Investigating the involvement of mTOR and the integrated stress response in regulating oligodendrocyte maturation in multiple sclerosis

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Abstract

Multiple sclerosis (MS) is an immune-mediated demyelinating disorder of the central nervous system (CNS) that leads to neurodegeneration. Remyelination is an essential reparative process in MS. While remyelination occurs efficiently during the early stages of the disease, it significantly declines with disease progression. This is despite the presence of oligodendrocyte progenitor cells (OPCs) within the lesions with the ability to generate new myelinating oligodendrocytes (OLs). Understanding how OPC and OL are regulated in MS lesions is critical to promote remyelination in chronic MS lesions. Emerging studies indicate that a balanced activity of mTOR pathway is essential for normal differentiation and maturation of OLs. Other findings have suggested that the integrated stress response (**ISR**), which occurs upon phosphorylation of eIF2α, has a protective role in remyelination. In this study, we aimed to characterize the activity of mTOR and the ISR in OPCs and OLs during acute and chronic demyelination and remyelination by using a cuprizone mouse model of demyelination. We also used primary *in vitro* systems to assess the changes in OPC maturation under MS-like conditions. We found that mTOR and ISR activity is increased in OPCs and OLs during both acute and chronic demyelination in cuprizone mice, and this expression is sustained during the remyelination period. *In vitro,* we confirmed that rapamycin-inhibition of mTORC1 results in decreased OPC proliferation and differentiation and inhibit OL maturation and morphological complexity *in vitro*, while increasing their lysosomal activity. Treating OPCs with MS-relevant cytokines also decreases OPC proliferation acutely and reduces their differentiation while promoting lysosomal activity. This study provides initial data that suggest the involvement of mTOR and ISR in oligodendrocyte behavior in demyelinating lesions. Future studies are required to determine the role of these pathways in impaired remyelination during chronic MS.

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List of Abbreviations

CC, corpus callosum

CIS, clinically isolated syndrome

CNS, central nervous system

CPZ; cuprizone

EAE; experimental autoimmune encephalomyelitis

eIF2*α*; eukaryotic initiator factor 2-alpha

GFP; green fluorescent protein

IFNγ, interferon gamma

ISR, integrated stress response

MS, multiple sclerosis

mTOR, mechanistic target of rapamycin (previously known as mammalian target of rapamycin)

OLs, oligodendrocytes

OPC, oligodendrocyte precursor cell

p-eIF2*α*, phosphorylated eukaryotic initiator factor 2 subunit alpha

pmTOR, phosphorylated mTOR

PPMS, primary progressive multiple sclerosis

pS6, phosphorylated ribosomal protein S6

RRMS, relapsing-remitting multiple sclerosis

SPMS, secondary progressive multiple sclerosis

TNF*α*, tumor necrosis factor subunit alpha

UPR unfolded protein response

WB, Western blot

1. Introduction

1.1 Pathophysiology of multiple sclerosis (MS)

1.1.1 Epidemiology and clinical impact

Multiple sclerosis **(MS)** is an autoimmune disease of the central nervous system **(CNS)** characterized by immune-mediated myelin damage (demyelination) and consequent loss of myelin-forming oligodendrocytes and axons (nerve fibers) that result in progressive neurodegeneration[1]. According to an epidemiology study in 2020 by the MS International Federation, there are around 2.8 million people who live with MS worldwide[2]. MS is highly prevalent in Canada. According to the MS Society of Canada, there are currently over 90,000 people with this disease and 12 individuals are diagnosed with MS each day in Canada[3]. The disease onset is typically between the ages of 20-49 years, and due to its long-term effects, it often affects physical function, cognition, quality of life, and employment, leading to a major socioeconomic impact on both patients and the healthcare systems[3]. MS is a multi-factorial disease, and while the causes remain unclear, there are some risk factors attributed to the development of the disease, such as genetic and environmental factors, vitamin D deficiency, and previous Epstein-Barr virus infection as reported recently[4]. The disease is highly prevalent among the female population, as 75% of MS individuals are women. While the underlying reasons are not fully understood, it has been suggested that women have a higher predisposition to autoimmune diseases due to sexual dimorphism in the immune function[5]. Interestingly, a study conducted on an MS animal model reported that female mice were more susceptible to developing experimental autoimmune encephalomyelitis (**EAE**) than their male counterparts[6].

Some differences in MS and EAE susceptibility have been attributed to chromosomal effects as the Y-chromosome is shown to influence the development of autoimmune diseases in both male and female mice, making the latter more susceptible to autoimmunity[7]. Sex hormones can also influence the production of pro-inflammatory factors which increases the risk of developing MS and its progression. As reviewed in the literature, female hormones, estrogen and, progesterone can have pro-inflammatory effects while male hormones, androgens, seem to play a more protective role in autoimmunity, which could explain the higher prevalence of MS in women[8].

1.1.2 Pathophysiology of MS

The pathophysiology of MS is characterized by the peripheral activation and infiltration of leucocytes into the CNS tissue, where they interact with resident glial cells and induce an inflammatory response that results in immune-mediated demyelination[9]. The onset of MS is caused by autoreactive lymphocytes (T-cells) in the periphery that infiltrate into the CNS through the blood-CNS barrier causing local inflammation and demyelination[10]. This infiltration is assisted by the expression of adhesion molecules and chemokines that promote integrin activation, causing immune cells to enter the CNS[11]. Once inside the CNS, infiltrating immune cells are recognized by antigen-presenting cells (**APCs**), including CNS innate immune cells, microglia and astrocytes. Interestingly, studies of *in vitro* and *in vivo* models of inflammatory demyelination show that oligodendrocyte precursor cells (**OPCs**) can also have a role as APCs[12].

T-cells are then reactivated by microglia, dendritic cells, and B cells, causing the clonal expansion of CD4+ T helper subsets like Th1 and Th17, which both secrete proinflammatory cytokines such as TNFα, IFNγ, IL-17, GM-CSF, IL-1β, among others. These interactions help mediate the autoimmune response in MS. Infiltrated immune cells along with resident glial cells collectively secrete soluble neurotoxic and oligotoxic substances such as reactive oxygen species, reactive nitrogen species, nitric oxide, glutamate, matrix metalloproteinases (**MMPs**) and inflammatory cytokines that create a pro-inflammatory environment that promotes demyelination and further increases neuronal damage[13]. In normal conditions, myelin provides insulation to axons hence promoting faster conduction of action potentials in a process called saltatory conduction[14]; however, during MS the myelin is lost causing slower conduction, redistribution of sodium channels, and redistribution of paranodal and juxtaparanodal axonal proteins along the denuded axon[15], making them vulnerable and prone to axonal injury and axonal loss. Multiple focal areas of demyelination in the CNS are called plaques or lesions, which are the hallmark of MS[16]. Lesions can be found in optic nerves, spinal cord, brain stem, cerebellum and white matter regions and they are divided into acute and chronic plaques. Acute active plaques are frequently found in cases of relapsing-remitting MS (**RRMS**) display signs of remyelination while chronic plaques are mainly found in progressive cases of MS and are demyelinated sites where usually there is no remyelination[13]. Due to all the autoimmune attacks, myelin loss, and neurodegeneration, people with MS present a range of symptoms that can include fatigue, vision problems, impaired mobility, pain, and cognitive impairment, just to mention a few of them[3]. Most of the currently available treatments for MS are targeted toward the inflammatory response, there is an unmet need to identify restorative treatments.

1.1.3 Types of MS

There are different types of MS, which are characterized by their duration and clinical course, these types are:

- Clinically isolated syndrome (**CIS**) is a single episode of neurological symptoms caused by inflammation or demyelination that is suggestive of MS. People who experience CIS may or may not develop MS[3,17].
- Relapsing-remitting MS (**RRMS**) is characterized by exacerbations of existing symptoms defined as relapses. Demyelination occurs during the relapses, followed by short periods of myelin restoration (remyelination) where recovery takes place. Majority of people with MS start their disease as RRMS.
- Primary progressive MS (**PPMS**) is characterized by a slow progression of demyelination, without defined relapses nor periods of remission, that leads to an accumulation of disability. Around 12% of MS patients are diagnosed with this type of MS.
- Secondary progressive MS (**SPMS**): This stage of the disease is characterized by a progressive worsening with occasional relapses and minor episodes of remission[3]. It is estimated that around 85% of people with RRMS transform into SPMS, which results in a persistent neurological decline without recovery periods[18,19].

The prognosis of MS varies and depends on the type and aggressiveness of the disease; patients can either go on a lasting remission with undetectable symptoms, or develop paralysis, neurological dysfunction, blindness, and even death. Therefore, MS is considered a heterogenous condition with features that can vary among patients[18].

