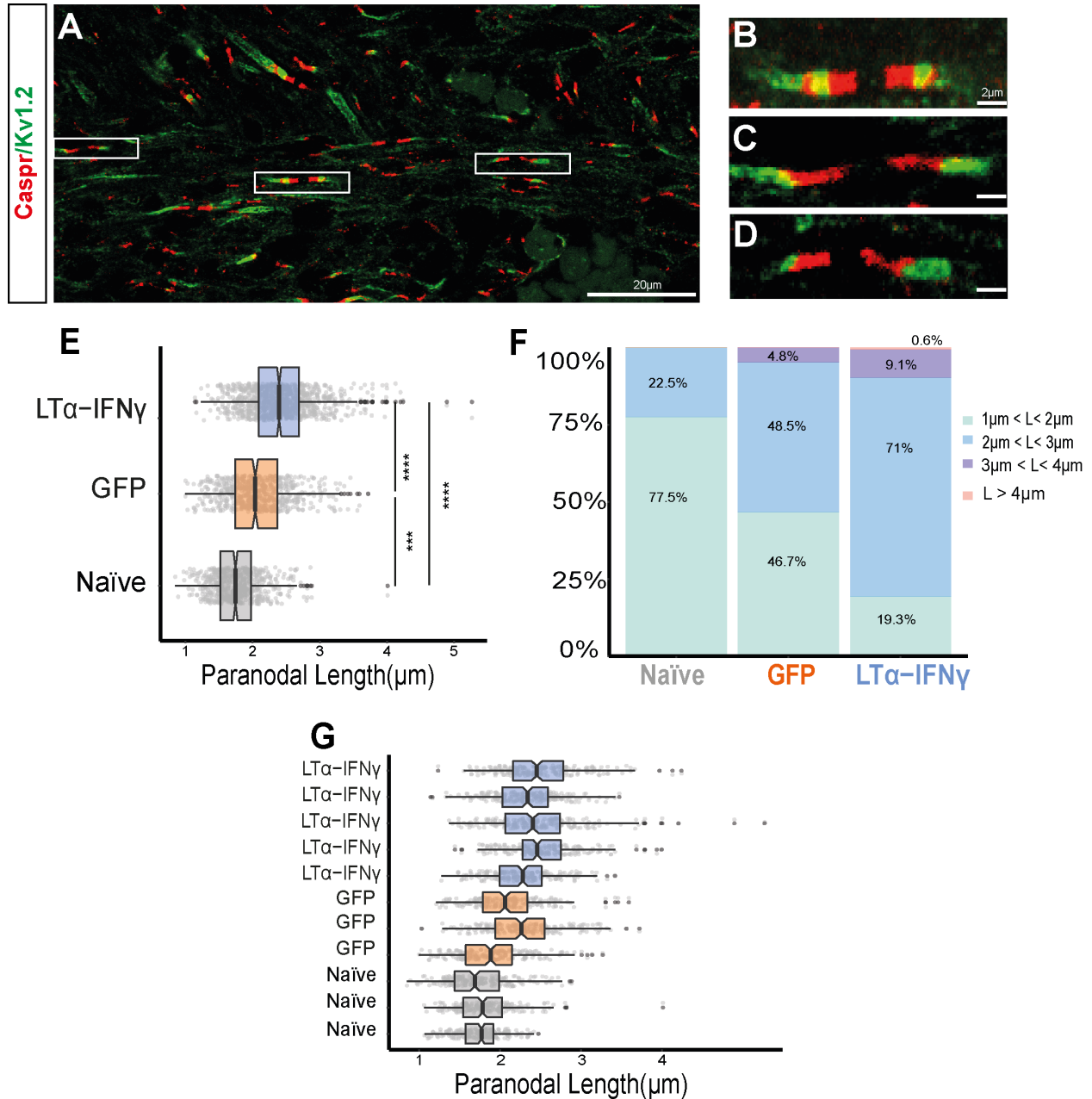


Figure 4.4: LTα/IFNγ rat tissue contained a higher proportion of elongated Caspr1-paranodes compared to the GFP and naïve. (A) Confocal image of a Caspr1-Kv1.2 stained paranodes and juxtaparanodes. (B,C,D) Confocal images of single Caspr1-Kv1.2 stained paranodes/juxtaparanodes. (E) Caspr1-measured paranodal length distributions of LTα/IFNγ (blue), GFP (orange) and naïve (grey) rat groups (**** p < 0.0001, Mann-Whitney test). (F) Paranodal length data was divided for each group in different length ranges. The results were represented in a bar plot. LTα/IFNγ group contains a larger proportion of paranodes longer than 3 μm and 4 μm than the GFP and naïve groups. (G) Paranodal length distributions per rat from the LTα/IFNγ, GFP and naïve groups.

4.3.2 Voltage-gated $K_v1.2$ dislocation

To assess the dislocation of $K_v1.2$ channels and its relationship to paranodal disruption, RGB intensity profiles of all the axons imaged were acquired (figure 4.5A). The dislocation of the channels was quantified in the same way as in the post-mortem human tissue: the RBG signal for Caspr1 was subtracted from the $K_v1.2$ signal for every single axon to analyse the proportion of overlapping regions (the purple circles in figure 4.5A denote the overlapping regions). Subsequently, when the difference between the two signals was lower than a intensity threshold (θ), that value was considered an overlapping region. Finally, the proportion of axons with overlapping regions was calculated over the total number of axons studied in the $LT\alpha/IFN\gamma$, GFP and naïve groups respectively.

$$S(\theta) = \{axon | overlap < \theta\} \quad (4.1)$$

$$overlap(axon) = \sum_{i=1}^n |Caspr1(x_i) - K_v1.2(x_i)| \quad (4.2)$$

$$overlap = \frac{|S(\theta)|}{axons} \quad (4.3)$$

Varying the intensity threshold(θ), figure 4.5 B shows that the $LT\alpha/IFN\gamma$ group contained a larger proportion of axons with overlapping regions at every threshold analysed (thresholds analysed from 0 to the maximum of the intensity signal). Therefore, $LT\alpha/IFN\gamma$ group had more damaged axons with dislocated voltage-gated $K_v1.2$ channels (figure 4.5 B, blue), and therefore disrupted PNJs than the GFP and naïve groups (figure 4.5 B, orange and grey), as it is also observed in the immunofluorescence images of figure 4.5 A, where Caspr1 and $K_v1.2$ are co-localising. Further, when the intensity threshold was set to 50, the proportion of overlapping regions were significantly different between $LT\alpha/IFN\gamma$ and GFP or naïve groups (* $p < 0.05$, Mann-Whitney test). Moreover, the proportion of overlaps ($\theta=50$) correlated with the average paranodal length (figure 4.5 C, $r=0.827$, ** $p < 0.01$) and with the proportion of paranodes $>3 \mu m$ per rat ($r=0.765$, ** $p < 0.01$). These results suggest that paranodal lengthening and $K_v1.2$ dislocation

4.3. Results

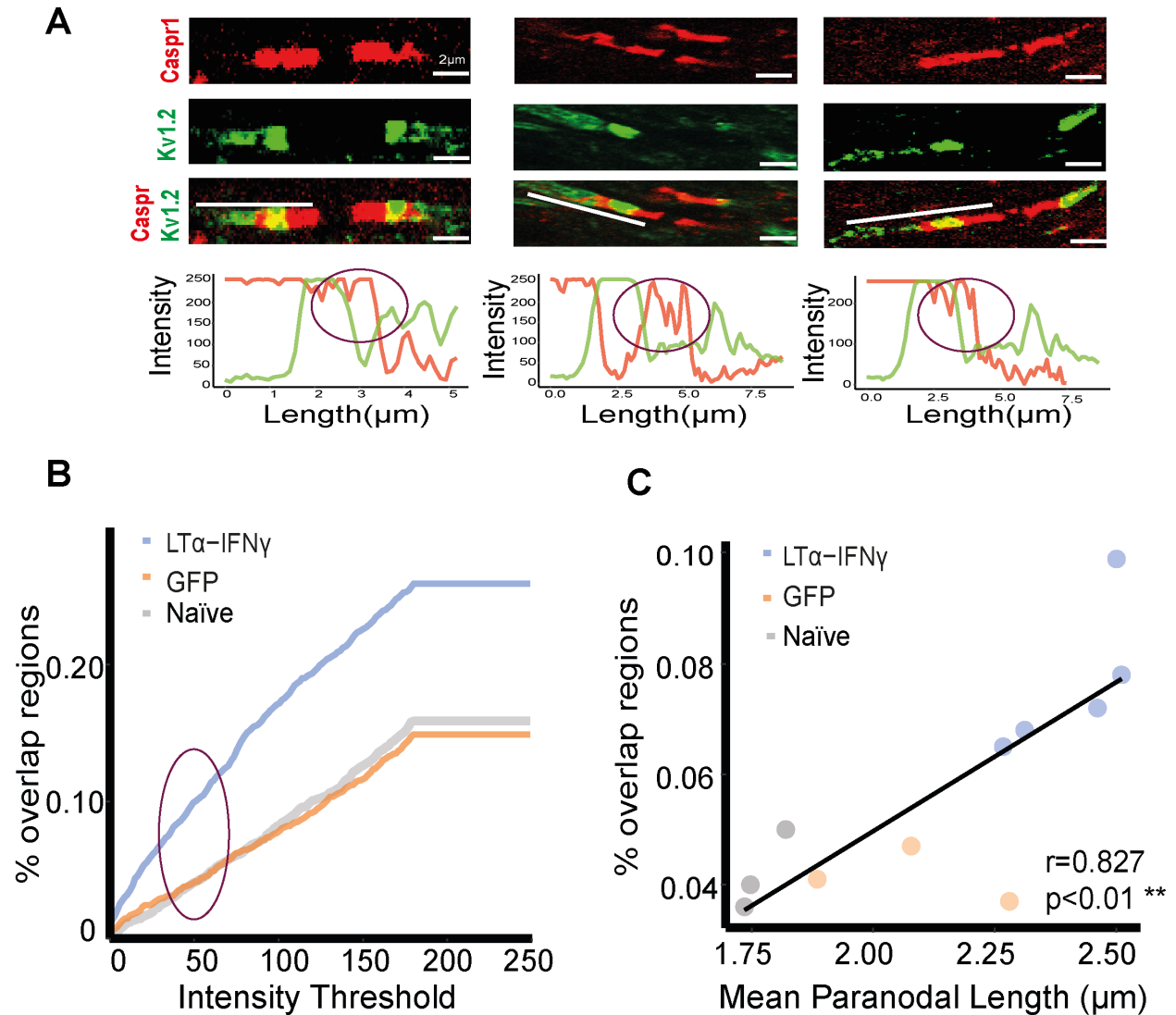


Figure 4.5: LT α /IFN γ rat tissue contained a larger proportion of regions where Caspr1 and Kv1.2 channels were overlapping compared to the GFP and naïve. (A) Confocal images in which Caspr1-stained paranodes (red) co-localised with Kv1.2-stained juxtaparanodes (green). In order to quantify the displacement of the channels, the RGB intensity profiles of each paranode/juxtaparanode were acquired. The purple circles denote regions where both signals were co-localising. (B) Graph showing that the proportion of overlapping regions between Caspr1 and Kv1.2 RGB signals was larger at every intensity threshold in the LT α /IFN γ (blue) group compared to the GFP (orange) and naïve groups (grey). (C) Mean paranodal length correlated significantly with the proportion of overlapping regions when the intensity threshold was set to 50 ($r=0.825$, $**p<0.01$, Spearman's rank correlation test).

of ten occurred simultaneously. Additionally, this data might imply that the integrity of the paranodal axo-glial junctions in the LT α /IFN γ rats was compromised in a greater

extent than in the GFP and naïve indicating that surrounding inflammation could have a role in this pathology (figure 4.5 A, B).

4.3.3 Microglia and astrocytes contribute to the axo-glial junction disruption

In this model the rats injected with lentiviral vectors for $LT\alpha$ / $IFN\gamma$ genes had an extensive recruitment of microglia and astrocytes throughout the brain. Thus, in order to examine if microglia and astrocyte could be potential mediators in the disruption of the paranodal axo-glial junctions locally, the number of microglia and astrocytes were measured in the surroundings of the paranodes and juxtaparanodes analysed (figure 4.6 A and figure 4.7 A). In fact, average paranodal length correlated to the number of microglia in the 10 ROIs of NAWM analysed (figure 4.6 C, $r=0.62$, $*p < 0.05$). It also correlated to the proportion of paranodes longer than $3\ \mu\text{m}$ per rat ($r=0.63$, $*p < 0.05$). Furthermore, in order to find the relationship between the proportion of overlapping regions between *Caspr1* and *Kv1.2*, and the number of microglia, the intensity threshold was set to 50 (figure 4.5 B). The number of microglia correlated to the percentage of overlapping regions at this intensity threshold (figure 4.6 D, $r=0.778$, $**p < 0.01$).

The average paranodal length per group also correlated to the number of astrocytes in the 10 ROIs of the NAWM regions analysed (figure 4.7 B, $r=0.69$, $*p < 0.05$) and with the proportion of paranodes longer than $3\ \mu\text{m}$ per rat ($r=0.75$, $**p < 0.01$). The proportion of overlapping regions between *Caspr1* and *Kv1.2* also correlated with the number of astrocytes when the intensity threshold was set to 50 (figure 4.7 C, $r=0.75$, $*p < 0.05$). Moreover, the number of microglia and the number of astrocytes correlated significantly in our tissue ($r=0.75$, $**p < 0.01$). This data supports our hypothesis that points to microglia and astrocytes as major mediators of the paranodal axo-glial junction disruption in myelinated axons.

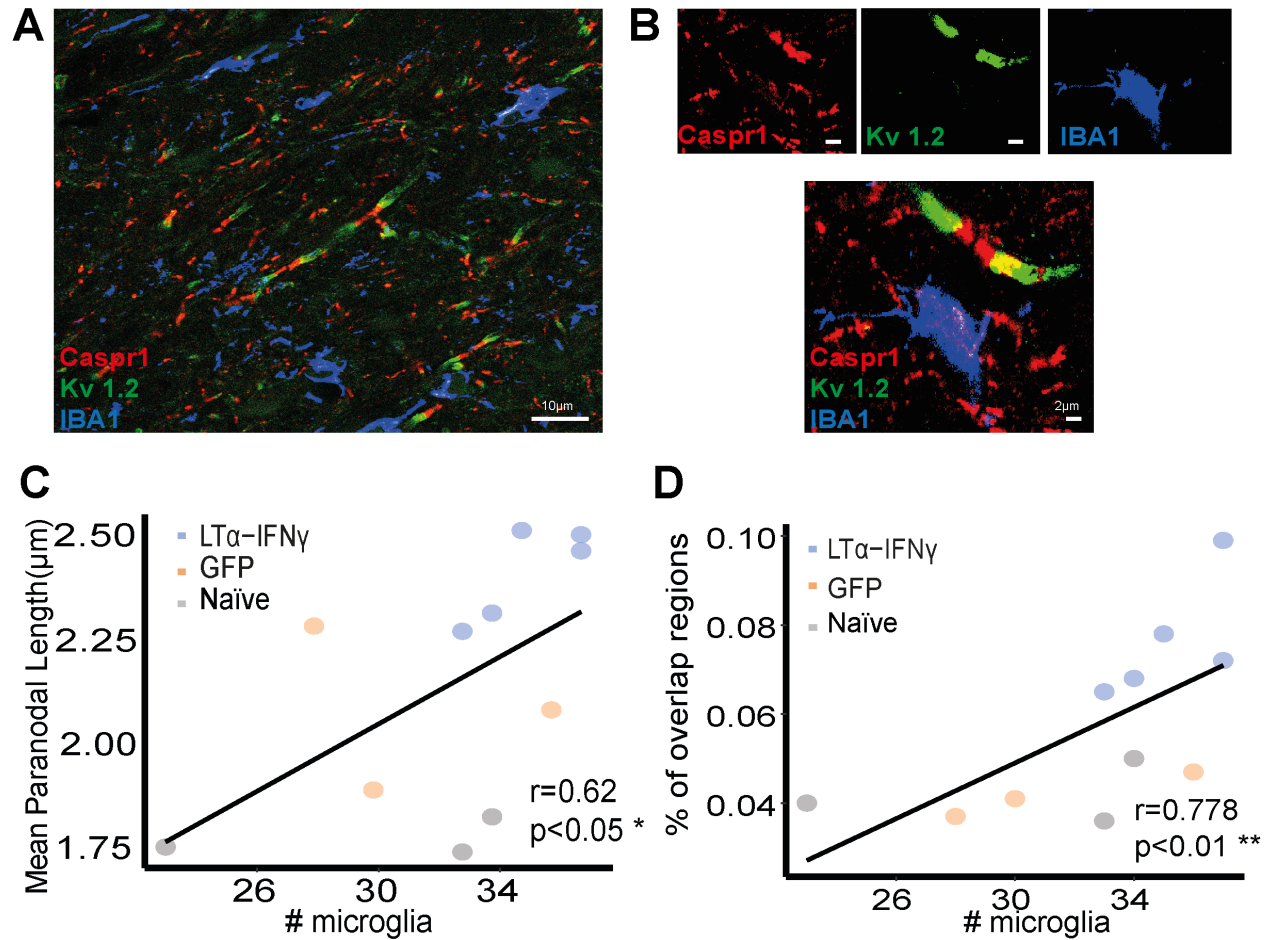


Figure 4.6: Paranodal elongation and the proportion of overlapping regions between the paranodal Caspr1 signal and the juxtaparanodal $K_v1.2$ signal were associated with a higher number of local microglia in $LT\alpha/IFN\gamma$, GFP and naïve rat tissue. (A) Confocal image of IBA1-stained microglia (blue), Caspr1-stained paranodes (red) and $K_v1.2$ -stained juxtaparanodes (green). (B) Confocal image of a single paranode disrupted in which voltage-gated $K_v1.2$ channels are co-localising with Caspr1-stained paranodes. (C) The number of microglia correlated to the mean paranode length per rat ($r=0.62$, $*p< 0.05$, Spearman's rank correlation test). (D) Moreover, the number of microglia correlated to the proportion of overlapping regions between Caspr1 and $K_v1.2$ when the intensity threshold was set to 50 ($r=0.778$, $**p< 0.01$, Spearman's rank correlation test).

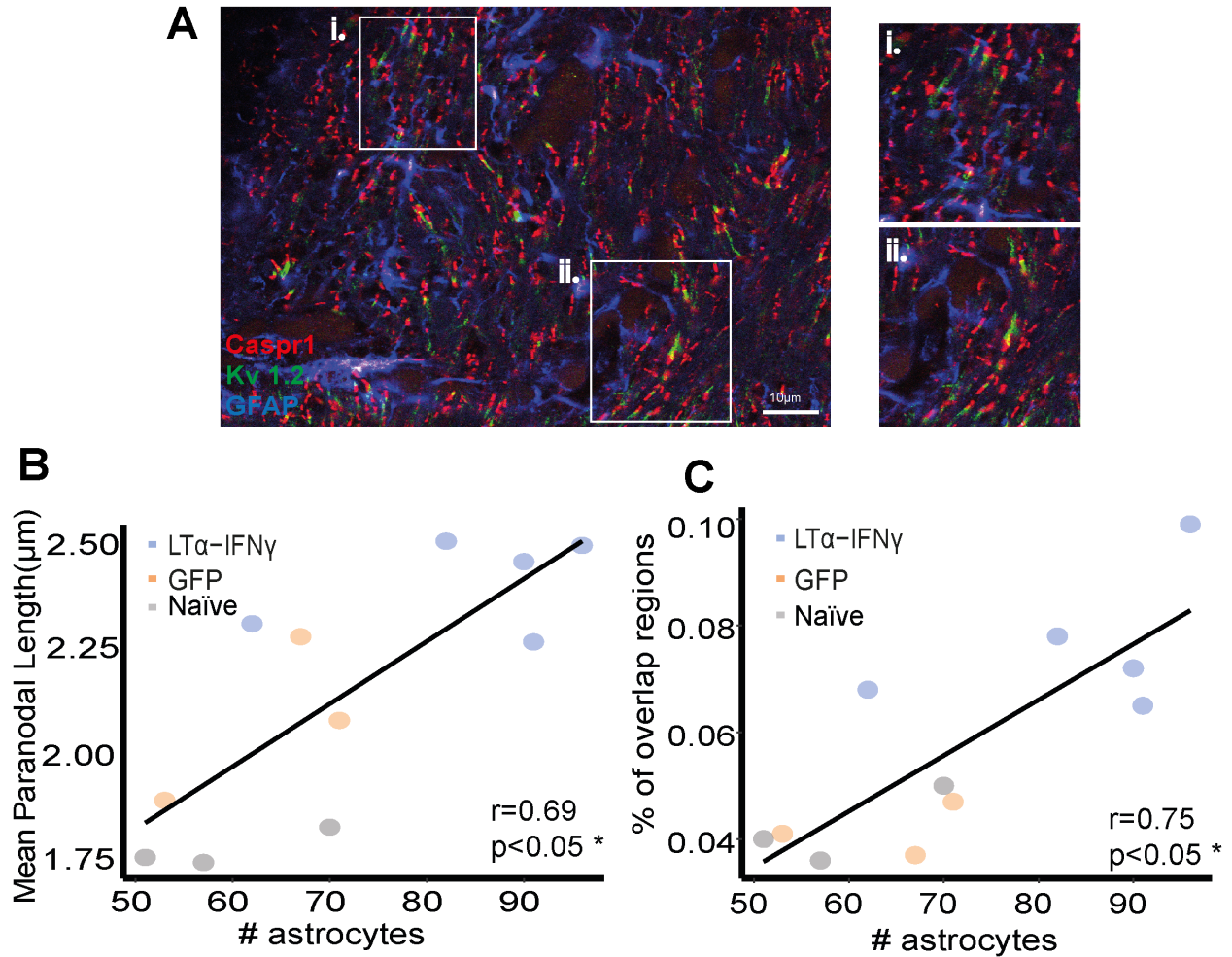


Figure 4.7: Paranodal elongation and the proportion of overlapping regions between the paranodal Caspr1 signal and the juxtaparanodal $K_v1.2$ signal were associated with a higher number of local astrocytes in $LT\alpha/IFN\gamma$, GFP and naïve rat tissue. (A) Confocal image of GFAP-stained astrocytes (blue), Caspr1-stained paranodes (Caspr1) and $K_v1.2$ -stained juxtaparanodes (green). (B) The number of astrocytes correlated to the mean paranode length per rat ($r=0.69$, $*p<0.05$, Spearman's rank correlation test). (C) The number of astrocytes correlate to the proportion of overlapping regions between Caspr1 and $K_v1.2$ when the intensity threshold is set to 50 ($r=0.75$, $*p<0.05$, Spearman's rank correlation test).

4.3.4 Presence of de-phosphorylated neurofilament (SMI32)

In order to assess axonal stress as discussed in the post-mortem MS NAWM tissue, double Caspr1-SMI32 staining was performed to observe the presence of de-phosphorylated neurofilament in association with the PNJ structure. However, the compacted axonal density, which characterises the corpus callosum, cingulate and external capsule made

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difficult to examine the association between the length of Caspr1-stained paranodes and the presence of SMI32+ axons quantitatively. Nonetheless, the presence of SMI32 was slightly greater in the $LT\alpha/IFN\gamma$ group compared to the GFP and naïve, from a qualitative point of view (figure 4.8 A, B, C).

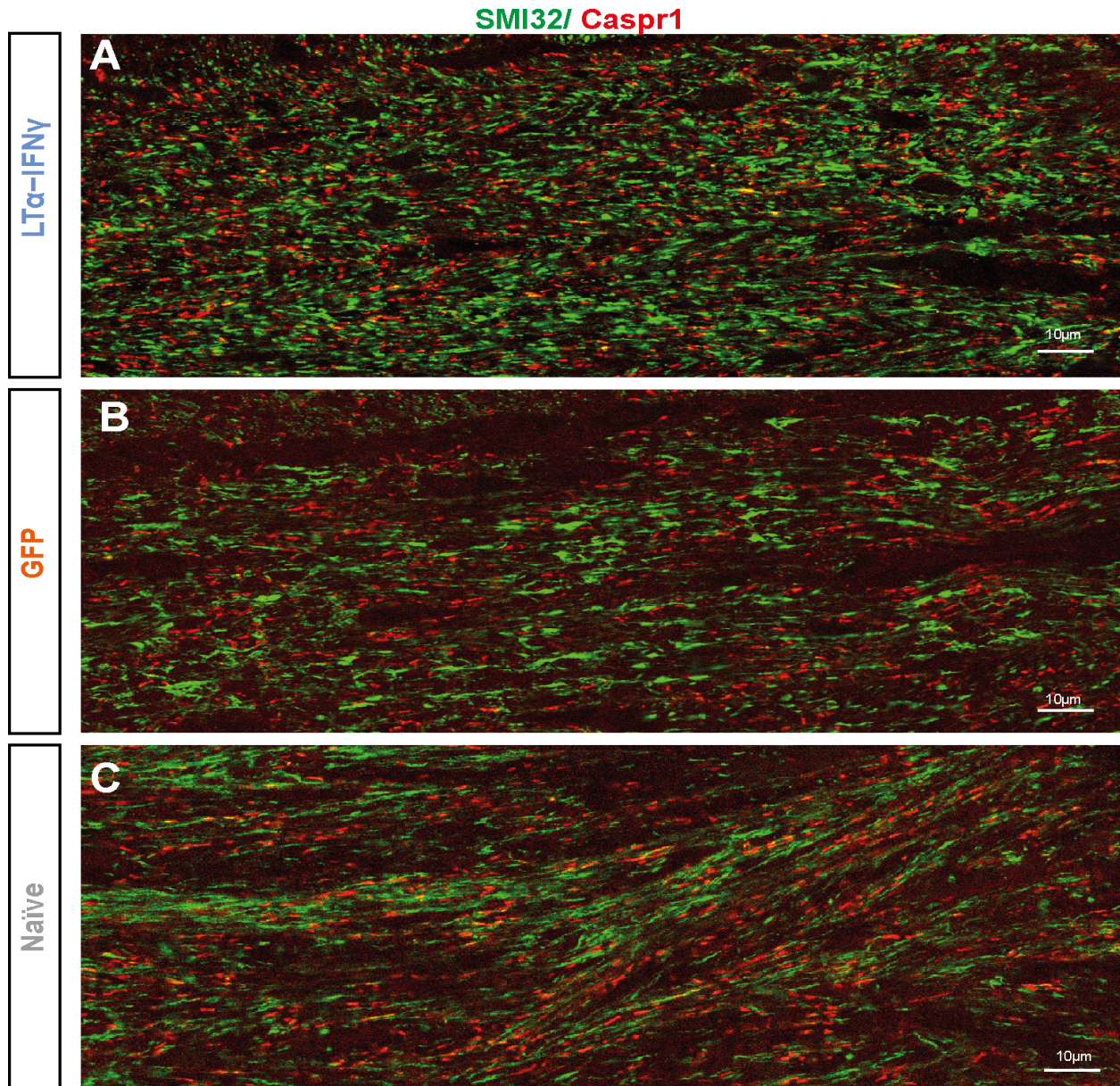


Figure 4.8: Caspr1-SMI32 immunofluorescence in $LT\alpha/IFN\gamma$, GFP and naïve rat tissue. (A) Confocal images of Caspr1-stained paranodes (red) and SMI32+ axons (green) in the $LT\alpha/IFN\gamma$ group, (B) GFP and (C) naïve.

4.3.5 Average profiles of Caspr1 and $K_v1.2$ RGB signals

In order to compare the average intensity distribution between the $LT\alpha/IFN\gamma$, GFP and naïve groups (figure 4.9 A,C,E), the overall mean and standard errors of all the RGB intensity profiles for Caspr1 and $K_v1.2$ signals along the paranodal and juxtaparanodal length were computed. Moreover, the intensity of each profile was scaled by the maximum length to observe the mean intensity distribution of Caspr1, $K_v1.2$ independently of length (figure 4.9 B,D). Assuming that the intensity of the signal is proportional to the density of the protein, the average Caspr1 intensity profile of the $LT\alpha/IFN\gamma$ group was higher at every length bin compared to the GFP and naïve groups (figure 4.9 A). The average $K_v1.2$ intensity profile was slightly lower than the GFP and naïve groups along the juxtaparanodal length (figure 4.9 C). When distributions were scaled, there was much more noise (figure 4.9 B,D). From this data, we can observe that the overall mean intensity of Caspr1 was larger in the $LT\alpha/IFN\gamma$ group compared to the GFP and naïve groups (figure 4.9 B), and the mean intensity of $K_v1.2$ between the three groups followed the same pattern (figure 4.9 D).

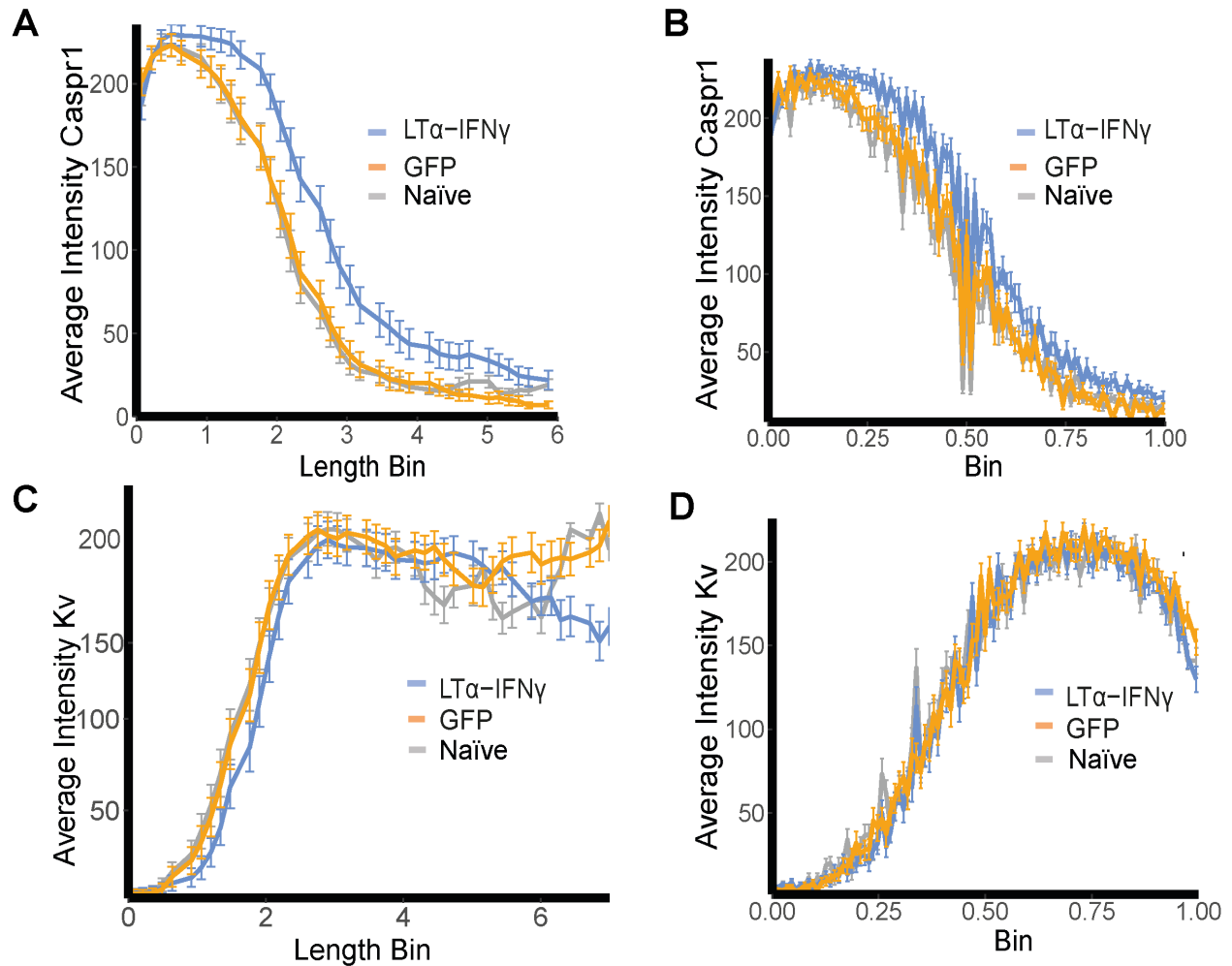


Figure 4.9: The average intensity profile of the Caspr1, $K_v1.2$ RGB signals suggested that in LT α /IFN γ tissue Caspr1 and $K_v1.2$ could have diffused along the axolemma. (A) Average intensity $\pm$ standard errors profile of all the Caspr1-stained paranodes along length. (B) Average intensity profile $\pm$ standard error of Caspr1-stained paranodes scaled by the maximum length per profile. (C) Average intensity $\pm$ standard error profile of all the $K_v1.2$ -stained juxtaparanode along length. (D) Average intensity $\pm$ standard error profile $\pm$ standard error of all the $K_v1.2$ -stained scaled by the maximum length per profile. The LT α /IFN γ tissue profiles were plotted in blue, the GFP in orange and the naïve in grey.

