**Universidade do Minho**
Escola de Ciências

Diogo Alexandre Guimarães Monteiro

# Characterization of the ventricular and subventricular zone in a multiple sclerosis mouse model

janeiro de 2023

Characterization of the ventricular and subventricular zone in a multiple sclerosis mouse model
Diogo Alexandre Guimarães Monteiro
UMinho | 2023

**Universidade do Minho**
Escola de Ciências

Diogo Alexandre Guimarães Monteiro

# Characterization of the ventricular and subventricular zone in a multiple sclerosis mouse model

Dissertação de Mestrado
Mestrado em Bioquímica Aplicada

Trabalho efetuado sob a orientação da
**Dra. Ana Luísa Mendanha Falcão**
e
**Prof. Dra. Paula Margarida Vidigal Soares Teixeira Ferreira**

janeiro de 2023

## DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS



**ACKNOWLEDGEMENTS**

Prior to everything else, I would want to thank my supervisor, Ana Falcão, for her dedication during the whole project. It has been a privilege to learn from your experience, which has helped me enhance my scientific skills. I am certain I will apply all your advice in my future career.

Also during this year, I met brilliant scientists who assisted me in completing my project. I would want to extend my gratitude to Sara Silva, Daniela Monteiro, Daniela Garcia, and Patrícia Gomes.

To my labmates Rúben Silva, Mónica Fernandes, and Sara Ferreira in particular for the lengthy conversations on the hopes and prospects of our generation. I hope that we may realize all of our dreams one day.

This work was co-funded by the Life and Health Sciences Research Institute (ICVS), by National funds, through the Foundation for Science and Technology (FCT) - project UIDB/50026/2020 and UIDP/50026/202, and by ICVS Scientific Microscopy Platform, member of the national infrastructure PPBI - Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122). Ana Mendanha Falcão was supported by the Portuguese Foundation for Science and Technology (2020.02753.CEECIND), a fellowship from "la Caixa" Foundation (ID100010434) with the fellowship code LCF/BQ/PI19/11690005, and a Swedish Research Council (grant no. 2019-02030).




## STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

## RESUMO

**Título:** Characterization of the ventricular and subventricular zone in a multiple sclerosis mouse model.

A Esclerose Múltipla (EM) é uma doença neurodegenerativa autoimune do Sistema Nervoso Central (SNC) caracterizada pela desmielinização dos axónios neuronais que leva a sintomas altamente incapacitantes nomeadamente em jovens adultos. A incidência praticamente duplicou desde 2013, sendo que urge uma necessidade maior da sua investigação.

A zona subventricular (ZSV) corresponde a um nicho de células estaminais do SNC que compreende a formação de novos neurónios na idade adulta e de células da glia, tais como os oligodendrócitos que têm a sua origem na diferenciação prévia de células percursoras de oligodendrócitos (CPOs). Este revela-se como de grande importância em fases de desmielinização e remielinização de modo a perceber a sua dinâmica em EM. Deste modo, foi proposta uma abordagem à investigação desta doença com recurso a um modelo desmielinizante e de morte de oligodendrócitos maduros (PlpCreERT;ROSA26-eGFP-DTA) após injeção com tamoxifeno.

A utilização de ferramentas de análise de imagem amparadas por técnicas laboratoriais como a marcação celular tais como a imunofluorescência, permitiu uma comparação entre animais que sofreram desmielinização (DTA+) e controlos em termos de número de CPOs, células em proliferação e perfis de dupla marcação.

A análise dos resultados revelou que não há diferencas estatisticamente significativas entre a ZSV de controlos e animais desmielinizados nos parâmetros de presença de CPOs nem de células em proliferação no período correspondente ao dia 21 após injeção de tamoxifeno (pico de desmielinização). No entanto uma abordagem com um valor de n superior de animais poderá traduzir resultados diferentes uma vez que o n utilizado foi reduzido (n=3).

Torna-se assim importante o estudo dos fatores associados aos mecanismos biológicos da EM de modo a poder revelar o seu perfil molecular e avançar no futuro com novos dados para a construção de terapias dirigidas ao desenvolvimento de agentes que possam melhorar a qualidade de vida dos doentes.



**Palavras chave:** Desmielinização Esclerose Múltipla, Oligodendrócitos, Oligodendrogénese, Zona Subventricular;

## ABSTRACT

**Title:** Characterization of the ventricular and subventricular zone in a multiple sclerosis mouse model.

Multiple Sclerosis (MS) is an autoimmune neurodegenerative disease of the Central Nervous System (CNS) characterized by demyelination of neuronal axons that results in highly severe symptoms, especially in young individuals. Since 2013, the incidence has nearly doubled, and there is a heightened need for its examination.

The ventricular and subventricular zone (V-SVZ) relates to a niche of CNS stem cells that includes the formation of new neurons in adulthood and glial cells such as oligodendrocytes that derive from the differentiation of oligodendrocyte precursor cells (OPCs). Understanding the dynamics of MS during the demyelination and remyelination stages is of vital relevance. In order to assess this illness, a model of demyelination and death of mature oligodendrocytes (PlpCreERT; ROSA26-eGFP-DTA) following tamoxifen administration was performed.

Using image processing methods supplemented by laboratory procedures such as cell labelling immunofluorescence, it was possible to compare the number of OPCs, proliferating cells, and dual labelling patterns between demyelinating mice (DTA+) and control animals.

Data analysis indicated that there are no statistically significant differences between the V-SVZ of control and demyelinated mice in the parameters of OPCs cellular densities or proliferating cells on the 21$^{st}$ day after tamoxifen administration (demyelination peak). Nonetheless, an increase in the number of animals is needed to make stronger conclusions, as n=3 is small.

Therefore, it is crucial to examine the aspects linked with the biological processes of MS in order to expose its molecular profile and move forward in the future with novel data for the development of targeted treatments or therapies that can enhance the quality of MS patients' lives.



**Keywords:** Demyelination, Multiple Sclerosis, Oligodendrocytes, Oligodendrogenesis, Subventricular zone

**Index**

## Abbreviations List

(APC) – Astrocytic Neural Progenitors

(BBB) - Blood-Brain Barrier

(B-CSF) – Blood Cerebrospinal Fluid Barrier

(BrdU) - Bromodeoxyuridine

(CNS) – Central Nervous System

(CP) – Choroid Plexus

(CSF) - Cerebrospinal Fluid

(dpf) – days after fertilization

(dpi) – days post infection

(dpi) – days post infection

(DTA) – Difteria Toxin – A

(EAE) – Experimental Autoimmune Encephalomyelitis

(EBv) - Epstein-Barr virus

(GC) – Glicocorticoid

(GFAP) – Glial Fibriliary Acidic Protein

(GM-CSF) – Grabulocyte – Macrophage Colony – Stimulating Factor

(IFN-) – Interferon

(IL-17) – Interleukin 17

(MBP) – Myelin Basic Protein

(MHC) - Major Histocompatibility Complex

(MO) – Mature Oligodendrocytes

(MOG) – Myelin Oligodendrocyte Glycoprotein

(MRI) - Magnetic Resonance Imaging

(MS) – Multiple Sclerosis

(NSC) – Neural Stem Cell

(NSPC) – Neuronal Stem Percursor Cells

(OB) – Olfactory Bulb

(OL) – Oligodendrocyte

(OPC) – Oligodendrocyte Percursor Cell

(PCR) – Polymerase Chain Reaction

(PLP) – Proteolipid Protein

(PSA-NCAM) – Polysialylated form of Neural Cell Adhesion Molecule

(RMS) – Rostral Migratory Stream

(SCH) – Spinal Cord Homogenate

(SGZ) - Subgranular Zone

(Th) – T helper

(TMEV-IDD) – Theiler's Virus Infection Mouse Model

(TNF-) – Tissue Necrosis Factor

(V-SVZ) – Ventricular-Subventricular Zone

**List of Figures**

**Index of Tables**

# Introduction

## 1. Multiple Sclerosis

### 1.1. Historical perspective

Multiple Sclerosis (MS) is a neurological disorder that has aroused the interest of researchers over the past century due to its unknown molecular processes, origins, and dynamics (Stys & Tsutsui, 2019).

MS was first described in text in the 14th century. The characteristics of St. Lidwina of Schiedam's disease, which she suffered from 1380 to 1433 (Medaer. R, 1979), remarkably resemble those of MS but it was in the 19th century that the "father of Neurology", Jean-Martin Charcot (1825-1893) drew the first connections between the post-mortem pathological changes and the clinical characteristics of MS. Charcot was the first person to diagnose MS on a living patient. In reality, he developed a triad for MS diagnosis (nystagmus, intention tremor, and scanning speech). Though lacking in precision, it is significant since it was an attempt to distinguish this condition from comparable diseases affecting the nervous system (Kumar et al., 2011).

### 1.2. Symptomatology

Currently, from a clinical perspective, MS is defined as a Central Nervous System (CNS) autoimmune disease characterised by chronic inflammation, demyelination, gliosis (scarring), and neuronal loss; the course can be relapsing and remitting or progressive (Brownlee et al., 2017). MS lesions in the brain often develop at different times and in different CNS sites, as such MS is considered to be time and spatially dispersed. This disorder is also distinguished by a wide spectrum of symptoms, which may occasionally lead to a different diagnosis. The most common are: sensory loss, optic neuritis, weakness, paresthesias, diplopia, ataxia, vertigo, paroxysmal attacks and bladder. Other more rare symptoms include lethermitte, general pain, dementia, visual loss, facial palsy, impotence, myokymia, epilepsy and falling (Hauser & Goodin, 2014).

MS patients may be effectively treated and free of current symptoms, but their quality of life remains poor due to patient-centred variables such as depression, incapacity to work, and relationship woes - patients, their families, and society as whole face challenges as a result of the diagnosis. There are several 'hidden'

difficulties associated with MS that may be more difficult to discuss at the time of diagnosis or later in the disease's progression. These concerns may include but are not limited to, tiredness, sexual dysfunction, treatment adherence, bladder disorders, social isolation, and cognitive impairments (Lysandropoulos & Havrdova, 2015).

## 1.3. Epidemiology

In terms of disease epidemiology, it is estimated that 2.8 million people globally suffer from MS (35,9 per 100.000 population) in 2020, and unfortunately MS prevalence has increased throughout every world region since 2013, although there are still gaps in incidence rates, the projection is 30% greater than in 2013 (Aarli et al., 2014). Fortunately, awareness of paediatric-onset MS has increased significantly, with 47 countries reporting 30,000 occurrences of MS diagnosed in children under the age of 18. Again, in 2013, 34 countries reported 7,000 cases, indicating that this issue has been prioritised in terms of communicating data to national statistics. Also, females are twice as likely as males to have MS worldwide, which is congruent with previous studies and data gathering. Nonetheless, in certain nations, the gender ratio is as high as 4:1, and in others, it has more than doubled since 2013. (Walton et al., 2020)

Another interesting aspect related to the epidemiological data is that Northern Europeans appear to be the most vulnerable to MS. The condition is diagnosed based on clinical findings and supporting evidence from magnetic resonance imaging (MRI) of the brain and cerebrospinal fluid (CSF) analysis. MS most commonly affects adults and young people aged 20 to 45, but it can also affect seniors in their late middle years. (Goldenberg, 2012).

## 1.4. Etiology

Although evidence suggests that MS is a complex feature caused by an interplay of genetic and environmental variables, little is known about its aetiology or the factors contributing to its erratic course throughout its development (Noseworthy, 2000).

Even though the actual cause of MS is uncertain, the identification of genetic variants linked to the disease development has increased fairly quickly in recent years. Before the advent of genome-wide association studies, despite decades of candidate-gene-based attempts, little progress was achieved in identifying

significant, related risk alleles outside the major histocompatibility complex (MHC). The sole significant advancement was the detection of a connection with the SNP rs6897932 from the IL7R gene, which was indicated by pooling information from multiple data sources (ie, genomic convergence) and corroborated by typing a large number of cases and controls. The findings of the first genome-wide association investigation MS were published at the same time and revealed a connection with variants in both IL7R and IL2RA. (Sawcer et al., 2014)

In addition to the genetic origins of MS, environmental variables play an essential role in the main causes of MS. These environmental factors have been investigated and their relationship to MS episodes has been established.

Smoking has a well-known effect on the immune system: smokers (and ex-smokers) have increased levels of C-reactive protein, fibrinogen, and inflammatory cytokines (e.g., IL-6), and they may also have a dysregulation of B-cell and T-cell homeostasis (Mandia et al., 2014). Other environmental variables include sun exposure, vitamin D deficiency, and personal diet.

One of the most widely recognised hypotheses about the origin of MS is that the disease is triggered by prior exposure to an infectious pathogen through the immune system.

Epstein-Barr virus (EBV) is a ubiquitous virus, infecting more than 90% of individuals globally and causing lifelong infection. Despite its often asymptomatic persistence, EBV has been found in a variety of cancers. EBV has also a significant impact on the immune system and is the most prevalent cause of infectious mononucleosis as well as deadly lymphoproliferative diseases in a variety of immunosuppressive circumstances(Soldan & Lieberman, 2022). It is becoming increasingly clear that EBV is a key risk factor for various autoimmune illnesses, most notably MS.

A recent study was based on cases of MS in a cohort of active-duty US military personnel between 1993 and 2013, a racially varied group of more than 10 million people, throughout the course of a 20-year collaboration with the US military (Bjornevik et al., 2022). Herein, it was carried out the most comprehensive epidemiology investigation on the relation between viruses and MS. Individuals who converted to EBV seropositivity had a higher risk of MS diagnosis than those who remained seronegative. This study strongly suggests that EBV infection is required for the future emergence of MS.

Although the source of MS remains undetermined, there are numerous approaches in terms of different therapies to address the associated symptomatology.

Depending on the disease's course through time neurologists can identify different forms of Multiple Sclerosis (Goldenberg, 2012).

### 1.5. Types of MS

In 2013, the International Advisory Committee for Clinical Trials of Multiple Sclerosis identified four fundamental MS disease courses (Lublin et al., 2014) (also known as kinds or phenotypes):

1. Relapsing-remitting MS: the most frequent kind, involving about 85% of MS patients. Flareups (relapses or exacerbations) of symptoms are followed by periods of remission when symptoms improve or cease .

2. Secondary progressive MS: arises following relapsing-remitting courses. Treatment with disease-modifying drugs can assist to slow reaching secondry progressive MS. The illness progresses with or without phases of remission or symptom intensity levelling off (plateaus).

3. Primary progressive MS: affects around 10% of all MS patients. From the onset, the symptoms worsen progressively. There are no relapses or remissions, but there may be plateaus from time to time. This kind of MS is more resistant to the pharmaceuticals often used to treat it.