During the early stages of MS, there is marked inflammation and demyelination, with a variable degree of demyelination and axonal loss, and possible remyelination. While the progressive stage is characterized by low-grade inflammation and microglial reactivity, which leads to further demyelination and neurodegeneration and low remyelination[20]. Early studies have demonstrated that OPCs can be found around chronic lesion sites, however, they fail to successfully re-myelinate these areas[21]. Thus, it has been suggested that some cellular and molecular mechanisms may be responsible for remyelination failure in the progressive stages of the disease.

1.1.4 Myelin and its role in the CNS

Myelin is a lipid-rich substance, produced by OLs in the CNS, that wraps around axons providing insulation and allowing the fast transmission of action potentials along the axon in a process called "saltatory conduction"[22]. When OPCs differentiate and mature into myelinating OLs they undergo morphological changes where they extend and retract their processes to engage axons in myelination. Once an axon has been engaged, changes in the plasma membrane of the OL are induced, and their processes flatten and spread along the axon, extending, and twisting in a corkscrew motion until the axon is wrapped in a multi-layered stack of membranes that form the myelin[23,24]. Myelin composition is unique, it is made up mainly of cholesterol, long-chain fatty acids, and sphingolipids. Myelin structure is stabilized by adhesion proteins. The myelin basic protein (**MBP**) is essential for the compaction and adhesion of the cytoplasmic membrane surfaces[25,26].

MBP interacts with adjacent membranes through electrostatic forces and closes the membrane by binding to cytoplasmic surfaces, this leads to the ejection of cytosol and the formation of a narrow protein phase that limits the entry of proteins and unwanted substances into the myelin sheaths[22]. Other important myelin proteins that provide structure are the proteolipid protein (**PLP**), myelin-associated glycoprotein (**MAG**), and myelin-oligodendrocyte glycoprotein (**MOG**)[27]. Myelin is not only an insulator, but it also provides trophic support to axons. Just the lipid and protein synthesis alone require a significant input of energy, hence it has been hypothesized that OLs provide support to axons, which could explain the maintenance of the energy metabolism[28]. In fact, studies have demonstrated that there are many types of trophic support provided by OLs, such as the "oligodendrocyte-axon lactate shuttle" where OLs metabolize glucose and export lactate to the axons[29]. Myelin has unique features that make it particularly susceptible to injury. When myelin and myelinated fibers are damaged, their structure and function are affected. In the following sections, there will be a further description about demyelination, remyelination, and the importance of OPCs in restoring the myelin sheath.

1.1.5 Demyelination and remyelination in acute and chronic MS lesions

MS is characterized by the presence of acute inflammatory demyelination accompanied by axonal damage. As mentioned previously, immune-mediated myelin damage (demyelination) and loss of myelin-forming oligodendrocytes result in disturbed conduction of action potentials and modification of axonal structure that ultimately results in neurodegeneration[13]. An early study demonstrated that axons can be damaged in two different ways depending on the stage of the disease.

In acute demyelination, axons are most likely damaged by the rapid effects of inflammatory mediators; while in the progressive stage, there is low-grade but constant axonal loss with lesions that may persist for years. Ongoing axonal degeneration occurs as a result of residual inflammation and the lack of trophic support provided by myelin and OLs, which altogether contribute to the progression of the disease[30]. Hence, it is thought that axonal injury during the acute phase of MS is inflammatory, while the axonal loss in chronic stages is more degenerative[31].

The information about the mechanisms of axonal injury and loss in MS is still scarce, as not all axons are affected in the same manner, i.e., small axons are more susceptible to damage and thick axons being more resistant[32]. There are two main phases of axonal degeneration: the initial trigger of axonal damage and the activation of downstream pathways that lead to degeneration. Axonal injury in MS can be initially triggered by T-cell cytotoxicity, where restricted T-lymphocytes target axons and transect them in an antigen-dependent manner. This initial trigger activates a series of downstream events that affect the permeability of the axoplasmic membrane, which results in an uncontrolled ion influx into the axoplasm causing the degradation of cytoskeletal proteins and impairing axonal transport. Eventually, voltage-gated calcium channels accumulate at sites where axonal transport is disturbed, allowing a major influx of calcium ions into the axons which cause nano ruptures and axon degeneration[33,34]. The degree of axonal damage in chronic demyelination is severe, with a 60-70% reduction in axonal density as compared to normal tissue[35]. Altogether, axonal injury in MS is caused by loss of trophic support from OLs, direct T-cell injury, and energy deficiency caused by pro-inflammatory factors[36].

As demyelinated axons become vulnerable in MS, it is essential to restore myelin to prevent further degeneration. The process in which new myelin sheaths are generated is called remyelination that can restore neurological function and provide neuroprotection. Early studies showed that remyelination occurs efficiently in the early stages of the disease (remission)[37]. Additionally, more recent studies have demonstrated that acutely damaged axons are remyelinated in MS and that remyelination helps preserve axons over time; while in chronic demyelinated MS lesions, there is less axonal density. This suggests that remyelination may be key for axonal protection[38]. However, remyelination becomes a challenge as the disease progresses because differentiation and maturation of OPCs into myelinating OLs is impaired in chronic MS lesions[39,40].

1.1.6 Oligodendrocyte precursor cells (OPCs): Lineage progression and their role in maturation

Evidence suggests that some mature oligodendrocytes can survive after a demyelinating event and even participate in remyelination by producing new myelin and ensheathing demyelinated axons[41], however, they do so to a lesser extent as compared to newly formed oligodendrocytes. Another study showed that while surviving oligodendrocytes can support ensheathment in denuded axons, new oligodendrocytes exhibit more abundant myelination hence having a greater capacity for regeneration[42]. New oligodendrocytes are generated from an endogenous population of OPCs in a process called oligodendrogenesis. Adult OPCs comprise around 2-8% of glial cell population in the CNS[43].

After a demyelinating event, OPCs proliferate and migrate to the lesion site, where they differentiate and mature into myelinating oligodendrocytes[21] **(Figure 1.1)**. OPC differentiation is characterized by a rapid change in morphological complexity, which means that the cell increases its number of branches that will later ensheath axons with myelin[44]. The newly formed oligodendrocyte (**OL**) will then extend its multiple processes to remyelinate axons. Due to their remyelinating capacity, OPCs have been studied extensively in an effort to identify the mechanisms of myelin repair and therapeutic targets for MS.

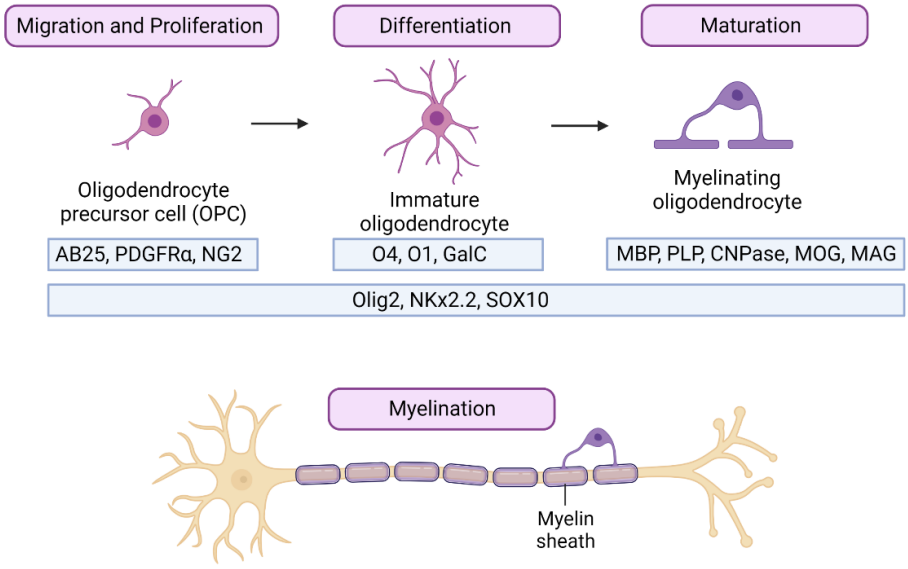


Figure 1.1: Oligodendrogenesis, from OPCs to oligodendrocytes. Oligodendrogenesis is the process in which new myelinating OLs are formed. There are three canonical stages of differentiation: the OPC, the pre-myelinating OL (pre-OLs) or immature OL, and the mature, myelinating OL. The immature OLs belong to a cell population that are no longer OPCs and are in process of differentiating to mature OLs, however, they do not produce myelin yet. Conversely, mature myelinating OLs have different transcriptional profiles than pre-OLs, making them a heterogeneous population. There are established markers for each lineage; just to mention a few of them: OPCs are usually identified by their expression of A2B5, PDGFRα, NG2; pre-OLs markers include O4, O1, GalC; and myelinating OLs are labeled against PLP, MBP, CNPase, MOG, MAG. All oligodendroglial lineage cells can be identified by their expression of Olig1/2, NKx2.2 and SOX10. Figure created in BioRender.