4.4 Discussion

4.4.1 Chronic meningeal inflammatory model

Several animal models have been developed over the years to replicate the molecular mechanisms underlying the pathological features of MS. Myelin mutants and chemically-induced lesions models to study myelination and remyelination, viral models to investigate aetiology and autoimmune EAE models for neuroinflammation research. However, it is the autoimmune chronic EAE the one that has been used widely and has shaped the research direction and therapeutic strategies for MS in the last decades (Baker et al., 2011). The chronic model of meningeal inflammation presented in this chapter should be classified as a neuroinflammatory model as it replicates the inflammation and cortical pathology of MS, although it cannot be classified as EAE. The advantages of using this model to examine the paranodal axo-glial junctions include the absence of white matter demyelination and chronic microglia/macrophages activation throughout the cerebrum even 90 days post-injection. Previous models that mimicked cortical pathology consisted of MOG-immunised animals, in which pro-inflammatory cytokines were injected directly into the subarachnoid space or cortex. These models showed that acute inflammation resolved post-injection rapidly and cortical demyelination occurred only in animals immunised with MOG (Merkler et al., 2006; Gardner et al., 2013; Üçal et al., 2017; Lagumersindez-Denis et al., 2017; Silva et al., 2018). Another neuroinflammatory model that lacks demyelination is the acute EAE, usually performed in Lewis rats immunised with MBP. In this model immune cell infiltration and activated microglia/macrophages are restricted to the brain stem and spinal cord, but inflammation resolves in the recovery phase after 17-21 days (Shin et al., 2012). Therefore, a strong and chronic microglia activation response throughout the brain without focal white matter demyelination is unique to our model (figure 4.3 F), and comprises the perfect platform to test if chronic activation of the CNS innate system would be sufficient to generate pathology at the nodes of Ranvier.

4.4.2 Axo-glia junction disruption in neuroinflammatory models

Previous studies with a MOG-induced EAE mouse model showed that paranodal elongation measured with Nf155 was present in the spinal cord of the animals, and could be reversed via the administration of the antibiotic minocycline, which reduces the activity of microglia and macrophages (Howell et al., 2010). This study has been the only one that described PNJ pathology in a neuroinflammatory model of MS. However, paranodal disruption has also been observed in experimental allergic neuritis, which is an animal model for Guillain-Barre syndrome (Lonigro and Devaux, 2008). In this case, Lewis rats were immunised against peripheral myelin (EAN-PM) and against a neuritogenic P2 peptide (EAN-P2), and they found dispersed juxtaparanodal voltage-gated *Kv1* channels at nodes and paranodes in the PNS. Axonal degeneration and loss, as one of the main predictors of neurological disability in MS has been modelled extensively in EAE models. In fact, a previous study in a MOG-induced EAE mouse model demonstrated focal axonal degeneration (FAD) in myelinated axons at spinal cord lesions. FAD was characterised by axonal swellings and transections closed to the nodes of Ranvier, which were as well, surrounded by activated microglia and macrophages (Nikić et al., 2011). Additionally, they found that FAD could be reversible by NO scavengers administration. A follow-up study pointed to calcium influx through plasma membrane nanoruptures as the main underlying cause (Witte et al., 2019). This data contributed to the hypothesis that axonal degeneration can be initiated at the nodes, as susceptible regions of injury, driven by local activated microglia/macrophages.

Although the chronic MOG-induced EAE model is highly reproducible and enables the use of transgenic strains (Mendel et al., 1995), the pathology is confined to the spinal cord with very low, if any, pathology in the brain (Lassmann and Bradl, 2017). Another issue is that axons differ greatly in caliber if we compare the brain to the spinal cord. For instance, electron microscopy studies of human and macaque brains at the superior longitudinal fascicle showed that the average axon diameter was of 1 μm (Liewald et al., 2014). Moreover, other studies have pointed to axons as thin as 0.1 μm in the cerebellar cortex (Sultan, 2000). Human spinal cords morphometric studies of the lateral corticospinal

tracts showed average diameters of 3 μm in controls of age between 40 and 59 (Zhou et al., 1997), and axons of more than 10 μm have also been described in the saphenous nerve of cats (Gasser and Grundfest, 1939). Further, previous neuropathological studies of MS tissue observed that small axons were more prone to injury (DeLuca et al., 2006). In fact, the study about FAD showed that axons smaller than 1.5 μm in diameter were more likely to be transected (Nikić et al., 2011). Thus, these data suggest that axons can undergo different degeneration routes depending on their size and surroundings, and focal axonal degeneration driven by inflammation might be then more relevant in the brain than in the spinal cord. Consequently, spinal cord axonal injury mechanisms elucidated in EAE models should not be always extrapolated to the thin axons of the brain. Furthermore, it is difficult to discard Wallerian (Dziedzic et al., 2010) or retrograde (Schaumburg et al., 1974) degeneration from distant lesions as the main cause of axo-glial disruption and eventual axon degeneration due to the presence of demyelinating lesions in the spinal cord of the EAE model.

4.4.3 PNJ disruption and voltage-gated K_v channels dislocation

The main objective of the current chapter was to examine the integrity of the paranodal axo-glial junctions in the chronic model of meningeal inflammation and compare the results to the inflamed MS NAWM tissue of Chapter 3. The longitudinal Caspr1-stained paranodes measured at the corpus callosum, cingulate and external capsule of the $\text{LT}\alpha/\text{IFN}\gamma$ rats were significantly longer than the ones from the GFP and naïve groups (figure 4.4 E), with 80.7 % of the paranodes in the $\text{LT}\alpha/\text{IFN}\gamma$ rats being longer than 2 μm , compared to the 22.5 % of the naïves (figure 4.4 F). Paranodal length distribution of the GFP was different from the naïve, which suggests that the stereotactic injection of the lentiviral vectors could have contributed partially to the damage (figure 4.4 E). Nonetheless, it was significantly different from the $\text{LT}\alpha/\text{IFN}\gamma$ injected rats (figure 4.4E and F). If we take the average paranodal length of the naïve (1.77 μm) as a representative value for paranodal length in a healthy animal, then 92.2 % of the paranodes in the $\text{LT}\alpha/\text{IFN}\gamma$ injected rats were longer. In the post-mortem human tissue, the paranodal length of the non-neurological control was 2.33 μm and 62.8 % of the paranodes in the MS NAWM

tissue were longer. Therefore, in terms of paranodal length chronic inflammation is sufficient for axonal injury.

The mean intensity profile of Caspr1 signal was larger compared to the GFP and naïve at every length, specifically at lengths longer than 1 μm (figure 4.9 A). Moreover, the same pattern was observed when the profile was scaled by distance (figure 4.9 B). Therefore, this data supports the idea that the PNJ disruption might be due to the diffusion of Caspr1 proteins towards the juxtaparanode, and not because of a difference in protein concentration as we have suggested in the post-mortem human tissue.

Dislocation of the voltage-gated juxtaparanodal $K_v1.2$ channels towards the paranode was also present in the model (figure 4.5 A). The $\text{LT}\alpha/\text{IFN}\gamma$ group had a larger proportion of overlapping regions compared to the GFP and naïve groups. The maximum proportion of overlapping regions in the $\text{LT}\alpha/\text{IFN}\gamma$ was 25.9 %, while in the naïve was 15.8 % and in the GFP 14.9 %. Therefore, there was an increment of 10 % of overlaps in the $\text{LT}\alpha/\text{IFN}\gamma$ group compared to the naïve and 11 % compared to the GFP (figure 4.5B). On the other hand, the maximum proportion of overlaps in the MS NAWM tissue was 68.9 %, which is an increment of 15.8 % compared to the non-neurological control (53.1 %) and 43 % compared to the $\text{LT}\alpha/\text{IFN}\gamma$. However, this difference in values could be due to the fact that paranodes of human axons are longer than those of the rat; the average paranodal length in the non-neurological control in the post-mortem human tissue was 2.33 μm , whereas in the naïve rats was 1.77 μm . Also, the inflammation in this model lasted 90 days, whereas, in the post-mortem MS NAWM tissue, the average duration of the disease in our cases was 28.3 years. Our data suggests that most of the axo-glial pathology, in terms of K_v1 displacement, is associated with a direct effect of focal and chronic inflammation, and occurs simultaneously with paranodal lengthening (figure 4.5 C).

Caspr1-stained paranodal elongation (figure 4.4) and voltage-gated $K_v1.2$ channel dislocation (figure 4.5) could translate structurally to an increment of peri-axonal space and diffusion of these proteins along the axolemma, as discussed in the previous chapter. This inferred structural arrangement was also seen in the chronic model of meningeal inflammation in this chapter. These diffuse structural changes at the nodes could result in both

physical and cognitive deficits in the animals since the peri-axonal space of the paranodes and juxtaparanodes will increment in width, decreasing significantly the resistance of these compartments and making the juxtaparanodal K_v1 channels more exposed to the ECM. In fact, a cat walk analysis of locomotion in rats injected with lentiviral vectors expressing TNF and IFN γ genes detected subtle changes in the gait of the rats (Dr. James, unpublished results). They had decreased body speed, regularity index, which is associated with coordination, and an increment in the stance phase which indicated that they stepped the ground for a shorter amount of time during each step cycle. However, in order to detect subtle cognitive deficits such as fatigue, extensive behaviour analysis should be performed. A recent study has demonstrated that fatigue-like behaviour induced in mice by abdominal irradiation can be inferred from measuring changes in physical activity passively and continuously without disrupting the animals over time with simple activities such as running wheels, video home cage monitoring, and telemetry (Wolff et al., 2018).

Mice with conditional knockouts of the axo-glial proteins, Caspr1 (Bhat et al., 2001), GPI-linked contactin (Boyle et al., 2001) and Nf155 (Pillai et al., 2009) had disrupted paranodal junctions in the PNS, and exhibited tremors, paresis, ataxia and reduced conduction velocity. Other animals with aberrant paranodal junctions such as the shiverer mice have tremor and convulsions (Kirschner and Ganser, 1980), mice deficient in myelin sulfatides develop neurological deficits after six weeks of life (Ishibashi et al., 2002), and mice lacking gangliosides showed motor disability on ageing (Wu et al., 2011). Moreover, mice with conditional null mutations in the genes for the paranodal cytoskeletal proteins, such as β II spectrin, had reduced grip strength and latency to fall times on rotarod and wirehang tests (Zhang et al., 2013). Mice lacking α II spectrin died before 1 month of age and exhibited seizures (Huang et al., 2017), and mice lacking 4.1.B showed neuronal hyperexcitability. Although the severity of these physiological symptoms cannot be compared to the chronic model of meningeal inflammation, they emphasize the importance of the function of the paranodal axo-glial junctions as physical and electrical barriers. Therefore, subtle structural changes could have a big impact in conduction and therefore in overall physiology.

4.4.4 Contribution of glia to paranodal pathology

TNF, which shares the TNFR1 receptor with $LT\alpha$, is known to drive processes such as apoptosis, necroptosis and inflammatory cytokine production. A previous study compared these two cytokines and obtained similar effects on inflammation and cell death mechanisms (Etemadi et al., 2013). Our data suggests that $LT\alpha$, like TNF, can cooperate with $IFN\gamma$ to induce the expression of many gene products during inflammation, such as STAT1 and NF- κ B (Ohmori et al., 1997), that then contribute to the gliosis observed in this chronic model of meningeal inflammation.

Paranodal lengthening and the proportion of overlaps at a $\theta=50$ correlated significantly with the number of surrounding microglia (figure 4.6 C, D). In fact, three clusters corresponding to each animal group were generated (blue, orange and grey), suggesting that the paranodal length might depend on the number of local microglia. Activated microglia can produce increasing amounts of neurotoxic molecules such as reactive oxygen and nitrogen species, glutamate and TNF. For instance, co-activation of TLR4 and $IFN\gamma$ receptors in microglia can result in nitric oxide production and neural dysfunction and death in the hippocampus (Papageorgiou et al., 2016). In our analysis throughout the corpus callosum, cingulate and external capsule, microglia were in the proximity of disrupted axo-glial junctions (figure 4.6 B), suggesting that they might be responsible for these effects. However, since in this model the persistent expression of pro-inflammatory cytokines in the subarachnoid space gives rise to a proportion of neuronal loss in the upper layers of the cortex, it remains possible that axonal injury precedes microglia recruitment and activation.

The number of astrocytes also correlated to the average paranodal length and to the proportion of overlapping regions at a $\theta=50$ (figure 4.7 B, C). Astrocytes are the most numerous cell type throughout the CNS and can perform a wide range of functions to maintain homeostasis such as axon guidance, synaptic support, control of the BBB and blood flow, regulate neuronal signaling and protect neurons from oxidative stress (Blackburn et al., 2009). However, recent studies have identified that activated microglia can induce reactive astrocyte phenotype (A1) by secreting $Il1\alpha$, TNF and C1q, and that it is the reactive

astrocyte that promotes neuronal and oligodendrocyte death, although the toxic species released by the astrocytes are unknown. Furthermore, A1 reactive astrocytes expressing C3 were identified in MS demyelinating lesions (Liddel et al., 2017).

Astrocytes and microglia are often in close proximity to one another. In our data the number of astrocytes and the number microglia correlated significantly, in fact, there were two astrocytes per microglia in every ROI. Therefore it is plausible that they can both modulate neuroprotective/neurotoxic mechanisms. For instance, glutamate excitotoxicity is one the most plausible mechanisms that could lead to PNJ pathology and both cell types could have a role in causing this. In-vitro studies with primary microglia showed glutamate release from hemichannels after TNF stimulation in an autocrine manner via TNFR1 (Takeuchi et al., 2006). A study with astrocyte culture showed that cytokines such as TNF and IFN γ can inhibit astrocytic glutamate uptake through a pathway that involves the liberation of nitric oxide, which activates microglia at the same time (Ye and Sontheimer, 1996). This way, glutamate excitotoxicity could then lead to the splitting of the paranodal loops and axonal injury could be initiated.

4.5 Conclusions

In conclusion, our results have demonstrated a significant elongation in the Caspr1-stained paranodes in the LT α /IFN γ rats compared to the GFP and naïve, indicating that chronic inflammation could be sufficient for PNJ disruption independently of demyelination. Further, paranodal elongation and voltage-gated K_v1 channels dislocation from the juxtaparanodes to the paranodes occurred simultaneously and correlated with the local number of microglia and astrocytes. Therefore, these results suggest that a chronic presence of a pro-inflammatory milieu in the CSF space can give rise to diffuse glial activation and pathology at the nodes of Ranvier.

5

A computational model of the effects of axo-glial pathology on AP conduction

5.1 Introduction

Most of the CNS axons larger than $0.2\ \mu\text{m}$ in diameter are myelinated. Myelin is a multilamellar membrane that concentrically surrounds the axon (Waxman et al., 1995; Sherman and Brophy, 2005) reducing the capacitance and increasing the resistance of the internodal axolemma. These biophysical properties enable APs to be regenerated at the nodes of Ranvier, as the only non-myelinated sites along axons. Moreover, this configuration establishes fast and reliable conduction over long distances without the requirement for large axonal diameters, which has been crucial throughout evolution. Therefore,

the main advantage of myelinated axons, in biophysical terms, is the rapid conduction (e.g., whereas the speed of myelinated axons is proportional to the diameter, for non-myelinated axons it is to the square root of the diameter (Rushton, 1951)).

Biophysical mechanisms underlying voltage membrane differences between the inside and outside of a cell necessary to generate an AP were unknown until the 1950s when Nobel prize winners Hodgkin and Huxley (HH) published their model. They combined mathematics and experimental data to build a biophysical framework to describe AP propagation through the kinetics of voltage-gated channels in the axon of a giant squid (Huxley and Stämpeli, 1949). The mathematical abstractions of their experimental observations led them to a set of differential equations that are widely known as the Hodgkin and Huxley model. Moreover, this discovery led to the exploration of new techniques such as patch clamping, developed by Nobel prize winners Neher and Sakmann, that allowed whole-cell and single-channel voltage-clamp studies of ion channels in mammalian tissue (Neher and Sakmann, 1976; Hamill et al., 1981).

HH equations can be applied to model AP regeneration across the axolemma. However, in order to model the current flow longitudinally, the cable equation is usually used. Furthermore, since different segments along the axon, such as the nodes, paranodes, juxtaparanodes and internodes have different biophysical and structural properties, compartmental models are utilised. These models consist of different compartments connected, in which each compartment can have different biophysical and structural characteristics. In order to integrate these equations, compartmental models are generated in software tools such as the widely used simulator NEURON (Hines and Carnevale, 2006).

5.2 MS AP conduction

Traditionally MS studies have focused on the changes in AP propagation of demyelinated axons. One of the first conduction deficits shown in demyelinated axons was conduction block (McDonald and Sears, 1969), which has been linked to devastating MS symptoms such as paralysis (Waxman, 1988) and blindness (Ulrich and Groebke-Lorenz, 1983). These types of studies were usually performed in experimental animal models such as

EAE in Lewis rats (Pender, 1988), or in demyelinating models generated by injecting toxins such as diphtheria (McDonald and Sears, 1969; Harrison et al., 1972a), detergent-like substance lysophosphatidyl choline (Smith et al., 1981) and the nucleic acid chelating agent ethidium bromide (Felts and Smith, 1992). Moreover, in these models, conduction block was observed mainly at body temperature, and above, and measurements were generally performed in the dorsal columns of the spinal cord, which contain large-diameter axons. However, it is important to note that these axons could have been degenerating by Wallerian type degeneration due to the toxins (Harrison et al., 1972a) and the effects measured on AP conduction might not have been linked entirely to demyelination. Nonetheless, conduction block of demyelinated axons is expected as the density of voltage-gated Na_v channels is low ($< 25/\mu m^2$, (Shrager, 1989)) underneath the myelin.

In biophysical terms, conduction block can be explained in two ways. Firstly, the local currents are no longer constrained to the node, they dissipate over the large area of the demyelinated internode, and the current needed to depolarise any membrane area is severely reduced. Secondly, the demyelination of the internode means that these regions have less resistance and more capacitance, and consequently more current is necessary to depolarise the same axonal membrane. However, in the experimental models mentioned above, they also observed that after a period of time, the demyelinated axons compensate for the damage and restore conduction by increasing the Na_v channel density and the contacts with glial cells, mainly astrocytes (Blakemore and Smith, 1983; Rosenbluth and Blakemore, 1984; Black et al., 1991). Other studies showed that the re-establishment of conduction preceded remyelination via foci of inward membrane current, which were considered precursors of sites for Na_v channel aggregation (nodes) (Bostock and Sears, 1978; Smith et al., 1982).

In focal demyelinated plaques, demyelination is not uniform; it includes segmental demyelination, paranodal demyelination, myelin thinning and remyelination (Harrison et al., 1972b,a). Each of these structural changes can contribute differently to conduction deficits. Regarding paranodal demyelination or disruption in the structural integrity, conditional knockouts of the paranodal proteins Caspr1 (Bhat et al., 2001), GPI-linked

contactin (Boyle et al., 2001) and Nf155 (Pillai et al., 2009) produced severe neurological defects such as hypomotility, tremor accentuated with movement, wide-based gait suggestive of a cerebellar defect, balance issues, motor paresis and spasms. Furthermore, electrophysiological measurements in the sciatic nerves of these animals showed a significantly reduced compound action potential conduction velocity and amplitude. However, the effects of human disease morphological changes, such as paranodal axo-glial junction disruption in the NAWM MS tissue, on AP conduction are rarely studied. In this chapter, using the histopathological information from the human post-mortem tissue, we will explore the AP conduction deficits generated by this pathology by building a biologically plausible model using NEURON software.

5.2.1 Aims

The following computational simulations aimed to build a novel biologically plausible axonal model of the CNS affected by neuroinflammation, using our observations from the human post-mortem tissue in Chapter 3 and from the in-vivo chronic meningeal inflammatory rat model in Chapter 4. Therefore, the specific aims of the study were:

- To simulate in-silico paranodal axo-glial disruption in a biologically plausible biophysical model using NEURON software and published morphometric data of CNS axons;
- To estimate changes in conduction velocity, amplitude and width of the AP propagation under different structural disruptions at the node of Ranvier;
- To predict changes in AP conduction after disrupting a patch of nodes.

5.3 Axon model

In our model, we simulated the functionality of an axon membrane made up of 4 types of compartments: nodes, paranodes (PNJ), juxtaparanodes and internodes. The nodes clustered a high density of fast Na_v channels ($Na_v1.6$), persistent Na_v channels and slow K_v channels (figure 5.1 A, purple). Immediately flanking the nodes, the paranodes were built

as compartments with no active conductances, and the sites where the myelin end loops connect with the axolemma (figure 5.1 A, dark grey). Next to the paranodes, the juxtaparanodes contained fast K_v channels (figure 5.1 A, medium grey). Finally, the internodes were sections surrounded by myelin with a low density of ion channels (figure 5.1 A, light grey). Seven fibre diameters were simulated: 0.5, 0.8, 1.1, 1.3, 1.6, 1.8, 3.5 [μm] (figure 5.1 D). Although the diameter of the nodes is usually smaller than the diameter of the internodal region, the same core diameter (d_{core}) was kept constant over the length of the axon since this constriction is narrower in small axons of the CNS than in large nerve fibres of the PNS (Rydmark, 1981; Berthold and Rydmark, 1983)(figure 5.1 D).

Most of the published computational models simplify axons as long cables with biophysical properties of high resistance and low capacitance in the compartments surrounded by myelin, and opposite properties in the nodal compartments (Goldman and Albus, 1968; Colbert and Pan, 2002; Moffitt et al., 2004; Gold et al., 2006a). However, the biophysical features that distinguish nodes, paranodes, juxtaparanodes and internodes are ignored. These differences should not be neglected as pathophysiological cascades could start at these sites (Simons et al., 2014), and therefore, they are essential to simulate pathological features from neurodegenerative diseases such as MS and Alzheimer's disease. Some of the first computational models that defined the different axolemma domains within the nodes of Ranvier were Richardson et al. (2000) and McIntyre et al. (2002). These models used a double cable core-conductor circuitry to represent the axonal membrane, the periaxonal space and the myelin sheath, separately. However, the average axon diameter of their simulations was much larger than 1 μm , which is the average axon diameter of the CNS (Liewald et al., 2014). Therefore, the biophysical characteristics of the small-calibre axons were ignored.

5.3.1 Methods

A double cable core model was built and solved numerically in NEURON with the backward Euler integration method (figure 5.1 E). Simulations across the different diameters were initiated and allowed to reach the resting potential for 1 ms before a current of 2 nA

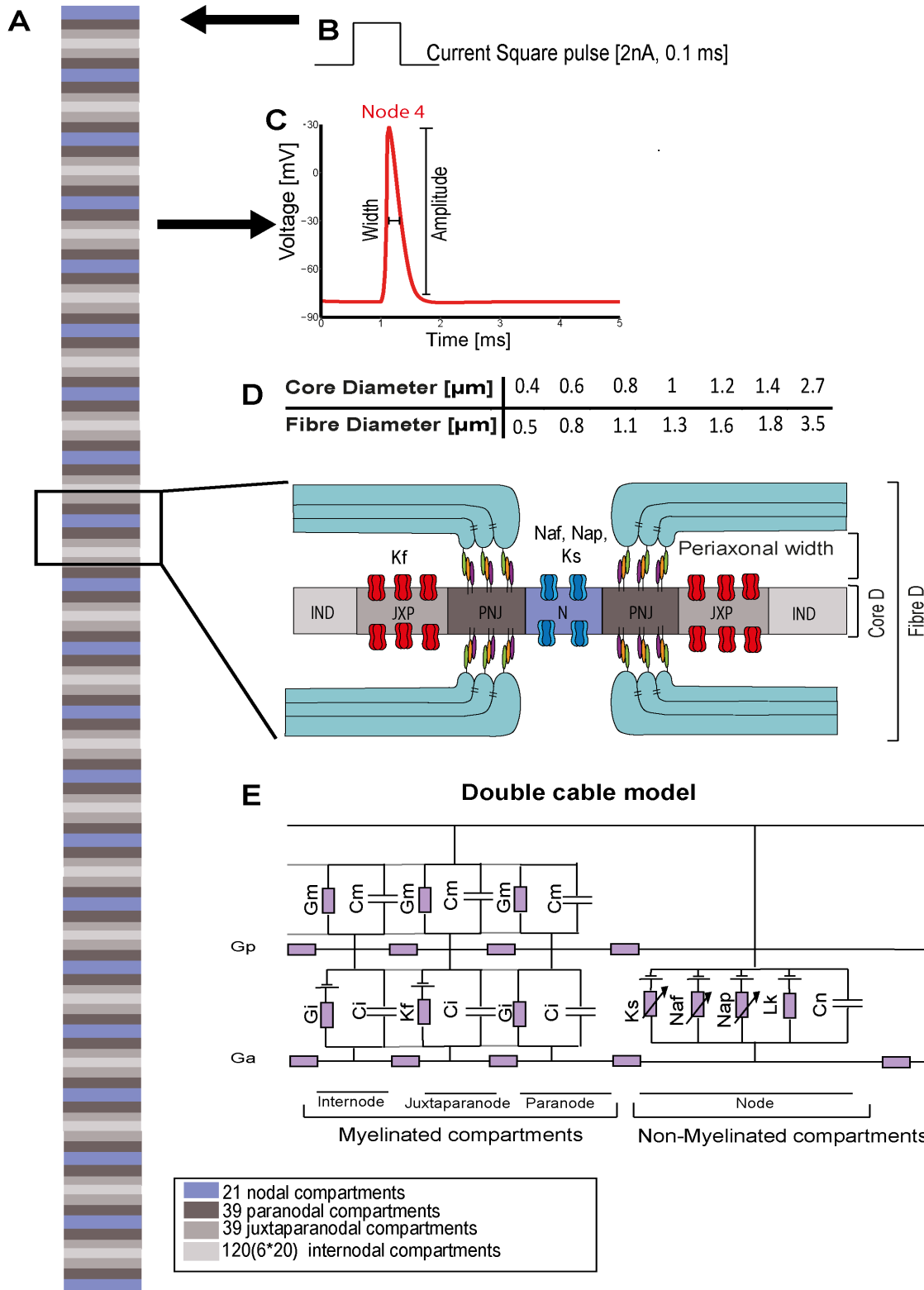


Figure 5.1: Axon model description. (A) 21 nodes, 39 paranodes, 39 juxtaparanodes and 20 internodes. (B) A current of 2 nA was injected at node 1 as a square pulse of 0.1 ms duration. (C) AP signal recorded in node 4; amplitude, width and velocity were measured. (D) Seven fibre diameters were simulated. (E) Double cable circuit of the model to represent the axolemma (Ga), the peri-axonal space (Gp) and the myelin sheath (Gm).

was injected in the mid-point of node 1 as a square pulse of 0.1 ms duration (figure 5.1 B). The propagation of the AP along the axon was measured up to 10 ms (2000 points plotted/ms). Measurements were recorded at node 4 and 16 of the 21 nodes created. AP amplitude, width and conduction velocity were measured. Amplitude [mV] was described as the voltage difference between the most negative voltage during hyperpolarisation afterpotential and $V_{\max}$. Width [ms] was defined as the time difference at half the amplitude, and conduction velocity [m/s] as the distance between $V_{\max}$ of the spikes at node 4 and 16.

5.3.2 Anatomical and Biophysical parameters

The anatomical parameters for the myelinated axon were acquired from our histological data (Chapter 3) and published anatomical data. The inner diameters of axons were chosen from measurements from an electron microscopy study of the superior longitudinal fascicle in the frontal plane of a macaque brain (Liewald et al., 2014). This study found a linear correlation between the myelin sheath thickness (mst [μm]) and the inner diameter of axons in axons greater than a d_{core} of $0.6 \mu\text{m}$. Furthermore, the number of myelin lamella (nl) was calculated from the myelin periodicity value of $0.0156 \mu\text{m}$ (Agrawal et al., 2009) measured with X-ray diffraction in mouse optic nerve (Table 5.1).

$$mst[\mu\text{m}] = 0.0156 * nl$$

$$d_{\text{fibre}}[\mu\text{m}] = d_{\text{core}} + 2 * (mst)$$

Core Diameter [μm]	0.4	0.6	0.8	1	1.2	1.4	2.7
Myelin sheath thickness [μm]	0.04	0.09	0.12	0.15	0.18	0.2	0.4
Number of myelin sheaths (nl)	3	6	8	10	12	13	26
Fibre Diameter [μm]	0.5	0.8	1.1	1.3	1.6	1.8	3.5

Table 5.1: Myelinated compartments anatomical properties.

The relationship between the axon core diameter and the fibre diameter is defined as the g-ratio ($d_{\text{core}} / d_{\text{fibre}}$). The optimal g-ratio was initially predicted theoretically for PNS axons to be around 0.6 (Rushton, 1951). However, experimental data and recent theoretical

predictions of myelinated CNS axons indicated a g-ratio between 0.77 and 0.8 (von Keyserlingk Graf and Schramm, 1984; Chomiak and Hu, 2009; Mohammadi et al., 2015). In fact, the g-ratios of our simulations lie in this range (Table 5.2).

Core Diameter [μm]	0.4	0.6	0.8	1	1.2	1.4	2.7
Nodal Diameter	0.4	0.6	0.8	1	1.2	1.4	2.7
Paranodal Diameter	0.4	0.6	0.8	1	1.2	1.4	2.7
Juxtaparanodal Diameter	0.4	0.6	0.8	1	1.2	1.4	2.7
Internodal Diameter	0.4	0.6	0.8	1	1.2	1.4	2.7
g-ratio	0.8	0.75	0.72	0.77	0.75	0.78	0.77
Fibre Diameter [μm]	0.5	0.8	1.1	1.3	1.6	1.8	3.5

Table 5.2: Core and Fibre Diameters of the axonal compartments.

Regarding the lengths of each of the nodal domains, the node-to-node length was taken from the linear relationship measured from rat nerve fibres ($117.52 + 30.47 * d_{fibre}$) (Ibrahim et al., 1995). The juxtaparanodal length was extrapolated from the diameter dependent scaling relationship from the ventral root of cats ($19.6 + 2.58 * d_{fibre}$) (Berthold and Rydmark, 1983; McIntyre et al., 2002). The paranodal length was determined from the average value of Caspr1 staining measured from our non-neurological control cases in Chapter 3. The internodal length was derived from the subtraction of two paranodes and juxtaparanodes lengths from the node-to-node length across the seven diameters. Although nodal length can change within the same axon even more than across axons and might also depend on nodal sodium channel density (Arancibia-Cárcamo et al., 2017), in this model, the nodal length ($\approx 1\mu\text{m}$) was kept constant across diameters for simplicity reasons (Table 5.3).