4. Progressive-relapsing MS: an infrequent variant that affects less than 5% of people. From the onset, it is progressive, with sporadic flare-ups of deteriorating symptoms along the process. There are no remission periods. (Goldenberg, 2012)

### 1.6. Treatment

Based on a specialist's evaluation, each patient's treatment should be individualised (Gafson et al., 2017). The history of MS therapy is a good illustration of the effective translation of research into treatment strategies and improvements in clinical outcomes. The first treatments for (MS) were launched in the 1990s with IFN-1β (Betaseron®). IFN-1β was the first immunomodulatory therapy approved for the treatment of relapsing–remitting MS in 1993 (Vosoughi & Freedman, 2010) and after more than three decades, MS might be the first chronic autoimmune illness to be treated, considering the progress made thus far (Hauser, 2021). Most MS treatments rely on modulation of immune system (Olek, M., 2007). In addition to the availability of a variety of innovative therapy options, high-efficacy therapeutic drugs have developed, and progress is now being made in the treatment of progressive MS. Despite these

developments, research into novel drugs, such as the histamine receptor antagonist GSK239512 and cell-based treatment, continues. Consequently, it is anticipated that the therapeutic pipeline will continue to evolve, and new insights will need to be incorporated into the routine care of MS patients. In the meanwhile, research into completely personalised treatment should be prioritised (Tintore et al., 2019).

## 2. MS mechanisms of action

### 2.1. Immune cell invasion in MS

MS pathophysiology is predicated on the hypothesis that early immunological dysregulation leads to autoreactivity against myelin-sheath components, which eventually results in blood-brain barrier (BBB) rupture. Encephalitogenic T-cells (Mockus et al., 2021) then invade the CNS, creating the localized inflammation and demyelination that characterize MS lesions (**Figure 1**). It has been estimated that such lesions are approximately five times more common than bouts of clinical deterioration. Disruption of the BBB normally lasts for approximately a month and subsequently resolves, leaving behind an area of damage that may be detected with a standard MRI.

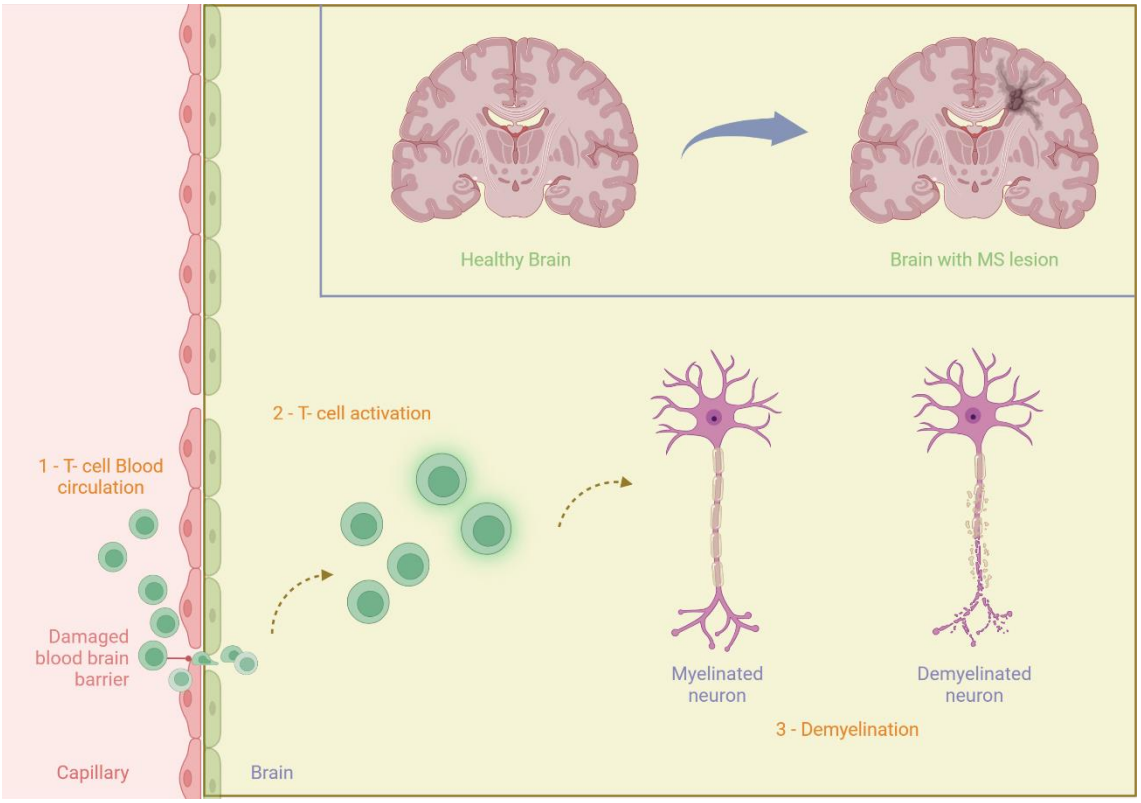
### 2.2. Oligodendrocyte loss

Myelin, the multilamellar sheath essential for saltatory transmission in nerves, is produced by the oligodendrocyte processes that develop around axons (Johns & Bernard, 1999). Encephalitogenic T cells stop recognizing myelin as a self protein and begin to target it for destruction (Melzer et al., 2009). In acute lesions, there is most likely variation in the extent of myelin than axon damage and the immunological and genetic susceptibility characteristics of CNS tissue may affect the fate of acute lesions (McFarland & Martin, 2007). Importantly, scientific data suggests that oligodendrocyte loss and myelin abnormalities are present in the brains of MS patients even when there are no apparent signs of inflammation. Consequently, the loss of oligodendrocytes and subsequent demyelination might result in autoreactivity to myelin antigens as well as inflammation and demyelination (Traka et al., 2015).

### 2.3. Remyelination potencial of the CNS

The CNS contains oligodendrocytes progenitors (OPC) that are capable of differenting into oligodendrocytes that will generate myelin. OPCs comprise around 5–8% of the CNS's glial cell population.

Their function in the intact CNS is not only forming new oligodendrocytes. It has been shown that their processes interact with nodes of Ranvier and synapses, indicating a regulatory role at these sites. OPC are slowly dividing cells and account for 70% of labelled cells following a pulse injection of bromodeoxyuridine (BrdU) (Horner et al., 2000). In response to demyelination, OPC divide and are believed to differentiate to produce new oligodendrocytes to replace those that have been destroyed. Nonetheless, remyelination fails in the later stages of MS, and it is unclear whether this is due to a lack of adult OPCs, inhibition within the glial scar, or axonal injury that precludes remyelination. Adult OPCs are also triggered and multiply in response to various kinds of CNS damage, including mechanical injury, excitotoxicity, and viral infection (Levine et al., 2001).




.

**Figure 1- Dysregulation of the BBB can lead to a T Cell infiltration within the CNS and consequent autoimmune reaction –** T cells can move from the bloodstream to the brain's capillaries. If the BBB epithelium is compromised, these cells can penetrate the CNS and, upon activation, trigger an autoimmune response that destroys the myelin sheets of healthy myelinated neurons. Behind the symptoms of MS lies a process that might progress over time. A brain MRI can reveal these abnormalities, which can aid in the diagnosis of MS in a patient. Created with *BioRender.com*

## 3. Neural stem cell niches of the brain

Neurons and macroglia of the CNS derive from self-renewing neuroepithelial progenitors. Neurogenesis is the process that refers to the formation of new neurons and gliogenesis refers to the formation of new glial cells. Adult neurogenesis is a recapitulation of brain development in the adult and consists of a set of sequential developmental processes required for the production of new neurons. The precursor cell population, could be recognized solely via the identification of their proliferative activity (which is going to be discussed further in this document) and the absence of mature neuronal morphological traits.

### 3.1. Subgranular zone

The precursor cells, from which adult neurogenesis arises, exist in the subgranular zone (SGZ), a short strip of tissue between the granule cell layer and the hilus. The SGZ contains a suitable microenvironment for neuronal development to occur. This milieu is referred to as the neurogenic "niche" since it is similar to other stem cell systems in the body. The niche consists of precursor cells, their immediate progeny and immature neurons, additional glial cells and endothelia, immune cells, microglia, and macrophages, and an extracellular matrix (Kempermann et al., 2015).

### 3.2. Ventricular and subventricular zone

The largest neurogenic niche inside the adult CNS is the ventricular and subventricular zone (V-SVZ). Neural stem cells (NSCs) of the V-SVZ create olfactory interneurons throughout life and maintain the inherent potential to form oligodendrocytes (OLs) (Butt et al., 2022), the CNS myelinating cells which are highly relevant in an MS context, as other glial cells.

#### 3.2.1. Cell types in the V-SVZ

NSCs are multipotent cells that can self-renew and differentiate into all types of neural cells, including neurons, astrocytes, and oligodendrocytes. These multipotent cells may be found in the adult mammalian brain, although they are confined to certain niches like the V-SVZ cell layer. Slowly dividing astrocytic

neural progenitors (APC), commonly known as Type-B cells, are found in the V-SVZ. These germinal astrocytes give birth to Type-C cells, which are actively proliferating transit-amplifying cells that produce immature neuroblasts (Type-A cells). The ability of SVZ Type-B cells to create myelinating oligodendrocytes was also proven already (Gonzalez-Perez & Alvarez-Buylla, 2011).

NSCs from the adult V-SVZ may be grown as a cell suspension in the presence of growth factors. Under these in vitro conditions, NSCs generate spherical structures known as neurospheres, which contain cells that may not only self-renew but also differentiate into neurons, astrocytes, and OLs.

The pool of NSCs inside the V-SVZ is not static, instead, this pool of stem cells is directed to create new neurons that migrate anteriorly through the rostral migratory stream (RMS) to the olfactory bulb, where they develop into several types of interneurons. It has been proven that, in mice, the V-SVZ stem cell niche is not topographically and functionally homogenous; in fact, the V-SVZ niche extends to more dorsal regions of the ventricle walls (and to the RMS). In vitro studies demonstrate a considerable fluctuation in the number of neurosphere-forming cells isolated from consecutive brain slices along the anterior-posterior axis, which further supports the notion that the V-SVZ NSCs population is heterogeneous (Falcão et al., 2012).

### 3.2.3. Oligodendrogenesis in V-SVZ in health and in response to demyelination

Axons can be remyelinated upon oligodendrocyte loss through the differenciation of OPCs. Loss of adult OPCs and their restricted capacity to move into demyelinating lesions and convert into mature OLs are amongt the possible reasons for impaired endogenous remyelination (Butti et al., 2019). It has been reported that CNS can form new oligodendrocytes from the V-SVZ following an injury. Demyelinating lesions in the surrounding white matter can surprisingly stimulate the generation of OPCs from SVZ progenitors (Maki et al., 2013).

Remyelination accomplished by new oligodendrocytes produced from OPCs and NSCs differs significantly. Notably, axons remyelinated by NSCs show a typical G-ratio, as contrasted to the sparsely myelinated axons that are characteristic of remyelination by OPCs (Samanta et al., 2015). In addition, there may be disparities between remyelination by aged local OPCs and newly produced OPCs by NSCs, which is consistent with the restricted remyelination reported in aging brain OPCs. While aged OPCs have a limited capacity for remyelination, NSCs retain their potential for proliferation and differentiation in the aging

brain, although their numbers decline with age, highlighting the need to enhance remyelination by NSCs. Furthermore, inhibiting NSC-mediated remyelination by ablating them results in axonal loss, demonstrating their significant functional contribution to remyelination (Segel et al., 2019). Collectively, these data imply that remyelination by NSCs may provide persistent axonal protection, and that boosting remyelination by NSCs may be advantageous for avoiding axonal deterioration, hence giving an approach for neuroprotective treatment in MS and other neurological illnesses (Radecki & Samanta, 2022).

In the aged brain, the oligodendrogenesis differentiation via parenchymal OPCs is restricted. The possibility of greater oligodendrogenic potential of NSC-derived OPCs compared to parenchymal OPCs in both aged and demyelinated settings may be essential for identifying pharmacological strategies to either specifically maximize oligodendrogenesis from the V-SVZ, or endow parenchymal OPCs with characteristics typical of V-SVZ-derived counterparts. In order to establish more effective treatment techniques, it is necessary to conduct high-throughput "omics" research (Butt et al., 2022) and create three-dimensional ex *vivo* human V-SVZ models.

Although several studies demonstrate that the adult human brain contains highly plastic cells, the majority of newly produced cells stay in a relatively immature state within the V-SVZ. Thus, in MS, V-SVZ-derived progenitors would "choose" differentiation/recruitment above survival. Future research should seek to elucidate the mechanisms underlying the migration, recruitment and differentiation of V-SVZ-derived progenitors in the demyelinated brain. Some data suggest that the extent of remyelination in MS varies with clinical course, disease duration and proximity to the lateral ventricle (Patrikios et al., 2006). However, a more comprehensive study is required to determine how the expansion of the V-SVZ fluctuates with clinical course and disease duration, and whether a correlation can be deduced between V-SVZ expansion and the extent of remyelination in ventricular lesions (Nait-Oumesmar et al., 2008).

The V-SVZ area has the particular microenvironments that allow Neuronal Stem Percursor Cells (NSPCs) to interact with V-SVZ components in an efficient and dynamic manner. Under physiological situations such as MS, the fate of NSPCs is carefully controlled by the combined activities of intrinsic and extrinsic stimuli. After demyelination, the homeostasis would be altered to encourage endogenous repair attempts during oligodendrogenesis. In contrast, as the illness progresses, the NSPC-OPC-OL transition is disturbed, mostly as a result of a decrease in pro-oligodendrogenesis signals and an increase in anti-oligodendrogenesis signals (Maki et al., 2013b).

To study the proliferation ratio within the V-SVZ (Mecha et al., 2013) studied a Theiler's virus infection mouse model (TMEV-IDD) . At 35 dpi (days post injection), BrdU was injected and integrated into cells in

the lateral ventricles in close proximity to the corpus callosum, striatum and septum. There was a considerable increase in the number of BrdU+ cells in infected animals in all three of these regions compared with wild-type mice. Moreover, a brief BrdU pulse at 35 dpi increased the number of cells that incorporated BrdU in the corpus callosum of infected mice at 45 dpi, indicating that the OPCs had migrated from the SVZ to the demyelinating locations. Indeed, double labeling for BrdU and APC, a marker for mature oligodendrocytes, revealed an increase in the frequency of BrdU+/APC+ cells in TMEV-IDD animals. Their findings emphasize the significance of V-SVZ to the remyelination process, which is confirmed by the enhanced proliferation ratio of this germinative niche, as demonstrated by the BrdU incorporation study.

Also, (Picard-Riera et al., 2002.) found out that in response to Experimental Autoimmune Encephalomyelitis (EAE) mouse model once neural progenitors are transplanted into the V-SVZ of the shiverer mouse, they migrate to the OB (Olfactory bulb) and develop into neurons and OLs. Without excluding the potential that oligodendrocytes are produced locally in the OB, particular signals such as hypomyelination or demyelination appear to induce the V-SVZ-derived neural progenitors to serve as an additional source of oligodendrocytes for the OB. In response to environmental signals, endogenous neural progenitors have the capacity to diverge from their initial fate, much as transplanted neural progenitors.