OPCs can repopulate and remyelinate acute demyelinating lesions partially or completely in MS animal models. However, in chronic progressive MS, although OPCs are present in the lesions, their capacity to mature into myelinating OLs declines considerably resulting in remyelination failure and accumulation of neurodegeneration and disabilities[21]. While the underlying mechanisms of remyelination failure have not been fully elucidated yet, evidence suggests it is lesion-stage dependent. As described by Heβ, et.al., OLs are preserved in active/ demyelinating lesions, but their numbers decrease in the center of inactive lesions. Furthermore, despite the high numbers of mature OLs in these lesions, only a few of them are remyelinated[45]. Another study reported that OPC differentiation into OLs is blocked, causing remyelination failure in chronic MS[46,]. It is shown that OLs processes retraction may underlie impaired remyelination[47], as it is associated with the induction of the integrated stress response by the cytokines TNFα and IFNγ[48]. Recent findings show that neuroinflammation caused by TNFR2 signaling shifts OPCs into an immunomodulatory phenotype, which decreases their proliferating and differentiating capacity[49].

It has been proposed that while OPCs can repopulate the lesion site in chronic MS, their maturation is blocked. This could be due to aging or cellular senescence, which might be triggered by oxidative stress and mitochondrial dysfunction[50]. Recent findings have shown that intracellular mechanisms, such as lysosomal activity are essential for regulating OL differentiation, as an increase in lysosomal pH prevents their maturation[51]. Investigating the cellular mechanisms involved in OPC maturation is key to finding novel restoration therapies. In the following sections, I will further review the role of lysosomes as well as two pathways that are important for OL maturation and myelination: the mammalian target of rapamycin (mTOR) pathway and the integrated stress response (ISR).

1.2 Role of Lysosomes in cell regulation

Lysosomes are membrane-bound organelles involved in various cellular processes such as homeostasis, secretion via exocytosis, cell signaling, apoptosis, autophagy, and energy metabolism. These vesicles have an acidic lumen limited by a single lipid bilayer. Lysosomes contain hydrolytic enzymes, which degrade macromolecules and other substrates[52]. The lysosome acts as a signaling center, where multiple cellular processes are regulated by signaling pathways that are initiated on the lysosomal surface. Lysosomes can participate in cell death and respond to endolysosomal damage under oxidative stress, proteases, photo-damage, certain lipids, and apoptotic regulators[53]. In the presence of these factors, lysosomes are permeabilized and ruptured, which leads to the release of cathepsins resulting in lysosome-dependent cell death[54]. Early studies have reported that TNFα, a relevant cytokine in MS, is associated with lysosomal permeabilization[55].

Lysosomes are key regulators of the three types of autophagy: macro-autophagy, chaperone-mediated autophagy (CMA), and micro-autophagy. In CMA and micro-autophagy, the unwanted cellular materials are directly transported to the lysosome via chaperones and membrane invagination respectively. On the contrary, macro-autophagy (here referred to as **autophagy**) requires the formation of an additional vesicle called autophagosome, which then fuses with the lysosome in order to degrade its contents[56].

Emerging studies indicate that lysosomal activity is essential for the development of OPCs and their differentiation into mature OLs, and myelination. It has been reported that autophagic activity increases during OL differentiation and autophagosome formation is observed in developing myelin sheaths, and inducing autophagy promotes myelination while inhibition of autophagy does the opposite[57].

Recent findings from Aber et.al. demonstrate that OLs perform autophagy to degrade and maintain existing myelin sheaths, hence allowing a myelin remodeling process that is essential for maintaining the CNS. This study also showed that autophagic myelin turnover was disrupted when Atg5 (autophagy-related protein 5) was conditionally knocked out from CNP+ OLs in mice. Additionally, the inhibition of autophagy in Atg5 cKO model resulted in structural changes of the myelin sheath, impaired neural function, and neurodegeneration[58]. More evidence indicates that autophagy is necessary for OLs survival and function. By using a rat model (Long-Evans shaker or "*les*" model) with mutations in MBP, Smith et.al. reported an accumulation of lysosomes and cytoplasmic vesicles in OLs; along with an upregulation in autophagy which was observed after the increase in autophagosomes and autolysosomes in the OLs of *les* rats. Additionally, it was found that *les* rats expressed more LC3-II (an autophagosome marker) than their controls. Interestingly, autophagy did not precede OL death in the "*les*" rats; on the contrary, it was found that OLs survived after the period of autophagy. Furthermore, autophagy increased the proportion of myelinated axons and myelin thickness in their animal model. To replicate the *in vivo* findings, OLs from the *les* rats were cultured and placed under glucose and serum deprivation to induce autophagy *in vitro*; and it was found that induction of autophagy in these cells aided in survival and promoted membrane extension[59].

Altogether, these studies have demonstrated that mTOR activity and autophagy are essential for OL differentiation and maturation. Dysregulated lysosomal activity and autophagy have been associated with several myelin disorders, including MS. In a human study in 2017, increased levels of autophagy marker, Atg5, and Parkin (a marker for protein degradation) were found in cerebrospinal fluid (CSF) samples from people with MS, which suggest a role for autophagy in MS[60].

Considering all the above-mentioned findings, the relationship between autophagy and neurodegenerative diseases seems unclear. While some studies report that autophagy is protective and necessary for myelination, other studies indicate the opposite. Due to these controversies, more investigation is warranted to elucidate the role of lysosomes and autophagy in myelination and myelin disorders.

1.3 Role of mTOR pathway in OL regulation and myelination

The mTOR (mechanistic Target of Rapamycin) pathway is a key regulator of cellular metabolism that integrates the input from upstream pathways to regulate cell growth, protein synthesis, gene expression, and protein turnover[61]. mTOR signaling contributes to neuronal and neuroglial development in the central nervous system. Recently, it has been found that mTOR has a key role in the pathophysiology of MS[62]. This study showed that mTOR signaling promoted efficient remyelination in the mouse brain after cuprizone-induced demyelination. Interestingly, it was found that the conditional deletion of mTOR in adult OPCs (Ng2-MtorcKO) resulted in delayed remyelination after CPZ.

Their *in vitro* studies showed that OPCs undergoing differentiation had a reduced metabolic function, with low mitochondrial oxidative phosphorylation and glycolysis after treatment with CPZ or rapamycin. Hence it was suggested that delayed remyelination could be related to mTOR-mediated metabolic function[62]. Additionally, this pathway has been demonstrated to have an emerging role as a regulator for OL development and maturation, hence suggesting that mTOR signaling has a role in remyelination after MS[63].

mTOR is a serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase-related kinase family, known to be expressed on the surface of lysosomes of eukaryotic cells, including neural cells[64]. mTOR pathway is activated upstream through the PI3K/Akt pathway by extracellular factors such as the insulin-like growth factor 1 (IGF-1), which activates Akt leading to protein synthesis[65]. mTOR downstream signaling is regulated by two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), both share mTOR as part of their structure[64]. In the past, mTORC1 was known for being the only complex sensitive to rapamycin; however, recent studies have shown that long-term rapamycin treatment can also inhibit the activity of mTORC2[66].

mTORC1 has three main components: Raptor (regulatory protein associated with mTOR)[67], mLST8 (mammalian lethal with Sec13 protein 8 or GβL)[68], and mTOR itself. mTORC1 also contains the inhibitory subunits PRAS40 (proline-rich Akt substrate of 40 kDa) and DEPTOR (DEP domain-containing mTOR interacting protein)[64]. The main function of mTORC1 is to regulate the production of lipids, proteins, and nucleotides, while negatively regulating autophagy[69] **(Fig. 1.2)**. mTORC1 is regulated by the presence or absence of nutrients. In the presence of amino acids, mTORC1 promotes the translation of mRNAs related to protein synthesis, cell growth, and lipid synthesis[70,71].

Activation of mTORC1 results in the phosphorylation of the ribosomal protein 6 kinase (**pS6**), which in turn promotes the activation of the S6 ribosomal protein and the eukaryotic translation initiation factor 4B (EIF4B or 4E-BP), both required for the initiation of mRNA translation and subsequent protein synthesis[70]. mTORC1 is also involved in lipid synthesis.