Core Diameter [μm]	0.4	0.6	0.8	1	1.2	1.4	2.7
Node to node Length	132.76	141.9	151.04	157.13	166.27	172.37	224.17
Internodal Length	86.38	93.97	101.56	106.62	114.22	119.28	162.31
Paranodal Length	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Juxtaparanodal Length	20.89	21.66	22.44	22.95	23.73	24.24	28.63
Nodal Length	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Fibre Diameter [μm]	0.5	0.8	1.1	1.3	1.6	1.8	3.5

Table 5.3: Compartment Lengths.

The peri-axonal space dimensions of the paranode, juxtaparanode and internode were

5.3. Axon model

based on the data measured by following dextran tracers in myelinated fibres of mouse sciatic nerves (Mierzwa et al., 2010). They found that the tracers can flow following three different pathways underneath the paranode (figure 5.2). For pathway 1, the dextran moved axially, for pathway 2 obliquely and for pathway 3 between the myelin end loops resulting in the formation of a small extracellular space with a triangular x-sectional area of around 170 nm^2 . The width of the space was 2-4 nm and adjacent myelin loops. Furthermore, the peri-axonal space of the juxtaparanode and internode was estimated to be 10-20 nm. In our model, the peri-axonal x-sectional area of the paranode, which determines the axial resistance of this compartment, was set to be the area of the axial pathway plus the area between the myelin loops ($170[\text{nm}] * (nl - 1)$) (Arancibia-Cárcamo et al., 2017). The peri-axonal space widths of the paranodes (axially) were set to $0.002 \mu\text{m}$ while in the juxtaparanodes and internodes was $0.02 \mu\text{m}$ (Table 5.4).

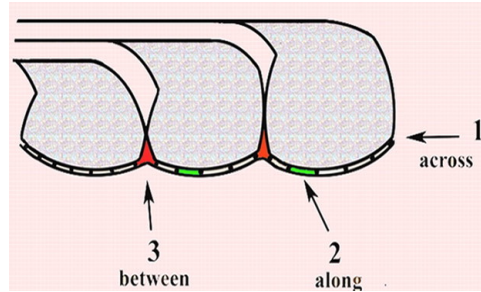


Figure 5.2: Paranodal peri-axonal space. The image has been modified from Mierzwa et al. (2010).

Core Diameter [μm]	0.4	0.6	0.8	1	1.2	1.4	2.7
IND peri-axonal space width [μm]	0.02	0.02	0.02	0.02	0.02	0.02	0.02
JXP peri-axonal space width [μm]	0.02	0.02	0.02	0.02	0.02	0.02	0.02
PNJ peri-axonal space width [μm]	0.002	0.002	0.002	0.002	0.002	0.002	0.002
Fibre Diameter [μm]	0.5	0.8	1.1	1.3	1.6	1.8	3.5

Table 5.4: Peri-axonal space widths.

Axon capacitance ($1 \mu\text{F}/\text{cm}^2$) was based on data from rat ventral roots (Bostock and Sears, 1978), as this value had been widely used in previous axon simulations (Richardson et al., 2000; McIntyre et al., 2002; Gow and Devaux, 2008). Myelin capacitance and leak conductance per lamella were based on the frog sciatic nerves (Tasaki, 1955). Published studies used myelin resistivity between $10\text{-}1000 \text{ Ohm} * \text{cm}^2$ (Tasaki, 1955; Barrett and Barrett, 1982;

Halter and Clark, 1991; McIntyre et al., 2002; Gow and Devaux, 2008; Boucher et al., 2012). In this model the resistivity was set to $1000 \text{ Ohm} * \text{cm}^2$ for each myelin lamella (Table 5.5).

Biophysical Parameters	Values	Units
Nodal Capacitance	1	$[\mu\text{F} / \text{cm}^2]$
Internodal Capacitance	1	$[\mu\text{F} / \text{cm}^2]$
Myelin Capacitance	0.1	$[\mu\text{F} / \text{cm}^2]$
Axoplasmic resistivity	70	$[\text{Ohm} * \text{cm}]$
Periaxonal resistivity	70	$[\text{Ohm} * \text{cm}]$
Paranodal conductance (g_{pas})	0.0005	$[\text{S} / \text{cm}^2]$
Juxtaparanodal conductance (g_{pas})	0.005	$[\text{S} / \text{cm}^2]$
Internodal conductance (g_{pas})	0.005	$[\text{S} / \text{cm}^2]$
Myelin resistivity	1000	$[\text{Ohm} * \text{cm}^2]$
Myelin capacitance	0.1	$[\mu\text{F} / \text{cm}^2]$

Table 5.5: Biophysical parameters from the axolemma, peri-axonal and myelinated compartments.

The axon membrane possesses a highly ordered molecular structure across the node, paranode, juxtaparanode and internode, which includes a non-uniform distribution of ion channels (Waxman and Ritchie, 1993). In this model four types of voltage-gated channels were included: Fast Na_v channels, Persistent Na_v channels, Slow Persistent K_v channels, Fast K_v channels and Leakage channels. The following voltage-gated channel kinetics were implemented in NEURON using .MOD files.

Fast Na_v channels

Fast Na_v channel nodal density was quantified in a rabbit axon to be between 1000-2000 channels/ μm^2 (Howe and Ritchie, 1990). However, gating current measurements suggested a maximum nodal density of 1500 channels / μm^2 (Chiu and Ritchie, 1981). Other studies estimated that the maximum nodal Na_v channels density was 1000 channels / μm^2 (Shrager, 1989; Waxman and Ritchie, 1993), which is the value used in this model. Human nerve electrophysiological studies measured a single conductance of a fast Na_v channel of 15 pS (Scholz et al., 1993). Taking this value, the maximal conductance in our model was set to $1.5 \text{ S} / \text{cm}^2$. Fast Na_v channel (mainly $\text{Na}_v1.6$) gating was measured experimentally from a human nerve by Schwarz et al. (1995) at 20°C . The following gating dynamics

5.3. Axon model

were based on these experimental data, and the temperature change was adjusted with a $q_{10}=2.2$ (Richardson et al., 2000; McIntyre et al., 2002; Coggan et al., 2011).

$$I_{Na} = g_{Na} * m^3 * h * (V_m - E_{Na})$$

$$\alpha_m = 6.57(V_m + 20.4) / (1 - \exp(-(V_m + 20.4)/10.3))$$

$$\beta_m = 0.304(-(V_m + 25.7) / (1 - \exp((V_m + 25)/9.16)))$$

$$\alpha_h = 0.34(-(V_m + 114)) / (1 - \exp((V_m + 114)/11))$$

$$\beta_h = 12.6 / (1 + \exp(-(V_m + 31.8)/13.4))$$

Persistent Na_v channels

Persistent Na_v channel conductance was based on the data from rat ulnar nerves (Bostock and Rothwell, 1997). The estimated density was much smaller than the fast sodium channels, 6.5 channels/ μm^2 , with a conductance of a single channel of 20 pS. Thus, the maximum conductance in this model was set to 0.01 S/cm². This value was taken from previous myelinated axon computational studies (Richardson et al., 2000; McIntyre et al., 2002). Since there was little experimental data for these channels (Honmou et al., 1994; Bostock and Rothwell, 1997; Smith et al., 1998; Caldwell et al., 2000), the membrane gating dynamics used were the same as previous computational models (Richardson et al., 2000; McIntyre et al., 2002; Volman and Ng, 2013).

$$I_{Nap} = g_{Nap} * p^3 * (V_m - E_{Na})$$

$$\alpha_p = 0.0353(V_m + 27) / [1 - \exp(-(V_m + 27)/10.2)]$$

$$\beta_p = 0.000883(-(V_m + 34)) / [1 - \exp((V_m + 34)/10)]$$

Slow Persistent K_v channels

Slow K_v channels have an estimated density of $110/\mu\text{m}^2$ (Safronov et al., 1993). From human nerve electrophysiological studies, the single conductance of a channel was quantified to be between 7-10 pS (Scholz et al., 1993; Safronov et al., 1993; Reid et al., 1999). In this model, the single conductance of a slow K_v channel was set to 8 pS. Thus, with a density of 110 channels $/\mu\text{m}^2$ and the maximum conductance was $0.088 \text{ S}/\text{cm}^2$.

$$I_{Ks} = g_{Ks} * s * (V_m - E_k)$$

$$\alpha_s = 0.3 / (1 + \exp((V_m + 53)/5))$$

$$\beta_s = 0.03 / (1 + \exp((V_m + 90)/-1))$$

Fast K_v channels

The density of fast K_v channels was estimated to be $12 \text{ channels}/\mu\text{m}^2$ (Safronov et al., 1993) while the single conductance of a channel measured in the rat peripheral nerve was 17 pS (Röper and Schwarz, 1989; Safronov et al., 1993). Thus, the maximum conductance in our model for the fast K_v channels was set to $0.02 \text{ S}/\text{cm}^2$. These values have been used in previous computational studies (Safronov et al., 1993; McIntyre et al., 2002).

$$I_{kf} = g_{kf} * n^4 * (V_m - E_k)$$

$$\alpha_n = 0.00798(V_m + 93.2) / (1 - \exp(-(V_m + 93.2)/1.1))$$

$$\beta_n = 0.092(-(V_m + 76)) / (1 - \exp(-(V_m + 76)/10.5))$$

Leakage channels

Leakage conductances are like static pores, they do not depend on time and/or voltage:

$$I_l = g_l * (V_m - E_l)$$

The leakage conductance of the node was set to $g_l=0.007 \text{ S/cm}^2$ (Bostock and Rothwell, 1997) while the leakage conductance of the paranode was $g_l=0.0005 \text{ S/cm}^2$, the juxtaparanode and internode conductances were $g_l = 0.005 \text{ S/cm}^2$ (Chiu and Schwarz, 1987).

The leakage equilibrium potential was set to -80 mV , and the simulations were run at a temperature of 37°C .

5.4 Model Validation

5.4.1 Conduction velocity

Boyd and Kalu (1979) experimentally derived the velocity for small diameter axons as $V = 4.6 * d_{\text{fibre}}$ from cat hind limb nerves (dotted gray line). The velocity in the model showed a linear relationship with the fibre diameter ($V[m/s] = 4.52 * d_{\text{fibre}} [\mu\text{m}]$) (blue line), which reproduced faithfully previous experimental results (figure 5.3 A, B).

5.4.2 Amplitudes and Widths

AP amplitudes in this model were slightly higher in larger fibre diameters (106.9, 108.2, 109.1, 109.6, 109.8, 110.1, 111.01) (figure 5.3 C). These values resembled the amplitudes reported by Schwarz et al. (1995) for a human myelinated axon at room temperature or for a rat axon, both at 25°C and 37°C by Schwarz and Eikhof (1987).

In this model, the widths of the AP across diameters agreed with the AP widths measured at the nodes of Ranvier of rats at 37°C by Schwarz and Eikhof (1987) at approximately 0.2 ms . This value is lower than the width measured in human myelinated axon by Schwarz et al. (1995) or in lizard motor axons by David et al. (1997) at 25°C . This variability is due to the change in ionic channel kinetics with temperature (Schwarz and Eikhof, 1987) (figure 5.3 D).

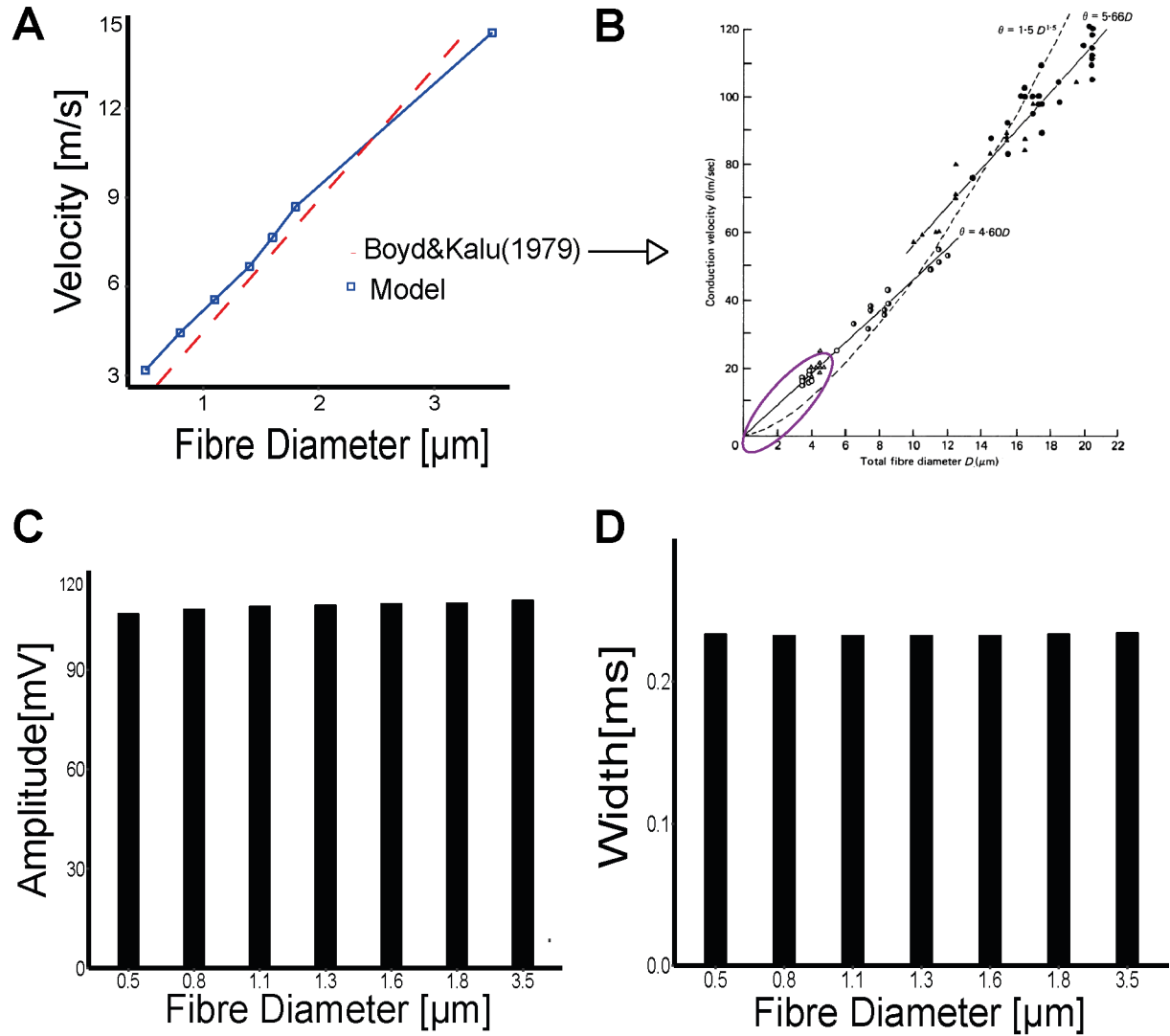


Figure 5.3: Model validation. (A) Conduction velocity of the model across the seven fibre diameters simulated. (B) Conduction velocity data measured in cat hind limb nerves (Boyd and Kalu, 1979). (C) AP amplitudes [mV] of the seven fibre diameters simulated. (D) AP widths [ms] of the seven fibre diameters simulated.

5.5 Simulations

In order to simulate paranodal axo-glial junction disruption, the resistance of the paranodal and/or juxtaparanodal compartments was decreased, and the juxtaparanodal K_v channels were dislocated. The resistance was decreased by increasing the peri-axonal space of both compartments progressively. Furthermore, juxtaparanodal K_v channels were dislocated to the paranode, and their conductance was increased proportionally to the increment in peri-axonal space of the paranode. The velocity (V/V_0), amplitude and width were quantified in each of the conditions. In summary, the following structural arrangements were simulated:

1. Proportional increment of the paranodal peri-axonal space.
2. Proportional increment of the paranodal and juxtaparanodal peri-axonal space.
3. Proportional increment of the juxtaparanodal fast K_v channels conductance and the paranodal peri-axonal space.
4. Proportional increment of the juxtaparanodal fast K_v channels conductance, and the paranodal and juxtaparanodal peri-axonal space.

5.5.1 Proportional increment of the paranodal peri-axonal space

The conduction velocity decreased as the paranodal peri-axonal space increased in all the axon diameters (50%, 100%, 200%, 500%, 1000%, figure 5.4 A, B). If the data was fitted to a logarithmic curve ($y = a * \ln(x) + b$), the velocity of the axons decreased faster in the smaller-diameter axons as the paranodal peri-axonal space was increased than in the larger-diameter axons. For instance, in the 0.4 μm diameter axon, the total velocity decrease was 52.6 % ($y = -0.126 * \ln(x) + 1.3245, r^2 = 0.99$), whereas in the 2.7 μm axon was 37.7 % ($y = -0.09 * \ln(x) + 1.2373, r^2 = 0.99$) (figure 5.4 A). The amplitude of the AP also decreased at every axon diameter as the paranodal peri-axonal space increased (figure 5.4 C). This data could also be fitted to a logarithmic curve, and it showed that the amplitude of larger-diameter axons decreased more than thinner ones. For example, the

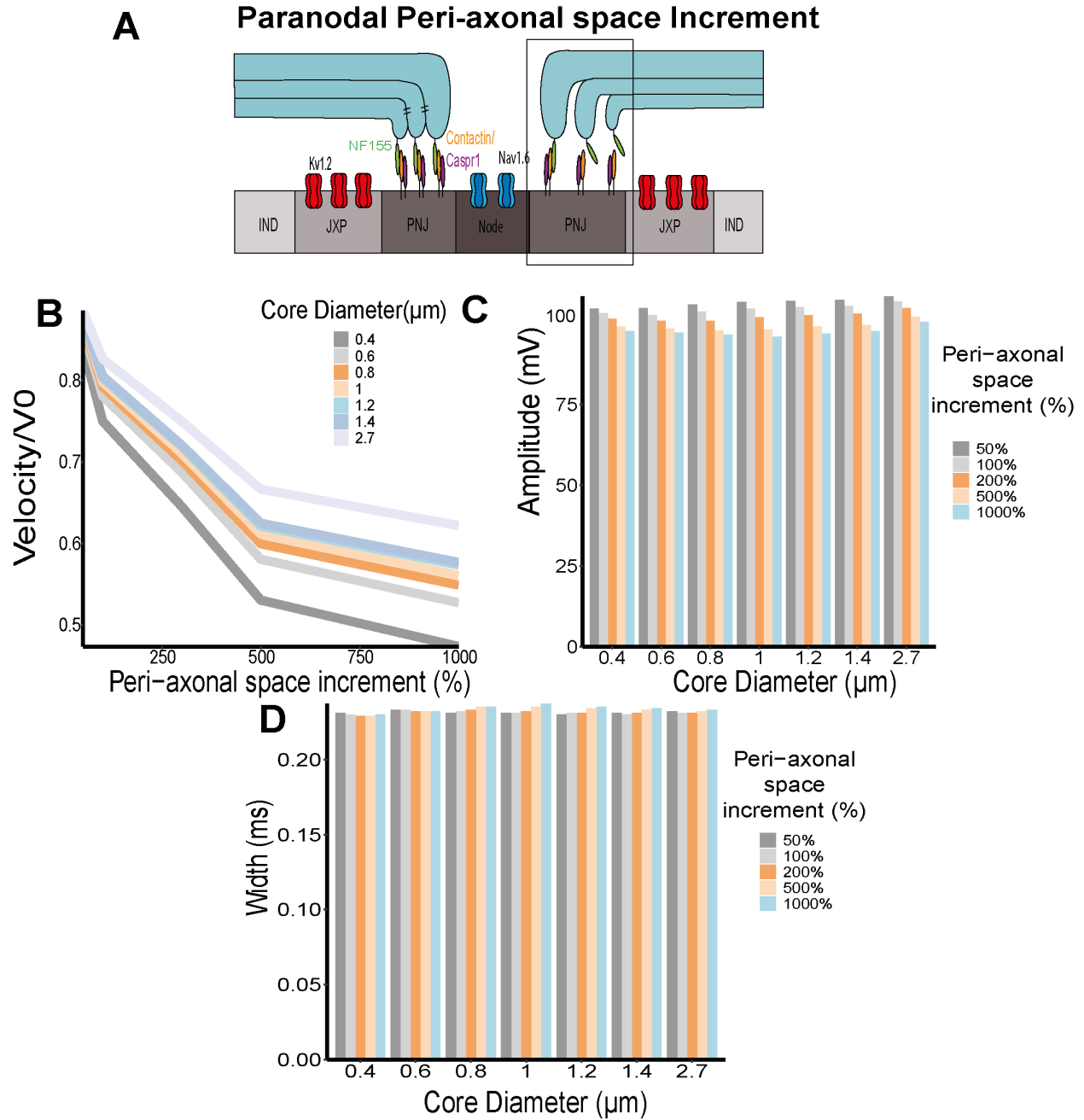


Figure 5.4: Paranodal Peri-axonal space increment induces a reduction of AP velocity and amplitude. (A) Diagram representing the increment of the paranodal peri-axonal space in the node of Ranvier. (B) Normalised velocity plot at every core diameter as the paranodal peri-axonal space increases. (C) Bar plot of the amplitude (mV) of the AP at every core diameter as the paranodal peri-axonal space increases. (D) Bar plot of the width (ms) of the AP at every core diameter as the paranodal peri-axonal space increases.

amplitude decay in $0.4 \mu\text{m}$ axon was 8.5% ($y = -2.414 * \ln(x) + 114.36, r^2 = 0.99$),

whereas in the 2.7 μm axon was 9.4% ($y = -2.755 * \ln(x) + 119.49, r^2 = 0.99$). However, the width of the AP did not change significantly as the paranodal peri-axonal space of the axon was increased (figure 5.4 D).

5.5.2 Proportional increment of the paranodal peri-axonal space and K_v1 dislocation

In this condition, the paranodal peri-axonal space was increased progressively, and the juxtaparanodal fast K_v channels were dislocated to the paranodal compartment. Further, their conductance was increased proportionally to the peri-axonal space increment to simulate the higher exposure of these channels to the extracellular space (figure 5.5 A). Under this disruption, the conduction velocity decreased faster in the small-diameter axons, as we saw in the previous section (figure 5.5 B). In fact, if the data was fitted to a logarithmic curve, the total velocity decay of the 0.4 μm was 59.1 % ($y = -0.147 * \ln(x) + 1.4167, r^2 = 0.99$), while in the 2.7 μm , the decay was 40.9 % ($y = -0.102 * \ln(x) + 1.289, r^2 = 0.99$). The amplitude also decreased similarly to the last condition (figure 5.5 C). The amplitude decay of the 0.4 μm axon was 10.1 % ($-2.695 * \ln(x) + 114.92, r^2 = 0.99$), and in the 2.7 μm axon was 10.9 % ($y = -3.001\ln(x) + 119.73, r^2 = 0.99$). However, the width of the AP decreased significantly compared to the last condition as the K_v1 channel conductance was increased (figure 5.5 D). In fact, if we fit the width data to a logarithmic curve in each axon diameter, all the axons had similar width decrements. For instance, in the 0.4 μm , the total width decrease was of 20.6 % ($y = -0.012 * \ln(x) + 0.2691, r^2 = 0.94$), whereas in the 2.7 μm axon was 19.2 % ($y = -0.011 * \ln(x) + 0.263, r^2 = 0.95$).

5.5.3 Proportional increment of the paranodal and juxtaparanodal peri-axonal space

The third condition that was simulated consisted of a progressive increment of the paranodal and juxtaparanodal peri-axonal spaces (figure 5.6 A). In this case, the axon with a core diameter of 0.4 μm failed to conduct when the paranodal and juxtaparanodal peri-axonal spaces were increased by 500% (the paranodal peri-axonal space was 0.012 μm and

the juxtaparanodal $0.12 \mu\text{m}$, figure 5.6 B). Meanwhile, the axons with d_{core} of $0.6 \mu\text{m}$ and $0.8 \mu\text{m}$ also failed to conduct when the paranodal and juxtaparanodal peri-axonal spaces were increased by 1000% (the paranodal peri-axonal space was $0.022 \mu\text{m}$ and the juxtaparanodal $0.22 \mu\text{m}$, figure 5.6 B). Larger-diameter axons had a significant velocity decay. For instance, the velocity of the axon with a core diameter of $2.7 \mu\text{m}$ decreased 78.47 % ($y = -0.209 * \ln(x) + 1.6457, r^2 = 0.99$) (figure 5.6 B). AP conduction failure occurred in the smaller-diameter axons, which suggests that they might be more susceptible to paranodal and juxtaparanodal disruption than larger-diameter axons. The amplitude of the AP also decreased significantly as the paranodal and juxtaparanodal peri-axonal spaces were larger. When the amplitude data was fitted to a logarithmic curve, the larger the axon-diameter, the higher the amplitude decrease. For instance, the amplitude decreased in $1 \mu\text{m}$ was of 29.6 % ($y = -8.892 * \ln(x) + 140.28, r^2 = 0.96$), while in the $2.7 \mu\text{m}$ axon was 33.02 % ($y = -10.71 * \ln(x) + 150.32, r^2 = 0.93$) (figure 5.6 C). The width increased predominantly in the larger-diameter axons, making the AP broader as the peri-axonal spaces were increased. For instance, in the axon of core diameter of $1 \mu\text{m}$, the width increased 6.9 % ($y = 0.004 * \ln(x) + 0.2114, r^2 = 0.98$), whereas in the $2.7 \mu\text{m}$, 11.9% ($y = 0.0072 * \ln(x) + 0.1956, r^2 = 0.91$) (figure 5.6 D).

5.5.4 Proportional increment of the paranodal and juxtaparanodal peri-axonal space and K_v1 dislocation

The last structural arrangement that was simulated included a proportional increment of the paranode and juxtaparanode peri-axonal space and K_v1 displacement from the juxtaparanode to the paranode (figure 5.7 A). In this condition, conduction failure happened as in the last section, for the axons with a core diameter of $0.4, 0.6$, and $0.8 \mu\text{m}$ (figure 5.7 B). The larger-diameter axons also experienced a significant velocity decrease. For example, the total velocity decay of the axon with a core diameter of $2.7 \mu\text{m}$ was 79.26 % ($y = -0.209 * \ln(x) + 1.6457, r^2 = 0.99$). The amplitude also decreased as the peri-axonal spaces were increased to a similar extent as in the last condition figure 5.7 C). For instance, the amplitude decreased in the $1 \mu\text{m}$ by 31.4 % ($y = -9.085 * \ln(x) + 140.6, r^2 = 0.99$) and

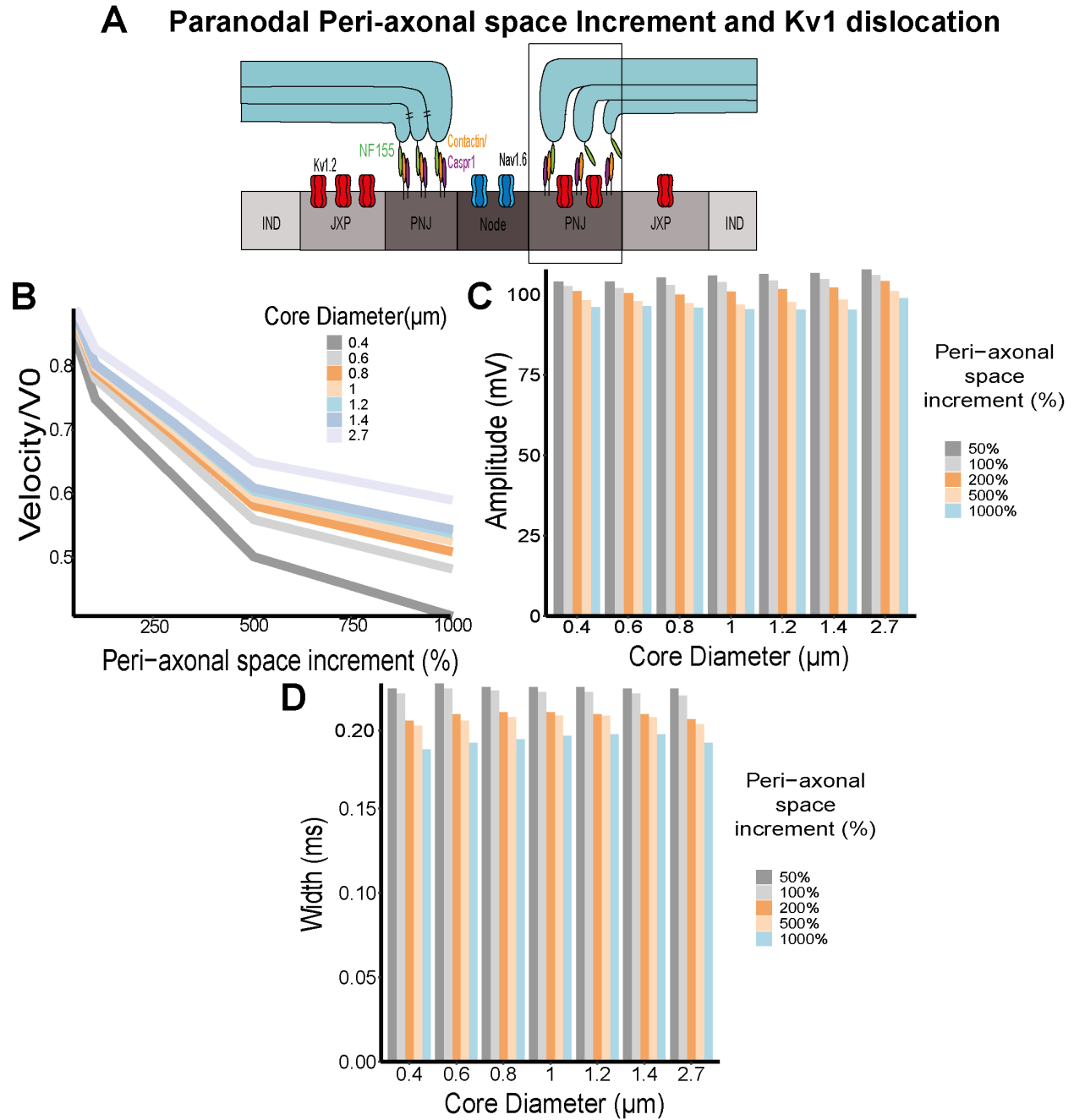


Figure 5.5: Paranodal Peri-axonal space increment and K_v1 dislocation induces a reduction in AP velocity, amplitude and width. (A) Diagram representing the increment of the paranodal peri-axonal space and K_v1 dislocation. (B) Normalised velocity plot at every core diameter as the paranodal peri-axonal space increases. (C) Bar plot of the amplitude (mV) of the AP at every core diameter as the paranodal peri-axonal space increases. (D) Bar plot of the width (ms) of the AP at every core diameter as the paranodal peri-axonal space increases.