The ”choice” to differentiate into a glial cell like the oligodendrocytes from a NSC can be explained by the presence of various markers and growth factors. The V-SVZ endures in the adult human brain and contains cells expressing the same markers as in rodents, including PSA-NCAM (polysialylated form of neural cell adhesion molecule), GFAP (glial fibrillary acidic protein), nestin and the epidermal growth factor receptor (Lindvall & Kokaia, 2015). (Nait-Oumesmar et al., 2007) revealed evidence that the V-SVZ is activated in MS, resulting in the production of PSA-NCAM progenitors in the ependymal–stem cell area. Early progenitors were also discovered in the periventricular region of MS lesions. PSA-NCAM progenitors' expression of early markers such as Sox9, Sox10, and Olig2 demonstrated their commitment to a glial rather than neuronal fate. The predominance of PSA-NCAM progenitors in lesions close to the V-SVZ and the discovery of progenitors with bipolar profiles imply that some of them move into neighboring lesions, where they may be one of the origins of the previously identified oligodendrocytes progenitors. Based on the increase in cell density and proliferation in MS groups over controls, their findings demonstrated a definite expansion of the V-SVZ.

(Butti et al., 2019b) demonstrated the role of NPCs in the cuprizone-induced toxic demyelination model mice that is also accompanied by axonal loss. The work demonstrates that SVZ-NPCs mobilized after a first demyelinating phase may partially restore the pool of oligodendrocyte precursors and lower the activation status of microglia. In turn, this latter impact may induce some neuroprotection of corpus callosum axons at later stages. In disorders such as stroke, epilepsy, and gliomas, NPCs can detect inflammatory signals and minimize tissue damage by producing neuroprotective chemicals, therefore contributing to CNS homeostasis.

NSC's cell fates are influenced by a further growth factor that has not yet been disclosed. EGF is the *epidermal growth factor*. EGF is essential for the proliferation and migration of mature NSCs. According to accumulating evidence, overstimulation of the EGF signalling pathway in the adult V-SVZ substantially increases the number of OPCs (Gonzalez-Perez et al., 2009). High migratory capacity and remyelination potential are two crucial traits to consider when creating cell repair therapies for demyelinating diseases such as MS.

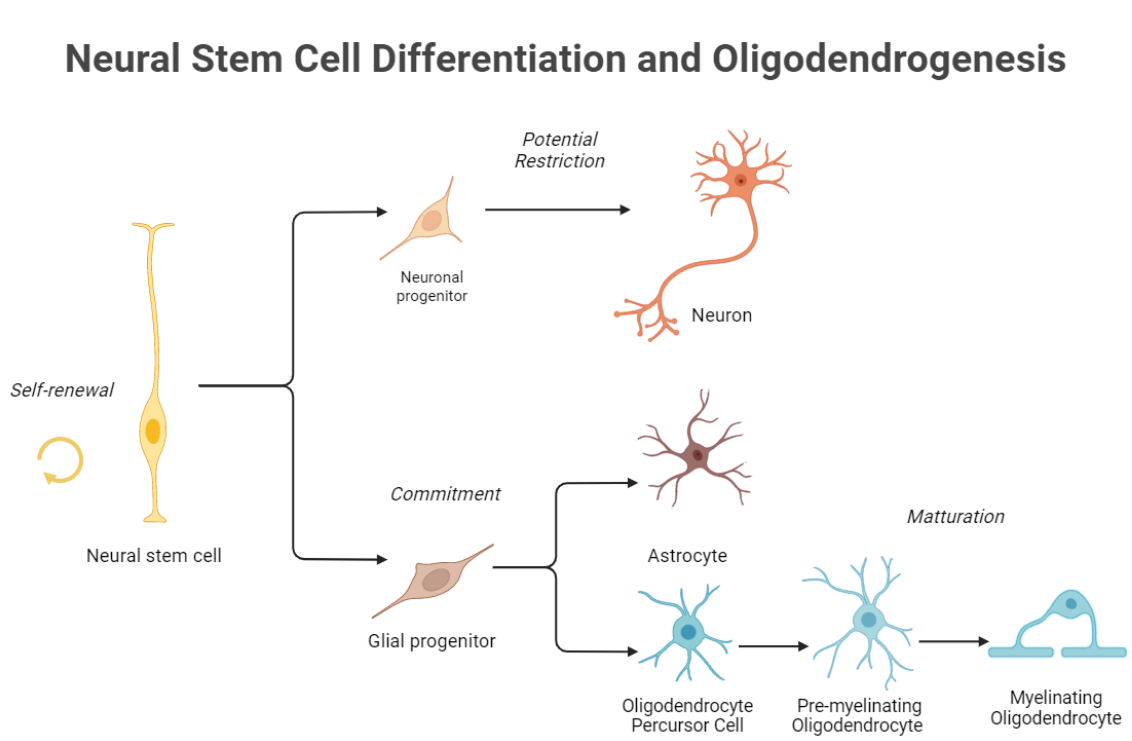


**Figure 2** – **Mature Oligodendrocytes, OPCs and other Macroglia have their origin in Neural Stem cells –** Neural Stem Cells are pluripotent cells that can generate Neuronal Progenitors whose potential restriction can lead to the formation of new Neurons in adulthood. On the other hand, they can also form Glial Progenitors to form glial cells like Astrocytes. In these figures specifically, it's given a particular interest in Oligodendrogenesis – the process to form new myelinating oligodendrocytes from the OPCs throughout the maturation process. Created with *BioRender.com*

## 3.3. Modulation of V-SVZ cells

### 3.3.1. BBB

Blood-Brain Barrier (BBB) ensures homeostasis of the CNS and along with the Blood-Cerebrospinal Fluid (B-CSF) barrier and the arachnoid barrier, performs a crucial function by controlling the bidirectional exchanges between the circulatory system and the CNS in a very selective manner. So, BBB is a complex structure composed of endothelial cells of CNS microvessels and other structures that are essential to its optimal operation (Schreiner et al., 2022).

The selective nature of the BBB is defined by tight connections between endothelial cells that only permit specific neuronal communication, resulting in blood exchange across the CNS. Although the barrier consists of endothelial cells, it is bordered by pericytes, a basement membrane, and astrocyte end-foot connections. Due to the formation of tight connections between particular cell types, only specific chemicals can cross the BBB and reach the brain (Rice et al., 2022). In addition, a multitude of circulatory chemicals, such as inflammatory agents and hormones, are important for maintaining the physiological selective permeability of the BBB (Rice et al., 2022).

The BBB and the peripherical vasculature play an important role in the regulation of the V-SVZ. Juxtacrine signals and paracrine factors from vascular endothelial cells (Ephrin-B2, Jagged-1, NT-3) collaborate with cell-intrinsic programs to keep type-B cells in a quiescent, undifferentiated state that is sensitive to proliferative stimuli. In contrast, active type-B cells and their progeny, type-C cells, detect and respond to paracrine vascular stimuli that encourage their proliferation and neuronal differentiation into type-A cells. Paracrine substances like BDNF and SDF1 drive the migration of Type-A neuroblasts along blood capillaries to the OB (Ottone & Parrinello, 2015).

### 3.3.2. CP

The Choroid Plexus (CP) is a highly vascularized tissue situated in each ventricle of the brain, whose primary function is the generation of cerebrospinal fluid (CSF). CP secretes growth factors, polypeptides, several hormones and biologically ative molecules. It has been reported that molecules produced by the CP have a role in the proliferation, differentiation, and survival of NSC in V-SVZ (Silva-Vargas et al., 2016; Talhada et al., 2020).

Under normal physiological settings, the CP is able to express many genes encoding proteins known to enhance neural progenitor cell proliferation, differentiation, and survival. These proteins are released into the CSF, which serves as a delivery pathway for CP-derived proteins/molecules to the V-SVZ. Importantly, under situations that disrupt normal CP homeostasis, the CSF protein level will be altered, and this will eventually have an effect on the V-SVZ. Also, this effect may serve as a possible rescue mechanism for brain parenchyma lesions (such as those caused by stroke, Parkinson's disease, or MS) (Falcão et al., 2012).

## 4. Multiple Sclerosis mouse models

MS is a neurodegenerative illness of great biological complexity whose diagnosis results in a decline in patients QoL. Consequently, it is imperative to know the causes and underlying processes of this illness. As such, many MS models have been developed to study this disease. However, how to decide on the best suitable model for research? Which features should be considered while approving a biological model that permits researchers to acquire meaningful results?

Biological systems are extraordinarily complex. There are intricate interconnections between the body's cells, tissues, organs, and organ systems, and even slight alterations at the molecular level can have tremendous effects on the organism as a whole. Due to the complexity of MS, it is challenging to recreate the disease using in *vivo* models. Despite this, a variety of models have been designed that can mimic various phases of the disease's progression.

## 4.1. The EAE mouse model for MS

Experimental autoimmune encephalomyelitis (EAE) is a T-helper (Th) cell-mediated autoimmune disorder characterised by T-cell and monocyte infiltration in the CNS together with local inflammation. The autoimmune molecular targets are proteins produced by myelin-producing oligodendrocytes. In EAE, primary demyelination of axonal tracks decreases axonal conduction in the CNS, and gradual paralysis of the hind limbs are the results. EAE is frequently used as a model of MS and, as such, has proven a potent instrument for investigating disease aetiology and possible therapeutics. There are several pathophysiologic variants of EAE with variable clinical presentation patterns based on the animal species and strain, priming protein/peptide, and vaccination method. Thus, several models have been utilized to examine disease progression and particular histopathologic aspects pertinent to MS, as well as to unravel the processes behind prospective treatment approaches (Robinson et al., 2014). There are two primary methods to induce EAE: active EAE by immunization with CNS peptides and passive EAE via adoptive transfer of encephalitogenic T cells. Encephalitogenic peptides, including proteolipid protein (PLP), spinal cord homogenate (SCH), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), can produce EAE in susceptible strains such as SJL mice (Burrows et al., 2019). Subcutaneous injection of an emulsion containing encephalitogenic peptides induces the activation of myelin antigen-specific T cells, followed by their proliferation and differentiation into effector T cells. The presence of integrins on these effector T cells allows them to traverse the BBB. Once in the CNS, effector T cells produce pro-inflammatory cytokines, including interferon- (IFN-), interleukin-17 (IL-17), and tissue necrosis factor- (TNF-) granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, the generation of pathogenic chemokines draws T cells, macrophages, monocytes, and neutrophils into the CNS. These mechanisms contribute significantly to the breakdown of the myelin sheath mimicking MS basic pathology (Burrows et al., 2019)

EAE can be induced in Lewis rats that have a monophasic EAE disease course characterized by an initial onset and spontaneous recovery, which resembles the return of clinical symptoms observed in MS. This enables the analysis of a complete cycle of symptom aggravation and remission, making this animal model a commonly used tool for studying immunological and pathological aspects of MS. In addition, EAE in Lewis rats is a useful tool for the medication development of anti-inflammatory chemicals such as glucocorticoids (GCs), which are widely used clinically as a pulse treatment for acute relapses of MS. However, this EAE model cannot be utilized to investigate the beginning events that cause MS or the

demyelination that occurs in MS patients. In addition, unlike MS, the inflammation in EAE rats is mostly confined to the spinal cord. Thus, acute EAE in rats is not a comprehensive model for MS, but rather a valid animal model for examining the fundamental processes underlying T cell-mediated CNS inflammation (Schneider et al., 2009).

### 4.2. Zebrafish demyelination model

Zebrafish are developing as an attractive new animal model for several neurological illnesses, including MS and other demyelinating diseases. Zebrafish produce numerous embryos, grow swiftly, are transparent and susceptible to genetic modification. This makes them great model systems for studying neurological diseases and producing high-throughput drug screens, which is not achievable with in *vivo* models at present. The zebrafish CNS is a simplified counterpart of the mammalian nervous system and retains the majority of the cell types, connections, and structures of the mammalian brain and spinal cord. As in mammals, myelin is formed by OLs in a highly regulated and controlled manner in zebrafish, producing tight concentric wraps around the axons.(Burrows et al., 2019)

(Karttunen et al., 2017) developed a transgenic, drug-inducible model of oligodendrocyte ablation in larval zebrafish, with subsequent demyelination and remyelination. Authors exploit the superior capabilities of zebrafish larvae for imaging myelinated axons to track the demyelination response of individual axons over time. With this model, they were able to examine the response to demyelination of reticulospinal axons in the CNS of zebrafish, a well-defined identifiable population that is strongly myelinated along its length barely 5 days after fertilization (dpf).

### 4.3. Cuprizone model

The Cuprizone model, already accessed in this document, is also an important tool for the experimental study of MS.

This is an example of a model of neurotoxic demyelination. Young adult mice are given the copper chelator cuprizone in this model (bis-cyclo-hexanone oxaldihydrazone). This results in continuous demyelination. However, spontaneous remyelination can be detected as early as four days following neurotoxic removal, making the cuprizone model ideal for examining variables that prevent demyelination and encourage

remyelination. The delivery of cuprizone results in oligodendrocyte cell death, which is followed by demyelination (Torkildsen et al., 2008).

### 4.4. Theiler's virus model

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) was first reported by Theiler in 1937 and is a single-stranded RNA virus that belongs to the *Picornaviridae* family. TMEV is responsible for causing neurological and enteric diseases in susceptible strains of mice.

Infection of susceptible mouse strains with TMEV causes a biphasic condition of the CNS consisting of early acute disease and late chronic demyelinating disease that manifests 30 to 40 days post-infection (dpi). This late chronic demyelinating illness in the CNS of TMEV-susceptible mouse strains is one of the finest models for studying MS.

### 4.5. Oligodendrocyte ablation mouse model

Although there are reliable models for the study of MS a good part of the models have flaws and the research developed in this area has led to the emergence of new animal models as alternatives with specific characteristics based on the objectives designed for each investigation.

The specific model used for this work was developed to overcome some barriers such as toxicity and loss of cell specificity during the myelination and remyelination phases. This genetic model, the PlpCreERT; ROSA26-eGFP-DTA mouse model, was developed by Traka and collegues (Traka et al., 2010 ) and is centred in CNS demyelination and remyelination. Herein oligodendrocytes are eliminated by cell-specific expression of Difteria Toxin-A (DTA) expression in mice carrying two transgenes: PlpCreERT and ROSA26-eGFP-DTA. In the ROSA26-eGFP-DTA line the DTA coding sequence has been introduced into the ROSA26 locus and is preceded by a floxed eGFP gene neocassette, a strong transcriptional stop sequence which inhibits DTA production (**Figure 3**). The PLP/CreERT transgene promotes the expression of the tamoxifen-induced Cre recombinase under the transcriptional control of the abundantly expressed CNS PLP.The expression of DTA in myelinating oligodendrocytes leads to their death. As such, this model eliminates the potentially confounding non-cell-specific effects present in the toxin models such as cuprizone model or LPC model described above. As OPCs do not express the PLP, they are not eliminated

during tamoxifen treatment periods which can then replace the lost oligodendrocytes. Therefore, this model allows the study of remyelination processes through OPC differentiation.