Although the exact mechanisms remain to be elucidated, it is known that mTORC1 can promote the production of SREBP (sterol-responsive element binding protein) transcription factors, which participate in the *de novo* lipid synthesis[71]. Activated mTORC1 inhibits autophagy. The first step of lysosomal-dependent autophagy is the formation of the autophagosome, which is initiated by the formation of the ULK-1/Atg13/FIP200 complex. In the presence of amino acids, mTORC1 phosphorylates ULK-1 (unc-51-like autophagy-activating kinase 1), hence inhibiting the formation of the complex and the initiation of autophagy[72,73]. Conversely, in cases of nutrient deficiency, mTORC1 activity is suppressed. This allows the dephosphorylation of the ULK-1 complex and the subsequent initiation of autophagy[73].

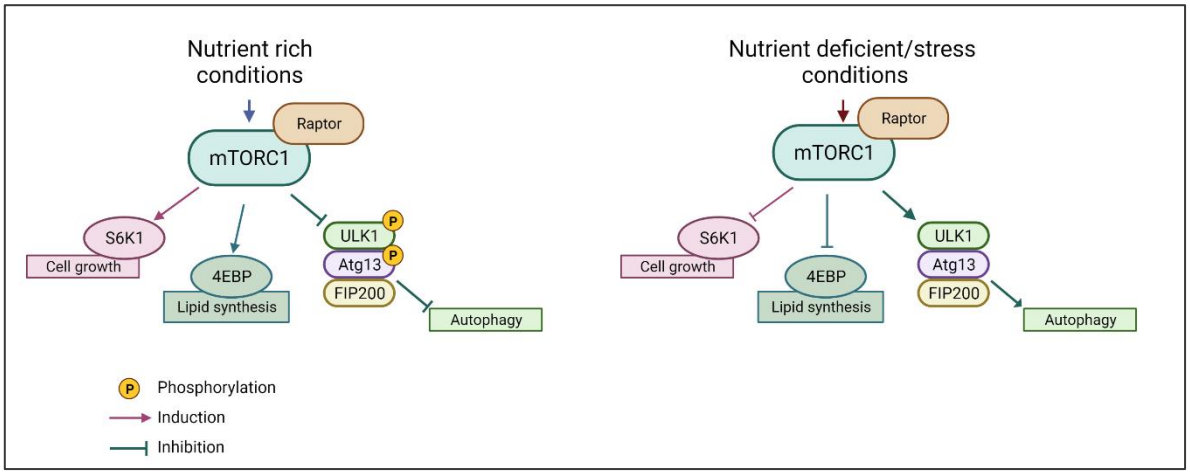


Figure 1.2: mTORC1 downstream activity. mTORC1 has different effects depending on the stimuli received upstream. The mTOR-Raptor complex, located on the surface of the lysosome, detects the nutrient conditions and determines growth factor and amino acid availability. In nutrient-rich conditions, mTORC1 promotes cell growth via phosphorylation of the ribosomal protein 6 (pS6) and 4E-BP, as well as lipid synthesis. mTORC1 also negatively regulates autophagy by phosphorylation of ULK1, which suppresses the formation of the autophagosome and hence inhibits the initiation of autophagy. In case of nutrient deficiency, mTORC1 is no longer maintained on the lysosomal surface, which allows the dephosphorylation of the ULK1 complex, leading to the induction of autophagy. Energy deprivation (low levels of ATP) can also promote autophagy through the activation of the AMPK pathway, which inactivates mTORC1 normal activity. Figure created in BioRender.

Due to its many roles in cell growth, there has been a growing interest in elucidating the role of mTOR in glial cells and myelination. Recent findings suggest that mTOR activity regulates the cytoskeleton and actin reorganization of OLs during developmental myelination[74]. It is known that mTORC1 is key for lipid biosynthesis, hence, extensive investigations have been conducted into the role of the mTOR pathway in myelin production. Recent findings from Khandker, et.al., show that mTOR is an important regulator for CNS myelination because it promotes the synthesis of cholesterol in OLs[75]. Interestingly, loss of mTOR resulted in a population of oligodendroglial cells with reduced capacity for lipid biosynthesis and dysregulated cytoskeletons.

These investigations have demonstrated that balanced mTOR activity is essential for myelin synthesis and maintenance[75]. Adequate mTOR signaling is essential to preserve axonal integrity and myelinating OLs through mTORC1-dependent lipid synthesis and the activation of the SREBP pathway[76]. Although the role of mTOR in myelination has been established, information regarding the activity of the mTOR pathway in MS and demyelinating diseases is still scarce. However, recent findings show that mTOR signaling regulates the metabolism of OPCs in cuprizone (**CPZ**)-induced demyelination and promotes efficient remyelination in the brain[62]. As described by Jeffries et.al., the selective inhibition of mTOR in oligodendroglial cells leads to delayed remyelination after CPZ-induced demyelination, but they reported no changes in a Lysophosphatidylcholine (**LPC**) models of demyelination. They also found a decrease in both oxygen consumption rate and glycolytic rate in mTOR-inhibited OLs, hence concluding that mTOR activity is required for OL metabolism[62].

The mTORC1 complex is also involved in lysosomal autophagy[68]. In a nutrient-rich environment, mTORC1 translocates to the lysosome, where it is activated. Then, it coordinates cellular growth while inhibiting catabolic processes such as autophagy, by suppressing the formation of the autophagosome[72,73]. Conversely, during starvation or nutrient deficiency, mTORC1 dissociates from the lysosome, becoming inactive. mTORC1 inactivation leads to the dephosphorylation of ULK-1, which allows the formation of the ULK-1/Atg13/FIP200 complex and the subsequent initiation of autophagy[73]. Evidence shows that activating mTOR is important for OL differentiation and myelination, and mTOR positively regulates the expression of myelin genes and proteins such as MBP and PLP[63].

The second mTOR complex, named mTORC2, is formed by mTOR, Rictor (rapamycin-insensitive companion of mTOR), and DEPTOR[77]. It was initially thought that mTORC2 was insensitive to rapamycin, but it was later identified that prolonged treatment of rapamycin inhibits the formation of this complex[66]. mTORC2 is known for promoting cell survival by activating Akt and cytoskeleton organization and being a key effector in regulating the insulin signaling pathway through the insulin receptor substrate-1 (IRS-1)[78]. As mTORC1 is phosphorylated after the activation of IRS-1, this would mean that mTORC2 regulates mTORC1 activity, hence the regulation of the mTOR pathway appears to be a delicate and complex process.

As reported by Dahl et.al., conditional deletion of mTORC2 in OPCs results in developmental hypomyelination and delayed myelination. By using PDGFRα-Cre x Rictor $^{fl/fl}$ mice, it was found that loss of mTORC2 resulted in the reduction of OL differentiation, and a reduced expression of myelin-related RNAs and proteins in the corpus callosum (**CC**) and spinal cord.

While these deficits in myelin were recovered in the spinal cord, in the CC there was severe hypomyelination and an increased number of non-myelinated axons, suggesting that mTORC2 plays a role in OL development in a region-specific manner[79]. Interestingly, this study found that loss of Rictor in OLs did not alter mTORC1 signaling in the CC during development, but it did reduce S6 phosphorylation, indicating that mTORC1 signaling was affected at later stages[79]. Others have reported similar findings by using another Rictor conditional knockout mouse model. In this study, Olig2-Cre+ mice were crossed with Rictor$^{fl/fl}$ mice to create a cKO of mTORC2 in oligodendrocyte lineage cells. Their findings suggest that loss of mTORC2 in OPCs delayed the expression of myelin-related proteins and caused a reduction in size of cerebral white matter tracts[80].

These findings show that the mTOR pathway, and its two complexes, are important for OL development and proper myelination. However, further investigation is required for placing these findings into the context of MS. Understanding the role of the mTOR pathway in oligodendroglial cells can provide insight into the pathophysiology of MS and identify novel approaches for remyelinating and regenerative therapies for MS.

1.4 Endoplasmic reticulum stress and oligodendrogenesis

The endoplasmic reticulum (ER) is an organelle made up of two subunits, the rough endoplasmic reticulum (RER) and the smooth endoplasmic reticulum (SER). The general role of the SER is to synthesize and store lipids and steroids, while the RER maintains cellular homeostasis by regulating intracellular calcium levels and aiding in protein synthesis. The RER is also responsible for the folding, modification, and transportation of new proteins[81].