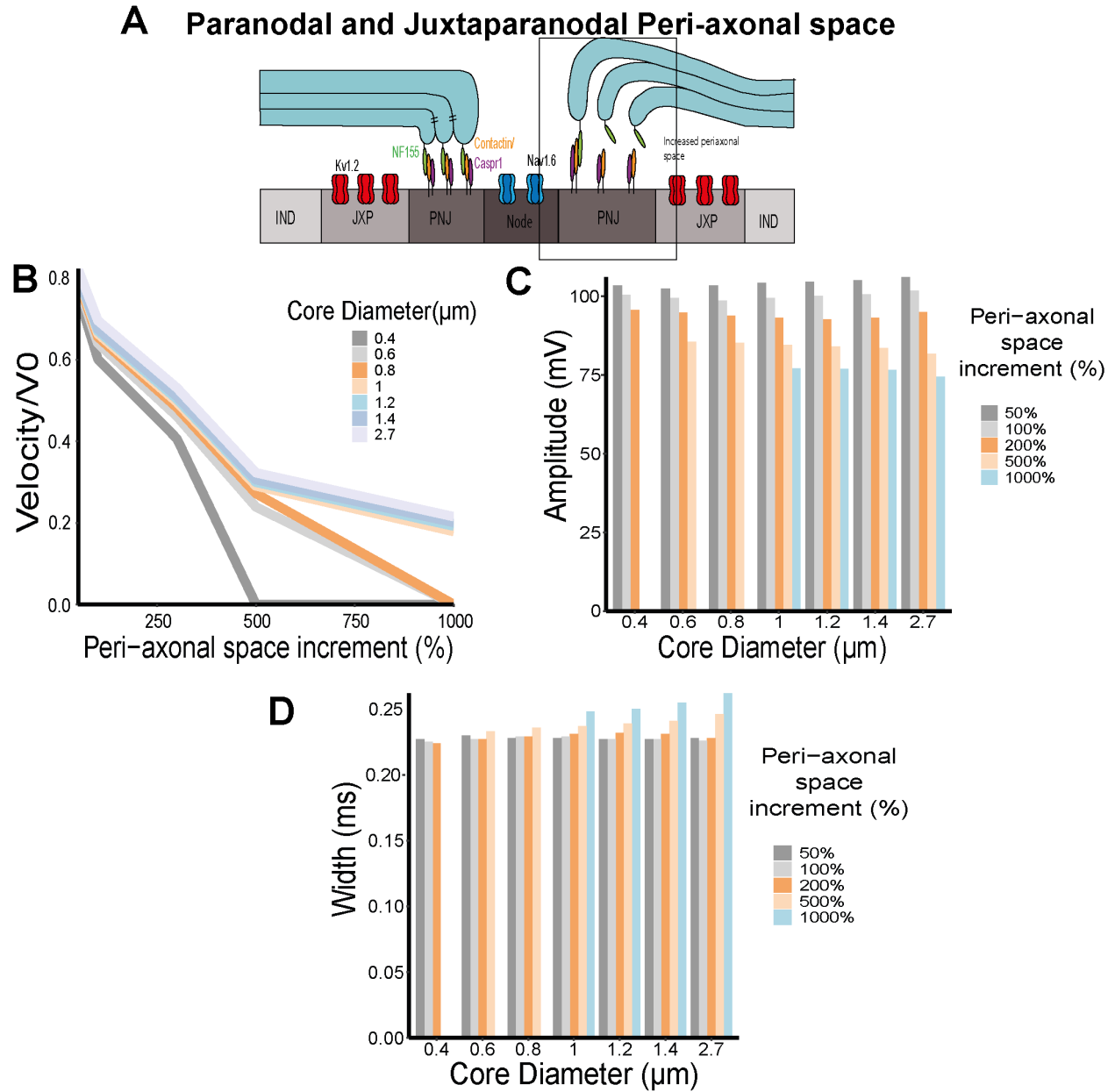


Figure 5.6: Paranodal and Juxtaparanodal Peri-axonal space increment induces AP velocity reduction and conduction failure in axons with a core diameter of 0.4, 0.6 and 0.8 μm . (A) Diagram representing the increment of the paranodal and juxtaparanodal peri-axonal space in the node of Ranvier. (B) Normalised velocity plot at every core diameter as the paranodal peri-axonal space increases. (C) Bar plot of the amplitude (mV) of the AP at every core diameter as the paranodal peri-axonal space increases. (D) Bar plot of the width (ms) of the AP at every core diameter as the paranodal peri-axonal space increases.

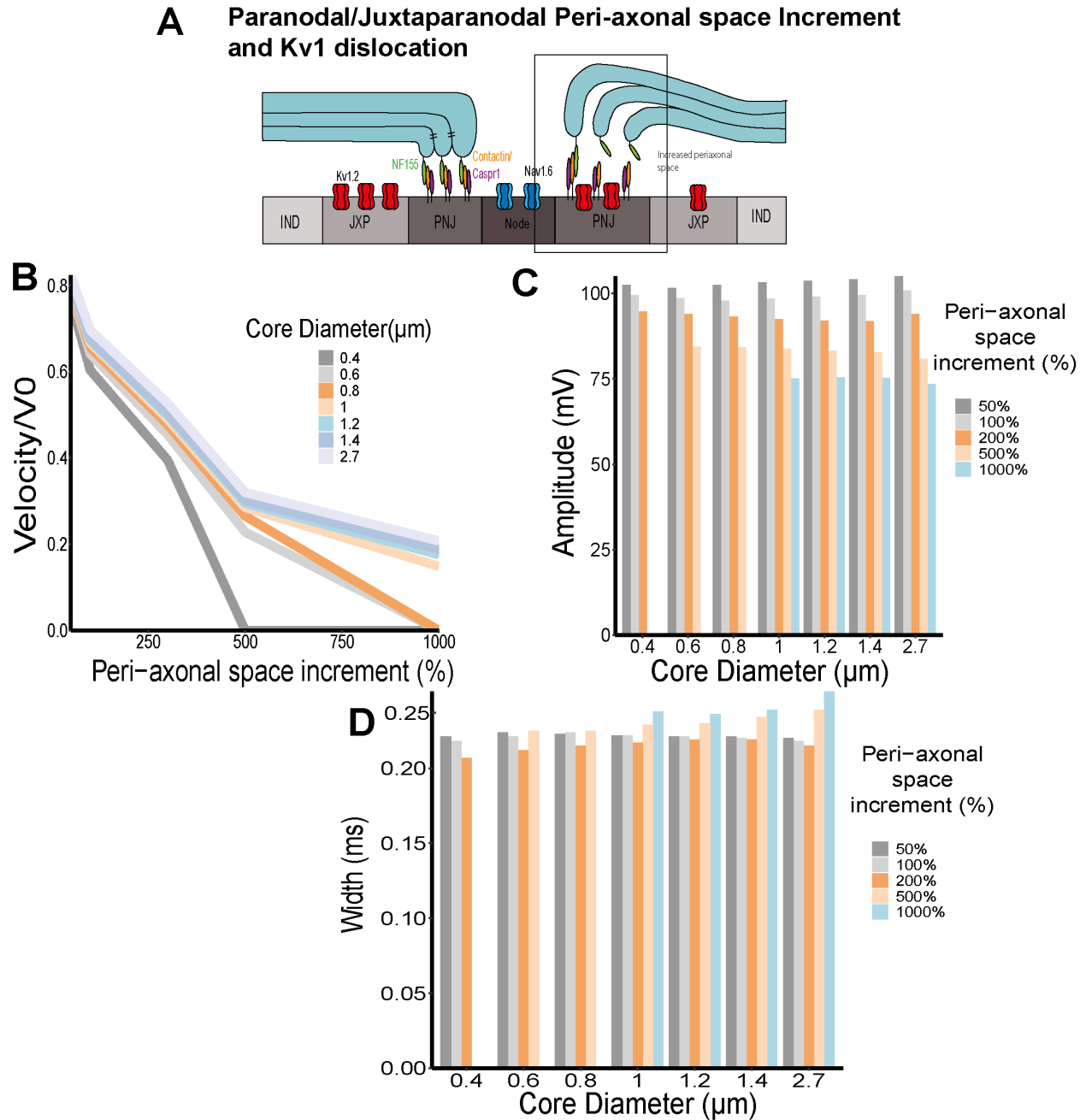


Figure 5.7: Paranodal and Juxtaparanodal Peri-axonal space increment and K_v1 dislocation induces AP velocity reduction and conduction failure in axons with a core diameter of 0.4, 0.6 and 0.8 μm . (A) Diagram representing the increment of the paranodal and juxtaparanodal peri-axonal and K_v1 dislocation. (B) Normalised velocity plot at every core diameter as the paranodal peri-axonal space increases. (C) Bar plot of the amplitude (mV) of the AP at every core diameter as the paranodal peri-axonal space increases. (D) Bar plot of the width (ms) of the AP at every core diameter as the paranodal peri-axonal space increases.

in the 2.7 μm axon by 33.83 % ($y = -11.04 * \ln(x) + 151.23, r^2 = 0.98$) (figure 5.6 C). However, the width did not increase as much as in the last condition due to the displacement of the K_v1 channels (figure 5.6 D). In fact, it increased in a linear way rather than logarithmic. For instance, in the axon of core diameter of 1 μm , the width increased 2.58 % ($y = 2 * 10^{-05}x + 0.2185, r^2 = 0.85$), whereas in the 2.7 μm , 7.26 % ($4 * 10^{-05}x + 0.2146, r^2 = 0.90$) (figure 5.7 D).

5.6 Proportion of disrupted nodes required for conduction failure depends on axon diameter

In the previous simulations, all 21 nodes of the axons were disrupted by increasing the peri-axonal space width and displacing the juxtaparanodal voltage-gated K_v channels. However, conduction failure only occurred in the small-diameter axons (d_{core} of 0.4, 0.6 and 0.8 μm) when the paranodal and juxtaparanodal peri-axonal spaces were increased 500 % in the 0.4 μm axon (from 0.002 to 0.012 μm) and 1000 % in the 0.6 and 0.8 μm axons (from 0.002 to 0.022 μm). In this section, we examined the number of consecutive nodes needed for conduction failure at these diameters, taking into consideration this structural disruption. Also, in the axons where conduction failure did not happen, the velocity decay was quantified after simulating conduction in an axon where patches of healthy nodes (figure 5.8 A, purple) were interspersed with patches of disrupted nodes (figure 5.8 A, orange).

In the axon with $d_{core}= 0.4 \mu\text{m}$, five consecutive nodes were needed for conduction failure when the paranodal and juxtaparanodal peri-axonal spaces width were increased to 0.012 and 0.12 μm respectively (figure 5.8 A). We then explored if damaging less than five nodes consecutively in different patterns would also cause conduction failure or a decrease in conduction velocity (figure 5.8 B). By simulating different patterns, we can observe that the total number of disrupted nodes along an axon did not determine if the conduction was going to fail. It depended on the number of consecutive nodes disrupted. For instance, when three disrupted nodes (figure 5.8 B, orange) were followed by two healthy

5.6. Proportion of disrupted nodes required for conduction failure depends on axon diameter

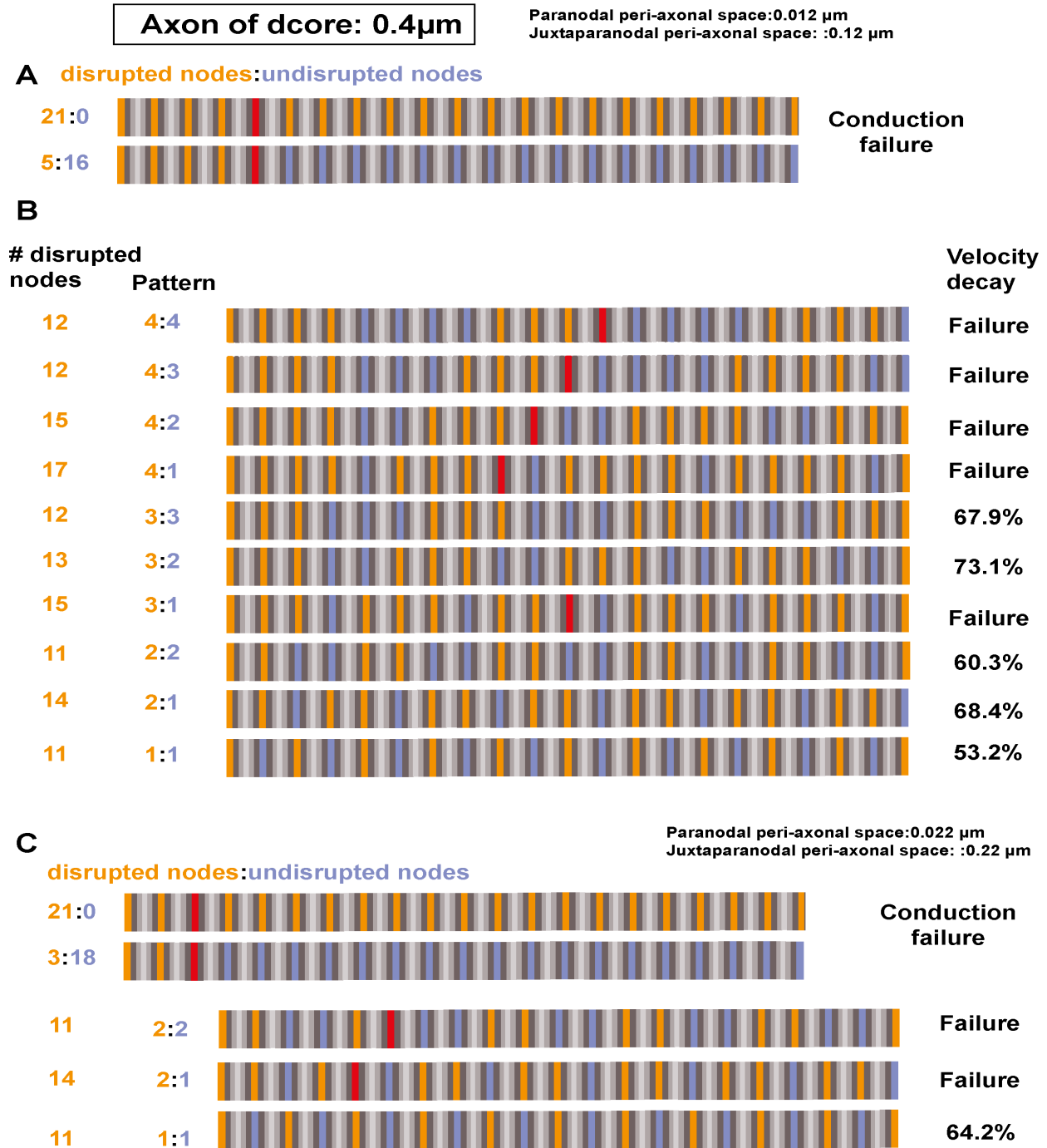


Figure 5.8: The difference in the proportion of disrupted paranodes within an axon of d_{core} of 0.4 μ m can provoke conduction failure and a variable degree of velocity reduction. (A) In axon model of 0.4 μ m core diameter, conduction failure occurred when five consecutive nodes were disrupted (orange), and the paranodal and juxtaparanodal peri-axonal space widths were increased up to 0.012 and 0.12 μ m, respectively. (B) Velocity decay and conduction failure of this axon model under different patterns of disruption (orange means disrupted node, purple, healthy node and, red denotes conduction failure). (C) In this axon model, conduction failure occurs after three consecutive nodes were disrupted when the paranodal and juxtaparanodal peri-axonal space widths were increased up to 0.022 and 0.22 μ m, respectively.

nodes (figure 5.8 B, purple) the conduction did not fail, it caused a velocity decay of 73.1 %, having a total of 13 out of 21 nodes disrupted. Meanwhile, in other conditions with less numbers of disrupted nodes, the conduction failed when four consecutive nodes were disrupted (figure 5.8 B).

In the axon with $d_{core} = 0.4 \mu\text{m}$, three consecutive nodes were needed for conduction failure when the paranodal and juxtaparanodal peri-axonal spaces were increased to 0.022 and $0.22 \mu\text{m}$ respectively (figure 5.8 C). In this case, only when one disrupted node was followed by a healthy one, the axon conducted signal, but with a velocity decay of 64.2 %.

In the axon with $d_{core} = 0.6 \mu\text{m}$, five consecutive nodes were needed for conduction failure when the paranodal and juxtaparanodal peri-axonal spaces width were increased to 0.022 and $0.22 \mu\text{m}$ respectively (figure 5.9 A). We then explored if damaging less than five nodes consecutively in different patterns would also cause conduction failure or a decrease in conduction velocity (figure 5.9 B). By simulating different patterns, our data showed that the total number of disrupted nodes along an axon did not determine completely if the conduction was going to be interrupted. It depended on the number of consecutive nodes disrupted. For instance, when three disrupted nodes (figure 5.9 B, orange) were followed by three healthy nodes (figure 5.9 B, purple) the conduction did not fail, it caused a velocity decay of 70.3 %, having a total of 12 out of 21 nodes disrupted. Meanwhile, in other conditions with less number of disrupted nodes, the conduction failed when four consecutive nodes were disrupted (figure 5.9 B).

In the axon with $d_{core} = 0.8 \mu\text{m}$, eleven consecutive nodes were needed for conduction failure when the paranodal and juxtaparanodal peri-axonal spaces width were increased to 0.022 and $0.22 \mu\text{m}$ respectively (figure 5.10 A). In our 21 node axon, if the disruption was made in less than eleven consecutive nodes, there were more than 56 combinations between healthy and disrupted nodes. Thus, a sample of them was explored (specifically, the same patterns as in the last sections to enable a comparison). In the simulations using this axon diameter, the velocity decay was lower than in the other diameters. Nonetheless, it followed a similar pattern: assuming that the same number of consecutive nodes

5.6. Proportion of disrupted nodes required for conduction failure depends on axon diameter

were disrupted, the velocity decay was higher as less healthy nodes remained in between.

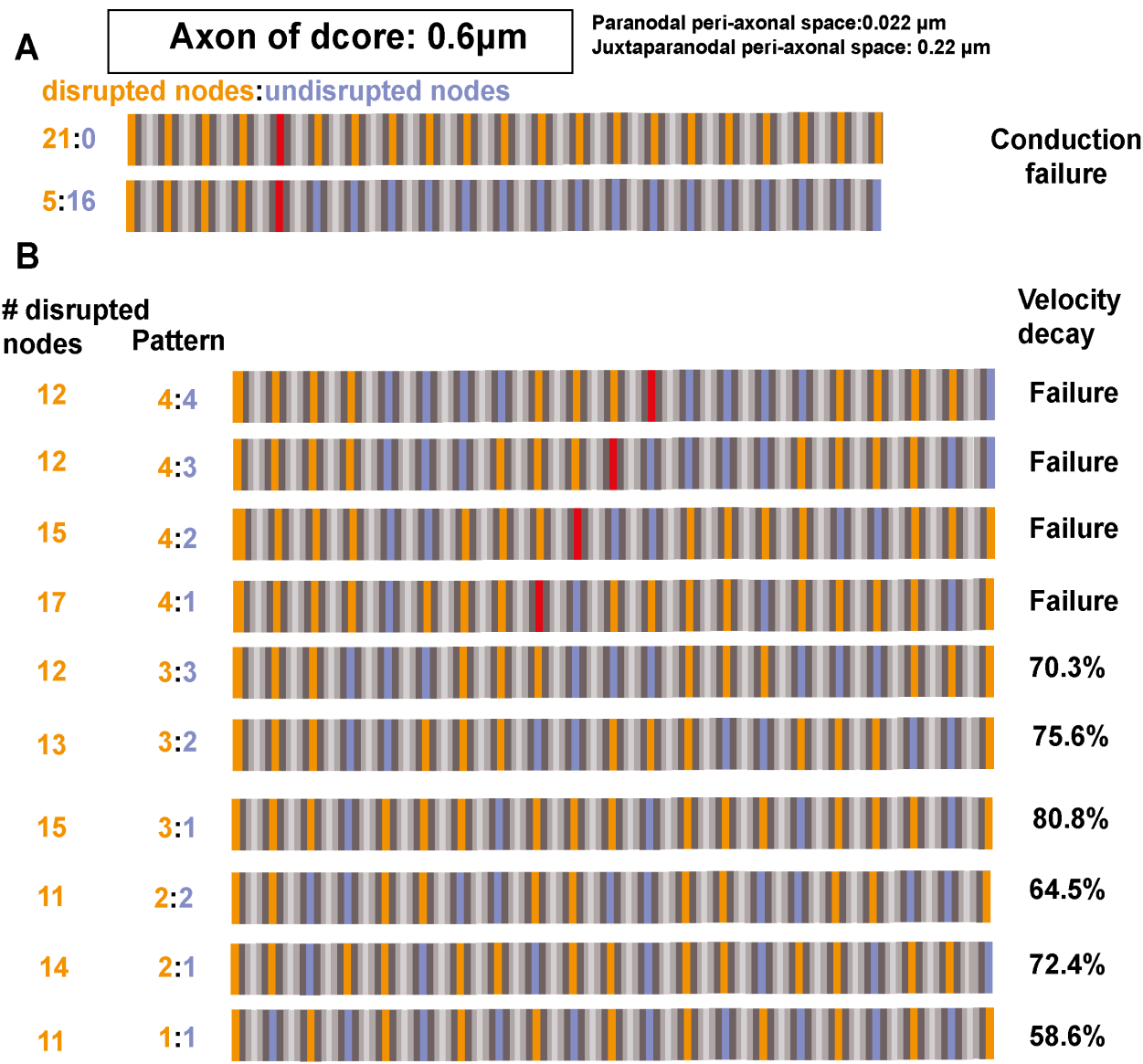


Figure 5.9: The difference in the proportion of disrupted paranodes within an axon of d_{core} of 0.6 μm can provoke conduction failure and a variable degree of velocity reduction. (A) In axon model of 0.6 μm core diameter, conduction failure occurred when five consecutive nodes were disrupted (orange), and the paranodal and juxtaparanodal peri-axonal space widths were increased up to 0.022 and 0.22 μm, respectively. (B) Velocity decay and conduction failure of this axon model under different patterns of disruption (orange means disrupted node, purple, healthy node and, red denotes conduction failure).

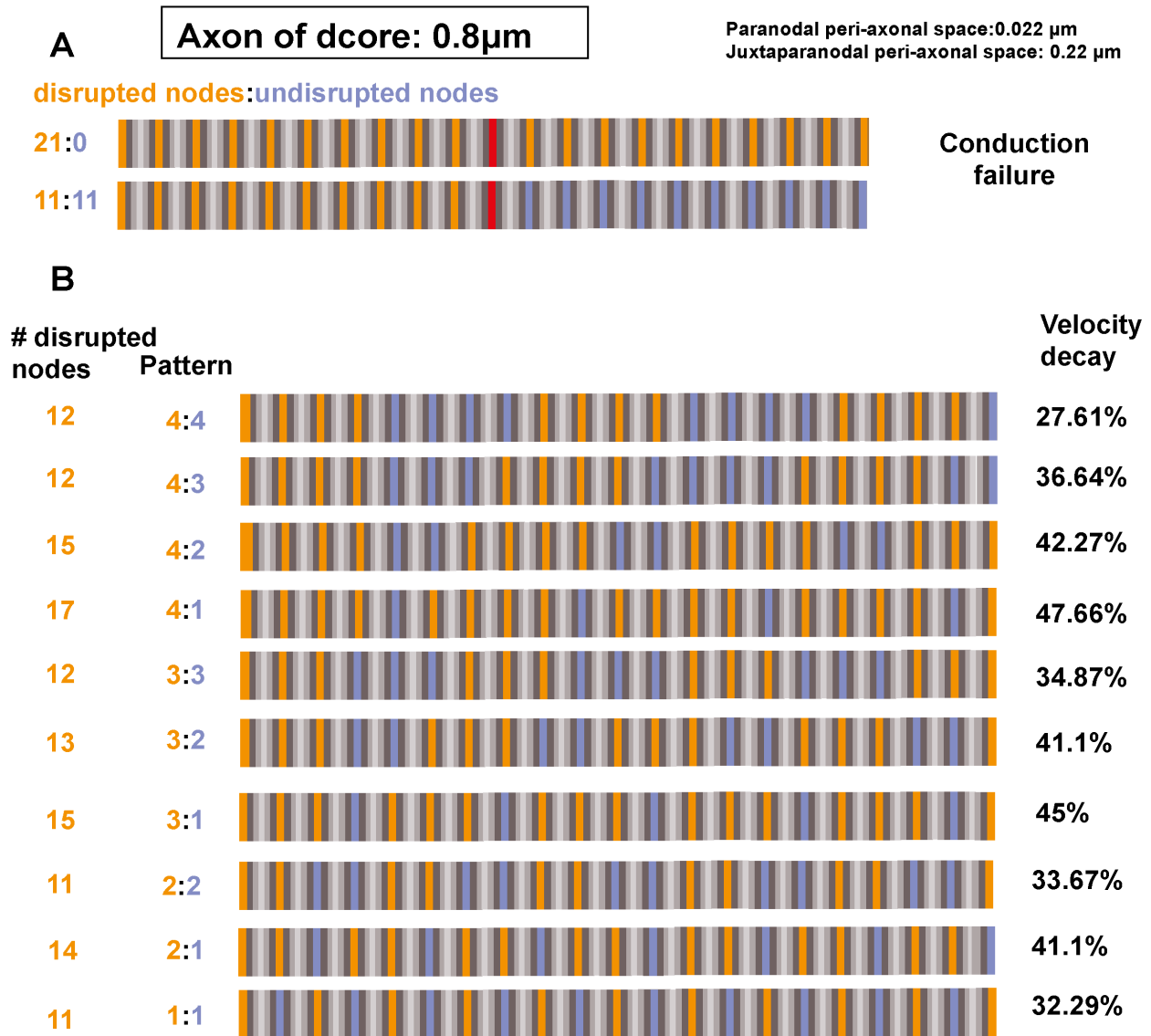


Figure 5.10: The difference in the proportion of disrupted paranodes within an axon of d_{core} of $0.8\mu m$ can provoke conduction failure and a variable degree of velocity reduction. (A) In axon model of $0.8\mu m$ core diameter, conduction failure occurred when eleven consecutive nodes were disrupted (orange), and the paranodal and juxtaparanodal peri-axonal space widths were increased up to 0.022 and $0.22\mu m$, respectively. (B) Velocity decay and conduction failure of this axon model under different patterns of disruption (orange means disrupted node, purple, healthy node and, red denotes conduction failure).

5.7 Discussion

5.7.1 Computational models of paranodal axo-glial junction pathology.

In this study, a biologically plausible model of a CNS myelinated axon comprised of 21 successive nodes was built (figure 5.1), and seven different diameters that have been previously measured in axons in EM studies from macaque and human brains (Liewald et al., 2014) were used. This model enabled the correlation of structural changes observed in the axon, described in previous chapters, with functional disruption, to help understand and characterise the consequences of axon damage in the NAWM. Therefore, it is a platform that could enable the testing of potential structural targets to improve functional axonal recovery. Moreover, in this model, different structural axonal parameters, such as diameter and peri-axonal space width, were progressively modified, since previous literature has reported that they can affect signal conduction (Goldman and Albus, 1968; Waxman et al., 1995). Although some published computational models have included the presence of the paranodal and juxtaparanodal compartments in their simulations (Richardson et al., 2000; McIntyre et al., 2002; Gow and Devaux, 2008), paranodal axo-glial junction pathology has been simulated in just a few studies by different strategies (Gow and Devaux, 2008; Babbs and Shi, 2013; Volman and Ng, 2014; Kohan et al., 2017). Most computational models reduced the axonal compartments to the non-myelinated nodes and the myelinated internodes (Goldman and Albus, 1968; Colbert and Pan, 2002; Moffitt et al., 2004; Gold et al., 2006a). However, recent computational models have simulated paranodal retraction by increasing the nodal width (Babbs and Shi, 2013), equating the nodal resistance to the paranodal (Volman and Ng, 2014) or by reducing the number of myelin loops in the paranodal compartment (Kohan et al., 2017). However, these models only simulated these conditions in a single axon diameter (Babbs and Shi, 2013; Volman and Ng, 2014), or in a few of them (Kohan et al., 2017), and in axons that were usually $\geq 1\mu\text{m}$. In addition, the structural parameters were mainly acquired from the ventral cat nerve fibres (Berthold and Rydmark, 1983) and the pathology was not changed across different nodal patches as we have presented in the current chapter.

5.7.2 Axonal diameter influences conduction

In order to simulate paranodal axo-glia pathology, the paranodal and juxtaparanodal peri-axonal spaces widths were increased progressively, and the juxtaparanodal voltage-gate K_v channels were displaced towards the paranode (figure 5.4 A figure 5.5 A, figure 5.6 A figure 5.7 A). Hence, the resistance of the paranodal and juxtaparanodal compartments were significantly reduced. For example, the axial resistance of the paranodal peri-axonal space in our model changed from 24425264.4 to 237235.49 MOhm/cm in the axon of d_{core} 0.4 μm when the width was increased from 0.002 to 0.022 μm . The resistance was expressed in MOhm/cm in the NEURON simulator as it took into consideration the cross-sectional area of the peri-axonal space:

$$periaxonal_{xarea} = \left(\frac{d_{core}}{2} + width\right)^2 - axon_{xarea} \quad (5.1)$$

$$axon_{xarea} = \pi * r^2 \quad (5.2)$$

The conduction velocity decreased significantly following a logarithmic curve as the paranodal and/or the juxtaparanodal peri-axonal widths were increased in all the axonal diameters (figure 5.4 B figure 5.5 B, figure 5.6 B figure 5.7 B), since a greater amount of axonal membrane had to be depolarised. This result is consistent with previously published models of paranodal disruption (Babbs and Shi, 2013; Volman and Ng, 2014; Kohan et al., 2017). Furthermore, conduction failure occurred in the axon with a core diameter of 0.4 μm when the widths were increased by 500 % or more, and in the axons with core diameter of 0.6 μm and 0.8 μm , when the peri-axonal widths were increased by 1000 % (figure 5.6 B, figure 5.7 B). This data suggests that the severity of the paranodal axo-glia pathology depends on axon diameter. The smaller the diameter, the higher the velocity decay and the higher the probability of conduction failure. These results could be explained by the fact that the thickness of the myelin sheath (mst) is proportional to the axon diameter (Rushton, 1951; Goldman and Albus, 1968). Moreover, previous computational studies have suggested that increasing the number of myelin loops around an axon increases the conduction velocity (Castelfranco and Hartline, 2015). However, they esti-

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mated that this increment in conduction velocity stopped when the fibre diameter of an axon was $1\text{ }\mu\text{m}$ and was wrapped by 10 myelin loops (Castelfranco and Hartline, 2015). Therefore, the conduction velocity is more sensitive when the axons are en-sheathed by a lower number of myelin loops, such as in the small-diameter axons of this model (Table 5.1). This data would imply that because axons with small diameter have a lower myelin sheath resistance and a higher axoplasm resistance, they have a higher dependency on the structural integrity of the PNJ to shunt electrical currents and maintain fast and efficient conduction. Furthermore, these results also highlight the importance of modelling the paranodal and juxtaparanodal compartments, as well as, the nodal and internodal compartments, as they are highly sensitive regions. Additionally, it should be noted that our analysis of the MS and animal model tissue showed that the myelin covering the internodes remained intact in the NAWM regions, which further emphasizes the likely important role of changes at the paranode and juxtaparanode in causing conduction deficits in MS.

The velocity also decreased when the K_v channels were displaced from the juxtaparanode (figures 5.5 and 5.7). For example, in the axon of core diameter of $2.7\text{ }\mu\text{m}$, the velocity decreased by 37.7 % when the paranodal peri-axonal space was increased from 0.02 to $0.022\text{ }\mu\text{m}$ (figure 5.4 B), whereas it decreased by 40.9 % when the channels were also displaced (figure 5.5 B). When the juxtaparanodal K_v channels were dislocated towards the paranode, the repolarising potassium current increased above normal levels contributing more to the AP repolarization. This effect could have affected conduction velocity slightly since the hyperpolarising currents of the K_v channels could have slowed down the rate of depolarising current driven by the nodal Na_v channels.

The amplitudes of the AP were also decreased under these structural changes at the paranodes and juxtaparanodes (figure 5.4 C figure 5.5 C, figure 5.6 C figure 5.7 C). The amplitudes of the AP were decreased because of two reasons. Firstly, the re-polarizing K_v currents were increased above normal levels earlier in time. Secondly, a greater axonal area was needed to be depolarised for conduction to occur, since the capacitance of the paranodes and juxtaparanodes was increased. Therefore, a larger depolarising current

would be needed to reach the same AP amplitude as in the healthy axon.