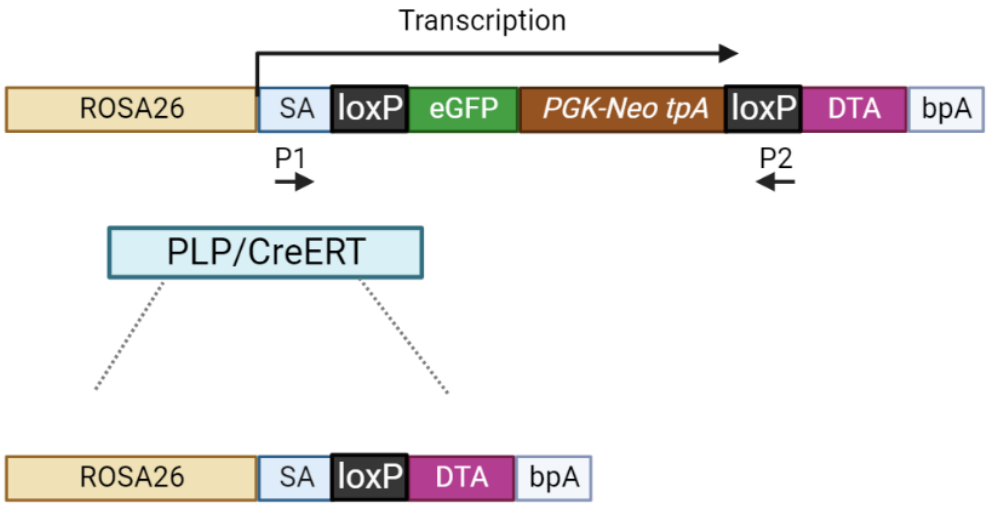



**Figure 3 – Injected tamoxifen promotes Plp-driven CreERT activity in DTA mice. -** oligodendrocytes produce DT-A due to the mediated deletion of the loxP-flanked eGFP, PGK-Neo, and the tpA coding sequences from the ROSA26-eGFP-DTA allele. Made with *BioRender.com*

## AIMS

The main goal of this thesis is to characterize the V-SVZ cell dynamics in response to MS.

**Specifically we aim to:**

### 1- Evaluate V-SVZ OPCs and cell proliferation rates in response to demyelination in a mouse model of MS

A mouse model where oligodendrocytes are massively and selectively ablated throughout the CNS in a tamoxifen-induced Cre-mediated expression of the DTA was used. This genetic mouse model, PLPCreERT; ROSA26-eGFP-DTA and controls ROSA26-eGFP-DTA is characterized by having initial robust demyelination followed by full remyelination promoted by endogenous progenitors. The contribution of the V-SVZ cells to the process of oligodendrocyte regeneration in this model remains undetermined. The brain was collected at the peak of demyelination (week 3 post-tamoxifen injection). Total cell proliferation, OPC and OPC proliferation in the V-SVZ was assessed in this time point by performing Immunofluorescence (IF) for KI67+, PDGFRa+, and PDGFRa+KI67+, respectively. Comparisons were performed between controls and demyelinating conditions.

### 2- Evaluation of the presence of OPCs and proliferating cells in the vicinity of the V-SVZ in response to demyelination in a mouse model of MS

Another part of this work focused on the migraton pattern of OPC's and an overall of their proliferation in the vicinity of the V-SVZ. Total cell proliferation, OPC and OPC proliferation in the V-SVZ was assessed in this time point by performing immunofluorescence for KI67+, PDGFRa+, and PDGFRa+KI67+, respectively. Comparisons were performed between controls and demyelinating conditions

### 3- Characterization of the OPCs and proliferating cells in the different ventricular walls of the V-SVZ in controls

Finally, with the same conditions of the previous tasks, a statistical study was accessed to underline the different aspects of total cell proliferation, OPC and OPC proliferation in the V-SVZ different walls (dorsal, septal and lateral). Comparissons were done between walls of the control groups.

## Materials and Methods

### 1. Ehtical Concerns and use of animals regulation.

All animal procedures previously performed by the supervisor of this thesis were conducted in accordance with European regulations (European Union Directive 2010/63/UE). The animal facility and the people directly involved in animal experiments were certified by the Portuguese regulatory entity — Direcção Geral de Veterinária (DGV).

The 3R's policy were adapted – (refinement, reduction and replacement) and all animal experiments were undertaken in accordance with Directive 2010/63/EU of the European Union.

In accordance with FELASA (Federation of European Laboratory Animal Science Associations) requirements, the Specified Pathogen Free health status was certified by a sentinel two weeks following the animal's arrival at the animal facility (UBIOT) in the host institution.

Each mouse was kept in the same animal housing room. To minimize any kind of stress on the animals It was essential to establish humane endpoints, which included: a 20 % reduction in body weight as measured by a regular dynamic weight measurement, the presence of eventual fighting wounds, or signs of dehydration or sub-nutrition.

It is essential to refer that until I got my certificate in LAS (Laboratory Animal Science), the protocols and procedures that include the manipulation of animals in this work were performed by the supervisor Ana Falcão.

### 1.2. The animal facility conditions

The UBIOT housing conditions for the mice used in this study include: - Artificial 12 h light/dark cycle, with lights on from 8:00 am to 8:00 pm, 21°C ambient temperature, and 50–60% relative humidity. The mice were fed a regular 4RF25 diet throughout the gestation and postnatal periods, and a 4RF21 diet after weaning (3 weeks of age; Mucedola SRL) in addition to ad libitum water. Mice were also kept in a maximum group of six in polysulfone cages 267 x 207 x 140 mm (370 $cm^2$ floor space) covered with a tissue filter (Tecniplast) and corncob bedding at weaning (Scobis Due, Mucedola SRL). Finally, to

encourage natural nesting behaviour, the environment was enriched with soft tissue paper and paper shreds.

## 2. Mouse model used

We used the ROSA26-eGFP-DTA (full name: *Gt(ROSA)26Sortm1(DTA)Jpmb*/J, purchased from The Jackson Laboratory, stock No: 006331) previouly described in this work which has a STOP sequence flanked by loxP (EGFP::PGK-neo::3xpolyA) upstream of the diphtheria toxin A subunit (DTA) gene. Mutant animals have extensive EGFP expression, but DTA transcription is blocked by a strong transcriptional stop sequence (Ivanova et al., 2005). When mated to mice PlpCreERT (full name*: B6.Cg-Tg(Plp1-cre/ERT)3Pop/J*, purchased from The Jackson Laboratory, stock No: 005975) that express Cre recombinase under Plp promoter, the floxed-STOP cassette is deleted and DTA expression is triggered, resulting in the targeted elimination of cre-expressing cells which in this specific case were the mature oligodendrocytes of the mice CNS.

At 5–7 weeks of age, mice were injected intraperitoneally with 1mg of tamoxifen on three consecutive days to promote recombination of the ROSA26-eGFP-DTA allele and subsequent expression of DTA in oligodendrocytes. Mice were sacrificed at dpi 21 at the peak of demyelination.

## 3. Mice Genotyping

### 3.1 - Animal identification and mating

First, we crossed a DTAfl/fl female mouse with a PLPCreERT heterozygous male and from this crossing we obtained 2 different genotypes (**Figure 4**): wt/wt;DTAfl/wt (control group) and PLPCreERTtg/wt;DTAfl/wt (DTA+ group).

The animals were identified by making an ear punch with an appropriate scissor and a biopsy was collected for DNA extraction purposes. In addition, 4 weeks after the birth, the animals were separated according to the gender.

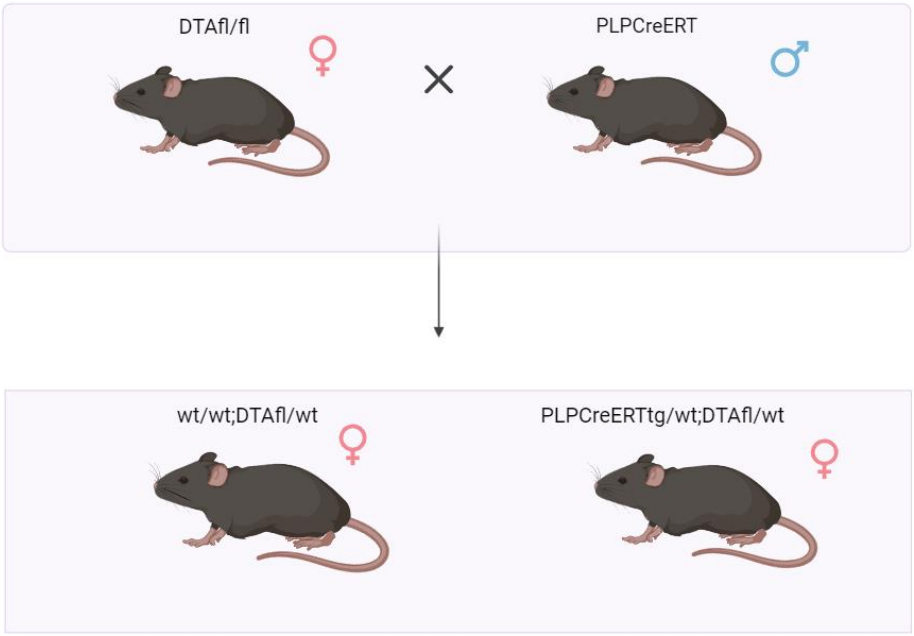



**Figure 4 – Mice mating –** In this work, DTAfl/fl female mice were bred with PLPCreERT male mice. After mating, mice with the appropriate genotypes for the experiment were acquired for the two research groups: wt/wt;DTAfl/wt – Control Group and PLPCreERTwt;DTAfl/wt – DTA group. For this experiment, only females were employed. Created with *BioRender.com*

### 3.2 - DNA extraction from mice ear biopsies

The first step of the genotyping process is the DNA extraction of ear biopsies. To accomplish this, 75 µL of 25mMNaOH/0.02mM EDTA was added to the biopsies followed by an incubation for one hour at 98 °C in a heatblock. The temperature associated with this alkaline solution allowed the lysis of the cell membranes. Next, 75 µL of Tris-HCl 1 mM, pH 5.5 was added to the cells to neutralize the solution. Finally, to separate the undigested tissue, we centrifuge for 3 minutes at 4000 rpm and collect the supernatant containing the DNA for further analysis.

1 µL of the supernatant was the volume used for the subsequent PCR procedure in each sample.

Note: If samples were not utilized immediately, they were stored at 4 C° in the lab fridge, short term, or -20°C long term.

### 3.3. - PCR amplification

To detect the presence or absence of a target DNA, we performed polymerase chain reaction (PCR) using the pre-made master mix DreamTaq (2x) (K1081, Termofisher). DreamTaq master mix contains DNA Polimerase, Dream Taq buffer, dNTP's and 4 Mm $MgCl_2$. Primers used to genotyping were chosen according to the mice supplier, The Jackson laboratory (**Tables 1 and 2**). Primers (final concentration 1uM) and $ddH_2O$ were added to the master mix (final concentration 1x) for both controls and samples. 1 µL of a DNA sample or ddH2O was added to the final reaction, for samples and negative control, respectively. A spin down of the sample was performed right after with a bench centrifuge.

**Table 1 – Primers used for DTA genotyping**

| Primer | Sequence 5' → 3' | Primer Type | Locus |
|---|---|---|---|
| oIMR8545 | AAA GTC GCT CTG AGT TGT TAT | Forward | Rosa26 |
| oIMR8546 | GGA GCG GGA GAA ATG GAT ATG | Reverse | Rosa26 |
| oIMR8052 | GCG AAG AGT TTG TCC TCA ACC | Mutant Reverse | SA site |

**Table 2 – Primers used for PLPCre genotyping**

| Primer | Sequence 5' → 3' | Primer Type |
|---|---|---|
| oIMR3798 | AGG TGG ACC TGA TCA TGG AG | Transgene Forward |
| oIMR8346 | ATA CCG GAG ATC ATG CAA GC | Transgene Reverse |

Finally, we executed the PCR process in the thermocycler with a design protocol previously established ensuring that all PCR tubes were well sealed to the correct processing of the reaction (described in the table below):

**Table 3 – PCR steps used in the termocycler**

| Step | Temp °c | Time (min) |
|---|---|---|
| 1 | 95° | 3:00 |
| 2 | 95° | 0:30 |
| 3 | 59° | 0:30 |
| 4 | 72° | 1:00 |
| 5 | Go to Step 2 x34 times | |
| 6 | 72° | 5:00 |
| 7 | 12° | ∞ |

### 3.4. - Gel Electrophoresis

To visualize the PCR product, a 2 % agarose gel in 150 mL TBS 1x was warmed in the microwave for about 2 minutes. After dissolving the agarose, 4.5 μL of Greensafe Premium DNA stain (nzytech®), a safer alternative to ethidium bromide, was added. The gel solution was then poured into a support with the appropriate number of wells to accommodate the number of samples required for analysis. Within one hour, we allowed the gel to harden at room temperature.

2 μL of a 100-bp DNA ladder (Hyper ladder 100 bp – meridian bioscience®) was added as a standard in the first well. 6,25 μL of our PCR products were added to the wells. Cables were connected in the right

orientation (red – positive pole/black – negative pole) and configured the device to operate the gel at 120 V. The gels operate for approximately one hour with the same voltage supplied.

After one hour, we ultimately turned off the gadget and removed the gel from the support to be analysed in the the Chemidoc (Biorad).