Unfolded or misfolded proteins can accumulate in the ER lumen, leading to ER stress and the initiation of the integrated stress response (**ISR**)[82]. The ISR is an evolutionarily conserved signaling pathway that can be activated by cell-intrinsic stress like ER stress, and extrinsic factors like glucose or amino acid deprivation. The main goal of the ISR is to reprogram gene expression, maintain cellular proteostasis (which includes sustaining protein-folding capacity), support cellular differentiation, and respond to injury[82]. The ISR exerts its effects by reducing the rate of general translation and increasing the translation of specific mRNAs. If the ISR is not mitigated, apoptosis is triggered[83]. The ISR responds to different types of stress and is sensed by at least four kinases that phosphorylate the Ser51 site of eIF2α: 1) Gcn2 (general amino-acid control nonderepressible 2), which responds to amino acid deprivation; 2) HRI (heme-regulated inhibitor) that responds to heat shock, osmotic stress, and proteasome inhibition; 3) PKR (double-stranded RNA-dependent protein kinase, encoded by the gene EIF2AK) that can be activated by oxidative stress; and 4) PERK, which responds to the aggregated unfolded proteins in the ER[82].

ER stress can also result in the activation of the unfolded protein response (**UPR)**[84]. Unfolded proteins are sensed in the ER lumen by three ER localized transmembrane receptors: PERK, IRE1, and ATF6, that initiate the unfolded protein response (**UPR)**[85]. The main goal of the UPR is to activate a series of signaling pathways to maintain proteostasis. Hence, the three branches collaborate to upregulate the expression of genes involved in protein folding and degradation to decrease the number of unfolded proteins. The UPR also aims to increase the ER-folding capacity, thus there is also an upregulation of genes related to ER folding, lipid biosynthesis, and ER-associated degradation (ERAD).

Simultaneously, the UPR also targets selective mRNAs to promote degradation and translational repression to decrease the protein folding load. If the stress cannot be resolved, the UPR becomes cytotoxic, inducing apoptosis[85].

Both the ISR and UPR converge in the phosphorylation of eIF2α through PERK[82,85]. The distinct salient feature of ISR signaling lies in the formation of the ternary complex (**TC**), a heterotrimeric translation initiator factor that consists of eIF2 (with all the α, β, and γ subunits), guanosine 5′-triphosphate(GTP), and charged methionyl-initiator tRNA (Met-tRNAi). The formation of the TC complex is essential to reduce translation initiation and protein synthesis[82]. PERK activation induces the phosphorylation of the eukaryotic initiation factor 2 subunit-α (**eIF2α**) at the serine site 51, which in turn reduces mRNA translation initiation, hence reducing protein translation. At the same time, eIF2α phosphorylation regulates the selective translation of mRNAs encoding the transcription factor ATF4, a master regulator that controls the expression of pro-survival genes related to protein folding, autophagy and even pro-apoptotic genes in case the ER stress cannot be relieved[82]. ATF4 is also involved in a feedback loop to dephosphorylate eIF2α to restore protein synthesis via upregulation of the protein phosphatase 1 regulatory subunit GADD34[86]. Interestingly, the increase in protein synthesis mediated by ATF4 and CHOP can induce cell death. The switch from pro-survival to pro-apoptotic is most likely related to the duration and intensity of stress, as well as the amount of CHOP being expressed. This means that ATF4/CHOP can promote an initial survival response; however, if the stress is prolonged CHOP expression will initiate cell death to restore homeostasis[87].

The PERK-ATF4-CHOP pathway can promote cell death by binding to the death receptor pathway and upregulating the expression of death receptors 4 and 5 (DR4 and DR5). DR5 can promote extrinsic apoptosis through sensitizing cells to reactive oxygen species (ROS)[88]. Additionally, persistent ER stress can result in the accumulation of DR5 in the ER and Golgi apparatus, which results in a ligand-independent multimerization of different DR5 splice variants (DR5L). DR5L then promotes the rapid formation of the death-inducing signalling complex, activating caspase-8 and resulting in cell death through the cleavage of BID into tBID[88]. Another studied mechanism of ER stress related cell death is the regulation of different BCL-2 proteins. Under ER stress, CHOP downregulates the expression of the anti-apoptotic gene BCL-2 (which sensitizes the cells to apoptosis) and upregulates some pro-apoptotic BH3-only proteins like BIM, PUMA and NOXA[89]. ER stress is a feature of several diseases, many of which are inflammatory disorders.

In MS, several inflammatory mediators and cytokines come into play, generating an inflammatory response in tissue-resident cells and infiltrated cells that can result in ER stress[90,91]. An early study in CPZ demyelinating mice demonstrated that IFNγ, a relevant cytokine in MS pathophysiology, induced an ER stress response in remyelinating OLs that inhibited CNS remyelination. It was also reported that administering IFNγ to heterozygous PERK deficient mouse under a CPZ demyelinating regimen resulted in reduced numbers of OLs and less remyelinated axons[92]. This was one of the first reports that identified a relationship between cytokine-induced ER stress and remyelination failure in a model of MS. Interestingly, a human MS study showed increased mRNA levels of the markers ATF4, BiP, and CHOP in post-mortem samples of normal-appearing white matter (NAWM) and demyelinating lesions[93].

Another study reported an increased expression of CHOP and XBP1 in OLs, astrocytes, T cells, and microglia in active MS lesions from post-mortem human samples, further indicating the involvement of ER stress activity in MS[94]. Due to these findings, OLs and MS have become a recent target for research related to ER stress, UPR and remyelination. Among the three UPR responders, the PERK signaling pathway has been the best-characterized branch in OLs, and demyelinating models followed by ATF6. However, there is not much information on IRE1 activity and its role in OLs.

An early study by Wensheng, et.al., suggested that IFNγ, a relevant cytokine in MS, may promote ER stress in myelinating OLs. The authors' direct *in vitro* studies in rat primary OLs indicated that apoptosis induced by IFNγ was associated with ER stress. Moreover, cultured immature OLs were found to be more sensitive to ER stress as compared to more mature OLs[95]. PERK activity in OLs has shown to be protective in an EAE model of MS[96]. It was reported that CNS-specific delivery of IFNγ before the onset of EAE ameliorated the course of the disease, preventing demyelination, axonal damage, and OL loss; these protective effects were related to the activation of PERK[96]. Due to the important findings on PERK signaling and its effects on OLs, further studies have been conducted to elucidate PERK's specific role in disease by using MS animal models. These studies have shown that conditional deletion of PERK in OLs does not have any developmental effects, but it does result in increased EAE severity and OL loss, suggesting the protective role of PERK activity.

Enhancing ISR is shown to promote OL survival in response to ER stress[97]. Extended ISR via prolonged phosphorylation of eIF2α can delay the onset of clinical symptoms in EAE model, reduce both OL and axonal loss and decrease the numbers of T cells in the CNS[98].

More recent findings show that prolonging the ISR can also increase remyelination of the CNS in an inflammatory setting[99]. In EAE and cuprizone mouse models, prolonged ISR protected OLs and increased remyelination. Use of the selective estrogen modulator, bazedoxifene (BZA), increased the number of remyelinating OLs in a CPZ model, and its combination with Sephin1 (an agent that prolongs ISR) enhanced remyelination[99]. Altogether, the ISR has become an interesting and relevant topic for CNS remyelination.

1.5 Brief overview of animal models to study MS, demyelination and remyelination

The most well-known model for MS is the experimental autoimmune encephalomyelitis (**EAE**) model. This model is designed to recapitulate the immune-mediated aspect of MS. EAE can be induced in all vertebrates; hence it is commonly used in mice, rats, and primates. In this model, animals are immunized with myelin proteins (or peptides) like MBP, PLP, or MOG or by inducing the passive transfer of T-cells to specifically target myelin antigens[100]. The antigen and genetic make-up of the animal will result in different types of EAE, this way the model can mimic a monophasic, relapsing-remitting, or chronic form of EAE [100]. Since EAE model is more physiologically relevant to MS; it has been used extensively in understanding MS pathogenesis, progression and treatment. However, there are other models that aid in investigating demyelination and remyelination, such as the cuprizone mouse model.

The cuprizone (**CPZ)** mouse model is one of the most used MS animal models for studying demyelination and remyelination. Cuprizone is added to the diet of rodents. It is a copper chelator that results in oligodendrocyte death, and consequent demyelination in the

white-matter regions of the brain, like the corpus callosum[101]. The model was first standardized in 1998 when it was found that a low dose of 0.2% w/w of CPZ caused demyelination in C57BL/6 mice[102]. The mechanism of action for CPZ-induced toxicity is still under investigation; however, the main and most accepted theory is that it causes mitochondrial disturbances in oligodendrocytes that lead to their death[103]. Cuprizone can be administered for 4 – 6 weeks for acute demyelination and up to 12 weeks for chronic studies. This model mainly induces demyelination in the brain, the corpus callosum, cerebellar peduncles, hippocampus, and other myelinated areas[104]. When the cuprizone diet is removed and replaced with normal rodent chow, spontaneous remyelination can happen. Hence, this is an advantageous model to study both demyelination and remyelination[23].