The widths of the AP decreased when the juxtaparanodal voltage-gated K_v1 channels were displaced to the paranodal compartment (figure 5.5 D). This decrease was provoked as these channels contributed more significantly to re-polarise the AP (they opened their channel gates earlier), while in the healthy axon their effect was minimal as they were located underneath the myelin sheath. However, when both paranodal and juxtaparanodal peri-axonal widths were increased substantially (by 500 % - 1000 %), the width increased slightly making the AP broader (figure 5.6 D, figure 5.7 D). This effect could be explained by the fact that under these conditions, the amplitude was highly decreased because not enough current was administered to depolarise the axonal membrane. Therefore, when the membrane voltage did not attain a certain level, the driving force ($V_m - E_K$) of the voltage-gated channels decreased, and the repolarising phase was slower.

5.7.3 Relevance of the axonal model in MS

Chronically activated microglia and macrophages have been found scattered throughout the NAWM tissue in progressive MS (Trapp et al., 1998; Graumann et al., 2003; Kutzelnigg et al., 2005b; Howell et al., 2010; Moll et al., 2011). Our data from human post-mortem tissue and the chronic model of meningeal inflammation showed that local activation of microglia and astrocytes was associated with paranodal axo-glial junction pathology (Chapter 3 and 4). However, the distribution and density patterns of activated microglia and macrophages can vary significantly throughout the WM tracts of the brain and spinal cord in MS. Taking this heterogeneity into consideration, we simulated different patterns of disrupted (paranodes and juxtaparanode width increment) and healthy nodes to characterise the effect of a highly variable paranodal junction disruption on AP conduction within a myelinated axon (figures 5.8, 5.9 and 5.10). In these figures, we can observe that the extent of conduction failure depended on diameter, the number of disrupted consecutive nodes and the extent of the paranodal and juxtaparanodal disruption. For example, regarding the importance of axon diameter, in the cases where we incremented the paranodal and juxtaparanodal peri-axonal widths by 1000 %, conduction failure happened

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after three nodes were disrupted in an axon of core diameter of $0.4\ \mu\text{m}$ (figure 5.8 C). In contrast, in the $0.6\ \mu\text{m}$ diameter axon five consecutive nodes were needed (figure 5.9), and in the $0.8\ \mu\text{m}$ diameter axon eleven consecutive nodes (figure 5.10). In the rest of the diameters, conduction failure did not occur even when the 21 nodes of the axon were affected (figures 5.6 and 5.7). Another parameter that determined the conduction failure in these axons was the number of disrupted consecutive nodes. The extent of velocity decay was greater as fewer healthy nodes were positioned in between patches of consecutive disrupted nodes (figures 5.8, 5.9 and 5.10). This data suggests that the proportion of disrupted nodes needed to generate conduction failure is proportional to the axon diameter, since the importance of the integrity of the PNJ structure is higher in the small axons.

The conduction velocity decreased substantially under different patterns in the small-diameter axons. Therefore, these structural changes could provoke significant neurological deficits if it happens throughout the WM of the brain. In fact, previous histopathological studies in human post-mortem MS tissue (DeLuca et al., 2006) and in a mouse EAE model (Nikić et al., 2011) showed that the thinner axons were more prone to axonal injury and focal axonal degeneration (FAD) than the larger ones. Moreover, other experiments in brain tissue have also demonstrated that smaller axons were more susceptible to trauma than larger ones (Reeves et al., 2005; Tang-Schomer et al., 2012). The inverse proportionality of the axon diameter with the importance of the integrity of the PNJ for adequate conduction could be one of the explanations.

Furthermore, previous studies have demonstrated experimentally that blocking AP in CNS axons results in a decrease in the movement of mitochondria within the axoplasm, whereas enhancing neuronal firing increases the mitochondrial movement and the size of mitochondrial stationary sites in the nodal/paranodal axoplasm (Ohno et al., 2011). Therefore, if the conduction is blocked or the firing is reduced, the mitochondria movement could be disrupted, making the axon energetically deficient. In fact, a previous study in human post-mortem MS tissue demonstrated the presence of impaired antero-grade mitochondrial transport (Kim et al., 2010). Therefore, axons that conduct inefficiently due to a variable degree of PNJ disruption related to the chronically activated

microglia surrounding them could also be energetically deficient. This condition could be one of the underlying explanations of fatigue in MS. This fatigue affects most MS patients and interfere with their usual and desired activities (Christodoulou, 2005; Mills and Young, 2008). Therefore, reversing the paranodal axo-glial junction pathology could have a significant effect on brain conduction and the quality of life of patients. Hence, in the next chapter, we have explored different molecular mechanisms by which chronically activated microglia could be generating these disruptions at the nodes of Ranvier.

5.8 Conclusions

In conclusion, our simulations have demonstrated that AP conduction velocity can significantly decrease when the structure of the paranodal axo-glial junction is disrupted by decreasing the paranodal and juxtaparanodal peri-axonal space resistances. Further, our results pointed to axon diameter as the parameter that predicted best the extent of velocity decay and the probability of conduction failure. Furthermore, conduction failure of a myelinated axon might depend on the number of consecutive nodes disrupted and the number of healthy nodes in between patches of disrupted nodes. Therefore, these results suggest that diffuse paranodal axo-glial pathology in MS NAWM can significantly alter the efficiency and velocity of AP conduction, and consequently the overall neurological function in MS.

6

Inducing paranodal axo-glial pathology in cerebellar slices

6.1 Introduction

Progressive axonal injury in NAWM MS tissue has been associated with diffuse inflammation, which is characterised by chronic activation of microglia (Lassmann et al., 2012). Glutamate excitotoxicity and subsequent calcium overload within the axoplasm and myelin domains are suggested as one of the possible molecular mechanisms to explain this pathology throughout the NAWM regions. In order to investigate the molecular mechanisms underlying the paranodal pathology seen in MS tissues and our animal model, we used organotypic brain slices as they preserve the neuronal cytoarchitecture during weeks to months, with the possibility to easily perform pharmacological, biochemical and immunohistochemical experiments (Doussau et al., 2017). Moreover, organotypic cerebellar slices are widely used to study myelination processes in physiological

and pathological conditions such as MS (Marzban et al., 2015). Further, only a single type of myelinated axon is maintained in cerebellar slices: the axons of the Purkinje cells, whereas the axons of afferent fibres (mossy fibres and climbing fibres) or efferent fibres (deep cerebellar nuclei) degenerate after a few days in vitro (Zhang et al., 2011; Doussau et al., 2017) (figure 6.1 A (i., ii.)).

6.1.1 Activated microglia release glutamate

Microglia can be activated by a wide range of mechanisms, including pro-inflammatory cytokines. Previous studies have demonstrated the role of TNF in potentiating glutamate-mediated excitotoxicity by various mechanisms in microglia. For example, TNF can stimulate microglia via TNFR1 to release glutamate in an autocrine manner through hemichannels (Takeuchi et al., 2006; Yawata et al., 2008). Moreover, it has been observed that the stimulation of the group II metabotropic glutamate (mGlu2) receptors in microglia induces the release of TNF (Taylor et al., 2005), which then could stimulate the cell in an autocrine way via TNFR1. Microglia could also release glutamate via its cystine-glutamate antiporters (Xc-) (Domercq et al., 2007). The glutamate levels that microglia release can be amplified in the presence of TNF as previous studies have shown that, in astrogloma cells and rat primary astrocytes, it inhibits glutamate uptake transporters EEAT1/GLAST and EEAT2/GLT-1 by inducing downregulation of EAAT2/GLT-1 mRNA (Chao and Hu, 1994; Wang et al., 2003b; Sitcheran et al., 2005). Furthermore, TNF activated microglia can release oxidative species such as ROS and NO that can block glutamate uptake of GLAST and GLT-1 (Miralles et al., 2001). Lastly, TNF can also trigger the expression of AMPA receptors (Beattie et al., 2002) and NMDA receptors (Wheeler et al., 2009) in the synaptic membrane of neurons, modifying the synaptic strength (Stellwagen et al., 2005; Han and Whelan, 2010).

Glutamate is synthesised in microglia from its precursor glutamine through the enzyme glutaminase. An up-regulation of glutaminase and glutamate transporters has been observed in active lesions in MS tissue, which are usually characterised by large numbers of activated microglia and infiltrated macrophages (Werner et al., 2001). Previous studies

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have shown an up-regulation of the cystine glutamate exchanger (Xc-) in monocytes from MS blood samples (Pampliega et al., 2011).

The effects of TNF could be amplified if it works in synergy with the pro-inflammatory cytokine IFN γ , which is a potent inducer of both TNF and TNFR1 gene expression in microglia. IFN γ can also induce the expression of CD40, which is involved in the endogenous production of TNF (Nguyen and Benveniste, 2002). Also, both cytokines can act cooperatively to induce oxidative stress (Mir et al., 2009). Moreover, IFN γ can up-regulate molecules such as MHCII to maintain antigen-dependent T-cell activation and keep microglia persistently activated (Frei et al., 1987).

6.1.2 Microglia derived glutamate could disrupt PNJ

As presented in the previous chapters, paranodal axo-glial junction disruption is associated with microglial activation in progressive MS NAWM tissue. Glutamate derived from activated microglia and macrophages could disrupt these tight junctions slowly in progressive MS. In fact, previous studies with coherent anti-Stokes Raman scattering (CARS) imaging microscopy have seen that direct administration of glutamate in adult female guinea pig spinal cord can induce myelin paranode splitting (figure 6.1 B i.) and complete dislocation of the juxtaparanodal voltage-gated $K_v1.2$ channels (Fu et al., 2009) (figure 6.1 B iii.). Additionally, another study with CARS showed that high frequency stimulation (200 Hz) can also cause paranodal splitting (figure 6.1 B ii.), dislocation of the juxtaparanodal voltage-gated $K_v1.2$ channels and Caspr1-stained paranodal elongation (Fu et al., 2011) (figure 6.1 B iv.). Moreover, both studies suggest the presence of an underlying Ca^{2+} dependent mechanism as inhibitors for calpain1, glutamate and calcium receptors can reduce myelin paranodal retraction.

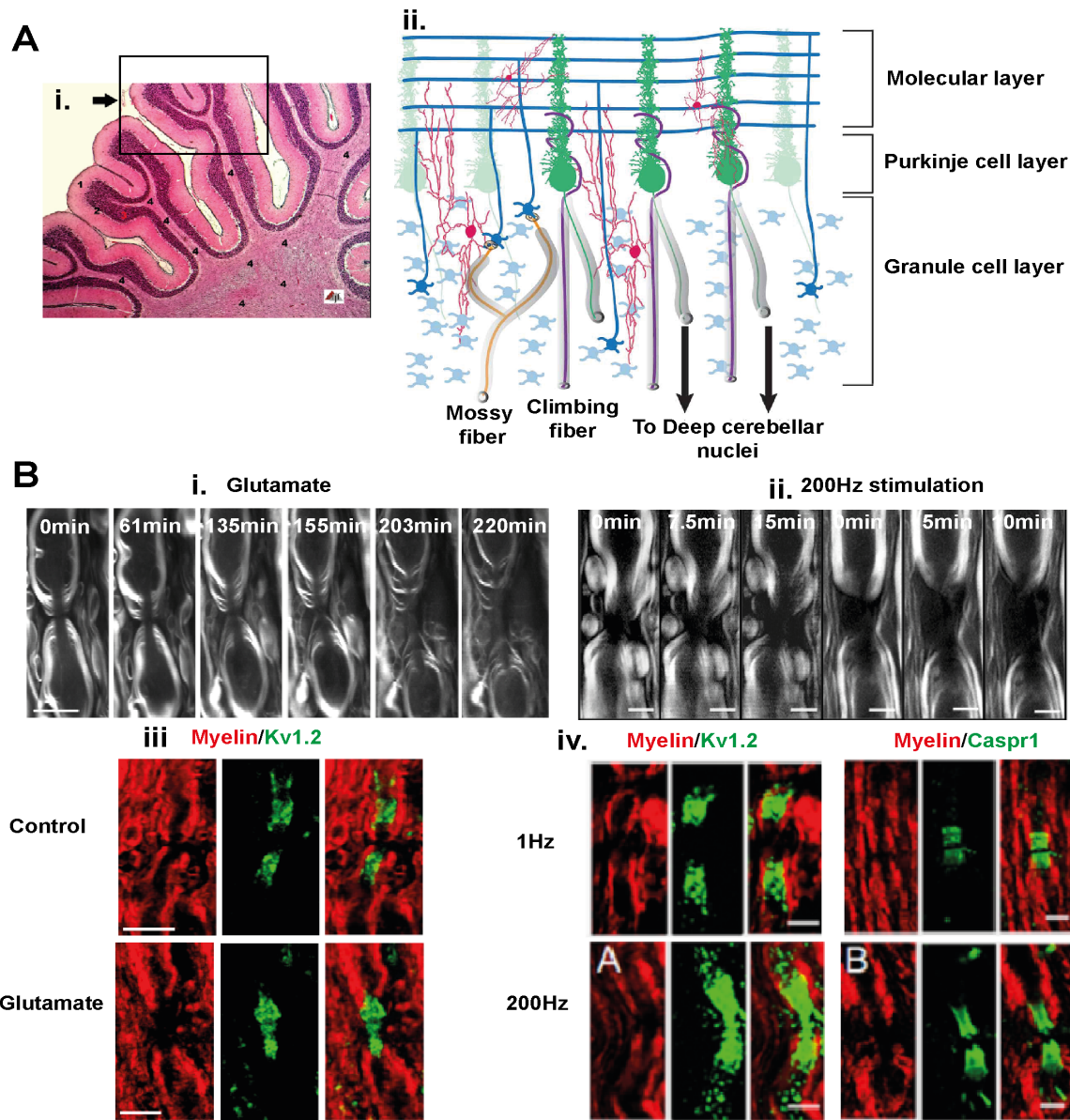


Figure 6.1: Cerebellar structure and paranodal splitting. (A) (i.) Saggital histological image of the cerebellum comprised of: (1) molecular layer, (2) Purkinje cell layer, (3) granular layer and (4) white matter (acquired from Department of Morphology UFJF). (ii.) The molecular layer contains stellate cells and basket cells, the Purkinje or ganglionic layer is formed of a single row of large Purkinje cells, and the granular layer contains small granule cells with dark-staining nuclei (Doussau et al., 2017). (B) (i.) CARS microscopy image of the paranodal myelin loop splitting over time via glutamate administration (Fu et al., 2009) and (ii.) 200 Hz high frequency stimulation during (0, 7.5 and 15 min) and after stimulation (0, 5, 10 min) (Fu et al., 2011). (iii.) Immunofluorescence image of myelin (red) and $K_v1.2$ (green) of guinea pig spinal cord axons of controls (top) and glutamate treated (bottom), in which the $K_v1.2$ channels appeared completely dislocated to the node (Fu et al., 2009). (iv.) Immunofluorescence image of myelin (red), $K_v1.2$ (green) and Caspr1 (green) of guinea pig spinal cord axons treated with 1 Hz stimulation (top) and 200 Hz high frequency stimulation (bottom). In the latter, the channels were completely dislocated and the Caspr1-stained paranodes were longer.

6.2 Aims

The following experiments aimed to identify an underlying mechanism that could explain the paranodal axo-glial junction pathology observed in the MS post-mortem human tissue (Chapter 3) and the chronic model of meningeal inflammation (Chapter 4) in a physiological environment such as the organotypic cerebellar tissue slices. Therefore, the specific aims of this chapter were:

- To culture primary rat microglia and activate them with pro-inflammatory cytokines such as TNF, IFN γ and LT α to determine the best strategy to trigger glutamate release in these cells;
- To set up an ex-vivo organotypic cerebellar tissue slice culture platform;
- To induce paranodal axo-glial pathology in organotypic cerebellar tissue slices; firstly, with direct exposure to pro-inflammatory cytokines such as TNF and IFN γ , secondly, with high glutamate microglial-conditioned medium, and thirdly, with direct glutamate administration;
- To quantify the paranodal axo-glial pathology under the different conditions mentioned with immunofluorescence staining and confocal microscopy;
- To reverse the induced pathology with an NMDA receptor antagonist.

6.3 Results

6.3.1 Cytokine activated microglia release high amounts of glutamate

Primary rat microglial cultures were treated with different concentrations of pro-inflammatory cytokines. Specifically, one acute treatment of 100 ng/ml or 200 ng/ml and two acute treatments of 100 ng/ml (24h apart) were performed (figure 6.2 G). The pro-inflammatory cytokines used include TNF, IFN γ , TNF+IFN γ , LT α and LT α +IFN γ . In all the cases, microglia were activated as their morphology transformed from a ramified

shape with long processes (figure 6.2 A) to a more activated amoeboid morphology with short and thicker processes (figure 6.2 B-F).

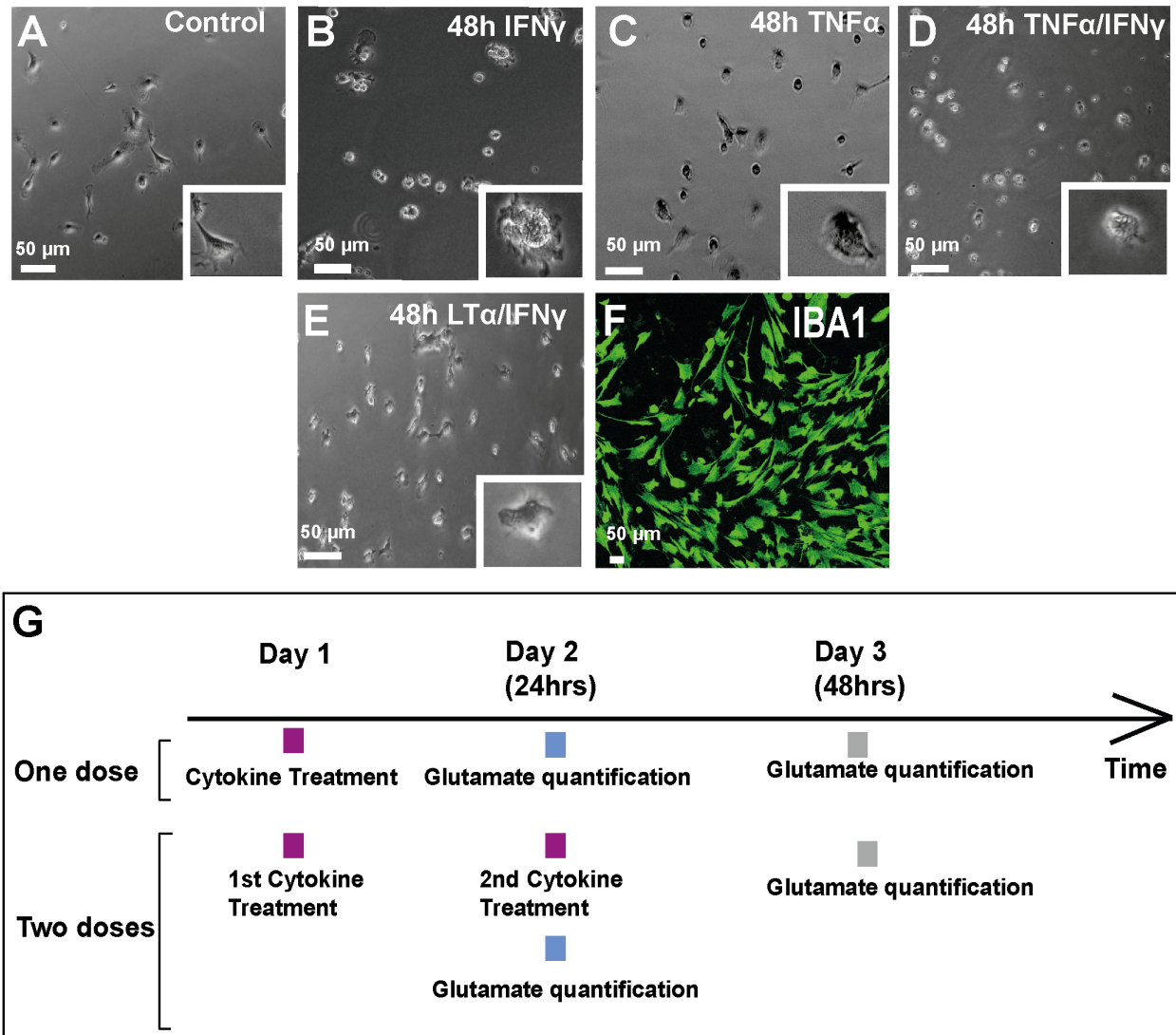


Figure 6.2: Primary rat microglial cultures images treated with the pro-inflammatory cytokines IFN γ , TNF and LT α . (A) Live image of non-treated microglia. (B) Live image of microglia treated with IFN γ after 48hrs. (C) Live image of microglia treated with TNF after 48hrs. (D) Live image of microglia treated with TNF/IFN γ after 48hrs. (E) Live image of microglia treated with LT α /IFN γ after 48hrs. (F) Confocal IBA+ immunofluorescent image of fixed microglia cultures. (G) Time line diagram showing the timings of the experiments. Microglia were either treated with one dose of cytokines or two doses provided 24 hrs apart. The glutamate in the supernatant was analysed in both cases after 24 hrs and 48 hrs.

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The role of TNF and IFN γ in microglial glutamate release

In order to assess if the glutamate release by microglia depended on the cytokine treatment (TNF, IFN γ and TNF+IFN γ) and the time lapsed after the treatment (24 hrs and 48 hrs), different concentrations were tested. With a 100 ng/ml dose, the glutamate levels across the cytokine treatment groups and timings were significantly different (**** $p=3.398e-09$, Friedman rank-sum test, figure 6.3 A). In order to examine which cytokine treatment was significantly different from non-treated cultures, Wilcoxon pair-wise comparisons with a Bonferroni correction were performed. TNF, IFN γ and TNF+IFN γ treated cultures were significantly different from the control group (* $p=0.013$). With a 200 ng/ml dose, the glutamate distributions were also significantly different (** $p=1.058e-09$, Friedman rank-sum test), and specifically the cultures treated with TNF (* $p=0.026$) and TNF + IFN γ (* $p=0.013$). The difference in glutamate levels between 24 hrs and 48 hrs across treatments was not significant. However, the glutamate levels induced by the cytokine treatments at both 100 ng/ml and 200 ng/ml after 48 hrs decreased for every group (figure 6.3 A, B (purple)). Specifically, in the 100 ng/ml group, the glutamate concentration decreased after 48 hrs an average of 17.97 % across all treatments, whereas in the 200 ng/ml an average of 21.52 %. In order to compensate for this effect, two acute treatments of 100 ng/ml were administered (figure 6.3 C). In this scenario, the glutamate levels across groups were also significantly different (**** $p=1.274e-09$, Friedman rank-sum test) with the TNF+IFN γ group the only one significantly (** $p=0.007$) different from the non-treated cultures. Furthermore, it was the only condition in which the glutamate concentration increased after 48 hrs. In fact, this treatment resulted in the highest concentration of glutamate at 48 hrs (an average of $96.046 \pm 9.92 \mu\text{M}$) and, therefore, was the most effective treatment.

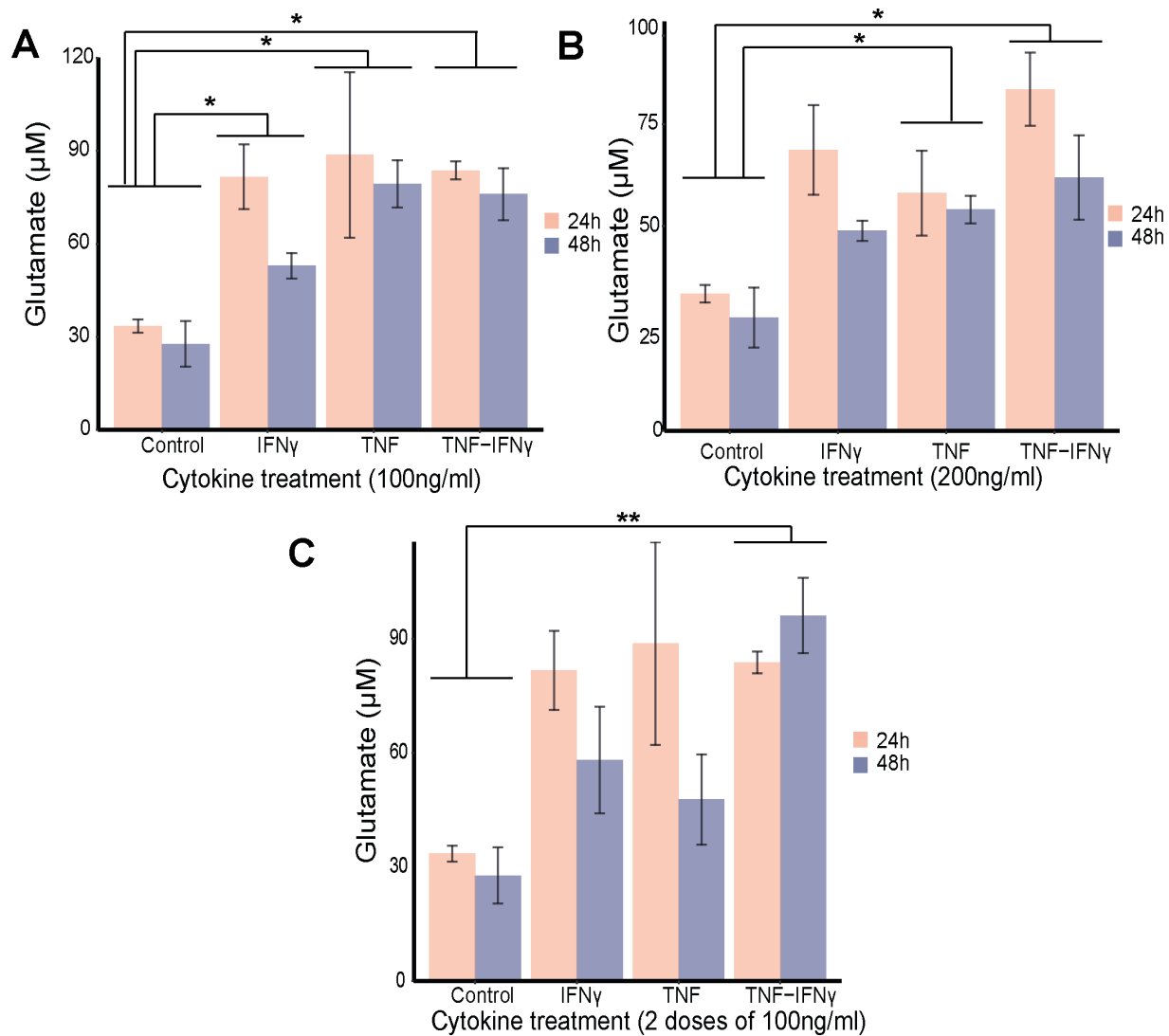


Figure 6.3: Two acute treatments of TNF+IFN γ to primary microglia induced a larger and more sustained release of glutamate than one treatment or an increment in dose. Mean $\pm$ SEM glutamate levels from replicates showing the statistical difference between controls and the cytokine treatments at different concentrations: (A) 100 ng/ml (n=3 Control, n=3 TNF, n=3 IFN γ , n=3 TNF+IFN γ), (B) 200 ng/ml (n=3 Control, n=3 TNF, n=3 IFN γ , n=3 TNF+IFN γ) and (C) two acute treatments of 100 ng/ml (n=3 Control, n=3 TNF, n=3 IFN γ , n=4 TNF+IFN γ). Non-parametric Friedman test was performed across cytokine groups and timings and post hoc paired-wised Wilcoxon tests to compare groups (* p<0.05, ** p<0.01).

LT α and IFN γ stimulation of microglial glutamate release

In order to study if the pro-inflammatory cytokine LT α had a similar effect on microglial glutamate release as TNF, primary microglia were treated with one acute and two acute

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treatments of 100 ng/ml with $LT\alpha$, $IFN\gamma$ and $LT\alpha + IFN\gamma$ (figure 6.4 A, B). With a 100 ng/ml dose, the glutamate levels across the cytokine treatments and timings were significantly different (**** $p=2.457e-08$, Friedman rank-sum test). However, using the Wilcoxon pair-wise comparisons, only $IFN\gamma$ was significantly (* $p=0.013$) different from the non-treated group (figure 6.4 A). When two acute treatments were administered, the glutamate levels across treatments were also significantly different (**** $p=3.2e-09$, Friedman rank-sum test) for $LT\alpha$ (* $p=0.013$) and $LT\alpha + IFN\gamma$ (* $p=0.026$) groups compared to the untreated cultures (figure 6.4 B). Moreover, both $LT\alpha$ and $LT\alpha + IFN\gamma$ treatments resulted in higher concentrations after 48 hrs.

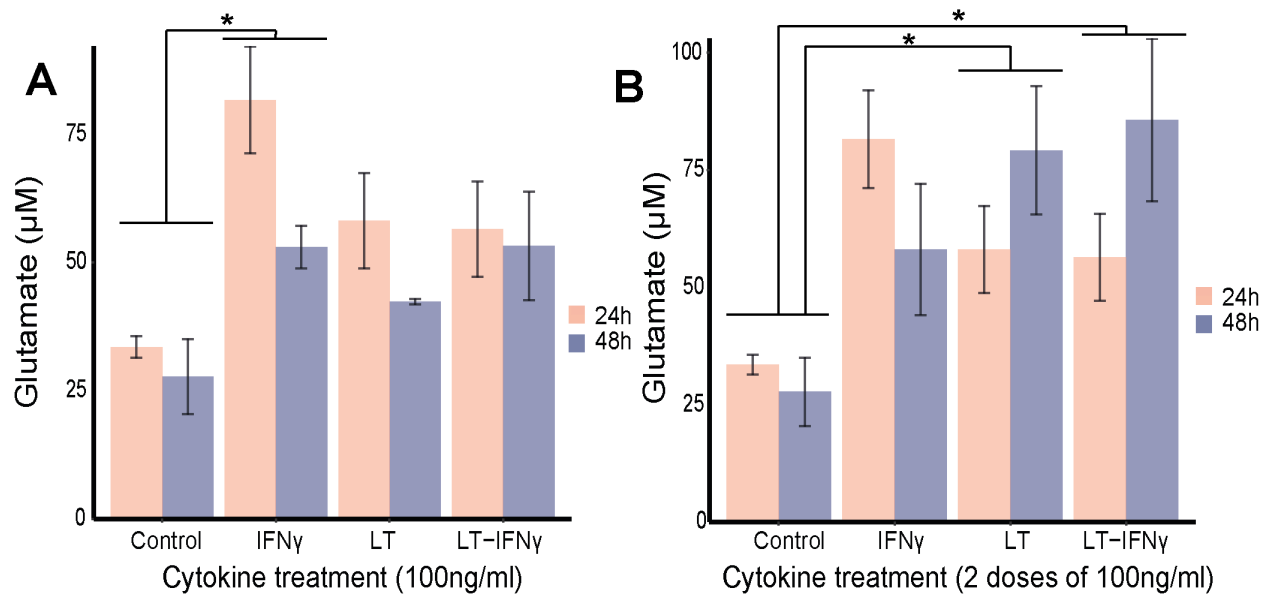


Figure 6.4: Two acute treatments of $LT\alpha+IFN\gamma$ to primary microglia induced a larger and more sustained release of glutamate than one treatment or an increment in dose. Mean $\pm$ SEM glutamate levels from replicates showing the statistical difference between controls and the cytokine treatments at different concentrations: (A) 100 ng/ml ($n=3$ Control, $n=3$ $LT\alpha$, $n=3$ $IFN\gamma$, $n=3$ $LT\alpha+IFN\gamma$) and (B) two acute treatments with 100 ng/ml ($n=3$ Control, $n=3$ $LT\alpha$, $n=3$ $IFN\gamma$, $n=3$ $LT\alpha+IFN\gamma$). Non-parametric Friedman test was performed across cytokine groups and timings and post hoc paired-wised Wilcoxon tests to compare groups (* $p<0.05$, ** $p<0.01$).