The resultant gel after analysis is represented bellow in **Figures 5** and **6**:

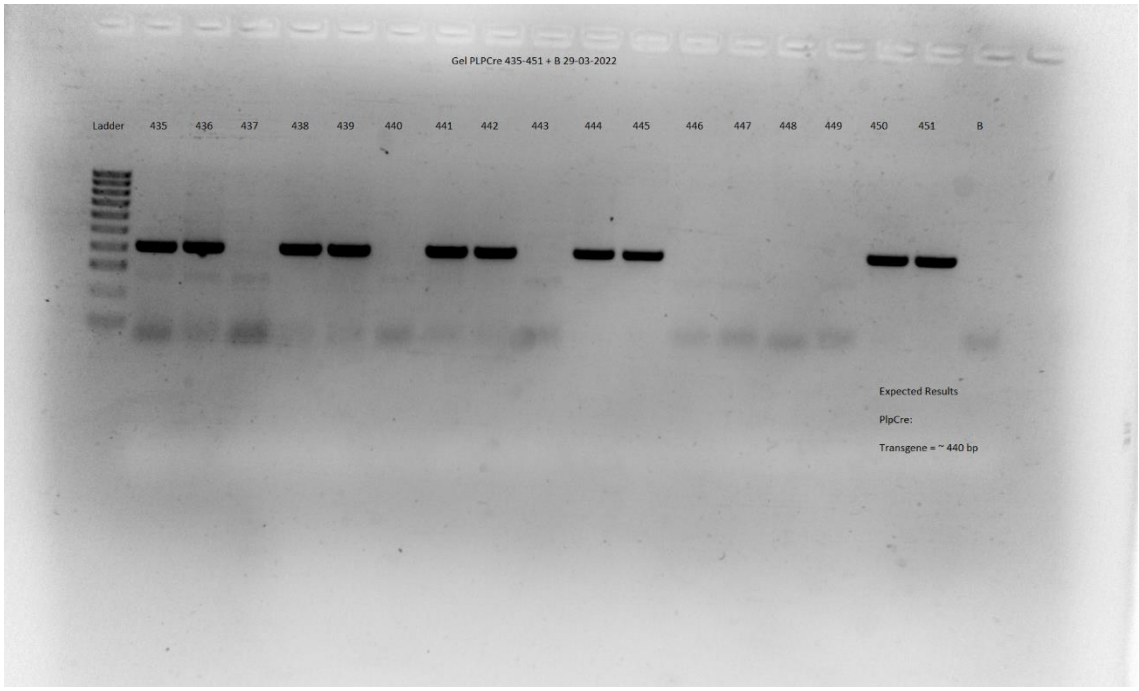



**Figure 5** - **Agarose Gel to confirm the genotype from animals 435-451 and a Control free from DNA**. The expected results show that the animals 435, 436, 438, 439, 441, 442, 444, 445, 450 and 451 have the mutant Transgene based on the band that appears with a Molecular weight of approximately 440 bp. The others are considered to be WT. Ladder - 100bp~~)~~

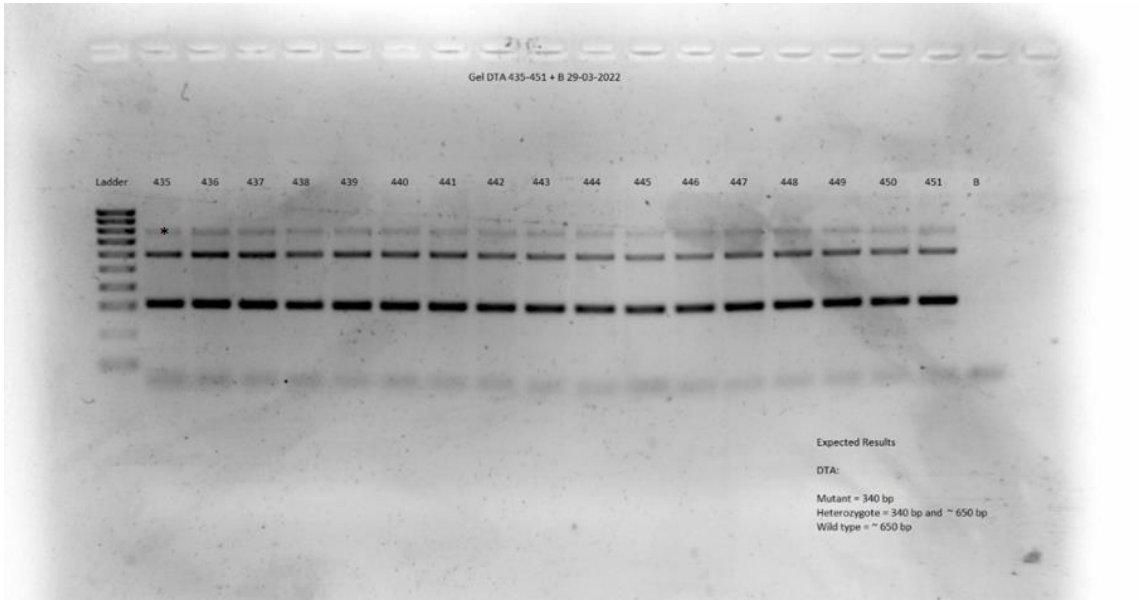



**Figure 6** - **Agarose Gel to confirm the genotype from animals 435-451 and a Control free from DNA**. The expected results show that all animals presented in this gel are Heterozygotic based on the bands that appear with a Molecular weight of 340 bp and 650 bp (Mutant and Wild Type Bands respectively) Ladder - 100bp. The band with (*) is non-specific.

### 4. Mice Sacrifice Procedure

To collect the brain of the mice at the demyelination peak and controls, mice were sacrificed at 21 dpi(**Figure-7**).

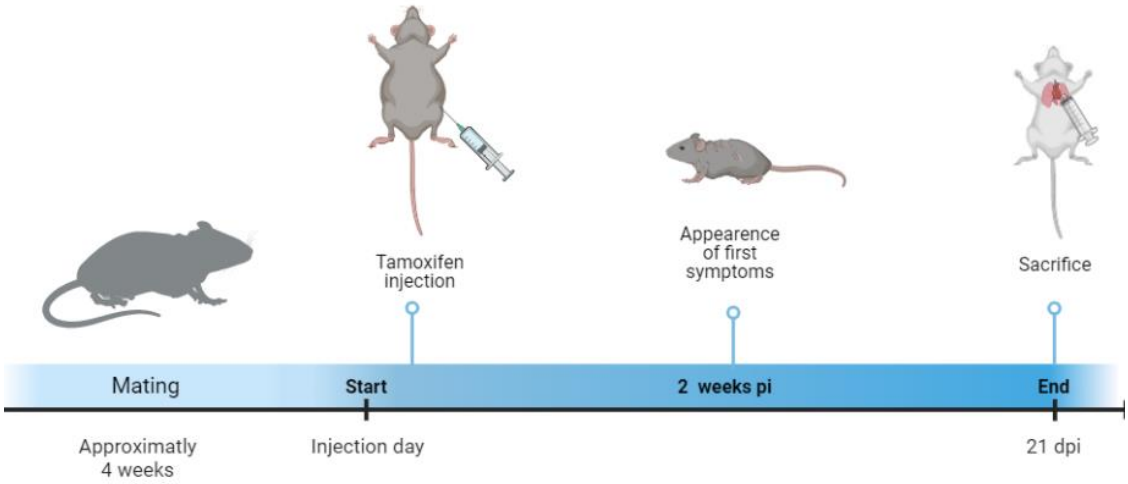


**Figure 7 – Timeline representing the experience from the mating to the sacrifice day** – After the mating process with the mice used, we genotyped them and inject Tamoxifen intraperitoneally. The symptoms started to appear after 2 weeks due to the death of mature oligodendrocytes, which lead to demyelination of the CNS neurons. In 21 dpi (3 weeks) , we sacrificed the mice to procede with 4% PFA perfusion and collect the brain and spinal cords.

### 4.1 - Anesthesia and Brain + Spinal Cord Removal

Mice were anesthesized by an intraperitoneal injection of the anesthesia solution (Imalgene, 75mg/kg and Dormitor, 1mg/Kg). The mouse is put down and we wait until the animal no longer responds to stimuli. A transcardiac perfusion with PBS followed by PFA 4% in PBS was performed in all mice in order to fix the brain tissue. The success of the perfusion is confirmed by both the lack of blood in the liver (from a red to a white colour transition) and by the mice body stiffness.

Another biopsy sample is taken from the base of the tail for genotyping confirmation purposes.

Brain was removed by fracturing the skull between the eyes, and then on the side between the ear canal and the eyes. We access the brain by removing the skull's top. Next, the brain was preserved in a Falcon tube with 7-8 mL of 4% PFA for 1-2 hours.

### 4.2. Brain cryopreservation

For brain cryopreservation the PFA 4% was removed and a 30% Sucrose solution in PBS 1x was added. The brain was left in this solution at 4°C until it sinks. Next, brains were embedded in O.C.T solution, frozen in dry ice and stored at -20°C.

### 5 - Cryostat cutting procedure

A cryostat was used to slice the brain. Cryostat settings were adjusted as the cryostat temperature must be a few degrees colder than the cutting support. The cutting range was adjusted to 20 μm and the blade was positioned correctly.

Slices were collected into superfrost glass slides previously marked with the animal identity and the slide serial number.

Finally, we preserved the slides at -20°C for subsequent examination.

## 6. Immunofluorescence

### 6.1. Antigen Retrieval

We used an EDTA 1% antigen retrieval solution to retrieve antigens obscured by the cross-linking fixative PFA.

We placed the slides into a cuvette containing an EDTA solution and heated at 96°C for 30 minutes in a double-boiling bath. When the time was up, we removed the cuvette and allowed it to cool for around 15 minutes. After that, the slides were prepared for the IF procedure.

### 6.2 – Primary antibody

Slices were washed in PBS three times, five minutes apart. In the meantime, a solution of primary antibody was prepared as follows: primary antibody was added to a solution of 10% donkey serum in PBS-0.5% Triton. The primary antibodies and the dilutions used are described in **Table 4**.

Following the removal of the PBS solution, the antibody solution was added to each slide and left overnight at 4°C in an humidified box.

**Table 4 – Antibodies**

| Antibody | Company, reference | Dilution Volume | Target |
|---|---|---|---|
| Primary goat anti-Pdgfra | R&D, AF1062 | 1:100 | OPCs |
| Primary rabbit anti-ki-67 | Invitrogen | 1:200 | Proliferative Cells |
| Secondary Donkey anti-goat 555 | Invitrogen | (1:1000) | Primary antibody: goat anti-Pdgfra |
| Secondary Donkey anti-rabbit 647 | Invitrogen | (1:1000) | Primary antibody: rabbit anti- ki-67 |

### 6.3 - Secondary antibody

Slices were washed in PBS three times, five minutes apart to remove the unbound primary antibody. The secondary antibody was prepared in a solution of 10% donkey serum in PBS-0.5% Triton. The secondary antibodies and the dilutions used are described in **Table 4.**

Secondary antibodies were added to the slides and incubated for 2h at room temperature.

### 6.4. DAPI application and finalizing the IF protocol

Slices were washed three times in PBS to remove unbound secondary antibody. DAPI solution, for nuclei staining, was added to each slide for 2 minutes.

At the conclusion, the mounting media added each slide and covered with a coverslip.

The slides were now prepared for confocal microscopy analysis.

## 7. Confocal imaging

After completing the steps of the IF methodology, we analyzed the slides using Fluorescence Microscopy to see if the process was carried out as intended. Then, we utilize an Olympus® LPS Confocal FV1000 microscope to acquire images of the required location - the V-SVZ - as well as the area around it (150 μm), in order to infer on the migration and organization of OPCs throughout the ventricular system.

## 8. Cell counting

For cell counting, Image J software was used and cell counter plugin to count the targeted cells of the IF protocol: PDGFR-a (marker of OPC'S), ki-67 (to show cells in proliferation), and the double positivity of both when this was the case in each wall (dorsal, septal, and lateral) (**Figure 8**) of each ventricular and in every animal, individually.

## 9. Statistical Report

Statistical Analysis GraphPad PRISM 8 program was used to analyze the data (GraphPad Software Inc., San Diego,CA). The study comprised of one-way analysis of variance (ANOVA) for single-factor multiple group comparisons between three groups and Student's t test for comparisons between two groups.

The statistical significance level was established at $p < 0.05$.

## Results

V-SVZ analysis in the PlpCreERT; ROSA26-eGFP-DTA mouse model is lacking. Therefore, a dynamic examination of total cell proliferation and OPC proliferation and migration was assessed at V-SVZ.

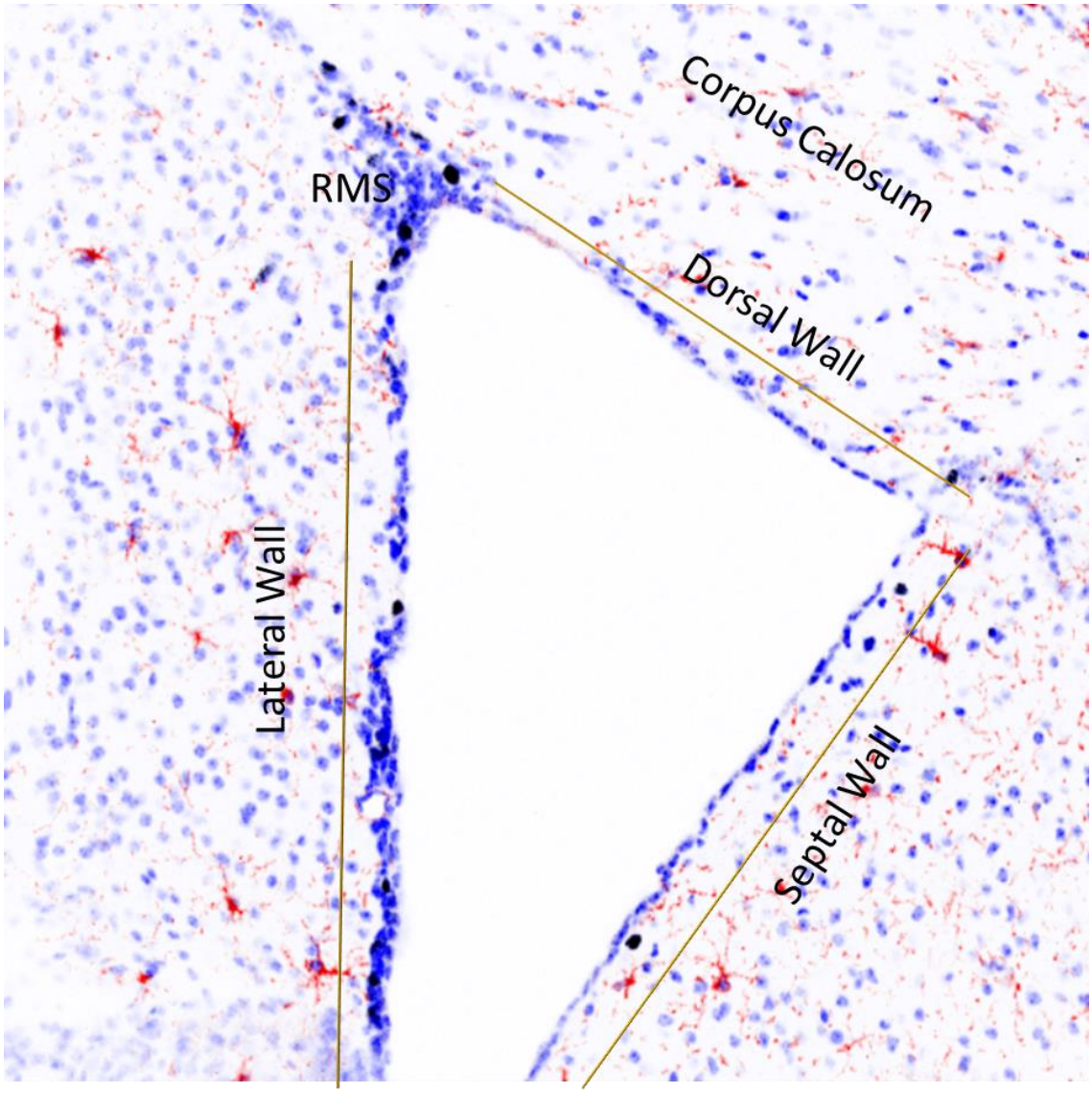



**Figure 8 – Schematic representation of the walls in the lateral ventricle –** This schematic image illustrates the ventricle's walls: dorsal, septal and lateral. Note that the dorsal wall lies below the corpus callosum. The RMS in depicted the ventricle's upper left quadrant. This image was processed from an image taken at the confocal microscope (Olympus® LPS Confocal FV1000), 20x magnification, with DAPI (blue) indicating the presence of nuclei and OPCs (red) using Alexa Fluor 555. The photo were processed with ImageJ® software. Made with *BioRender.com*

## 1. OPC density in the V-SVZ

To characterize OPCs in the V-SVZ, PDGFRα positive cells, a marker of OPCs, co-stained with DAPI, to visualize their nucleus, were quantified. OPC cell density was estimated by dividing the number of PDGFRα positive cells to the correspondent area. OPC density was estimated for all ventricular walls and a global comparison between control and the DTA mice was performed.