1.6 Study rationale, hypothesis, and objectives.

1.6.1 Study Rationale

MS is an autoimmune disease that results in neurodegeneration. MS is characterized by demyelination, loss of OLs, and axonal loss. While remyelination can occur during the acute stages of the disease, it is impaired during the chronic stages of MS. Successful remyelination depends on the formation of new OLs from the endogenous population of OPCs that exist in the adult CNS. Evidence suggests that OPCs can proliferate and repopulate the lesion site, but their maturation is blocked and hence remyelination fails in chronic MS. Unraveling how the potential of endogenous OPCs for remyelination is restricted in progressive MS is required to attenuate neurodegeneration and promote the repair processes. It has been reported that lysosomal activity is required for OL development.

One of the most relevant pathways located on the lysosome is the mTOR pathway, which has been shown to be important for myelination and OL maturation. Additionally, ER stress is a feature of MS that is known to activate UPR and ISR pathways that both converge in eIF2α phosphorylation which is shown to have a protective role in demyelination. In this thesis I sought to study whether the mTOR and ISR activity regulate OPC proliferation and their maturation into myelinating oligodendrocytes *in vitro* and characterize the expression pattern of these pathways in acute and chronic demyelinated lesions of CPZ mice.

1.6.2 General Hypothesis.

Activation of both mTOR and the ISR occur during acute and chronic remyelination, and these pathways are involved in promoting OPCs differentiation into mature oligodendrocytes.

1.6.3 Research Objectives

1. To characterize mTOR and ISR activity in a CPZ demyelinating mouse model.
2. To characterize the involvement of mTOR and ISR pathways in proliferation, and maturation of OPCs *in vitro*.

2. Materials and Methods

2.1 Reagents

The reagents used for this study were: Rapamycin (Sigma-Aldrich, 53123-88-9), Tunicamycin (Sigma-Aldrich, 11089-65-9), cytokine TNFα (R&D Systems, 410-MT-010) and IFNγ (FUJIFILM Irvine Scientific, 100-77-20UG) and LysoTracker Deep Red (ThermoFisher Scientific, L12492, ON, Canada).

2.2 *In vivo studies*

2.2.1 Animals

All animal protocols were approved by the University of Manitoba Animal Care Committee (Protocol number: 20-065) in accordance with the Canadian Council on Animal Care guidelines and policies. For *in vivo* studies a total of 21 adult male and female C57BL/6 were used. Mice were provided by Central Animal Care Facility at the University of Manitoba.

2.2.2 PDGFRα-CreER:Rosa26mGFP (mT/mG) mice

A double transgenic mouse model was used to track oligodendroglial lineage. Upon tamoxifen (Sigma, T55648) administration (2mg/kg) to PDGFRα-CreER:Rosa26mGFP (mT/mG) mice, the PDGFRα expressing OPCs are identified under the control of PDGFRα promoter (PDGFRα-CreERT2) and the membrane-tethered reporter, allowing expression of GFP (green fluorescent protein) in fine cellular processes of OPCs and myelinating oligodendrocytes. These mice are here referred to as PD-mice. Tamoxifen was administered at 6 weeks of age for 3 days (2mg/kg/day) followed by 14 days of clearance or "wash-out" period.

2.2.3 Cuprizone mouse model of demyelination

Demyelination was induced in PD mice by adding 0.2% of CPZ (Santa Cruz Biotechnology, CAS 370-81-0) to their grounded food for 5 weeks (acute) or 10 weeks (chronic). Then, the diet was changed back to normal chow to allow remyelination. For the acute timepoint, mice were placed under normal chow for 2 weeks after being 5 weeks under CPZ diet. For the chronic timepoint, mice were placed under normal chow for 2 weeks (short term recovery) and 4 weeks (long term recovery) after the 10 weeks under CPZ diet. CPZ diet was refreshed

twice a day (6 grams of food per mouse daily), with drinking water *ad libitum.* Pelleted food and drinking water were available *ad libitum.* To prevent dehydration, mice that lost 20% of their weight were administered 0.5 ml of normal saline solution subcutaneously until recovery. Mice were weighted every other day and inspected daily for signs of discomfort. See **Fig.2** for model description.