6.3.2 Characterisation of the cerebellar organotypic tissue cultures

To assess the preservation of the cytoarchitecture of the organotypic cerebellar slices, 8-10 DIV tissues were fixed and stained for MBP and Calbindin1 to observe the myelin

integrity and the morphology of the Purkinje cells (figure 6.5 D, E). In addition, the transparent flattened macroscopic appearance of the slice another method used to check the integrity of the slices, as well as the levels of PI+ cells (figure 6.5 A-C). The sagittal sections of the cerebellum exhibited the Purkinje axons longitudinally for adequate quantification of the paranodal axo-glial junction pathology. Caspr1 immunoreactivity in the axon was used to quantify the paranodal length and SMI32 to check the structural integrity of the axon (figure 6.5 F, G). One of the main limitations of this model was the lack of voltage-gated K_v1 channels at the juxtaparanodes. A number of different monoclonal antibodies for $K_v1.2$ channels and Caspr2 proteins (which co-localise in the juxtaparanode (Poliak et al., 1999)) were used under many experimental conditions by immunofluorescence without any visible signal localised within the juxtaparanode. Although there is not precise literature about the specific week in development in which these channels are expressed and clustered in the juxtaparanodal membrane underneath the myelin sheath, studies suggest that they might be present in the first weeks of development but they might not cluster in the membrane after at least one to two months of development (Sánchez-Ponce et al., 2012; Rama et al., 2017).

In order to induce paranodal axo-glial pathology, the organotypic cerebellar slices were treated with different molecules. Firstly, with direct administration of the pro-inflammatory cytokines TNF and $IFN\gamma$ at different concentration; secondly, with conditioned medium from TNF+ $IFN\gamma$ activated microglia; and thirdly, with the direct administration of glutamate at different concentrations (figure 6.5 H i., ii., iii.).

6.3.3 Inducing paranodal pathology with the pro-inflammatory cytokines TNF and $IFN\gamma$

In order to examine the effect of the direct administration of the pro-inflammatory cytokines, three doses of TNF and $IFN\gamma$ at a concentration of 50 ng/ml, and two doses at a concentration of 100 ng/ml were administered to cerebellar slices' culture medium every 24 hrs. In total, 1200 Caspr1-stained paranodes (200 per slice) from six cerebellar slices treated with three doses of 50 ng/ml, 800 Caspr1-stained paranodes from four cerebellar

6.3. Results

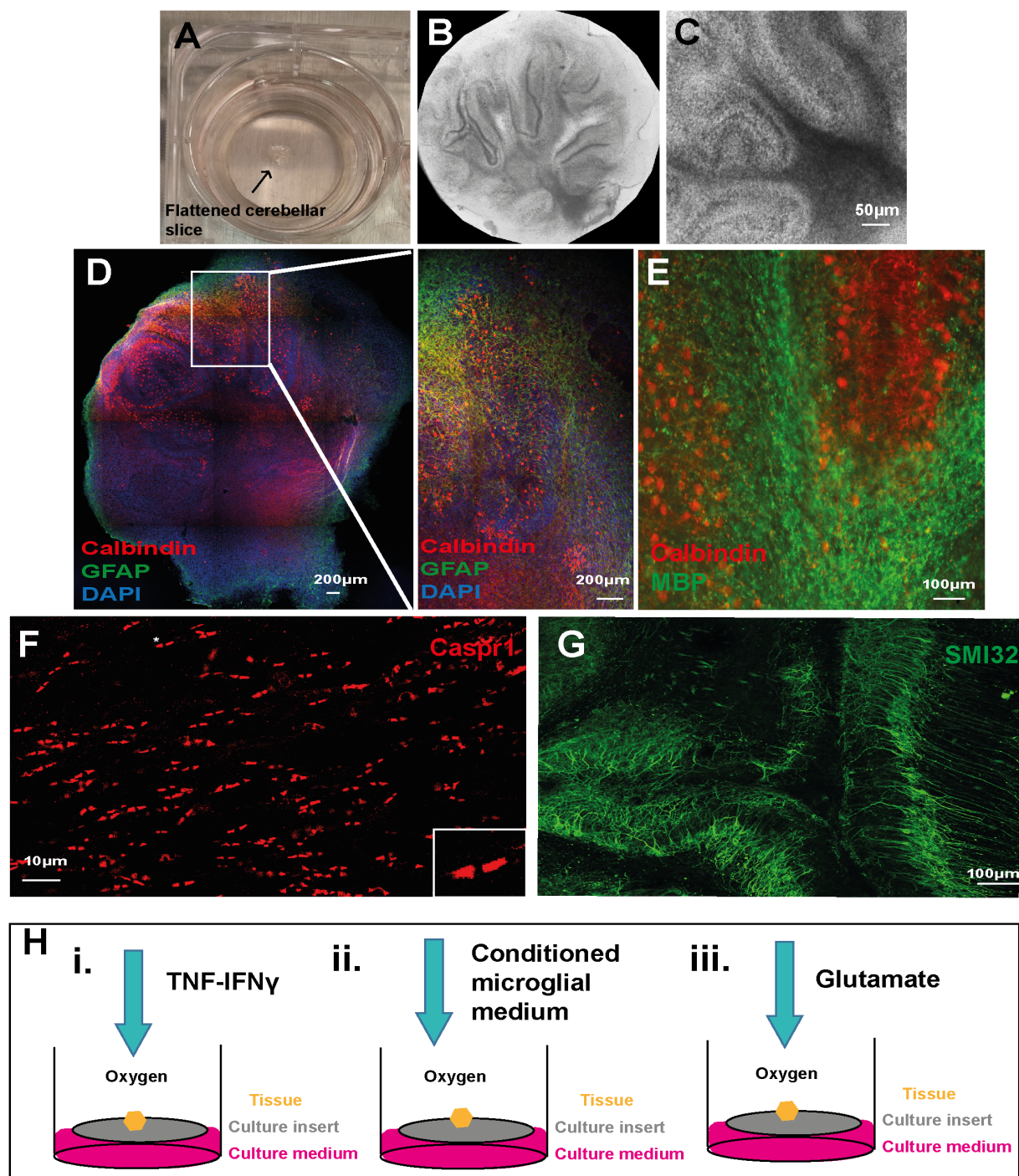


Figure 6.5: Characterisation of organotypic cerebellar tissue cultures with Calbindin, GFAP, Caspr1 and SMI32. (A) Image of a live flattened cerebellar slice. The slices were cut at 400 μm thickness and after 8-10 DIV healthy slices flatten to approximately 100 μm . (B, C) Live macroscopic images of cerebellar slices. (D) Confocal image of a cerebellar slice stained with Calbindin+Purkinje cells and GFAP+ astrocytes. (E) Confocal image of a cerebellar slice stained with MBP for myelin and Calbindin for Purkinje cells. (F) Confocal image of a cerebellar slice stained with Caspr1. (G) Confocal image of a cerebellar slice stained with SMI32. (H) Cerebellar slices were treated with the pro-inflammatory cytokines TNF+IFN γ (i.), microglial conditioned medium (ii.), and glutamate (iii.).

slices treated with two doses of 100 ng/ml, and 800 Caspr1-stained paranodes from four control cerebellar slices (figure 6.6 A) were analysed. Caspr1-stained paranodal length was 19.15 % longer on average in the 50 ng/ml group ($2.96 \pm 0.03 \mu\text{m}$; figure 6.6) than in the non-treated cerebellar cultures ($2.47 \pm 0.027 \mu\text{m}$; **** $p < 0.0001$; figure 6.6). Caspr1-stained paranodal length was 16.6 % longer on average in the 100 ng/ml group ($2.88 \pm 0.04 \mu\text{m}$; figure 6.6) than in the non-treated cerebellar cultures (**** $p < 0.0001$; figure 6.6). However, the paranodal length distribution between the different concentration and doses of TNF and IFN γ were not significantly different (figure 6.6 E). Nonetheless, when separating the paranodal length measurements by length ranges (figure 6.6 F), paranodal length distributions were slightly different. Although both of them had a smaller proportion of paranodes shorter than $2 \mu\text{m}$ compared to the untreated group, the proportion of paranodes longer than $2 \mu\text{m}$ and smaller than $4 \mu\text{m}$ was higher in the three doses 50 ng/ml group (figure 6.6 F, grey). There were similar proportions of highly disrupted long paranodes $> 4 \mu\text{m}$ (11.38% in the 100 ng/ml group and 11.34 % in the 50 ng/ml group figure 6.6 F, light and dark purple). Abnormally long Caspr1-stained paranodes can be seen in figure 6.6 C, D (asterisks). Moreover, for the purpose of observing if the levels of glutamate concentration in the supernatant of the tissues was similar to the microglial results, the supernatant of two cerebellar cultures treated with 100 ng/ml of TNF and IFN γ was quantified and they contained a mean glutamate concentration of $81 \mu\text{M}$.

6.3.4 Inducing paranodal pathology with microglial conditioned medium

In order to assess if the high-glutamate microglial conditioned medium could be sufficient to generate paranodal junction disruption, five cerebellar slices were treated with this media for 48 hrs. Specifically, they were treated with the microglial medium produced after two doses of 100 ng/ml of TNF+ IFN γ , as it was the condition which resulted in the larger glutamate release (Section 6.3.1). These cerebellar cultures had an average paranodal length of $3.23 \pm 0.04 \mu\text{m}$, which was 32.84 % longer on average than the non-treated ($2.47 \pm 0.027 \mu\text{m}$; **** $p < 0.0001$; 200 paranodes were analysed per cerebellar

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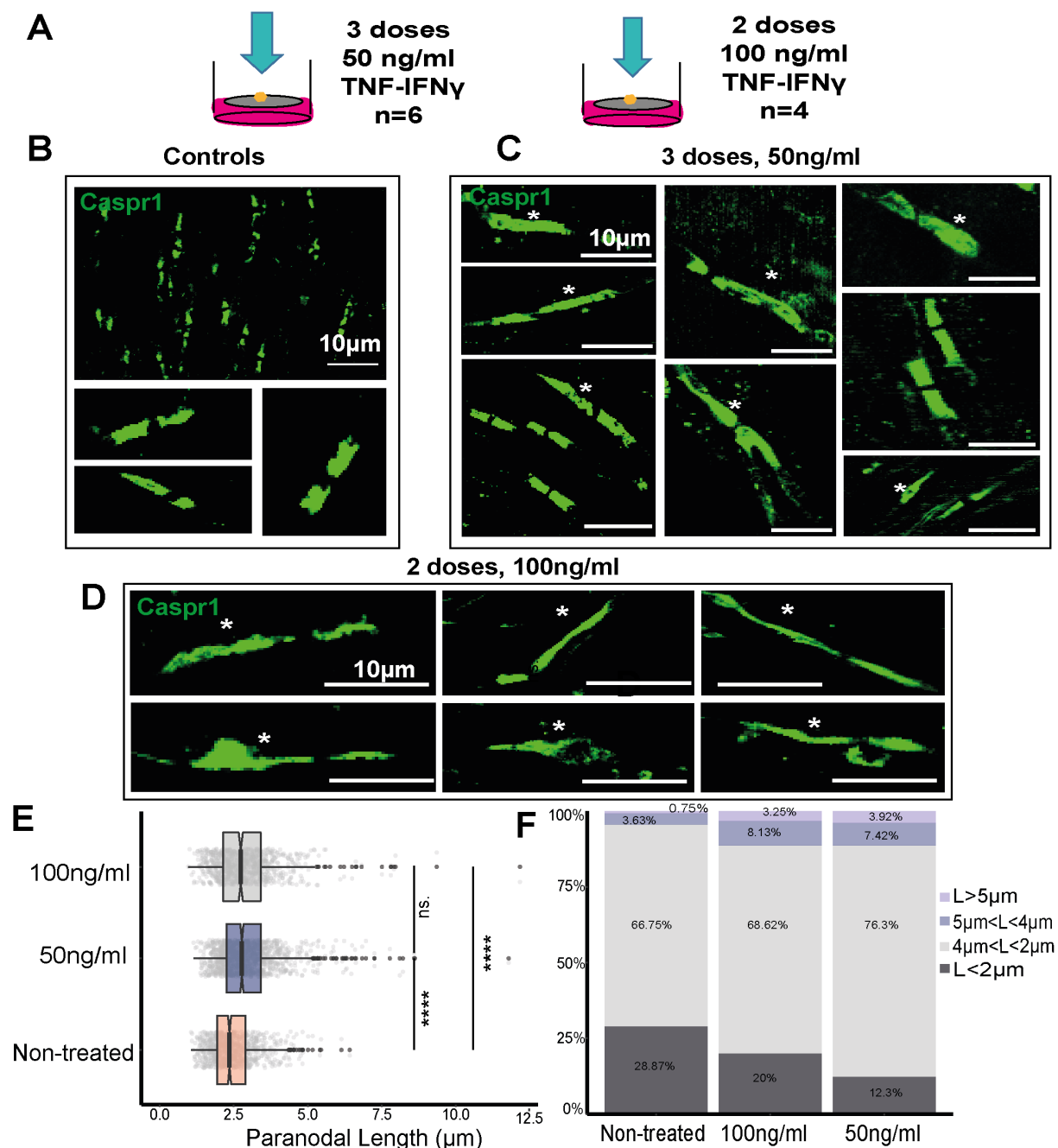


Figure 6.6: Cerebellar tissue slices treated with TNF-IFN γ contained a larger proportion of elongated paranodes compared to untreated slices. (A) Six cerebellar slices were administered 3 doses of 50 ng/ml TNF+IFN γ and four slices with 2 doses of 100 ng/ml. (B, C, D) Confocal images of Caspr1-stained paranodes from untreated cerebellar cultures (B), slices treated with 3 doses of 50 ng/ml (C) and slices treated with 2 doses of 100 ng/ml (D); asterisks point to long and disrupted paranodes. (E) Significantly different distributions of paranodal length between paranodes from cerebellar cultures treated with 100 ng/ml, 50 ng/ml and untreated (**** p < 0.0001, Mann-Whitney test). (F) The paranodal length distributions were separated into length ranges and represented in a bar plot. Slices treated with 100 ng/ml and 50 ng/ml had a larger proportion of paranodes longer than 4 μ m (purple) and 5 μ m (purple) than the non-treated cultures.

slice, figure 6.7 D). In order to examine if glutamate was one of the main cytotoxic factors in the microglial cytokine-conditioned medium, four cerebellar slices were treated with 75 μ M of glutamate. This concentration was chosen from our previous microglia cultures results; the average glutamate release concentration in the 100 ng/ml TNF+IFN γ group was $96.046 \pm 9.92 \mu$ M. However, the tissues were treated with 80 % of cytokine-conditioned medium and 20 % of the organotypic cerebellar nutrient medium, therefore, to give a final glutamate concentration of approximately 75 μ M. The cerebellar slices treated with 75 μ M of glutamate for 48 hrs also had longer paranodes (26.11 % longer on average) than the non-treated tissue slices ($3.12 \pm 0.027 \mu$ m; *** $p < 0.0001$; figure 6.7 D). Furthermore, the paranodal length distribution between the cultures treated with microglial medium versus the 75 μ M glutamate was not significantly different (figure 6.7 D). When separating the paranodal length measurements into length ranges (figure 6.7 E), the distributions were distinct, the microglial condition medium group had a larger proportion of paranodes longer than 4 μ m (17.8 % of the paranodes, light and dark purple, figure 6.7 B, C, E) in comparison to the glutamate group (11.26 % of the paranodes figure 6.7 E). Despite this result, both groups had a higher proportion of paranodes longer than 2 μ m, 4 μ m and 5 μ m compared to the non-treated group (figure 6.7 E).

6.3.5 Inducing paranodal pathology with direct administration of glutamate

In order to study the effect of glutamate administration on paranodal disruption, two cerebellar cultures were treated with two doses of glutamate at 100 μ M (in this group the paranodal length average was $3.44 \pm 0.14 \mu$ m) (separated by 24hrs). To compare all the distributions, a non-parametric Kruskal Wallis test was carried out (**** $p < 0.0001$, figure 6.8 A). Pairwise comparisons were calculated and all of the treatments were significantly different from the non-treated cultures (**** $p < 0.0001$, Wilcoxon rank-sum test, figure 6.8 A). Both groups treated with 50 ng/ml and 100 ng/ml of TNF+IFN γ were different from the microglia conditioned medium, 75 μ M and 100 μ M glutamate groups (**** $p < 0.0001$). Also, the data of these paranodal length distributions showed that the

6.3. Results

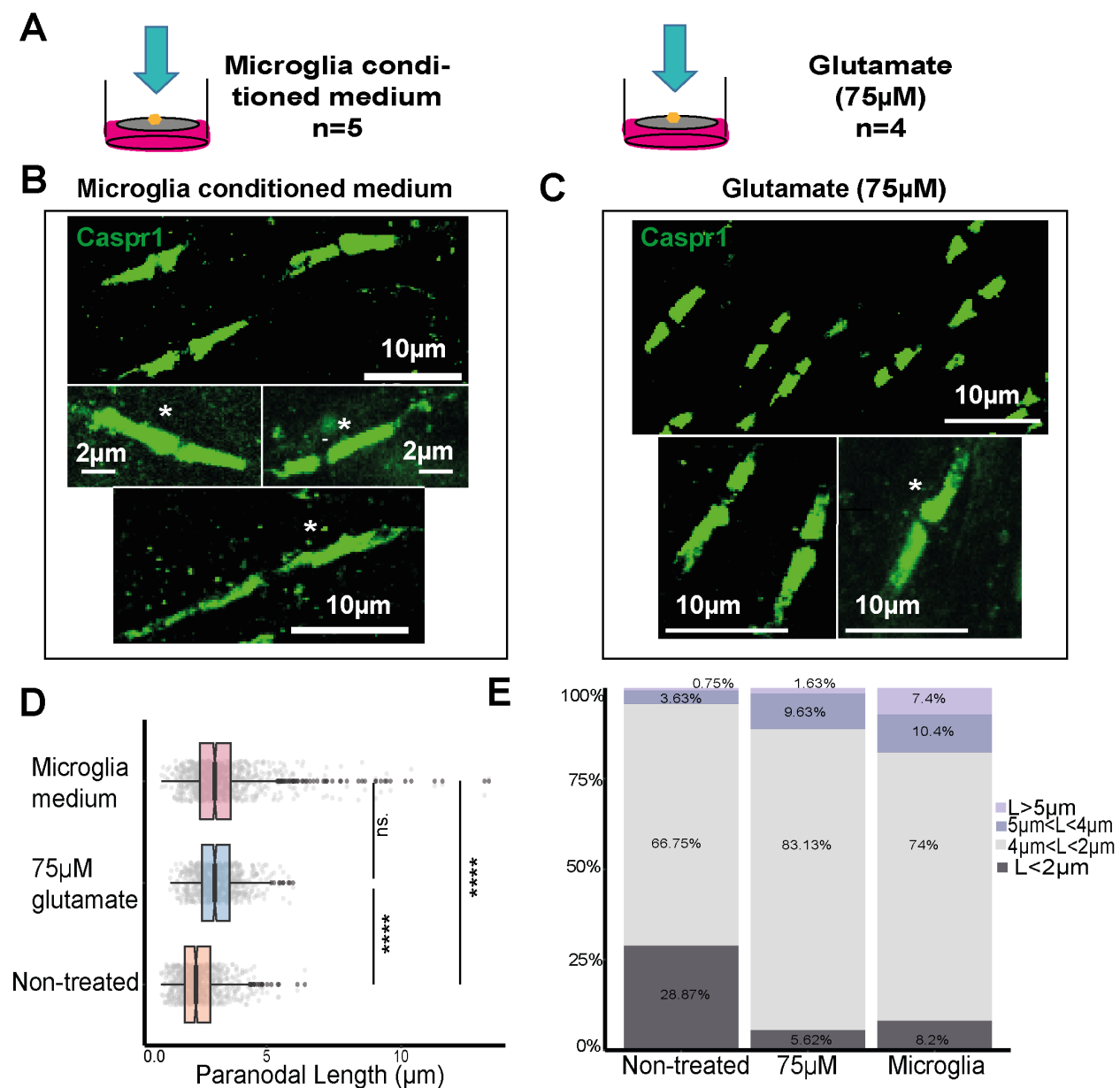


Figure 6.7: Cerebellar tissue slices treated with microglial-conditioned medium and a high concentration of glutamate contained a larger proportion of elongated paranodes compared to untreated slices. (A) Five cerebellar slices were treated with microglial conditioned medium and four with 75 μ M of glutamate. (B, C) Confocal images of Caspr1-stained paranodes of the Purkinje axons from cerebellar organotypic cultures treated with microglial medium and glutamate; the asterisks point to long and disrupted paranodes. (D) Significantly different distributions of paranodal length between Caspr1-measured paranodes in cerebellar cultures treated with microglial conditioned medium and 75 μ M of glutamate (**** $p < 0.0001$, Mann-Whitney test). (E) The paranodal length distributions were separated into length ranges and were represented in a bar plot. Microglia conditioned slices contained a larger proportion of paranodes longer than 4 μ m (purple) and 5 μ m (lighter purple) than the 75 μ M of glutamate group and the non-treated tissue cultures.

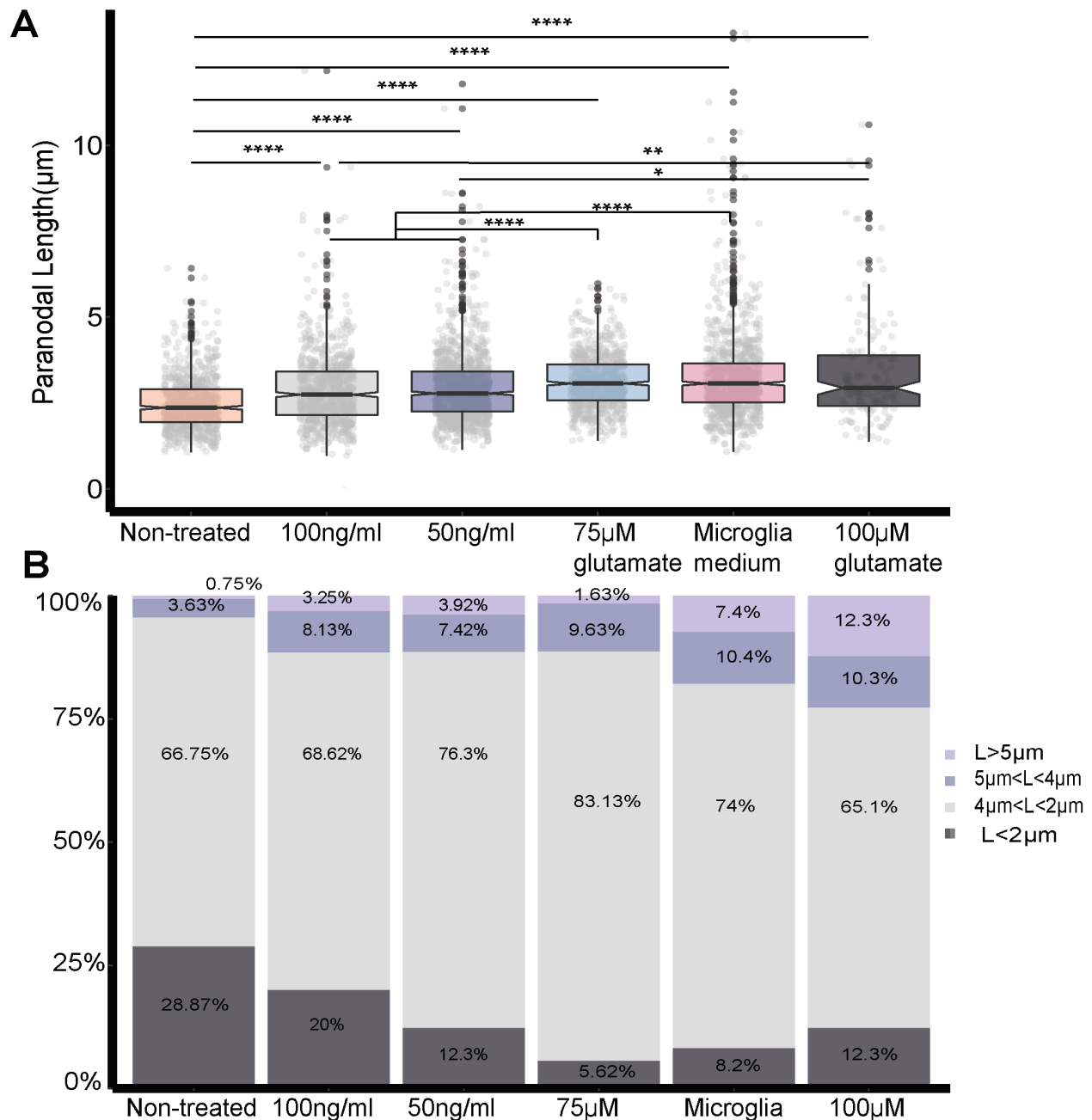


Figure 6.8: The proportion of elongated paranodes in cerebellar tissue slices treated with pro-inflammatory cytokines and a high concentration of glutamate was significantly larger than the untreated slices. (A) Box-plots showing the different paranodal length distributions between the treated and non-treated cultures (2 doses of 100 ng/ml- TNF+IFN γ , 3 doses of 50 ng/ml-TNF+IFN γ , 2 doses of 75 μM and 100 μM glutamate and microglial conditioned medium, **** $p < 0.0001$). (B) Bar plots of the same paranodal length distributions showing the proportion of paranodes in each data set of different lengths. The cultures treated with glutamate and microglia conditioned medium had larger proportions of paranodes longer than 4 μm (purple) and 5 μm (lighter purple) than the slices treated with cytokines.

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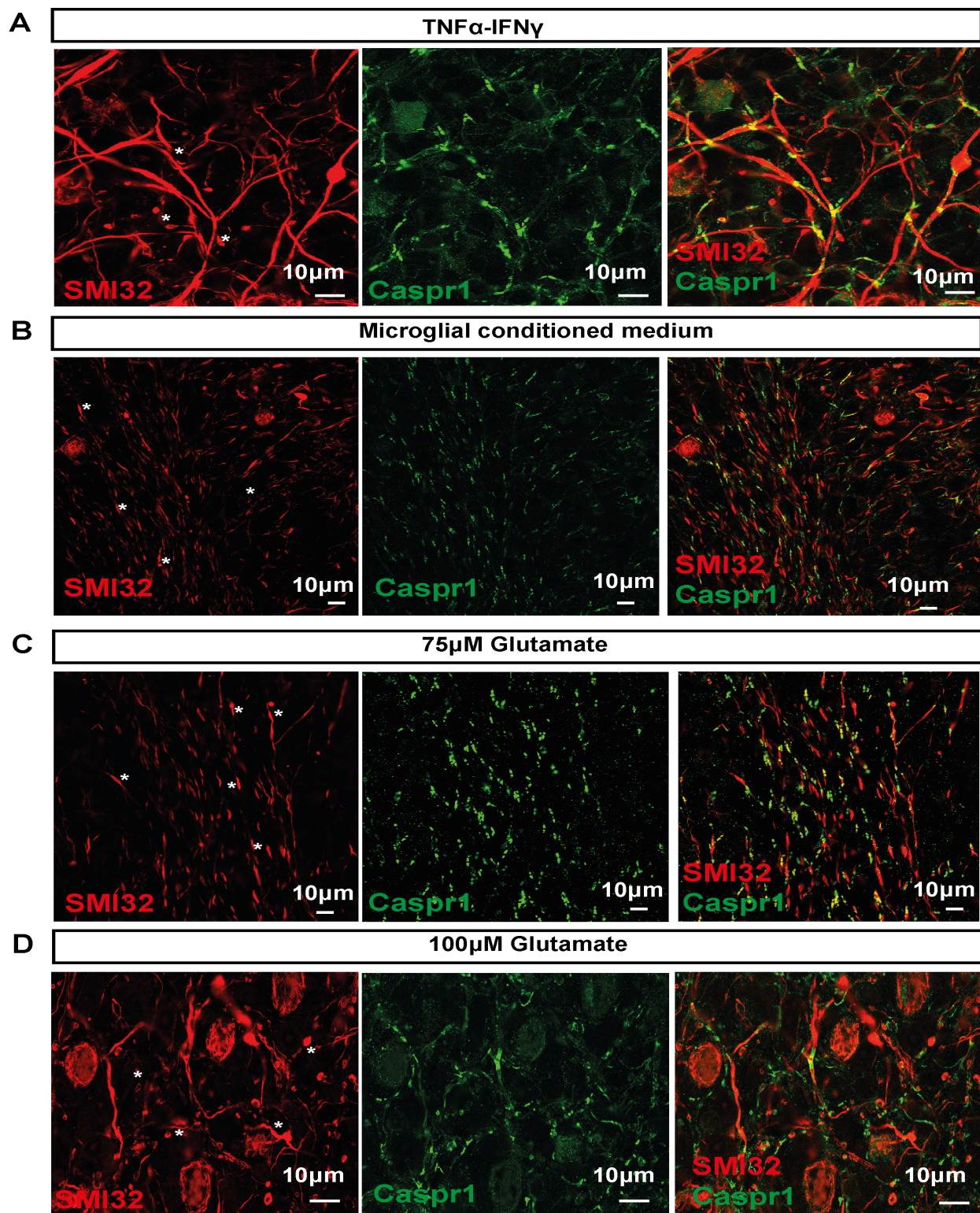


Figure 6.9: Axonal integrity in cerebellar tissue slices was compromised in the cerebellar slices treated with a high concentration of glutamate. Confocal images of Caspr1-SMI32 from cerebellar cultures treated with TNF+IFN γ (A), microglial conditioned medium (B), 75 μ M of glutamate (C) and 100 μ M of glutamate (D). The asterisks point the axonal transections and beadings.

cerebellar slices treated with glutamate or microglial conditioned medium contained larger proportions of paranodes longer than 4 μm than the slices treated with pro-inflammatory cytokines (figure 6.8 B). These results suggested that elevated glutamate levels can be highly toxic for axons. Although all the cerebellar slices contained axonal swellings (figure 6.9), the axons appeared more transected in the slices treated with higher levels of glutamate (figure 6.9 B, C, D). Notably, the axons from the slices treated with 100 μM glutamate were extensively degenerated and transected with a lot of beadings (figure 6.9 D).

6.3.6 NMDA antagonists

In order to definitively show that the effect of TNF+IFN γ was due to glutamate release, five slices were treated with two doses of 100 ng/ml of TNF+IFN γ and in the second dose the non-competitor NMDA antagonist MK-801 was administered (0.6 mM). MK-801 reversed or prevented paranodal damage as the paranodal length distribution of the slices treated with this antagonist had similar lengths to the non-treated slices (figure 6.10 A, B; the distributions were not significantly different). Moreover, the proportion of paranodes longer than 4 μm was greatly reduced in the MK-801 group compared to the cultures treated with the cytokines alone (from 11.38 % of the paranodes to 3.3 %, figure 6.10 C).