Specifically, an estimation of OPC cell density was made for the dorsal, septal and lateral ventricular walls, as well as a total OPC cell density estimation in the V-SVZ which comprised all ventricular walls (**Figure 9**). A t-student t test was performed to compare control and DTA mice and the p- value indicated that there are no statistically significant differences ($p<0.05$) in all comparisons made.

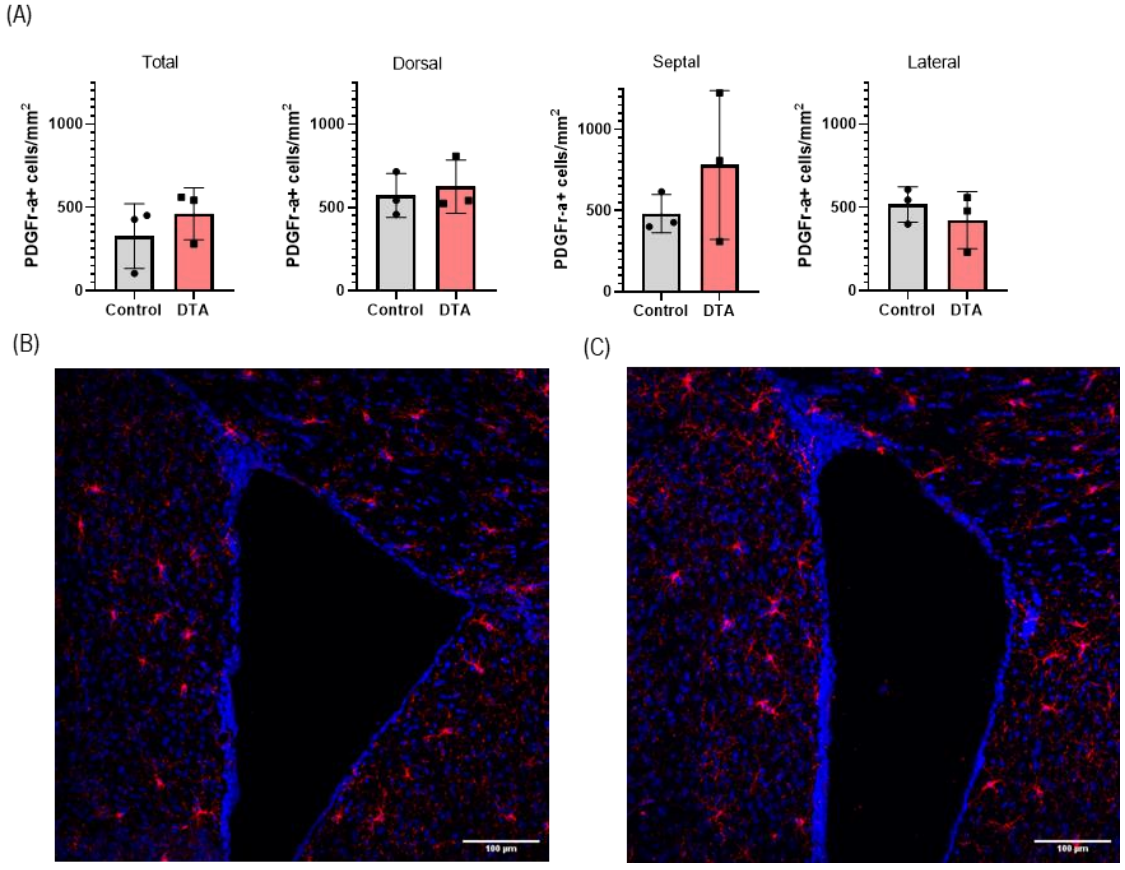


**Figure 9 - Analysis of Platelet-Derived Growth Factor Receptor alfa positive PDGFR $\alpha$ + cells by Immunofluorescence** – (A) Bar plot representation of PDGFRα+ cells densities in all ventricle walls, dorsal, septal, lateral and total. Densities are estimated as cells/área($mm^2$). N=3 in control and DTA mice. A student t-test was performed for comparisons between conditions, where p>0.05 is not significant. (B ) and (C) representative confocal images of the V-SVZ in control and DTA mice, respectively. Scale bar=100 μm, DAPI staining in blue and PDGFR $\alpha$ in red. The images were processed with ImageJ® software.

## 2. Proliferating cell density in the V-SVZ

To characterize the proliferative profile of the neural stem and precursor cells from the V-SVZ , an IF was performed for Ki-67, a canonical proliferation marker. The densities for the proliferating cells were estimated for the dorsal, septal and lateral ventricular walls. A total proliferation cell density comprising all ventricular walls was also estimated (**Figure 10**). A student's t test was performed to compare control and DTA mice and the p-value indicated that there are no significant differences (p>0.05) in all comparisons made.

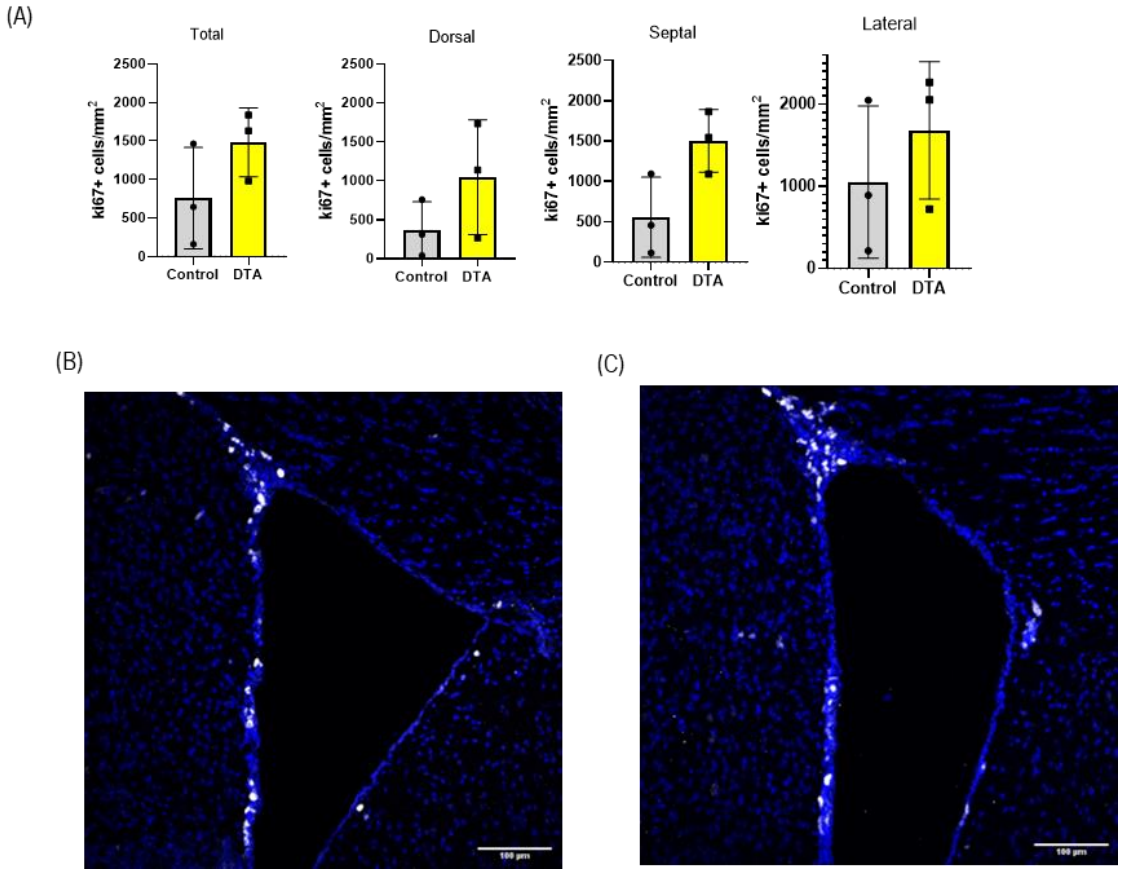


**Figure 10 - Analysis of ki-67 positive cells by Immunofluorescence – (A) – Different analysis of ki-67 + cells in the ventricle** - Bar plot representation of ki67+ cells densities in all ventricle walls, dorsal, septal, lateral and total. Densities are estimated as cells/area ($mm^2$). N=3 in control and DTA mice. A student t test was performed for comparisons between conditions, where p>0.05 is not significant. (B ) and (C) representative confocal images of the V-SVZ in a control and a DTA mice, respectively. Scale bar=100µm, DAPI staining in blue and ki67 in white. The images were processed with ImageJ® software.

### 3 – Proliferating OPC density in the V-SVZ

Proliferating OPCs display a double staining for Ki67 and PDGFR $\alpha$ +. We have quantified the number of Ki67+ PDGFR $\alpha$ +double positive cells and estimated the cell densities for the dorsal, septal and lateral ventricular walls. A total proliferation cell density comprising all ventricular walls was also performed. A student's t test was performed to compare control and DTA mice and the p-value indicated that there are no statistically significant differences (p>0.05) in all comparisons made.

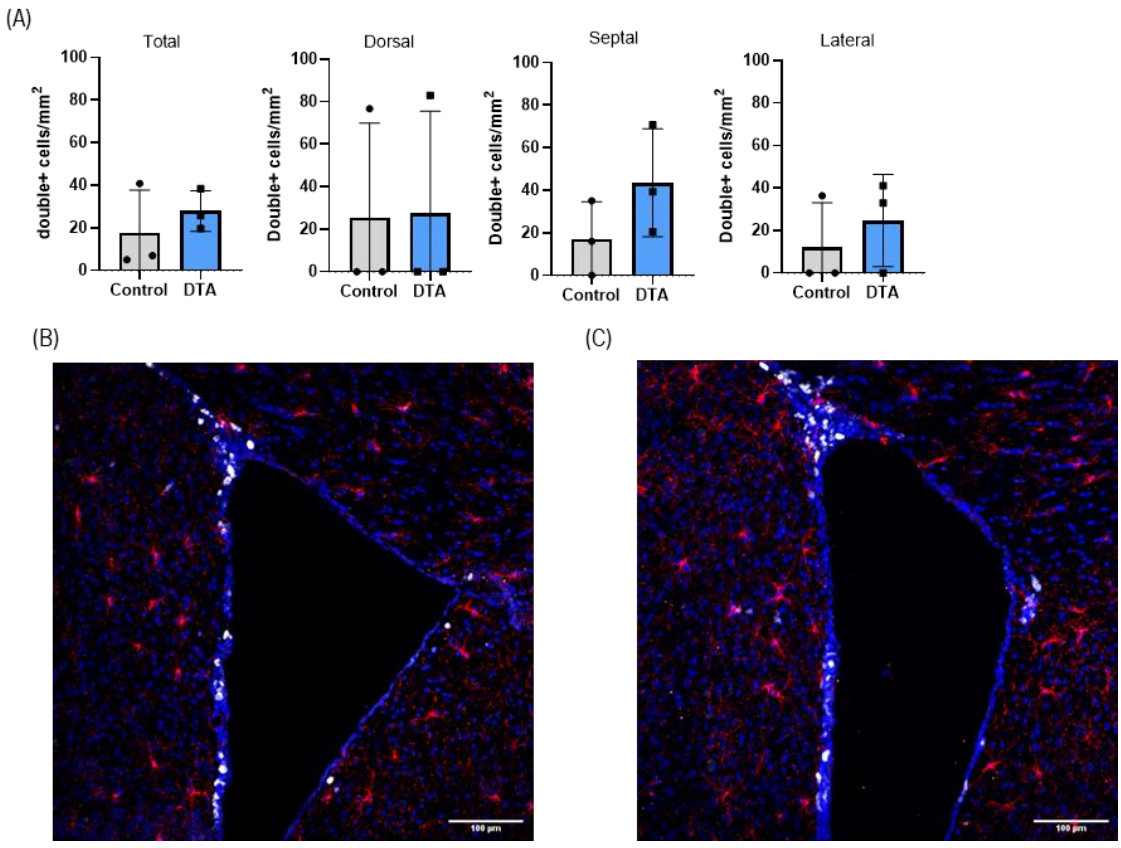


**Figure 11 - Analysis of double positive cells by Immunofluorescence – (A) – Different analysis of double + cells in the ventricle -** Bar plot representation of double+ cells densities in the all ventricle walls, dorsal, septal, lateral and total. Densities are estimated as cells/area(mm2). N=3 in control and DTA mice. A student t test was performed for comparisons between conditions, where p>0.05 is not significant. (B ) and (C) representative confocal images of the V-SVZ in a control and a DTA mice, respectively. Scale bar= 100 $\mu$ m, DAPI staining in blue, PDGFR $\alpha$ + in red and ki67 in white. The images were processed with ImageJ® software.

### 4 – Estimation of OPC in the vicinity of the V-SVZ

After addressing the OPC densities in the V-SVZ, OPCs lying in the vicinity of the V-SVZ, within 150 µm distance, were estimated. This estimation was performed in order to quantify OPC migration from the V-SVZ to the surrounding tissues. OPC were quantified within 150 µm of the dorsal, septal and lateral ventricle walls (**Figure 12**). A student's t test was performed to compare control and DTA mice and the p-value indicated that there are no significant differences (p>0.05) in all comparisons made.

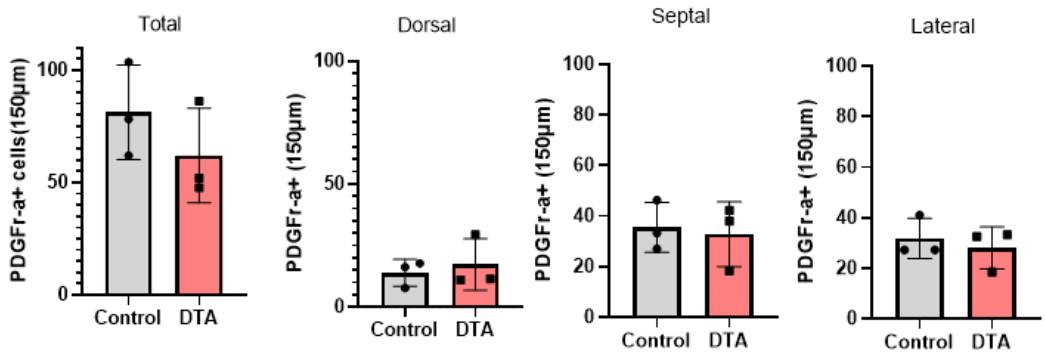



**Figure 12 – Estimation of PDGFR $\alpha$ + in the vicinity of the V-SVZ.** (A) – Bar plots representing the number of PDGFR $\alpha$ +cells within a distance of 150 µm from the total V-SVZ, dorsal, septal and lateral ventricle wall. N=3 in control and DTA mice. A student´s t-test was performed for comparisons between conditions, where p>0.05 is not significant.