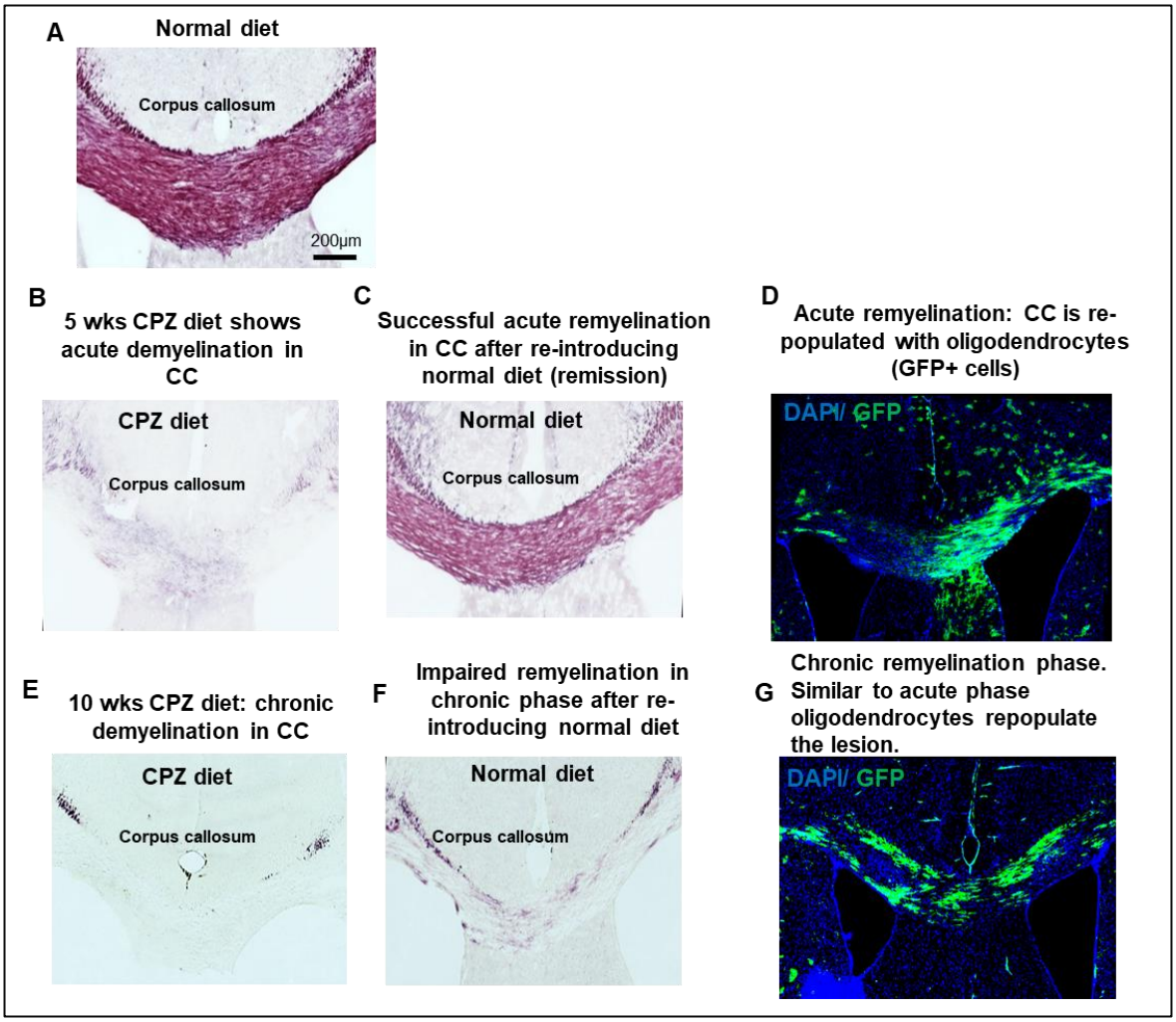


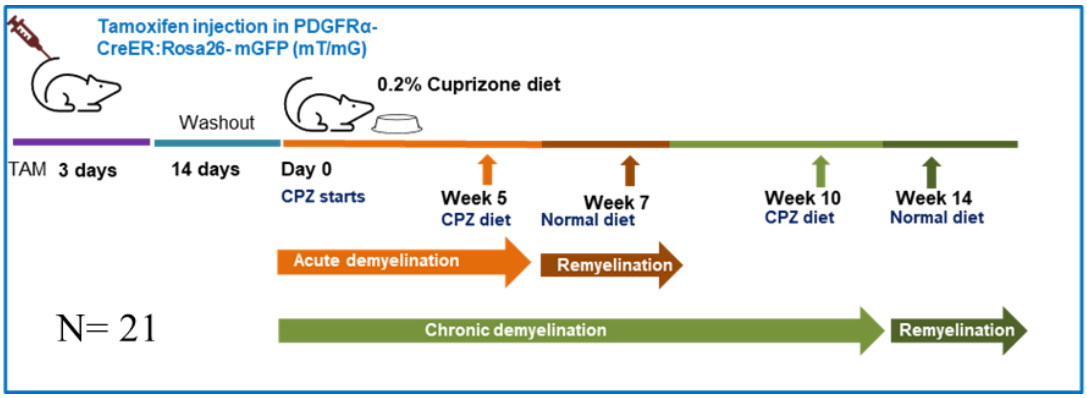
Figure 2. Remyelination fails in chronic lesions of Cuprizone mouse model of progressive MS despite the presence of oligodendrocytes. Black-gold complex specifically stains myelin within the central nervous system shows heavy myelination of axons in the corpus callosum (**CC**) of normal mice. **A)** CC with normal myelination. **B)** Acute demyelination, CPZ was given for 5 weeks to PD mice which caused loss of myelin in CC.

C-D) After mice were returned to normal diet for 1-2 weeks, spontaneous remyelination occurred with the presence of oligodendrocytes. **E)** For chronic demyelination, CPZ was given for 10 weeks, which resulted in a robust loss of myelin. **F-G)** After chronic CPZ diet, remyelination failed after mice were returned to normal diet for 4 weeks despite the presence of oligodendrocytes. This suggests that maturation of oligodendrocytes is impaired in chronic demyelinating lesions. Black Gold staining and images were provided by other members of Karimi's Lab.

2.2.4 Experimental groups

PD-mice received CPZ diet to induce acute and chronic demyelination. Experimental groups: 1) CPZ for 5 wk; 2) CPZ for 5 wk + 2 wks of normal diet; 3) CPZ for 10 wks; 4) CPZ for 10 wks + 2 wks of normal diet; 5) CPZ for 10 wks + 4 wks of normal diet; 6) Age match control for 7 and 12 weeks. Sample size: n=3-4 mice/group/sex. Information on experimental groups, endpoints and sample size is illustrated in Figure 3.

Figure 3. Experimental paradigms for PD-CPZ mice



2.2.5 Tissue processing

After each endpoint, mice were anesthetized with isoflurane and then transcardially perfused with ice-cold 3% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS).

Brains were post-fixed in a solution of 10% sucrose in 3% PFA overnight at 4°C and cryoprotected later with 20% sucrose in PBS for two more days at 4°C. The brains were later embedded in optimal cutting temperature compound OCT (TissueTek®, Electron Microscopy Sciences). Cross sections of 16μm were cut serially using a cryostat (Leica Biosystems GmbH) and mounted onto Superfrost® Plus Micro Slides (Fisher Scientific) and stored at -80°C until the immunostaining procedure. Some of the tissue was cut by other members and some tissue for acute and chronic time points was cut by me.

2.2.6 Immunohistochemistry on tissue sections.

Frozen cross-sections of brains were processed for immunohistological analysis. For immunostaining, slides were air-dried and permeabilized with 0.1M PBS. Later, slides were incubated with a blocking solution containing 5% non-fat milk, 1% Bovine serum albumin

(BSA) and 0.3% Triton X-100 in 0.1M PBS for 1 hour at room temperature. Blocking solution was removed and the sections were incubated with the respective primary antibodies overnight at 4°C (listed in Table 1) and then were later incubated with the appropriate secondary antibodies, including donkey anti-goat 647, goat anti-mouse 647 and anti-rabbit 405 Alexa Fluor (1:600; Invitrogen, Life Technologies Inc, ON, Canada) for 1 hour at room temperature. For double staining procedures, the tissue sections were treated sequentially with a second primary antibody followed by the incubation with its appropriate secondary antibody. Tissue sections were washed three times with 0.1M PBS during each period of incubation. Slides were cover-slipped with Mowiol mounting media (Sigma, 81381).

The specificity of all antibodies was validated through the implementation of negative controls (omitting primary antibody from the staining protocol).

Table 1. List of antibodies used for *in vitro* and *in vivo* studies.

Antibody	Targeted species	Application	Dilution factor	Source (CAT#)
p-mTOR	Rabbit	ICC/IHC/WB	1:100/1:200/1:1000	Cell Signaling (5536)
Total mTOR	Rabbit	WB	1:1000	Cell Signaling (2972)
p-eIF2α	Rabbit	ICC/IHC/WB	1:250/1:200/1:1000	Cell Signaling (3398)
Total eIF2α	Rabbit	WB	1:1000	Cell Signaling (5324)
p-S6 Ribosomal protein	Rabbit	ICC/IHC/WB	1:250/1:200/1:1000	Cell Signaling (2211)
S6 Ribosomal protein	Rabbit	WB	1:1000	Cell Signaling (2217)
CHOP	Mouse	ICC/ WB	1:200/1:1000	Cell Signaling (2895)
PDGFRα	Goat	IHC	1:200	R&D Systems (AF1062)
CC1	Mouse	IHC	1:50	Sigma (OP80)
NG2	Rabbit	ICC	1:300	Thermo Fisher (AB5320)
O4	Mouse	ICC	1:300	R&D Systems (MAB1326)
MBP	Rat	ICC	1:200	Millipore (MAB386)
Olig2	Rabbit	ICC	1:2000	Chemicon (AB9610)
GFAP	Mouse	ICC	1:800	Chemicon (MAB360)
OX42	Mouse	ICC	1:200	AbDSerotec (MCA711G)
LAMP1	Rat	ICC	1:200	Santa Cruz Biotechnology (sc-19992)

2.2.7 Imaging and tissue analysis.

Expression of mTOR and PERK-related markers in PD-CPZ tissue was assessed by co-immunostaining of CC1 (OLs) or PDGFRα (OPCs) and their respective marker (pmTOR, pS6, p-eIF2α, CHOP). The percentage of OPCs or OLs that were positive for the markers mentioned above was assessed in CC. For this purpose, the CC was imaged by Zen tiling software (Zeiss).

Cells that were CC1+ or PDGFRα+ were identified by their morphology, the expression of the oligodendroglial lineage markers, and their co-labeling with the GFP signal in the CC. DAPI was not used as per the limitations of the PD tissue, which only allows performing double staining since two channels are occupied with TdTomato and GFP. For this reason, two cell populations were considered: GFP+ and GFP-. The first one refers to all the OPCs and OLs that were successfully recombined after TAM administration, and the latter refers to OPCs and OLs that were either not recombined or represent other cell types (e.g. astrocytes) that may express the marker. All samples, including control and experimental conditions, were processed in parallel under the same condition and imaged using Zeiss AxioImager M2 fluorescence microscope (Zeiss) under similar exposure time. The specificities of all antibodies were determined with a negative control, omitting the primary antibody in our immunohistochemistry staining protocol (see Supplementary figure 2).

2.3 *In vitro* studies

2.3.1 Isolation and culture of primary OPCs from mice pups

Primary cells were isolated from the cerebral cortex of postnatal day 0-2 (P0-P2) CD-1 mice pups as described previously[105]. Cerebral cortices were dissected out, and olfactory bulbs, cerebellum, and meninges were removed. Cortices were dissociated enzymatically using papain (0.9mg/ml; Worthington Biochemical, New Jersey, United States) and DNase (10mg/ml; Sigma-Aldrich, ON, Canada) for 15 minutes at 37°C. The enzymatic reaction was stopped using Dulbecco's Modified Eagle Medium (DMEM) media (ThermoFisher, Gibco™, 31600091) and mechanic dissociation by pipetting was performed.