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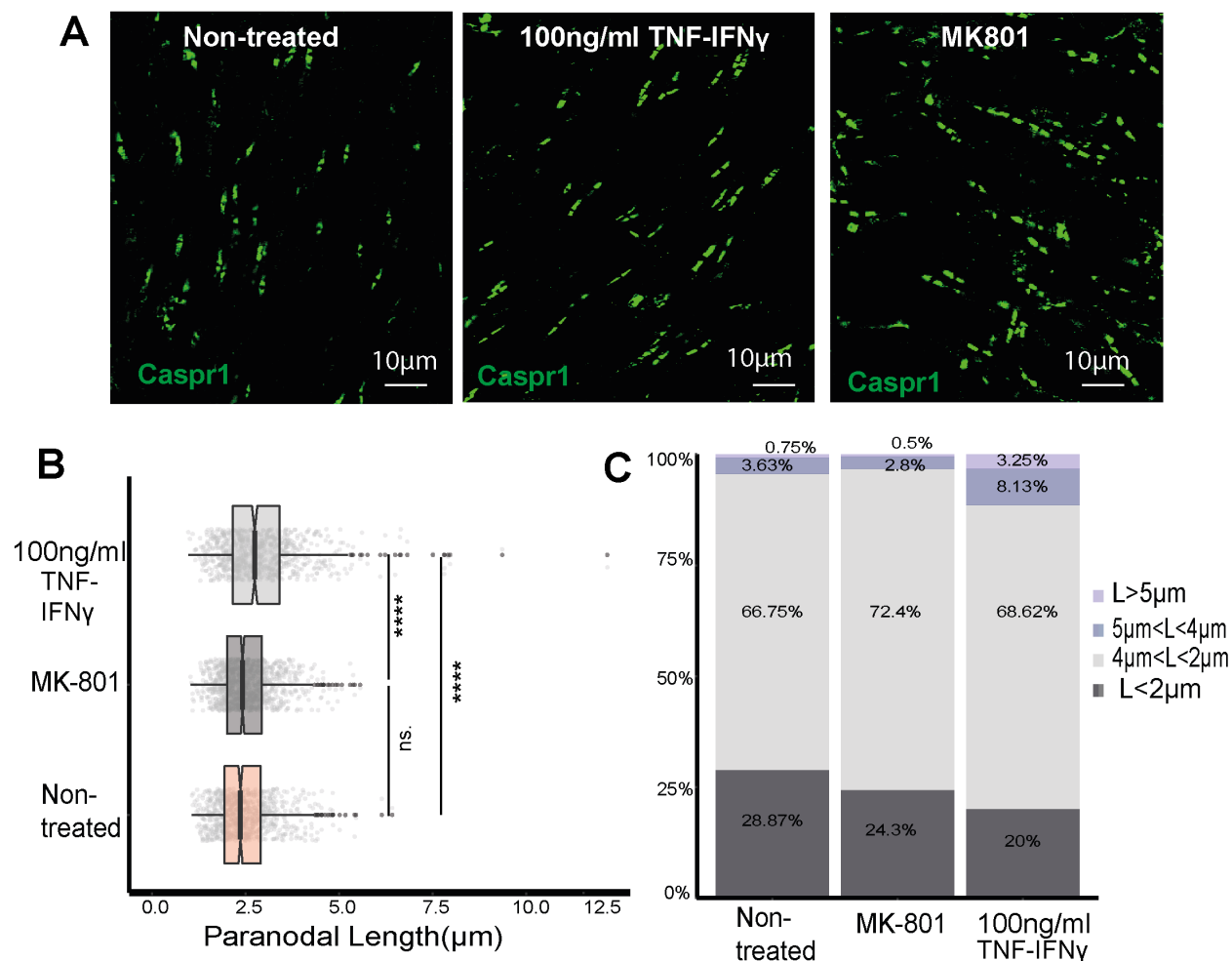


Figure 6.10: The proportion of elongated paranodes in cerebellar tissue slices treated with TNF+IFN γ was reversed after the administration of the NMDA antagonist MK-801. (A) Confocal images of Caspr1-stained paranodes from non-treated, two doses of 100 ng/ml TNF+IFN γ and MK-801 treated cultures. (B) Box-plot of the paranodal length distributions of the untreated (orange), MK-801 (dark grey) and two doses of 100 ng/ml TNF+IFN γ cultures (**** $p < 0.0001$, Mann-Whitney test). (C) Bar plot showing the same paranodal length distributions divided into different length ranges.

6.4 Discussion

6.4.1 Activated microglia release elevated levels of glutamate

As mentioned in previous chapters, microglia activation is one of the main pathological features observed in MS NAWM tissue. Microglia contribute to CNS homeostasis, and therefore abnormal activation of these cells is usually associated with the changes of surrounding cells and tissues, such as the pathological changes observed at the nodes of Ranvier. In this study, we hypothesised that microglia activation by pro-inflammatory cytokines could trigger an up-regulation of glutamate release, which is responsible for the pathological changes at the paranode. The primary microglia culture results confirmed an increase in glutamate release after the administration of the cytokines TNF, IFN γ and LT α compared to untreated microglia at different concentrations and timings. The combination treatment with 100 ng/ml TNF+IFN γ after 24 hrs induced more than double (2.89-fold) glutamate release, and after 48 hrs resulted in four times (4.088-fold) the untreated cultures. Nonetheless, the combination of LT α +IFN γ also resulted in high glutamate levels as LT α belongs to the same cytokine superfamily as TNF α and can bind to TNFR1. Thus, these results suggest that sustained exposure to a combination of pro-inflammatory cytokines generate higher glutamate levels than single acute treatments.

Microglia have been shown to express receptors for cytokines such as the IFN γ receptor (Tsuda et al., 2009), TNFR1 (Takeuchi et al., 2006) and TNFR2 (Veroni et al., 2010) and that they can initiate signal transduction cascades leading to the generation of more cytokines, chemokines and the activation of pro-inflammatory genes (Salim et al., 2016). The microglia cultures results have demonstrated that TNF and LT α have an enhanced effect on microglia when they work in synergy with IFN γ . In fact, multiple studies have characterised IFN γ as a potent inducer of TNF gene expression in microglia (Olmos and Lladó, 2014). For instance, IFN γ can induce the expression of CD40 in a cascade that involves the production of TNF (Nguyen and Benveniste, 2002). IFN γ can also promote the expression of MHCII molecules, along with CD40, to maintain persistent T-cell activation (Frei et al., 1987; Harms et al., 2013). Further, studies in Parkinson's disease have

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shown that IFN γ knock out (KO) mice were not much affected by a toxic pesticide that targeted TNF signalling (Mangano et al., 2012), and that the activation of microglia was reduced after treatment with a neurotoxic molecule (MPTP) compared to the wild type (Barcia et al., 2011). Furthermore, IFN γ can also up-regulate the expression of TNFR1 and TNFR2 (Wang et al., 2006). Although cooperation between LT α and IFN γ has not been as well characterised as TNF+IFN γ , our results in Chapter 4 have demonstrated the extensive activation of microglia triggered by these cytokines *in vivo*, and other studies in cerebral malaria showed a synergistic induction of CXCL10 in astrocytes by LT α and IFN γ (Bakmiwewa et al., 2016).

An increment in TNF levels and the up-regulation of TNFR1 and TNFR2 can increase the production of glutamate significantly as previous studies have demonstrated that TNF induces glutamate release by microglia via a gap junction/hemichannel system in an autocrine manner (Kuno et al., 2005; Takeuchi et al., 2006; Yawata et al., 2008). TNFR1 activation and an increment in glutaminase activity are part of this autocrine pathway (Takeuchi et al., 2006; Thomas et al., 2014). Microglia can synthesize glutamate by two main mechanisms. Firstly, via glutaminase hydrogenase which converts α -ketoglutarate to glutamate. Secondly, via glutaminase from extracellular glutamine brought into the cell via glutamine transporters (Newsholme and Calder, 1997; Nissim, 1999; Takeuchi and Suzumura, 2014). Pro-inflammatory cytokines such as TNF, IFN γ and LT α enhance specifically the expression of glutaminase and cell-surface hemichannels, such as connexin cx43 in activated microglia (Eugenín et al., 2001; Takeuchi et al., 2006). Additionally, other studies have shown that the cooperation of TNF+IFN γ not only induces glutamate release, it can also increase the production of reactive oxygen species via the activation of the transcription factor nuclear factor-kappa B (NF-kappaB) without affecting the viability of the cells (Mir et al., 2009).

The effects of these pro-inflammatory cytokines are highly relevant in the context of MS as recent studies have seen that TNF, IFN γ and LT α levels were increased in the CSF of progressive MS patients and correlated with meningeal inflammation, cortical demyelination (Matusevicius et al., 1996; Magliozzi et al., 2018) and activation of MHCII⁺ microglia

(Magliozzi et al., 2007, 2010). Furthermore, glutamate levels measured in the CSF of progressive MS patients with an increase of 1 or more points on the EDSS scale were also significantly higher than stable SPMS patients and controls (Sarchielli et al., 2003). The presence of an increased high pro-inflammatory cytokine profile in the CSF of progressive patients suggests that immune cells located in the meningeal subarachnoid space are releasing inflammatory factors within the CSF, creating an intracerebral milieu that sustains chronic compartmentalized inflammation and exacerbates pathology (Magliozzi et al., 2018). Therefore, it is likely that elevated levels of these pro-inflammatory cytokines would also be elevated in the blood (Navikas et al., 1996; Özenci et al., 2000). Active demyelination predominates around perivascular spaces (also known as the Virchow robin spaces), which are rich in CD20+ B and CD3+T infiltrates (Charcot, 1868; Serafini et al., 2004). Taking into consideration the extensive capillary density of the brain (Begley and Brightman, 2003), the diffusion of these pro-inflammatory cytokines into the surrounding brain parenchyma could generate some of the pathological features observed in NAWM MS tissue, such as microglial activation and axon injury (Graumann et al., 2003; Kutzelnigg et al., 2005a).

6.4.2 TNF and IFN γ induce paranodal disruption

The pro-inflammatory cytokines TNF and IFN γ can activate microglia as has been previously discussed. However, in cerebellar tissue slices, the presence of astrocytes, neurons and oligodendrocytes increases the system's complexity. The increment in length of Caspr1-stained paranodes in cerebellar tissue slices treated with three doses of 50 ng/ml (an average of 19.15 % increment) and two doses of 100 ng/ml (an average of 16.6 % increment) confirmed the significant role of these cytokines in the paranodal axo-glial pathology. Although TNF and IFN γ cannot have a direct effect on paranodal axo-glial pathology as no receptors have been found in the oligodendrocyte processes or the nodal axolemma, they can disrupt glutamate homeostasis, which is tightly regulated by astrocytes. Astrocytes generate lower glutamate levels in comparison to activated microglia but they are more effective in maintaining glutamate homeostasis (Liang et al., 2008).

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The effect of TNF in astrocytes can inhibit glutamate transporters by inducing downregulation of EEAT2/GLT-1 mRNA (Chao and Hu, 1994; Wang et al., 2003b; Sitcheran et al., 2005). TNF+IFN γ can also induce the release of high amounts of reactive oxygen species (Mir et al., 2009) that can block glutamate uptake by GLAST/GLT-1 receptors in astrocytes (Miralles et al., 2001). Furthermore, L-glutamate released from activated microglia downregulates astrocytic L-glutamate transporter expression in culture (Takaki et al., 2012). These facts are also relevant in MS and a previous analysis of the plasma from patients demonstrated that the presence of a polymorphism in the astrocytic promoter of EEAT2 was associated with higher glutamate plasma levels during a relapse (Pampliega et al., 2008). Therefore, the chronic activation of microglia and the suppression of astrocytic glutamate transporters would enhance extracellular glutamate concentration. In turn, glutamate could damage axo-glial junctions via NMDA receptors activation and calcium overload (Fu et al., 2011). In the interest of studying this effect, cerebellar slices were treated with glutamate and microglial conditioned medium containing high glutamate levels.

6.4.3 Glutamate induces cerebellar paranodal disruption

In our cerebellar tissues, activated microglia could have secreted other factors apart of glutamate, such as ROS or NO that could have also contributed to paranodal disruption. The slices treated with microglia conditioned medium had a higher proportion of paranodes longer than 5 μ m than the slices treated with 75 μ M of glutamate. However, it is important to note that this effect was not statistically significant. Thus, we can infer from the results that glutamate derived from chronically activated microglia was sufficient to trigger paranodal axo-glial pathology.

When cerebellar slices were treated with two doses of 100 μ M of glutamate, paranodes were disrupted, but axons appeared significantly transected, and full of neuritic beadings. The extent of transected axons in cerebellar cultures treated with high amounts of glutamate was higher than the ones treated with pro-inflammatory cytokines. This observation could be expected as cytokines also activate neuroprotective pathways, including

mitogen-activated protein kinase and the expression of nuclear factor- κ B (NF κ B) (Ghezzi and Mennini, 2001; Kamata et al., 2005). Moreover, the cytokine neurotoxic effect is indirect as they need to activate microglia first. Previous studies have demonstrated the presence of high levels of glutaminase expression in macrophages and microglia in active MS demyelinating plaques next to dystrophic axons (Werner et al., 2001). Also, glutamate derived from microglia has been linked to triggering neuritic beadings in primary culture neurons (Piani et al., 1991; Kim et al., 2010). Therefore, from all the treatments on the cerebellar tissue cultures, the pro-inflammatory cytokines might be the ones which mimicked best the pathology observed in MS NAWM regions due to the better preservation of the axon's integrity.

Establishing an inflammatory environment with the use of cytokines might be the best approach to model specific features of MS pathology, such as the paranodal axo-glial disruption. However, as mentioned before, glutamate might be the molecule causing damage to oligodendrocytes and myelin, which express glutamate receptors (Schwartz et al., 2003). In fact, elevated levels of glutamate have been related to oligodendrocyte death (Matute, 2007), generation of ROS (Bondy and Lee, 1993), reduction in compound action potentials (Li and Stys, 2000), paranodal splitting (Fu et al., 2009, 2011) and membrane nanoruptures (Witte et al., 2019). AMPA and NMDA are ion permeable glutamate receptors and whereas AMPA are located in the soma of the oligodendrocytes, NMDA can be found on their processes (Salter and Fern, 2005), specifically in the non-compacted myelinic channels which include the paranodal myelin loops (Micu et al., 2006; Saab and Nave, 2017). Furthermore, these channels can also be found clustered at the synaptic cleft, and therefore any modification in their function can lead to changes in AP generation. AMPA receptors are Na^+ permeable and their activation triggers depolarization, which in turn allows the removal of the NMDA receptor Mg^{2+} block (Nowak et al., 1984) which enables Ca^{2+} ions to flow in (Dingledine et al., 1999). High intracellular Ca^{2+} could be buffered in the neuronal soma by the endoplasmic reticulum (Subramanian and Meyer, 1997). But the only organelles located within the axoplasm at the nodes of Ranvier of long axons are mitochondria. A high concentration of Ca^{2+} within the nodal region can affect mitochondrial function by opening the transition pore (PTP) and leading to functional collapse (Su

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et al., 2009). Furthermore, it can activate proteins such as calmodulin-dependent protein kinase, which has been linked to the activation of nitric oxide synthase. In fact, a rise in intracellular NO can inhibit the mitochondrial respiratory chain complex IV, decrease ATP production and lead to energy failure, disrupted axonal transport and neuritic beading (Takeuchi and Suzumura, 2014). Interestingly, in a previous study in a mice EAE model, the earliest ultrastructural change within myelinated axons at lesions was mitochondrial pathology, which preceded axonal fragmentation, emerged closed to the nodes and was reversed by NO scavengers (Nikić et al., 2011).

Increased Ca^{2+} levels can also be very toxic to oligodendrocytes (Micu et al., 2006; Matute, 2007; Micu et al., 2016). If there is extensive Ca^{2+} overload, oligodendrocyte death can occur and might be prevented by glutamate receptor antagonists (Matute, 2007; Domercq et al., 2007; Matute, 2010). Any rise in Ca^{2+} levels could also activate calcium-sensitive proteases such as Calpain1 and phospholipase C triggering the degradation of cytoskeletal proteins and disrupting the integrity of paranodal junctions. Since paranodal axo-glial junctions are points of union between the axon and myelin, cellular cascades within the oligodendrocyte and the axoplasm triggered by glutamate should be considered as part of the system underlying the pathological changes observed in the cerebellar tissue cultures.

6.4.4 Therapy strategies: targeting NMDA receptors?

Previous studies in EAE models with Lewis rats have observed that the administration of NMDA antagonists such as MK-801 (Bolton and Paul, 1997), memantine (Paul and Bolton, 2002) and amantadine (Sulkowski et al., 2013) improved the disability scores, reducing axonal loss, BBB permeability, lesions and inflammatory cytokines. In contrast, antagonists for group I metabotropic glutamate receptors (mGluRs) did not have any effect (Sulkowski et al., 2013). Similar results have been shown with antagonists for AMPA or kainate receptors (NBQX) (Smith et al., 2000; Kanwar et al., 2004; Zhai et al., 2015). Furthermore, other studies with inhibitors for glutamate release, such as riluzole also showed reduced neurological deficits, leukocyte infiltration, axonal damage, and demyelination in a MOG-induced EAE mouse model (Gilgun-Sherki et al., 2003). In the cerebellar tissue

slices, the administration of the NMDA antagonist MK-801 along with TNF and IFN γ reversed or halted the paranodal pathology previously observed without the blocker. Moreover, the proportion of longer paranodes ($> 4\mu\text{m}$) was significantly reduced with the antagonist. Therefore, glutamate signalling should be considered as the key molecular cascade driving paranodal pathology in the NAWM MS tissue (Chapter 3). Our results also suggest that activated microglia, as both responders and generators of pro-inflammatory cytokines, might be the primary cell type triggering axo-glia pathology at the nodes of Ranvier. Thus, the interaction between microglia derived glutamate that is produced in response to activation by TNF and IFN γ and its direct association to paranodal axo-glia pathology in the context of MS is a novel and exciting result. This data could help in the development of new therapies to target these subtle changes at the paranodes, which are likely to have a significant impact on the quality of life of people with MS.

After the promising animal studies mentioned above, multiple clinical studies with glutamate signalling antagonists have been performed. One of the first ones was carried out with amantadine in 93 MS patients of all types, and they observed reduced fatigue (Krupp et al., 1995). However, various clinical trials performed with memantine did not find any significant difference (Lovera et al., 2010; Mehta et al., 2010; Saint Paul et al., 2016), except one that observed an improvement in visual acuity (Thurtell et al., 2010). Another one had to be halted due to worsening of neurological deficits (Villoslada et al., 2009). A clinical trial of riluzole on early stage MS patients did not find any significant difference (Waubant et al., 2014). However, most of these trials did not separate patients by types, which could have been an important factor obscuring the results as MS is a highly heterogeneous disease in which multiple molecular mechanisms may occur throughout different clinical phases. In addition, most of the studies had a small sample size and inadequate power (Macrez et al., 2016). Nonetheless, the results from a recent randomised, double-blind, placebo-controlled clinical trial with 440 SPMS patients (MS-SMART) with three different drugs: riluzole (glutamate blocker), amiloride (sodium channel blocker) or fluoxetine (serotonin reuptake inhibitor) (Nourbakhsh et al., 2015; Connick et al., 2018) did not show any improvements when brain atrophy was measured by MRI imaging,

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and cognitive deficits with cognitive tests and physician and patient perceptions of effectiveness. However, riluzole preferentially blocks voltage-gated Na_v channels preventing AP propagation and therefore glutamate release (Lazar and McIntyre, 2019). It does not have an effect on microglia but it can enhance glutamate uptake by astrocytes (dos Santos Frizzo et al., 2004).

These clinical trial results suggest that therapies targeting glutamate signalling should be cell-specific to be of clinical utility and to avoid highly adverse effects given the ubiquitous distribution of glutamate receptors in the CNS (Macrez et al., 2016). For instance, NMDA receptors expressed in myelin and oligodendrocytes are composed of GluN1 or GluN3 subunits, which are more sensitive to glycine than NMDA receptors containing NR1 and NR2 subunits (Stys and Lipton, 2007). Therefore, targeting sensitive glycine receptors might be most effective during demyelination episodes and have minimal effects on basal neurotransmission (Macrez et al., 2016). Another potential neuroprotective strategy would be reducing microglial glutaminase activity or blocking microglial hemichannels (Cx32, Cx36, and Cx43), especially when they are activated (Eugenín et al., 2001; Takeuchi et al., 2006).

An alternative approach would be to remove the inflammatory stimuli by blocking TNF, $IFN\gamma$ and $LT\alpha$ signalling pathways. Previous strategies directed to inhibit TNF included IgG monoclonal antibodies (infliximab, adalimumab, lenercept and etanercept) that sequester soluble TNF and transmembrane TNF (McCoy and Tansey, 2008) were ineffective in a clinical trial for MS and also initiated unexpected relapses in some patients (Arnason et al., 1999). This suggests that receptor specific antagonists may be needed as it is the TNFR1 receptor that is involved in its cytotoxic actions. Other therapies included the antibiotic minocycline, thalidomide and TACE inhibitors that block soluble TNF production. Minocycline decreases TNF synthesis, inhibits matrix metalloproteases, reduces COX-2 activity and prostaglandin E2 production, and attenuates apoptosis (Zhu et al., 2002; Wang et al., 2003a; Matsuki et al., 2003; Wang et al., 2004). In fact, minocycline as a microglial inhibitor was an effective therapy to reverse paranodal pathology in a previous EAE mouse model study (Howell et al., 2010).

6.5 Conclusions

In conclusion, our results have demonstrated that pro-inflammatory cytokines $\text{TNF}+\text{IFN}\gamma$ and $\text{LT}\alpha+\text{IFN}\gamma$ can induce a significant release of glutamate by activated microglia. Further, elevated glutamate levels would be sufficient to trigger paranodal axo-glial disruption and affect axonal integrity. Furthermore, this pathology could be reversed by blocking the glutamate signalling pathway, such as the NMDA receptors. However, based in our results and previous clinical trials, we can speculate that either targeting more specifically the microglial glutamate signalling pathways or reducing the inflammatory stimuli that activate them, would improve paranodal axo-glial pathology, and consequently the overall neurological function.

7

Final discussion

Our hypothesis for this investigation was: *the disruption of the structure of paranodal axo-glial junctions by microglial and macrophage activation in MS NAWM leads to an elongation of the non-compacted myelin loops from the axolemma, which in turn produces defects in AP conduction*. In this final chapter, I discuss the extent to which the presented results support or reject this hypothesis, some of the limitations of this work and the final conclusions of the study.

7.1 Paranodal Elongation and $K_v1.2$ channel dislocation in MS NAWM tissue

The histopathological analysis has shown systematically that a significant proportion of Caspr1 immunopositive elongated paranodes within the MS NAWM tissue were closely associated with the chronically activated microglia surrounding them. This change was

also associated with the displacement of the juxtaparanodal voltage-gated K_v1 channels towards the paranode (figure 7.1). Moreover, the dislocation of these channels was strongly associated in each tissue block with the proportion of Caspr1-stained elongated paranodes longer than 4 μm . The structural representation of this elongation could mean a complete or partial detachment of the myelin loops from the axolemma, and maybe between the loops themselves (figure 7.1). This was inferred from our RGB line profile data after computing the overall average Caspr1 profile between MS and non-neurological tissue; the intensity of this protein was greater along the quantified axolemma length, suggesting that some proteins might have lost their anchorage and diffused along the membrane (figure 7.1). Therefore, the elongation of the paranodal axo-glial junctions could indicate an ultra-structural disruption of their structural and functional integrity.

The paranodal axo-glial junctions are located at the intersection between the axon and the oligodendrocyte and it has been suggested that damage to this junction could be an initiating factor in both demyelination and axon damage. Therefore, both the oligodendrocyte and the axon are interdependent entities of the pathology and could play an essential role in PNJ disruption. For instance, myelin deficient rats (Arroyo et al., 2002), hypomyelinated shivered mice lacking MBP (Rasband et al., 1999), Jimpy mice lacking PLP (Jenkins and Bennett, 2002), as well as mice lacking certain galactolipids (Dupree et al., 1999; Poliak et al., 2001), sulfatides (Ishibashi et al., 2002) and gangliosides (Susuki et al., 2007) showed structurally aberrant paranodal axo-glial junctions. One of the mechanisms of oligodendrocyte injury at this site could be the disruption of the axo-glial transport along the myelinic channels of proteins that maintain the adequate structure and function of these junctions (Stassart et al., 2018). Another mechanism could be by disrupting mitochondrial physiology within oligodendrocytes. However, conditional Cox10 mutant mice, in which oligodendrocytes and Schwann cells fail to assemble mitochondrial cytochrome c oxidase (COX), showed no signs of demyelination or axonal pathology in the CNS, suggesting that oligodendrocytes can survive via glycolysis and provide lactate to myelinated axons (Fünfschilling et al., 2012). Alternatively, the changes to the structure of these junctions could be caused by the activation of calcium-sensitive proteinases, such as Calpain1, if glutamate levels are not kept at homeostatic levels.

7.1. Paranodal Elongation and $K_v1.2$ channel dislocation in MS NAWM tissue

Axoplasmic integrity could be also compromised. We have shown here that there is an association between elongated Caspr1 stained paranodes and the presence of dephosphorylated neurofilaments, which is consistent with previous studies of axonal degeneration in MS tissue within lesions and NAWM (Trapp et al., 1998; Howell et al., 2010). The phosphorylation of neurofilament proteins is considered necessary in the regulation of the axonal transport rate and stability (Shea et al., 2003). In fact, impaired mitochondrial movement has been previously observed in MS tissue and associated with the presence of glutamate and TNF (Kim et al., 2010). Furthermore, chronically activated microglia and macrophages, which characterise the NAWM studied here, could be secreting increased levels of ROS, NOS and glutamate that can lead to mitochondrial dysfunction (Lu et al., 2000; Van Horssen et al., 2008; Su et al., 2009; Haider et al., 2011).

Mitochondria produce ROS to perform oxidative phosphorylation and synthesise ATP. Thus, in environments where ROS levels are increased, mitochondrial DNA mutations can accumulate and compromise the oxidative phosphorylation efficiency. This damage can lead to failure of the energy dependent $\text{Na}^+/\text{K}^+-\text{ATPase}$, which leads to an increase in the intra-axonal Na^+ levels and therefore in the intra-axonal Ca^{2+} by reversing the function of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Stys et al., 1992). Therefore, axonal transport and conduction could be disrupted in these conditions, as they are highly energy-demanding processes. ROS and NOS can also activate enzymes including myeloperoxidase, xanthine and NADPH oxidases that can drive structural axonal degeneration (Gray et al., 2008). These enzymes could play a role in damaging the integrity of the cytoskeletal proteins that maintain adequate PNJ structure. In fact, mice with conditional null mutations in the genes of the paranodal cytoskeletal proteins βII spectrin (Zhang et al., 2013), αII spectrin (Huang et al., 2017), and 4.1.B (Cifuentes-Diaz et al., 2011; Buttermore et al., 2011) also displayed disrupted paranodal junctions and voltage-gated $\text{Kv}1$ channel displacement, showing the importance of these proteins in preserving structural integrity at these regions.

The disruption of the paranodes is thus likely to contribute to MS NAWM diffuse axonal injury that is characterised by activated microglia (Kutzelnigg et al., 2005a; Moll et al.,

2011; Singh et al., 2013). Overall, these results are especially relevant in progressive MS since paranodal pathology can affect the physiological integrity of axons and can lead to incremental deficits if the inflammatory stimuli persist.

7.2 Chronic neuroinflammation triggers paranodal axo-glial pathology

The next objective of this project was to investigate if chronically activated microglia and macrophages could generate the observed paranodal axo-glial junction changes in the MS NAWM tissue, in the absence of focal demyelinating lesions. Therefore, we continued studying this pathology in a chronic model of meningeal inflammation generated with lenti-viral vectors encoding the genes for the pro-inflammatory cytokines $LT\alpha$ and $IFN\gamma$. Elevated levels of these pro-inflammatory cytokines have been observed in the CSF and blood of MS patients (Rieckmann et al., 1994; Navikas et al., 1996) and within MS lesioned tissue (Selmaj et al., 1991). These cytokines are usually produced by B and T lymphocytes (Stein et al., 2018) and although the proportion of lymphocyte infiltrates in the WM parenchyma is small in MS tissue, a high density of lymphocytes has been observed in the perivascular spaces, and the subarachnoid space of the meninges of progressive MS brains (Serafini et al., 2004; Magliozzi et al., 2010, 2018). Moreover, elevated levels of pro-inflammatory cytokines such as TNF, $LT\alpha$ and $IFN\gamma$ in the CSF of progressive MS correlate with cortical demyelination (Matusevicius et al., 1996; Magliozzi et al., 2018) and widespread MHCII+ microglia and macrophage activation (Magliozzi et al., 2007, 2010). These pro-inflammatory cytokines activate microglia via the $IFN\gamma$ and TNFR1/TNFR2 receptors (Tsuda et al., 2009; Takeuchi et al., 2006; Veroni et al., 2010). Therefore, upon activation, they can initiate signal transduction cascades leading to the generation of more cytokines, chemokines, ROS, NOS, glutamate and the activation of pro-inflammatory genes (Salim et al., 2016).

This novel rat model which lacks WM demyelination, in contrast to the widely used EAE models enabled us to study the direct effect of chronic inflammation at the nodes

7.2. Chronic neuroinflammation triggers paranodal axo-glial pathology

of Ranvier. Previous data from this lab has shown that the persistent production of these cytokines in the subarachnoid space gives rise to a widespread chronic activation of microglial in both grey and white matter. The current data shows that the paranodal axo-glial pathology observed in MS NAWM tissue can be replicated in this model. Paranodal elongation and juxtaparanodal voltage-gated K_v1 channels displacement was observed in the corpus callosum, cingulate and external capsule of these animals, and correlated with the number of microglia and astrocytes that were surrounding them. The analysis of the paranodal pathology in the brain of these animals could be considered a more representative model of the human disease than others since the study was carried out within the brain and not the spinal cord and is not accompanied by significant immune cell infiltration, similar to the MS NAWM. In contrast, previous EAE and conditional knock-out models studied paranodal pathology in the large axons of the spinal cord (Pillai et al., 2009; Howell et al., 2010; Zhang et al., 2013). The results obtained from the brain and spinal cord axons can vary significantly due to axon size. In fact, studies in human post-mortem MS tissue and a mouse EAE model, have shown that small-diameter axons were more prone to axonal degeneration compared to larger ones (DeLuca et al., 2006; Nikić et al., 2011).

Regarding the underlying mechanisms triggering pathology, axonal loss in the spinal cord of EAE models is usually accompanied by the presence of CD4⁺ T-cells, which differs from the described pathology in human post-mortem MS NAWM tissue (Lassmann and Bradl, 2017). In this rat model, inflammation was initiated by chronic production of pro-inflammatory cytokines in the meninges. The extensive activation of microglia and the absence of focal demyelinating lesions in the WM after 90 days post-injection, demonstrates that it can be used as a model of chronic CNS inflammation.

In conclusion, these findings suggest that the chronic presence of a pro-inflammatory milieu in the CSF space can give rise to diffuse glial activation and pathology in the WM, in the absence of focal demyelination. Therefore, it is likely that microglia and astrocytes interact with one another to mediate paranodal axo-glial pathology, involving a change in the balance of neuroprotective and neurotoxic mechanisms.

7.3 The effect of axo-glial pathology on AP conduction

The described structural changes at the nodes of Ranvier are likely to have functional consequences for AP conduction, including slowing of AP propagation, alteration in AP waveform, increased ATP consumption and loss of metabolic support to the axon. Therefore, we hypothesised that these paranodal axo-glial changes observed throughout the NAWM in MS could give rise to AP disruption and in turn, to non-lesion associated symptoms such as fatigue. However, the contribution of paranodal axo-glial junction pathology to conduction deficits has been rarely explored. Although it could be expected that the conduction deficits provoked by these changes would be less severe than those generated by demyelinating lesions (Smith, 1994), they can still be sufficiently disruptive due to the critical role of the node in adequate AP conduction. The data generated by our biologically plausible computational axonal model comprising 21 nodes, demonstrated the pivotal role of the paranode/juxtaparanode in AP conduction. The progressive increment of the peri-axonal space width in the paranodal and juxtaparanodal compartments led to a rapid decrease in conduction velocity, with the smaller-diameter axons more susceptible than the larger ones (figure 7.1). The increment of the peri-axonal space width generated a progressive decrease in resistance and a progressive increment in capacitance at these regions to represent the ultra-structural changes observed in human post-mortem MS tissue in biophysical terms. The conduction velocity decreased significantly in the seven simulated diameters as a greater area of axonal membrane had to be depolarised. This velocity reduction is consistent with previous models of paranodal pathology (Babbs and Shi, 2013; Volman and Ng, 2013; Kohan et al., 2017).