### 5 – Estimation of proliferating cells in the vicinity of the V-SVZ

After addressing the ki67 densities in the V-SVZ, ki67+ lying in the vicinity of the V-SVZ, within 150µm distance, were estimated. This estimation was performed in order to quantify ki67 proliferation cells migration from the V-SVZ to the surrounding tissues. Ki67 were quantified within 150 µm of the dorsal,

septal and lateral ventricle walls (**Figure 13**). A student's t test was performed to compare control and DTA mice and the p-value indicated that there are no statistically < differences (p>0.05) in all comparisons made.

(A)

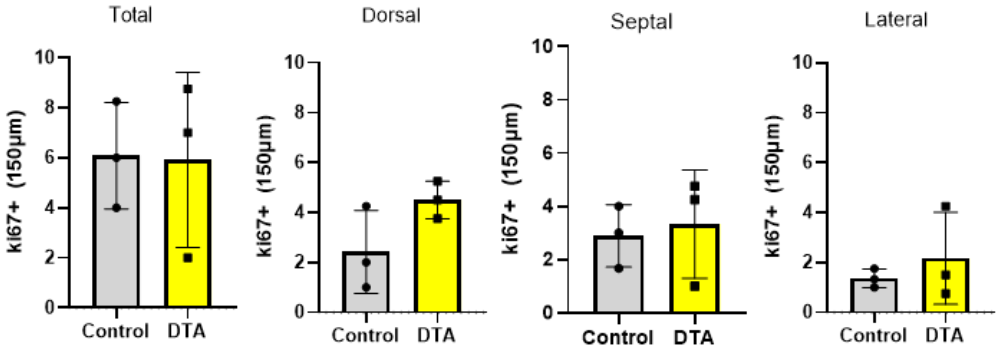



**Figure 13 - Estimation of ki67+ in the vicinity of the V-SVZ.** (A) – Bar plots representing the number of ki67+ cells within a distance of 150 µm from the total V-SVZ, dorsal, septal and lateral ventricle wall. N=3 in control and DTA mice. A student´s t test was performed for comparisons between conditions, where p>0.05 is not significant.

## 6 – Estimation of proliferating OPCs in the vicinity of V-SVZ

We also address the proliferating OPCs lying in the vicinity of the V-SVZ, within 150 $\mu$ m distance. This estimation was performed in order to quantify OPC's proliferation cells migration from the V-SVZ to the surrounding tissues. Proliferating OPC's were quantified within 150 $\mu$ m of the dorsal, septal and lateral ventricle walls (**Figure 14**). A student's t test was performed to compare control and DTA mice and the p-value indicated that there are no statistically significant differences (p>0.05) in all comparisons.

(A)

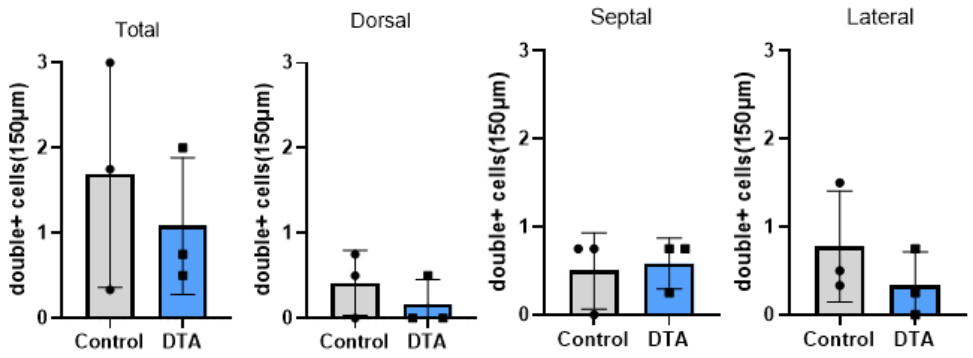



**Figure 14 - Estimation of Proliferating OPCs in the vicinity of the V-SVZ.** (A) – Bar plots representing the number of double+ cells within a distance of 150 µm from the total V-SVZ, dorsal, septal and lateral ventricle wall. N=3 in control and DTA mice. A student´s t-test was performed for comparisons between conditions, where p>0.05 is not significant.

## 7 – Characterization of the OPCs and proliferating cells in the different ventricular walls of the V-SVZ in controls

In order to estimate the existing differences in proliferating cells, OPCs and proliferating OPCs within the different regions of the V-SVZ, we have compared cell densities in the dorsal, septal and lateral ventricle walls of control mice.

A one way ANOVA statistical test was performed to compare the three conditions and the p-value indicated that there are no significant differences (p>0.05) in all comparisons made.

(A)

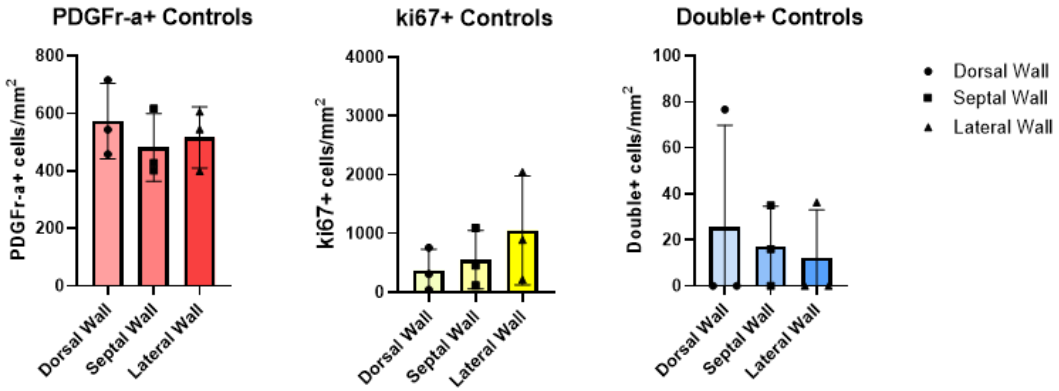


**Figure 15 – Comparison of cell densities between the different ventricle walls for both PDGFR $\alpha$ , ki-67+ and double+ cells in control Animals** – (A) Bar plots representing OPC, proliferating cells and proliferating OPCs (Ki67/ PDGFR $\alpha$ double positive)cell densities of the dorsal, septal and lateral wall of the lateral ventricle. N=3, a one-way ANOVA test was performed for comparisons between the three conditions, where $p>0.05$ is not significant.

## Discussion

In this study we have used a genetic mouse model of de and remyelination previously reported by Tracka and colleagues (Traka et al., 2010). In this mouse model oligodendrocytes are massively and selectively eliminated across the CNS by Cre-mediated expression of the DTA triggered by tamoxifen. This genetic mouse model, PLPCreERT; ROSA26-eGFP-DTA, is distinguished by early strong demyelination followed by complete remyelination induced by endogenous progenitors. Although the oligodendrocytes were eliminated, it has been demonstrated that OPCs may develop into new oligodendrocytes.

Herein we have characterize the response of V-SVZ cells at the peak of demyelination, where remyelination processes are beginning to be orchestrated, in order to evaluate if the V-SVZ could provide OPC and contribute to the remyelination process.

Our results indicate that OPC density in the V-SVZ does not change in response to demyelination in the model used in this study, as accessed by p value from student's t test (p-value > 0.05) (**Figure 9** A). Accordingly, differences in proliferating OPC densities were not observed for none of the ventricle walls (**Figure 11** A). Since the analysis was performed at the peak of demyelination which would corresponds to the beginning of the remyelination orchestration, it could be that if the analysis was performed at a later time point a difference could be observed.

By analysing the proliferation pattern in the V-SVZ, no statistically significant differences were observed from the dorsal, septal and lateral walls between controls and DTA mice indicating that the demyelination occurring in the CNS did not change the proliferation of the neural stem and progenitor cells from the V-SVZ (**Figure 11** A). Nonewithstanding, alterations in the proliferation of V-SVZ cells were reported before in other mouse models of MS such as in the described EAE mouse model (Picard-Riera et al., 2008).

In order to infer about the migration of OPCs, proliferating OPCs or proliferating cells out of the V-SVZ, it was also examined the number of these cells found within 150um distance of the V-SVZ walls, dorsal, septal and lateral. No statistically significant differences between controls and DTA mice were found in the number of cells found in the vicinity of the V-SVZ, suggesting that there is no difference in the migration of these cells in controls or DTA mice (Figure **12** and **13)**. Again, the same analysis performed in a later time point should be performed in order to confirm that cells from V-SVZ are not contributing for the remyelination at later stages.

In order to evaluate the heterogeneity in OPC, proliferating OPCs and proliferating cells in the distinct V-SVZ walls, cell densities were compared between dorsal, septal and lateral walls. No significant changes were observed from this analysis (**Figure 15**). A similar analysis was previously performed in rats showing that the proliferation cell density (assessed by Ki67 positive staining) was higher in the lateral wall when compared with the dorsal wall (Falcao et al. 2012). Although we haven't found any statistically significant result, our data indicates that there is a trend for increased proliferation in the lateral wall when compared to the septal and dorsal wall. This analysis would benefit if the n was increased.

A recent study has used a single-cell genomics technique to investigate the V-SVZ stem cell niche. In these study (Mizrak et al., 2019) also show that the septal wall was more prone to generate oligodendrocytes than the lateral (prone for neurogenesis) and the dorsal wall. Nevertheless, we could not find any difference statistically significant in the number of OPCs or proliferating OPCs in the septal wall when compared to the lateral and dorsal walls of the V-SVZ. Of notice, the marker used to label OPCs was different, Mizrak and colleagues used NG2 staining. Moreover, their differences were more pronounced in males, while our study was performed in females. Interestingly, these study also shows that OPCs exhibited pronounced gender difference being more enriched in males than in females.

## Conclusion

MS is a neurodegenerative illness with severely debilitating symptoms for patients whose genesis is not fully understood.

It is recognized that remyelination occurs in MS at earlier stages of the disease. Nevertheless, it fails at later stages and new sources of oligodendrocytes should be investigated.

The investigation of the dynamics of the V-SVZ cells as a potential source of oligodendrocytes to replenish the lost ones in MS is understudied. This thesis major goal was to investigate the potential of V-SVZ cells to generate OPCs and thus oligodendrocytes in response to a massive oligodendrocyte loss, as is the case of the genetic mouse model of oligodendrocyte deletion used herein.

Although with a low number of animals analysed (n=3), our data indicates that there is no activation of the V-SVZ niche to generate new oligodendrocytes, at least at the time point analysed, 21 dpi. Our study would benefit from an increased n in order to make more robust conclusions.

## Bibliography


Aarli, J. A., Abramsky, O., Browne, P., Chandraratna, D., Angood, C., Tremlett, H., Baker, C., Taylor, B. v, & Thompson, A. J. (2014). *Section Editors Global Perspectives ATLAS OF MULTIPLE SCLEROSIS 2013: A GROWING GLOBAL PROBLEM WITH WIDESPREAD INEQUITY*. http://www.msif.org/about-ms/publications-

Bjornevik, K., Cortese, M., Healy, B. C., Kuhle, J., Mina, M. J., Leng, Y., Elledge, S. J., Niebuhr, D. W., Scher, A. I., Munger, K. L., & Ascherio, A. (2022). MULTIPLE SCLEROSIS Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. In *Science* (Vol. 375). https://www.science.org

Brownlee, W. J., Hardy, T. A., Fazekas, F., & Miller, D. H. (2017). Diagnosis of multiple sclerosis: progress and challenges. In *The Lancet* (Vol. 389, Issue 10076, pp. 1336–1346). Lancet Publishing Group. https://doi.org/10.1016/S0140-6736(16)30959-X

Burrows, D. J., McGown, A., Jain, S. A., de Felice, M., Ramesh, T. M., Sharrack, B., & Majid, A. (2019). Animal models of multiple sclerosis: From rodents to zebrafish. In *Multiple Sclerosis Journal* (Vol. 25, Issue 3, pp. 306–324). SAGE Publications Ltd. https://doi.org/10.1177/1352458518805246

Butt, A. M., Rivera, A. D., Fulton, D., & Azim, K. (2022). Targeting the Subventricular Zone to Promote Myelin Repair in the Aging Brain. *Cells*, *11*(11). https://doi.org/10.3390/cells11111809

Butti, E., Bacigaluppi, M., Chaabane, L., Ruffini, F., Brambilla, E., Berera, G., Montonati, C., Quattrini, A., & Martino, G. (2019a). Neural stem cells of the subventricular zone contribute to neuroprotection of the corpus callosum after cuprizone-induced demyelination. *Journal of Neuroscience*, *39*(28), 5481–5492. https://doi.org/10.1523/JNEUROSCI.0227-18.2019

Butti, E., Bacigaluppi, M., Chaabane, L., Ruffini, F., Brambilla, E., Berera, G., Montonati, C., Quattrini, A., & Martino, G. (2019b). Neural stem cells of the subventricular zone contribute to neuroprotection of the corpus callosum after cuprizone-induced demyelination. *Journal of Neuroscience*, *39*(28), 5481–5492. https://doi.org/10.1523/JNEUROSCI.0227-18.2019

Falcão, A. M., Marques, F., Novais, A., Sousa, N., Palha, J. A., & Sousa, J. C. (2012). The path from the choroid plexus to the subventricular zone: Go with the flow! In *Frontiers in Cellular Neuroscience* (Vol. 6, Issue AUG). https://doi.org/10.3389/fncel.2012.00034

Falcão, A. M., Palha, J. A., Ferreira, A. C., Marques, F., Sousa, N., & Sousa, J. C. (2012). Topographical analysis of the subependymal zone neurogenic niche. *PLoS ONE*, *7*(6). https://doi.org/10.1371/journal.pone.0038647

Gafson, A., Craner, M. J., & Matthews, P. M. (2017). Personalised medicine for multiple sclerosis care. In *Multiple Sclerosis* (Vol. 23, Issue 3, pp. 362–369). SAGE Publications Ltd. https://doi.org/10.1177/1352458516672017

Goldenberg, M. M. (2012). DISEASE OVERVIEW Multiple Sclerosis Review. In *P&T®* (Vol. 37, Issue 3).

Gonzalez-Perez, O., & Alvarez-Buylla, A. (2011). Oligodendrogenesis in the subventricular zone and the role of epidermal growth factor. In *Brain Research Reviews* (Vol. 67, Issues 1–2, pp. 147–156). https://doi.org/10.1016/j.brainresrev.2011.01.001

Gonzalez-Perez, O., Romero-Rodriguez, R., Soriano-Navarro, M., Garcia-Verdugo, J. M., & Alvarez-Buylla, A. (2009). Epidermal growth factor induces the progeny of subventricular zone type B cells to migrate and differentiate into oligodendrocytes. *Stem Cells*, *27*(8), 2032–2043. https://doi.org/10.1002/stem.119

Hauser, S. L. (2021). Curing Multiple Sclerosis: How to Know When We're There. In *Annals of Neurology* (Vol. 90, Issue 4, pp. 539–541). John Wiley and Sons Inc. https://doi.org/10.1002/ana.26155

Hauser, S. L., & Goodin, D. S. (2016). *Harrison's Principles of Internal Medicine*.