Furthermore, the displacement of K_v channels from the juxtaparanode to the paranode decreased the velocity slightly in our model since the hyperpolarising currents of these channels could have decreased the rate of the initial depolarising Na^+ current. However, the dislocation of these channels was minimal as other K_v channels are located in the node to repolarise the signal. Regarding the waveform of the AP, the amplitude was decreased when the resistances of the paranodes and juxtaparanodes were decreased, and the K_v channels were dislocated. This decrement could be explained due to the earlier

7.3. The effect of axo-glial pathology on AP conduction

presence of the repolarising K^+ current, and since a greater area of axonal membrane had to be depolarised. The widths of the AP were decreased in the conditions where the K_v channels were dislocated as the repolarising current was greater than without channel displacement. However, the widths increased slightly, making the AP waveform broader, when the resistances of the paranode and juxtaparanode were significantly decreased.

The axon diameter is directly proportional to the myelin sheath thickness (Rushton, 1951; Goldman and Albus, 1968). Therefore, the smaller the axon diameter, the thinner its myelin sheath and the lower its resistance. Furthermore, previous studies have suggested that increasing the number of myelin loops around an axon increases the conduction velocity (Castelfranco and Hartline, 2015). However, they also showed that this increment in velocity reached a plateau when the fibre diameter was $1\text{ }\mu\text{m}$ and was wrapped by ten myelin loops. Therefore, the conduction velocity of smaller axon diameters, which are wrapped by a lower number of myelin sheaths is more sensitive to pathological changes such as paranodal disruption. This theory would mean that the smaller axons would be highly dependent on the adequate structure of the PNJ for current shunting and fast and efficient AP conduction. This dependence on diameter is consistent with previous studies in MS tissue (DeLuca et al., 2006) and in a mouse EAE model (Nikić et al., 2011), which determined that the thinner axons were more prone to transection. Furthermore, our data showed that the smaller the axon, the less the number of consecutive disrupted nodes were needed for conduction failure (figure 7.1). For example, when the peri-axonal space widths of the paranodal and juxtaparanodal compartments were increased, an axon with a core diameter of $0.6\text{ }\mu\text{m}$ needed the disruption of five consecutive nodes. In contrast, a $0.8\text{ }\mu\text{m}$ axon, the disruption of eleven nodes was necessary. Also, we have shown that the specific pattern of disrupted nodes along an axon can influence the conduction in different ways. Depending on the axon diameter, the number of consecutive disrupted nodes and the number of healthy nodes in between the disrupted nodal patches, the conduction velocity was reduced by different amounts and in some cases led to conduction failure, mainly in the small-diameter axons.

These results are relevant in the MS context as activated microglia and macrophages in

NAWM regions are usually scattered, as well as the diffuse axonal changes. Therefore, the different patterns of nodal injury that we have simulated could happen in different regions of the brain depending on the distribution and extent of microglial activation. Furthermore, these conduction deficits associated with paranodal structural changes could be accompanied by mitochondrial dysfunction in MS. If axons experience a significant velocity reduction and are energetically deficient, the conduction would be extremely inefficient. Therefore, this could be one of the underlying explanations of fatigue in MS patients.

7.4 Glutamate plays a neurotoxic role in PNJ pathology

The last objective of this study was to explore the molecular mechanisms underlying the paranodal axo-glial pathology changes. Previous literature has demonstrated that activated microglia and macrophages can release a wide range of molecules including ROS, NOS, glutamate and pro-inflammatory cytokines (Salim et al., 2016). We decided to study the effect of glutamate on PNJ pathology since NMDA receptors have been observed in the non-compacted myelin (Salter and Fern, 2005; Piña-Crespo et al., 2010; Micu et al., 2016; Saab and Nave, 2017), and elevated glutamate levels have been quantified in acute MS lesions and NAWM tissue compared to non-neurological controls in MRS studies (Srinivasan et al., 2005; Tisell et al., 2013; Azevedo et al., 2014), and in the CSF of MS patients (Sarchielli et al., 2003). Our hypothesis pointed to activated microglia as the cells provoking this pathology, and therefore as the main sources of glutamate (figure 7.1). In our study using primary microglial cultures, we showed that a combination and repeated effect of the pro-inflammatory cytokines TNF+IFN γ and LT α +IFN γ could lead to the release of high levels of glutamate. This data is consistent with previous studies that have demonstrated that the isolated effect of TNF activates the TNFR1 in microglia generating glutamate synthesis and glutamate release by a hemichannel in an autocrine manner (Takeuchi et al., 2006; Yawata et al., 2008). However, our data demonstrated that the effect of TNF or LT α on microglial glutamate release could be amplified if they work in synergy with IFN γ . This result is coherent with previous studies, which have demonstrated that

7.4. Glutamate plays a neurotoxic role in PNJ pathology

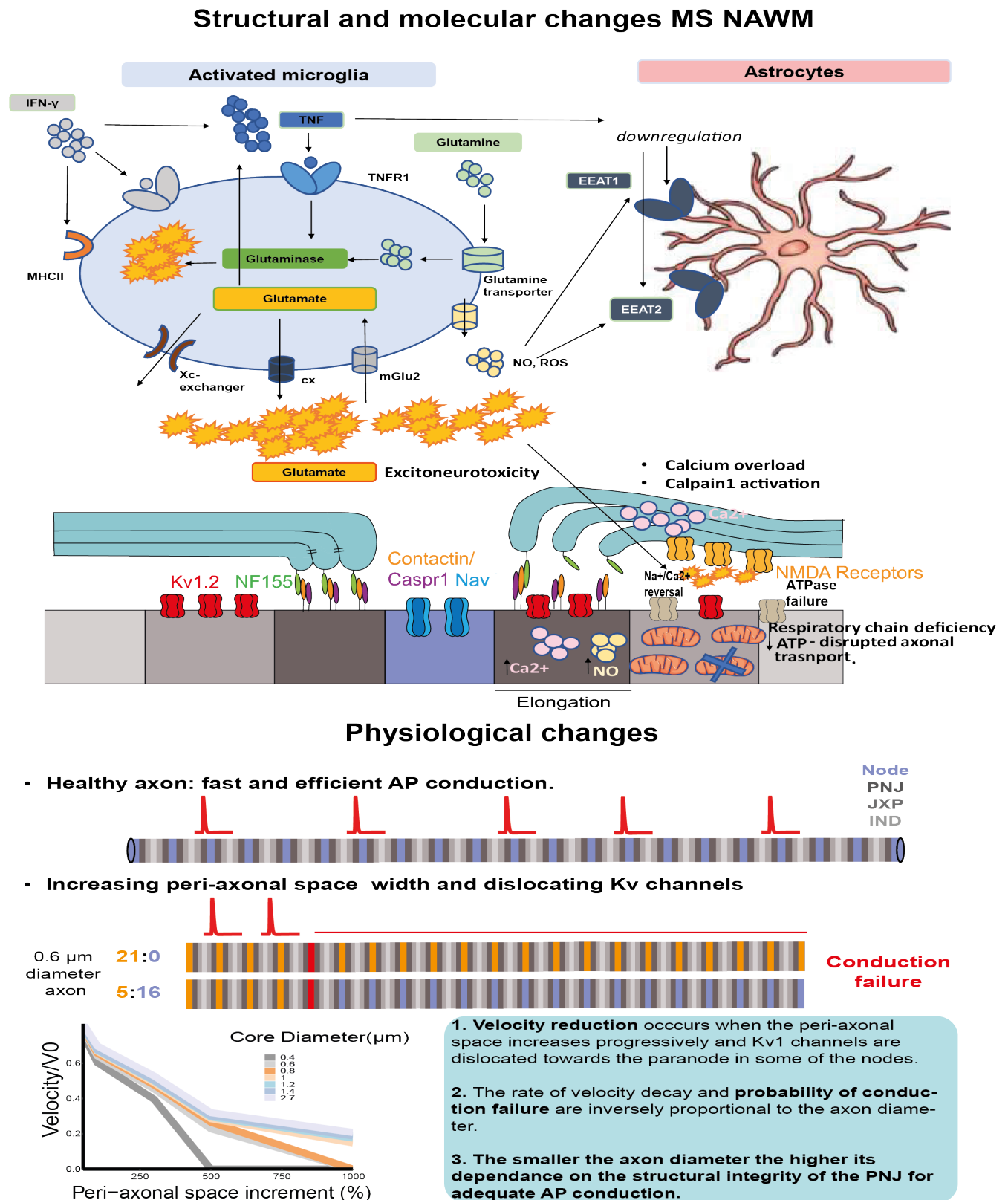


Figure 7.1: Chronically activated microglia by pro-inflammatory cytokines TNF and IFN γ generate elevated glutamate levels associated to PNJ pathology, which causes detrimental AP conduction effects.

IFN γ is a potent inducer of TNF gene expression in microglia (figure 7.1). Previous studies have also demonstrated that it promotes the expression of MHCII molecules (Nguyen and Benveniste, 2002) and up-regulates the expression of TNFR1 and TNFR2 (Wang et al., 2006). In order to assess the direct effect of glutamate on paranodal axo-glial junctions, organotypic cerebellar tissue slices were used. Our data showed a statistically significant paranodal elongation in cerebellar tissue slices treated with two 100 ng/ml doses, and three 50 ng/ml doses of TNF+IFN γ respectively, compared to non-treated slices. These results suggest that these pro-inflammatory cytokines could have activated microglia to release elevated glutamate levels. However, microglia upon stimulation with these cytokines could have also released ROS (Mir et al., 2009). Previous studies have demonstrated that ROS can inhibit glutamate uptake by the GLAST/GLT-1 transporters in astrocytes (Miralles et al., 2001; Takaki et al., 2012), and TNF can downregulate the expression of glutamate EAAT2/GLT-1 astrocytic transporters (Wang et al., 2003b; Sitcheran et al., 2005). Therefore, our data suggest that activation of microglia and suppression of the astrocytic glutamate transporters could have enhanced extracellular glutamate concentration and generated paranodal pathology in the cerebellar slices (figure 7.1).

Our data showed significant paranodal elongation in the organotypic cerebellar tissue slices when treated with microglial conditioned medium and high levels of glutamate, compared to the non-treated slices. However, paranodal disruption was not statistically different between them. Therefore, our results showed that glutamate derived from chronically activated microglia was sufficient to generate paranodal axo-glial pathology. Furthermore, when the slices were treated with an elevated glutamate concentration (100 μ m), the axons appeared more transected. Therefore, from all the treatments used, the pro-inflammatory cytokines might be the ones that represented best the pathology observed in MS NAWM regions due to the better preservation of the axonal integrity.

High concentrations of glutamate would be expected to lead to high intracellular concentrations of Ca^{2+} within the axon that could affect mitochondrial function by opening the PTP (Su et al., 2009). Also, it could activate nitric oxide synthase to synthesise NO, which would inhibit mitochondrial respiratory chain complex IV, disrupting axonal

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transport and generating neuritic beading (Takeuchi and Suzumura, 2014). Moreover, high glutamate can also develop elevated Ca^{2+} levels within the cytoplasmic regions of the oligodendrocyte and activate calcium-sensitive proteases such as calpain1, triggering the degradation of cytoskeletal and cell adhesion proteins that could alter the integrity of the PNJ (figure 7.1).

The administration of the NMDA antagonist MK-801 along with TNF+IFN γ reversed or halted the paranodal elongation previously observed in the organotypic cerebellar slices. Thus, our results suggest that activated microglia, as receptors and generators of the pro-inflammatory cytokines, might be one of the primary cell types triggering paranodal axo-glial pathology at the nodes of Ranvier. This data could add value to the development of new therapies to target these subtle changes at the paranodes, which indeed could affect the quality of life of patients significantly. Most of the negative results obtained in various clinical trials performed with memantine (Lovera et al., 2010; Mehta et al., 2010; Saint Paul et al., 2016) and riluzole (Waubant et al., 2014; Connick et al., 2018) did not separate patients by types, had a small sample size (Macrez et al., 2016), or use an indirect glutamate blocker such as riluzole, which preferentially blocks voltage-gated Na_v channels. Therefore, we suggest that therapies targeting the glutamate signalling should be cell-specific to be of clinical utility. Consequently, in the case of paranodal pathology, some of the strategies would involve reducing the microglial glutaminase activity or blocking microglial hemichannels, especially when they are activated to block excessive glutamate release.

7.5 Limitations of the study

The main limitation of the histopathological analysis of the samples acquired from the human MS NAWM tissue and the chronic rat model of meningeal inflammation was the imaging resolution. Ideally, scanning or conventional transmission electron microscopy would need to be performed to confirm whether structural changes had occurred to the paranodal axo-glial junctions. A scanning transmission electron microscope can achieve a 50 pm resolution in annular dark-field imaging mode (Erni et al., 2009), which is much

higher than the $0.8\ \mu\text{m}$ of confocal light microscopy. However, factors such as the long post-mortem delay of the human tissue, the extremely thin sections needed and the lack of an EM facility restricted the development of a systematic quantification study in a cohort of human post-mortem samples with this technique. An alternative would be super-resolution microscopy, which can achieve an imaging resolution of up to 50 nm, and the preparation of the samples is similar to that used here. Therefore, although we have observed the disruption of the PNJ, we cannot confirm that the paranodal loops do detach from the axolemma.

Regarding the chronic rat model of meningeal inflammation data, the histopathological analysis only looked at a single time point (90 dpi). Therefore, we did not present data about the progression of the observed paranodal axo-glial junction pathology and whether these changes could be reversible. In order to address this issue, the pathology of 90 dpi rats could be compared to that at 28 dpi. If disruption to the nodes of Ranvier is more severe at 28 dpi, it could suggest that there is a degree of repair as inflammation is dampened, giving support to the theory that this pathology might be reversible. However, as the model represents chronic inflammation due to the persistent long term presence of pro-inflammatory cytokines, it is unlikely that any evidence of reversal or repair will be present. Previous data from this laboratory using a mouse EAE model (Howell et al., 2010), suggests that the paranodal changes are likely to be reversible if it is possible to remove the stimulus to the inflammation. Also, we lack behavioural data to associate this pathology to changes in behaviour such as fatigue or tiredness. Producing an in vivo model that only exhibits paranodal pathology due to inflammation would be required in order to determine the behavioural consequences.

The results obtained from the computational model built with NEURON enabled us to predict potential effects and the complexity of this structural pathology on AP conduction along a myelinated axon. However, in order to confirm these results, electrophysiological studies to measure compound action potentials could be explored in the chronic rat model of meningeal inflammation to consolidate our results and also for further studies of neural networks. Also, more geometrically accurate models of CNS axons could be

7.6. Future Work

built to improve the accuracy of predictions in computational models. Nevertheless, the quantification of some of the axonal parameters is not physically feasible in human MS tissue. Therefore, the comparison between the computational results and experimental results from damaged axons might not be possible. On the other hand, NEURON is a deterministic simulator, which means that it takes the average effect, and stochastic channel fluctuations are ignored.

Regarding our in-vitro studies, we could argue that administering repeated inflammatory stimuli with cytokines is not fully representative of the sustained chronic inflammation observed in the MS tissue. An alternative could be to use lenti-viral vectors containing the genes for these cytokines to transduce meningeal cells and culture them beneath the tissues. However, the concentration of the cytokines would not be easily controlled. A limitation of using isolated primary cultures as we did with primary microglia, is that the interactions with other cells are ignored and therefore, interpretations of the results should be confirmed in another system. Although ex-vivo organotypic cerebellar tissue slices enabled us to observe the effects of cytokines, glutamate and conditioned medium in a multi-cellular platform that preserves cytoarchitecture, the slices were prepared from rat pups. Therefore, the maturity of the neuronal circuits and myelin is not fully representative of an MS brain. For instance, we could not quantify the displacement of the K_v1 channels as they were not expressed yet in the axolemma. Another methodology to overcome this issue could be to use adult rats for the preparation of the organotypic cerebellar tissues; nonetheless, it should be taken into consideration that the survival rate of those is much lower.

7.6 Future Work

In order to confirm the detachment of the paranodal myelin end loops from the axolemma in a cohort of MS human post-mortem tissue cases, super-resolution microscopy could be performed. Regarding the clinical symptoms that paranodal axo-glial junction pathology could generate, such as fatigue, behavioural studies could be done in our chronic rat model of meningeal inflammation. Furthermore, to study the details of the molecular

mechanism behind this pathology, live-imaging studies in the cerebellar tissue cultures could be made. This way, we could explore the order and rate in which paranodal end loops detached from the axolemma and if the movement of macromolecules such as exosomes through the myelinic cytoplasmic channels is altered. Additionally, transfected HEK cells with lentiviral vectors containing the genes of pro-inflammatory cytokines could be cultured underneath the cerebellar tissue slices to create a chronic inflammatory environment. Regarding the AP conduction deficits, electrophysiological studies in the chronic rat model of meningeal inflammation and in the cerebellar tissue slices could be performed to consolidate our results and for further studies of neural networks.

7.7 Final conclusions

In conclusion, our histopathological data has shown that MS NAWM tissue contained an apparent disruption in the structure of the paranodal axo-glial junctions, which positively correlated with activated microglia and was associated with axonal stress, indicating an underlying axonal pathology. The Caspr1-stained paranodal elongation was accompanied by a dislocation of juxtaparanodal voltage-gated K_v1 channels towards the paranodes, suggesting a disruption in the structural and functional integrity of these junctions as physical and electrical barriers. These changes were also observed in the chronic model of meningeal inflammation induced with $LT\alpha/IFN\gamma$ lenti-viral vectors, indicating that chronic inflammation throughout the brain could be sufficient for PNJ disruption independently of focal demyelination. Moreover, our results in primary microglia cultures demonstrated that pro-inflammatory cytokines $TNF+IFN\gamma$ and $LT\alpha+IFN\gamma$ could induce a significant release of glutamate, which can be highly toxic for axons in their vicinity. Elevated glutamate levels were sufficient to generate paranodal axo-glial disruption and affect axonal integrity in cerebellar tissue cultures, and an NMDA blocker to reverse it. Therefore, these results suggest that diffuse activation of microglia in MS generated by the presence of a chronic inflammatory milieu can trigger paranodal axo-glial junction pathology in myelinated axons. Consequently, targeting more specifically the activated microglial glutamate signalling pathways or reducing the inflammatory stimuli that acti-

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vate them, would improve paranodal axo-glial pathology.

These structural changes have functional consequences for AP conduction. Our simulations demonstrated that AP velocity significantly decreased when the structure of the PNJ was disrupted by decreasing the paranodal and juxtaparanodal peri-axonal space resistances. Also, our data pointed to axon diameter as the parameter that predicted best the extent of velocity decay and the probability of conduction failure. Furthermore, assuming that scattered activated microglia can damage a highly variable number of nodes throughout an axon, our simulations predicted that conduction failure might depend on the number of consecutive nodes disrupted and the number of healthy nodes in between patches of disrupted nodes. Therefore, these results suggest that diffuse paranodal axo-glial pathology in MS NAWM can significantly alter the efficiency and velocity of AP conduction, and consequently, the overall neurological function in MS.

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Appendix

A.1 Algorithm for the quantification of Caspr1-Kv or Caspr1-Na signal overlapping

```
rm(list =ls())
gc()
overall.wd = "C:/Users/pg2015/Desktop/Measurements/patients_K"
setwd(overall.wd)
file.list1 = list.files(path = ".") # list of all files
file.len1 = length(file.list1)
library(ggplot2)
```

```

total.data1 = c()

for(f in 1:file.len1){
  print(f)
  # get all files:
  setwd(paste0(overall.wd,sep = "/",file.list1[1]))
  folder.list1 = list.files(path = ".") # list of all files
  folder.len1 = length(folder.list1)
  for(i in 1:folder.len1){
    cat("i =", i, "\n")
    data1 = read.csv(folder.list1[i],header = TRUE)
    clean.data1 = data.frame('X0' = data1$X0,'Y0'= data1$Y0,'Y1'= data1$Y1,'Y2'=
      data1$Y2)
    total.data1 = rbind(clean.data1, total.data1)
  }
}

total.data1=total.data1

#establishing the overlap method
minus1 = abs(total.data1$Y0 - total.data1$Y2)
thresholdseq1=seq(0, max(max(total.data1$Y0),max(total.data1$Y2)),1.5)
cushion1=10000
overlaps1 = c()
tmin1 = 75

for (j in thresholdseq1){
  # for each threshold do:
  pair.store1 = c() # store pairs of start/end here
  count = 1 # start looking from first entry onwards
  ovlps1 = length(minus1[minus1 <= j & total.data1$Y0 > tmin1 & total.data1$Y2 >

```

A.2. NEURON computational model

```
        tmin1])/length(minus1)
overlaps1 = c(overlaps1, ovlps1)

}
```

A.2 NEURON computational model

```
proc model_globels() {
    celsius=37
    v_init=-80 //mV//
    dt=0.001 //ms//
    tstop=10
//Intracellular stimulation parameters//
    istim=2
    delay=1
    pw=0.1
//topological parameters//
    axnodes=21
    axparanodes=40
    axjuxtaparanodes=40
    axinternodes=120
    axontotal=221
//morphological parameters//
    fiberD=0.5//choose from 0.5, 0.8, 1.1, 1.3, 1.6, 1.8, 3.5 //um
    paralength1=2.3 //um
    nodelength=1.0 //um
    space_p0=0.002//um
    space_p1=0.002//um
    space_p2=0.02 //um
    space_i=0.02 //um
//electrical parameters//
    rhoa=70 //Ohm-cm//
```

```

axon_capacitance=1 //uF/cm2//
myelin_rhoa=1000 //Ohm-cm2//
myelin_capacitance=0.1 //uF/cm2//

}
model_globals ()

// the units of Rpn are Ohm.cm and not cm^2.

proc dependent_var() {
    if (fiberD==0.8) {axonD=0.6 nodeD=0.6 paraD1=0.6 paraD2=0.6 paralength2
        =2.58*fiberD + 19.6
node_node_L=117.52+30.47*fiberD interlength=(node_node_L-2*(paralength2+
    paralength1))/6 nl=6 thick_myelin=0.09 }
    if (fiberD==1.1) {axonD=0.8 nodeD=0.8 paraD1=0.8 paraD2=0.8 paralength2
        =2.58*fiberD+19.6
node_node_L=117.52+30.47*fiberD interlength=(node_node_L-2*(paralength2+
    paralength1))/6 nl=8 thick_myelin=0.12}
    if (fiberD==1.3) {axonD=1 nodeD=1 paraD1=1 paraD2=1 paralength2=2.58*
        fiberD+19.6
node_node_L=117.52+30.47*fiberD interlength=(node_node_L-2*(paralength2+
    paralength1))/6 nl=10 thick_myelin=0.15}
    if (fiberD==1.6) {axonD=1.2 nodeD=1.2 paraD1=1.2 paraD2=1.2 paralength2
        =2.58*fiberD+19.6
node_node_L=117.52+30.47*fiberD interlength=(node_node_L-2*(paralength2+
    paralength1))/6 nl=12 thick_myelin=0.18}
    if (fiberD==1.8) {axonD=1.4 nodeD=1.4 paraD1=1.4 paraD2=1.4 paralength2=2.58*
        fiberD+19.6
node_node_L=117.52+30.47*fiberD interlength=(node_node_L-2*(paralength2+
    paralength1))/6 nl=13 thick_myelin=0.2}
    if (fiberD==3.5) {axonD=2.7 nodeD=2.7 paraD1=2.7 paraD2=2.7 paralength2=2.58*
        fiberD+19.6

```


A.2. NEURON computational model

```
node_node_L=117.52+30.47*fiberD interlength=(node_node_L-2*(paralength2+
    paralength1))/6 nl=26 thick_myelin=0.4}
if (fiberD==0.5) {axonD=0.4 nodeD=0.4 paraD1=0.4 paraD2=0.4 paralength2=2.58*
    fiberD+19.6
node_node_L=117.52+30.47*fiberD interlength=(node_node_L-2*(paralength2+
    paralength1))/6 nl=3 thick_myelin=0.04}

//axon and myelin areas
axon_xarea=PI*(axonD/2)^2 //um2
node_surface_area=PI*nodelength*axonD //um2
para_surface_area=PI*paralength1*axonD //um2
juxta_surface_area=PI*paralength2*axonD //um2
inter_surface_area=PI*interlength*axonD //um2

node_periaxonal_xarea=(PI*(((axonD/2)+space_p0)^2))-axon_xarea
    //um2
para_periaxonal_xarea=((PI*(((axonD/2)+space_p1)^2))-axon_xarea)
    +(170*(10^-6)*(nl-1)) //um2 170nm2 is the third pathway xarea in the
    the paranodal space (Mierwa et al 2009)
juxta_periaxonal_xarea=(PI*(((axonD/2)+space_p2)^2))-axon_xarea
    //um2
inter_periaxonal_xarea=(PI*(((axonD/2)+space_i)^2))-axon_xarea
    //um2

para_myelin_surface_area=PI*(axonD+2*space_p1+thick_myelin)*paralength1
    //um2
juxta_myelin_surface_area=PI*(axonD+2*space_p2+thick_myelin)*paralength2
    //um2
inter_myelin_surface_area=PI*(axonD+2*space_i+thick_myelin)*interlength
    //um2
```

```
//axon resistance and capacitance
node_axon_resistance=(rhoa*nodelength)/axon_xarea
//Ohm cm/um

para_axon_resistance=(rhoa*paralength1)/axon_xarea
//Ohm cm/um

juxta_axon_resistance=(rhoa*paralength2)/axon_xarea
//Ohm cm/um

inter_axon_resistance=(rhoa*interlength)/axon_xarea
//Ohm cm/um


node_axon_capacitance=axon_capacitance*node_surface_area
//uF um2/cm2

para_axon_capacitance=axon_capacitance*para_surface_area
//uF um2/cm2

juxta_axon_capacitance=axon_capacitance*juxta_surface_area
//uF um2/cm2

inter_axon_capacitance=axon_capacitance*inter_surface_area
//uF um2/cm2


//Myelin resistance and capacitance

para_myelin_resistance=(myelin_rhoa*nl*2)/para_myelin_surface_area
//Ohm cm2/um2

juxta_myelin_resistance=(myelin_rhoa*nl*2)/juxta_myelin_surface_area
//Ohm cm2/um2

inter_myelin_resistance=(myelin_rhoa*nl*2)/inter_myelin_surface_area
//Ohm cm2/um2


para_myelin_capacitance=(myelin_capacitance*nl*2)/para_myelin_surface_area
//uF um2/cm2

juxta_myelin_capacitance=(myelin_capacitance*nl*2)/
juxta_myelin_surface_area //uF um2/cm2
```

A.2. NEURON computational model

```
inter_myelin_capacitance=(myelin_capacitance*nl*2)/
inter_myelin_surface_area      //uF um2/cm2

//periaxonal resistance
Rpn0=(rhoa/(node_periaxonal_xarea*10^-8))*0.000001
                                //Mohm/cm
Rpn1=(rhoa/(para_periaxonal_xarea*10^-8))*0.000001
                                //Mohm/cm
Rpn2=(rhoa/(juxta_periaxonal_xarea*10^-8))*0.000001
                                //Mohm/cm
Rpx=(rhoa/(inter_periaxonal_xarea*10^-8))*0.000001
                                //Mohm/cm
}
dependent_var()

objectvar stim

create nodes[axnodes], paranodes[axparanodes], juxtaparanodes[axjuxtaparanodes],
internodes[axinternodes]
access nodes[0] //default value when plotting

proc initialize(){
    for i=0,axnodes-1 {
        nodes[i]{
            nseg=1
            diam=nodeD
            L=nodelength
            Ra=rhoa // 70 Ohm.cm
            cm=1
        }
    }
}
```

```

        insert axnode
        insert extracellular xrxial=Rpn0 xg=10e9 xc=0 //xg(S/cm2)
            xc(uF/cm2)
        }
    }
for i=0, axparanodes-1 {
    paranodes[i]{
        nseg=1
        diam=paraD1
        L=paralength1
        Ra=rhoa
        cm=1

        insert pas
        g_pas=0.0005
        e_pas=-80
        insert extracellular xrxial=Rpn1 xg=1/(
            para_myelin_resistance*para_surface_area) xc=(
            para_myelin_capacitance/para_surface_area)
        }
    }
for i=0, axjuxtaparanodes-1 {
    juxtaparanodes[i]{
        nseg=1
        diam=paraD2
        L=paralength2
        Ra=rhoa
        cm=1
        insert axjuxtaparanode
        insert pas
        g_pas=0.005
        e_pas=-80
    }
}

```

A.2. NEURON computational model

```
        insert extracellular xrxial=Rpn2 xg=1/(
            juxta_myelin_resistance*juxta_surface_area) xc=(
            juxta_myelin_capacitance/juxta_surface_area)
    }
}
for i=0, axinternodes-1 {
    internodes[i]{
        nseg=1
        diam=axonD
        L=interlength
        Ra=rhoa
        cm=1
        insert pas
        g_pas=0.005
        e_pas=-80
        insert extracellular xrxial=Rpx xg=1/(
            inter_myelin_resistance*inter_surface_area) xc=(
            inter_myelin_capacitance/inter_surface_area)
    }
}
for i=0, axnodes-2 {
    connect paranodes[2*i](0), nodes[i](1)
    connect juxtaparanodes[2*i](0), paranodes[2*i](1)
    connect internodes[6*i](0), juxtaparanodes[2*i](1)
    connect internodes[6*i+1](0), internodes[6*i](1)
    connect internodes[6*i+2](0), internodes[6*i+1](1)
    connect internodes[6*i+3](0), internodes[6*i+2](1)
    connect internodes[6*i+4](0), internodes[6*i+3](1)
    connect internodes[6*i+5](0), internodes[6*i+4](1)
    connect juxtaparanodes[2*i+1](0), internodes[6*i+5](1)
    connect paranodes[2*i+1](0), juxtaparanodes[2*i+1](1)
    connect nodes[i+1](0), paranodes[2*i+1](1)
}
```

```

    }

    finitalize(v_init)
    fcurrent()
}
initialize()

//intracellular stimulus//
proc stimul() {
    nodes[0]{
        stim=new IClamp()
        stim.loc(.5)
        stim.del=delay
        stim.dur=pw
        stim.amp=istim
    }
}
stimul()

xpanel("Stimulus parameters")
    xvalue("Stimulus Amplitude (nA)", "istim", 1, "stimul()", 1)
    xvalue("Pulse Duration (ms)", "pw", 1)
    xvalue("Onset Delay (ms)", "delay", 1)
xpanel(100,100)

//recording and saving data from voltage node4
objref rect, recv
rect = new Vector()
recv = new Vector()

```

A.2. NEURON computational model

```
recv.record(&nodes[4].v(0.5))
rect.record(&t)

run()

recv.printf()

objref savdata

savdata = new File()

savdata.wopen("node4_2.7.txt")
savdata.printf("t node[4].v(0.5)\n")

for i=0,rect.size()-1 {
    savdata.printf("%g %g\n", rect.x(i), recv.x(i))
}

savdata.close()

//recording and saving data from voltage node16
objref recvtwo, recttwo
recvtwo = new Vector()
recttwo=new Vector()

recvtwo.record(&nodes[16].v(0.5))
recttwo.record(&t)
```

```

run()
recvtwo.printf()

objref savdatatwo

savdatatwo = new File()
savdatatwo.wopen("node16_2.7.txt")
savdatatwo.printf("t node[16].v(0.5)\n")
for i=0,recttwo.size()-1 {
    savdatatwo.printf("%g %g\n", recttwo.x(i), recvtwo.x(i))
}

savdatatwo.close()

//recording current node 4

objref recti, reci
reci = new Vector()
recti=new Vector()

reci.record(&nodes[4].ina_axnode(0.5))
recti.record(&t)

run()
reci.printf()

objref savdatai

savdatai = new File()
savdatai.wopen("node4_current_2.7.txt")
savdatai.printf("t node[4].ina_axnode(0.5)\n")

```


A.2. NEURON computational model

```
for i=0,recti.size()-1 {  
    savdatai.printf("%g %g\n", recti.x(i), reci.x(i))  
}  
savdatai.close()
```