Horner, P. J., Power, A. E., Kempermann, G., Kuhn, H. G., Palmer, T. D., Rgen Winkler, J., Thal, L. J., & Gage, F. H. (2000). *Proliferation and Differentiation of Progenitor Cells Throughout the Intact Adult Rat Spinal Cord*.

Ivanova, A., Signore, M., Caro, N., Greene, N. D. E., Copp, A. J., & Martinez-Barbera, J. P. (2005). In vivo genetic ablation by Cre-mediated expression of diphtheria toxin fragment A. *Genesis*, *43*(3), 129–135. https://doi.org/10.1002/gene.20162

Johns, T. G., & Bernard, C. C. A. (1999). The Structure and Function of Myelin Oligodendrocyte Glycoprotein. In *J. Neurochem* (Vol. 72).

Karttunen, M. J., Czopka, T., Goedhart, M., Early, J. J., Lyons, D. A., & de Castro, F. (2017). Regeneration of myelin sheaths of normal length and thickness in the zebrafish CNS correlates with growth of axons in caliber. *PLoS ONE*, *12*(5). https://doi.org/10.1371/journal.pone.0178058

Kumar, D. R., Aslinia, F., Yale, S. H., & Mazza, J. J. (2011). Jean-martin charcot: The father of neurology. *Clinical Medicine and Research*, *9*(1), 46–49. https://doi.org/10.3121/cmr.2009.883

Levine, J. M., Reynolds, R., & Fawcett, J. W. (2001). The oligodendrocyte precursor cell in health and disease Review Review. In *TRENDS in Neurosciences* (Vol. 24, Issue 1). http://tins.trends.com

Lindvall, O., & Kokaia, Z. (2015). Neurogenesis following stroke affecting the adult brain. *Cold Spring Harbor Perspectives in Biology*, *7*(11). https://doi.org/10.1101/cshperspect.a019034

Lublin, F. D., Reingold, S. C., Cohen, J. A., Cutter, G. R., Soelberg Sørensen, P., Alan Thompson, Dms. J., Wolinsky, J. S., Balcer, L. J., Brenda Banwell, M., Barkhof, F., Bebo, B., Calabresi, P. A., Clanet, M., Comi, G., Fox, R. J., Freedman, M. S., Goodman, A. D., Inglese, M., Kappos, L., … Polman, C. H. (2014). *VIEWS & REVIEWS Defining the clinical course of multiple sclerosis The 2013 revisions*.

Lysandropoulos, A. P., & Havrdova, E. (2015). "Hidden" factors influencing quality of life in patients with multiple sclerosis. In *European Journal of Neurology* (Vol. 22, pp. 28–33). https://doi.org/10.1111/ene.12801

Medaer, R. (1979). Does the history of multiple sclerosis go back as far as the 14th century?. Acta neurologica Scandinavica, 60(3), 189-192.

Maki, T., Liang, A. C., Miyamoto, N., Lo, E. H., & Arai, K. (2013a). Mechanisms of oligodendrocyte regeneration from ventricular-subventricular zone-derived progenitor cells in white matter diseases. *Frontiers in Cellular Neuroscience*, *7*(DEC). https://doi.org/10.3389/fncel.2013.00275

Mandia, D., Ferraro, O. E., Nosari, G., Montomoli, C., Zardini, E., & Bergamaschi, R. (2014). Environmental factors and multiple sclerosis severity: A descriptive study. *International Journal of Environmental Research and Public Health*, *11*(6), 6417–6432. https://doi.org/10.3390/ijerph110606417

McFarland, H. F., & Martin, R. (2007). Multiple sclerosis: A complicated picture of autoimmunity. In *Nature Immunology* (Vol. 8, Issue 9, pp. 913–919). https://doi.org/10.1038/ni1507

Mecha, M., Feliú, A., Carrillo-Salinas, F. J., Mestre, L., & Guaza, C. (2013). Mobilization of progenitors in the subventricular zone to undergo oligodendrogenesis in the Theiler's virus model of multiple sclerosis: Implications for remyelination at lesions sites. *Experimental Neurology*, *250*, 348–352. https://doi.org/10.1016/j.expneurol.2013.10.011

Melzer, N., Meuth, S. G., & Wiendl, H. (2009). CD8+ T cells and neuronal damage: Direct and collateral mechanisms of cytotoxicity and impaired electrical excitability. In *FASEB Journal* (Vol. 23, Issue 11, pp. 3659–3673). FASEB. https://doi.org/10.1096/fj.09-136200

Mizrak, D., Levitin, H. M., Delgado, A. C., Crotet, V., Yuan, J., Chaker, Z., Silva-Vargas, V., Sims, P. A., & Doetsch, F. (2019). Single-Cell Analysis of Regional Differences in Adult V-SVZ Neural Stem Cell Lineages. *Cell Reports*, *26*(2), 394-406.e5. https://doi.org/10.1016/j.celrep.2018.12.044

Mockus, T. E., Munie, A., Atkinson, J. R., & Segal, B. M. (2021). Encephalitogenic and Regulatory CD8 T Cells in Multiple Sclerosis and Its Animal Models. *The Journal of Immunology*, *206*(1), 3–10. https://doi.org/10.4049/jimmunol.2000797

Stoop P, Marcel P., et al. Multiple sclerosis-related proteins identified in cerebrospinal fluid by advanced mass spectrometry. Proteomics, 2008, 8.8: 1576-1585.

Nait-Oumesmar, B., Picard-Riéra, N., Kerninon, C., & Baron-Van Evercooren, A. (2008). The role of SVZ-derived neural precursors in demyelinating diseases: From animal models to multiple sclerosis. *Journal of the Neurological Sciences*, *265*(1–2), 26–31. https://doi.org/10.1016/j.jns.2007.09.032

Nait-Oumesmar, B., Picard-Riera, N., Kerninon, C., Decker, L., Seilhean, D., nter Hö glinger, G. U., Hirsch, E. C., Reynolds, R., & Baron-Van Evercooren, A. (2007). *Activation of the subventricular zone in multiple sclerosis: Evidence for early glial progenitors*. www.pnas.orgcgidoi10.1073pnas.0606835104

Noseworthy, J. H. (2000). *Progress in determining the causes and treatment of multiple sclerosis*. www.nature.com

Ottone, C., & Parrinello, S. (2015). Multifaceted control of adult SVZ neurogenesis by the vascular niche. *Cell Cycle*, *14*(14), 2222–2225. https://doi.org/10.1080/15384101.2015.1049785

Patrikios, P., Stadelmann, C., Kutzelnigg, A., Rauschka, H., Schmidbauer, M., Laursen, H., Sorensen, P. S., Brück, W., Lucchinetti, C., & Lassmann, H. (2006). Remyelination is extensive in a subset of multiple sclerosis patients. *Brain*, *129*(12), 3165–3172. https://doi.org/10.1093/brain/awl217

Picard-Riera, N., Decker, L., Cile Delarasse, C., Goude, K., Nait-Oumesmar, B., Liblau, R., Pham-Dinh, D., & Evercooren, A. B.-V. (n.d.). *Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adult mice*. www.pnas.orgcgidoi10.1073pnas.192314199

Radecki, D. Z., & Samanta, J. (2022). Endogenous Neural Stem Cell Mediated Oligodendrogenesis in the Adult Mammalian Brain. In *Cells* (Vol. 11, Issue 13). MDPI. https://doi.org/10.3390/cells11132101

Rice, O., Surian, A., & Chen, Y. (2022). Modeling the blood-brain barrier for treatment of central nervous system (CNS) diseases. In *Journal of Tissue Engineering* (Vol. 13). SAGE Publications Ltd. https://doi.org/10.1177/20417314221095997

Robinson, A. P., Harp, C. T., Noronha, A., & Miller, S. D. (2014). The experimental autoimmune encephalomyelitis (EAE) model of MS. utility for understanding disease pathophysiology and treatment. In *Handbook of Clinical Neurology* (Vol. 122, pp. 173–189). Elsevier B.V. https://doi.org/10.1016/B978-0-444-52001-2.00008-X

Samanta, J., Grund, E. M., Silva, H. M., Lafaille, J. J., Fishell, G., & Salzer, J. L. (2015). Inhibition of Gli1 mobilizes endogenous neural stem cells for remyelination. *Nature*, *526*(7573), 448–452. https://doi.org/10.1038/nature14957

Sawcer, S., Franklin, R. J. M., & Ban, M. (2014). Multiple sclerosis genetics. In *The Lancet Neurology* (Vol. 13, Issue 7, pp. 700–709). Lancet Publishing Group. https://doi.org/10.1016/S1474-4422(14)70041-9

Schneider, C., Schuetz, G., & Zollner, T. M. (2009). Acute neuroinflammation in Lewis rats - A model for acute multiple sclerosis relapses. *Journal of Neuroimmunology*, *213*(1–2), 84–90. https://doi.org/10.1016/j.jneuroim.2009.05.015

Schreiner, T. G., Romanescu, C., & Popescu, B. O. (2022). The Blood–Brain Barrier—A Key Player in Multiple Sclerosis Disease Mechanisms. In *Biomolecules* (Vol. 12, Issue 4). MDPI. https://doi.org/10.3390/biom12040538

Segel, M., Neumann, B., Hill, M. F. E., Weber, I. P., Viscomi, C., Zhao, C., Young, A., Agley, C. C., Thompson, A. J., Gonzalez, G. A., Sharma, A., Holmqvist, S., Rowitch, D. H., Franze, K., Franklin, R. J. M., & Chalut, K. J. (2019). Niche stiffness underlies the ageing of central nervous system progenitor cells. *Nature*, *573*(7772), 130–134. https://doi.org/10.1038/s41586-019-1484-9

Silva-Vargas, V., Maldonado-Soto, A. R., Mizrak, D., Codega, P., & Doetsch, F. (2016). Age-Dependent Niche Signals from the Choroid Plexus Regulate Adult Neural Stem Cells. *Cell Stem Cell*, *19*(5), 643–652. https://doi.org/10.1016/j.stem.2016.06.013

Soldan, S. S., & Lieberman, P. M. (2022). Epstein–Barr virus and multiple sclerosis. In *Nature Reviews Microbiology*. Nature Research. https://doi.org/10.1038/s41579-022-00770-5

Stys, P. K., & Tsutsui, S. (2019). Recent advances in understanding multiple sclerosis. In *F1000Research* (Vol. 8). F1000 Research Ltd. https://doi.org/10.12688/f1000research.20906.1

Talhada, D., Costa-Brito, A. R., Duarte, A. C., Costa, A. R., Quintela, T., Tomás, J., Gonçalves, I., & Santos, C. R. A. (2020). The choroid plexus: Simple structure, complex functions. In *Journal of Neuroscience Research* (Vol. 98, Issue 5, pp. 751–753). John Wiley and Sons Inc. https://doi.org/10.1002/jnr.24571

Torkildsen, Ø., Brunborg, L. A., Myhr, K. M., & Bø, L. (2008). The cuprizone model for demyelination. Acta Neurologica Scandinavica, 117, 72-76.

Tintore, M., Vidal-Jordana, A., & Sastre-Garriga, J. (2019). Treatment of multiple sclerosis — success from bench to bedside. In *Nature Reviews Neurology* (Vol. 15, Issue 1, pp. 53–58). Nature Publishing Group. https://doi.org/10.1038/s41582-018-0082-z

Traka, M., Arasi, K., Avila, R. L., Podojil, J. R., Christakos, A., Miller, S. D., Soliven, B., & Popko, B. (2010). A genetic mouse model of adult-onset, pervasive central nervous system demyelination with robust remyelination. *Brain*, *133*(10), 3017–3029. https://doi.org/10.1093/brain/awq247

Traka, M., Podojil, J. R., Mccarthy, D. P., Miller, S. D., & Popko, B. (2015). Oligodendrocyte death results in immune-mediated CNS demyelination. *Nature Neuroscience*, *19*(1), 65–74. https://doi.org/10.1038/nn.4193

Vosoughi, R., & Freedman, M. S. (2010). Therapy of MS. In *Clinical Neurology and Neurosurgery* (Vol. 112, Issue 5, pp. 365–385). https://doi.org/10.1016/j.clineuro.2010.03.010

Walton, C., King, R., Rechtman, L., Kaye, W., Leray, E., Marrie, R. A., Robertson, N., la Rocca, N., Uitdehaag, B., van der Mei, I., Wallin, M., Helme, A., Angood Napier, C., Rijke, N., & Baneke, P. (2020). Rising prevalence of multiple sclerosis worldwide: Insights from the Atlas of MS, third edition. *Multiple Sclerosis Journal*, *26*(14), 1816–1821. https://doi.org/10.1177/1352458520970841

## Attachments

### 1. Further Acnowledgments

To my family. Since I was a little lad aspiring to become a scientist, they have been a tremendous support. To my mother for being a great warrior and for showing me that we must always fight for ourselves and those we care about. To my sister, my own source of inspiration, strength, and courage. I hope to attain at least 1 per cent of your current person one day. To my brother for demonstrating to me that the world is filled with opportunities. To my brother-in-law Pedro, for the example of consistence and maturity and to my nieces, who remind me that science is driven by a curiosity in the smallest details, I wish for you to grow up healthy and strong, and that you may always take me as a positive example.

To my dad. Never will I forget the astronomy books you purchased for me. Thanks for always inspiring me to put out effort into all I do. I hope you may be proud of the person I've become, whomever you are.

To André Francisco. I cannot think of a more helpful reviewer or critic. We are aware of how much you meant to me.

To Ângela Costa – the authentic buddy. I have no idea how our insanity brought us here. I just hope that our friendship will endure indefinitely. Thank you for being there for me in each and every circumstance.

Thank you, Patricia Durão, Bruno Mendes, Tomás Felgueiras, Cristina Pires, Catarina Deseyve, Flávia Brás and João Roleira for the wonderful moments and hearty laughs. Besides that, I appreciate your patience during those times when I believe the world is conspiring against me. Our exceptional moments will continue to multiply.

To my “crocodiles” the strangest guys’ group and all the persons I have met during my college years – they say college is the most memorable step of our lives. With you, I couldn’t agree more!

To Ana Adelaide, my high school English teacher. You were the most motivational tutor, constantly encouraging us to follow your counsel and avoid being stuck in difficult situations. I wish every student could have a teacher as exceptional as you are.

My IKEA colleagues, you have been very supportive during this last year. And for me, it was a really difficult one. Your assistance, kind deeds, and each individual's smile make my workdays much more enjoyable.

Finally, I would want to acknowledge everyone I have interacted with over the past five years. I can affirm that each and every one of you has made me a better person. Thank you!