Digested tissue was filtered through a 70-µm cell nylon strainer (BD Biosciences, Mississauga, ON, Canada) and seeded on PDL-coated flasks in DMEM medium supplemented with 20% Fetal bovine serum (FBS) (Gibco, Life Technologies Inc, ON, Canada), 1% of Penicillin, Streptomycin, and Neomycin mixture or PSN (Invitrogen, LifeTechnologies Inc, ON, Canada), 30% glucose (Sigma-Aldrich, ON, Canada), L-glutamine (200 mM, Gibco, Life Technologies Inc, ON, Canada) and Sodium pyruvate (100 mM, Gibco, Life Technologies Inc, ON, Canada). The cultures were maintained at 37°C, and 5% CO_2, media was changed every 3 days. At 10 days, when confluency was reached, the cultures were shaken at 250 rpm at 37°C for 2 hours to remove microglia, and the medium was refreshed. The cultures were incubated for 6 hours at 37°C before they were shaken again at 250 rpm at 37°C to for 18 hours to obtain OPCs. The medium was then filtered through a 70-µm cell nylon strainer (BD Biosciences, Mississauga, ON, Canada) and centrifuged at 1500 rpm for 7 min. Cells were then seeded on non-coated Petri dishes (BD Biosciences, Mississauga, ON, Canada) and were kept for 1.5 hours at 37°C to remove adherent cells (microglia and astrocytes). After this time, the medium was collected and centrifuged at 1500 rpm for 7 min. to isolate OPCs. OPCs were plated onto PDL (0.1 mg/ml) coated multi-chamber glass slides (20,000 cells per chamber) (LabTek Fisher Scientific, ON, Canada) in OPC culture medium, which contained DMEM, insulin (10µg/ml), transferrin (50µg/ml), sodium selenite (5.2 ng/ml), hydrocortisone (18 ng/ml), putrescine (16 µg/ml), progesterone (6.3 ng/ml), biotin (10 ng/ml), N-acetyl-L-cysteine (5 µg/ml), BSA (0.1%), PSN (Invitrogen, Life Technologies Inc, ON, Canada), 1% FBS, and 5µM of forskolin. After 24 hours of plating, OPC medium was changed to either OPC differentiation medium which contained OPC medium and the growth factor T3 (20 ng/ml); or proliferation medium which contained OPC medium and the growth factors bFGF (20 ng/ ml) and PDGF-AA (20 ng/ ml).

2.3.2 Rapamycin, TNFα/IFNγ, and Tunicamycin treatment in OPC culture

OPCs were treated with: 1) rapamycin (Sigma-Aldrich, 53123-88-9) a concentration of 10 nM to suppress mTORC1, and 2) TNFα/IFNγ (R&D Systems, 410-MT-010) and (FUJIFILM Irvine Scientific, 100-77-20UG) in a concentration of 50 ng/ml and 40 ng/ml, respectively, as MS relevant cytokines. A third condition was also tried to induce UPR stress by using 0.25 μg/ml of Tunicamycin (Sigma-Aldrich, 11089-65-9). Assessment of overall cell viability was conducted by LIVE/DEAD assay according to the manufacturer's instructions (LIVE/DEAD® Viability/Cytotoxicity Kit; Thermo Fisher, L3224). LIVE/DEAD assay reagent was prepared with 2 mM Ethidium homodimer (EthD1) and 4 mM Calcein AM in 1X PBS. For cell viability assessments, primary OPCs under rapamycin and TNFα/IFNγ were kept for 48 hours at 37°C, after this time the cells were briefly washed twice with PBS. Then, 150 μl of the LIVE/DEAD assay reagent was added to each well of 8-well multi-chamber slides for 15 min at 37°C. Cells were washed with 1X PBS. Live imaging of 10 randomly chosen fields were taken under 20X objective using a Zeiss fluorescent microscope using optical filters for live cells (Calcein, 485 ± 10 nm, green) and dead cells (EthD1, 530 ± 12.5 nm, red). Cells were then quantified by counting the number of live cells among the total number of both live and dead cells. A gradient assay was performed for the Tunicamycin condition using the following concentrations: 0.1, 0.25, 0.5, 0.75, 1, 1.5 and 2 μg/ml. For this assessment, cells were kept at 37°C for 16 hours, and after this time LIVE/DEAD assay was performed as described above. Live imaging and quantification were performed as described above. Complementary to this survival assessment, an activity assay was conducted. Under various conditions, OPC proliferation and differentiation were assessed (N= 3 independent cultures).

For proliferation, OPCs were maintained for 48 hours and 7 days, and for differentiation for 7 and 14 days. For proliferation assessment, 5-ethynyl-2'-deoxyuridine or EdU (Life Technologies Inc, E10187) was added to the proliferation medium at the start of the experiments. The medium was refreshed every 2 days.

2.3.3 Western blotting

For immunoblotting-based assessments, primary OPCs were plated onto PDL (0.1 mg/ml) coated 6-well plates (Biolite, Fisher, ON, Canada) (1x10^6 distributed in 3 wells) in OPC culture medium (N=4 independent culture). After the addition of treatment, OPCs were incubated for 48 hours, and cell lysates were prepared with NP40 buffer containing protease inhibitor cocktail (Sigma). Protein content was determined in in the cell lysate and by Lowry assay (Sigma, L3540) and Western blot samples were prepared with 20µg of protein. Samples were loaded onto 12.5% Tris-glycine gels and transferred onto a nitrocellulose membrane (BioRad, Hercules, CA) using Trans-Blot® Turbo Transfer system (BioRad, 2.5V) for 7 mimutes. Membranes were then blocked for 1 hr at room temperature with 5% BSA in Tween-20 Tris-buffered saline (1% TBST) and incubated overnight at 4°C with different primary antibodies diluted in the blocking solution. Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:4000; BioRad). Membranes were then incubated in enhanced chemiluminescent immuno-blotting detection reagents (FroggaBio, Canada) and protein bands were detected with MicroChemi 4.2 (DNR Bio-imaging Systems).

2.3.4 Immunocytochemistry procedures

Purity of cortical OPC cultures was assessed by immunostaining against all three glial cell types: NG2 for OPCs, GFAP for astrocytes, and Iba1 for microglia. Maturity of OPCs into OLs was assessed by immunostaining against O4 for immature OLs, and MBP for mature OLs. Activity of mTOR was assessed by immunostaining against the markers pS6 and pmTOR; UPR activity was assessed by using a marker against peIF2α. Under Tunicamycin, OPCs were immuno-stained with the downstream UPR marker CHOP (Cell-signaling, 2895S). In all immunostaining procedures, cultured cells were fixed with 3% PFA for 20 min and washed with PBS at room temperature immediately after fixation. Cultures were then blocked for 1 hour at room temperature with 5% skim milk and 1% BSA (Sigma, A7030-100g) and 0.3% of Triton X in 0.1M PBS. Triton X was avoided in staining for surface markers (O4 and MBP). Cultures were incubated with primary antibodies for 1.5 hrs at room temperature, followed by incubation with appropriate secondary antibodies for 1 h, which included goat anti-rabbit 488, anti-mouse 568, anti-rat 647 (1:600, Invitrogen, Life Technologies Inc, ON, Canada). For double or triple staining, the multi-chamber slides were sequentially treated with a second primary antibody and then incubated with its respective secondary antibody. This process was repeated for a third marker. To label the nucleus, cells were incubated with 4, 6 diamidino-2-phenylindole (DAPI, Sigma D8417) for 15 min at room temperature, washed with PBS and cover-slipped with Mowiol (Sigma, 81381). Specificity of all antibodies was validated by using negative controls (omitting primary antibody from staining protocol). Fluorescence images were taken at 20X magnification, and 10-12 random fields were taken containing an average of 150 cells for each condition. The complete list of antibodies is listed on Table 1.

2.3.5 Assessments of OPC proliferation and differentiation

To assess purity of cultures, the percentage of microglia (Iba1+), astrocytes (GFAP+) and OPCs (NG2+) was determined by counting the number of cells positive for each of the markers mentioned above and normalizing them to DAPI+ cells. Assessments on proliferation were performed by adding EdU to live cells as described above. Once fixed, cells were permeabilized with 0.5% PBST for 30 min and washed, then EdU reaction mixture (double distilled water, 100 mM L-Ascorbic acid, 4mM copper sulphate, 2M Tris base and 5mM Azide Fluor 647) was added and incubated in the dark for 30 min at room temperature. Fluorescence images were taken at 20X magnification, and 10-12 random fields were taken containing an average of 150 cells for each condition. Proliferation assessment was performed by counting the number of EdU+/DAPI+ cells. To assess stages of maturation, the percentage of OPCs (NG2+), immature OLs (O4+), and mature OLs (MBP+) was determined by counting the number of cells positive for each of the markers and normalized to the total number of cells marked by DAPI. To assess the differences in the expression of MBP, fluorescence images were taken at 20X magnification, under the same exposure time for all conditions. Differences in expression were assessed by measuring the Integrated Density per cell, using the FIJI (previously known as ImageJ) software. Sholl analysis was performed to assess differences in morphological complexity of OLs; hence images of cells marked with O4 were used and Sholl was performed on each individual cell by using the neurite tracer tool of the FIJI software, 10 cells per condition per biological replicate were used for Sholl assessment (30 cells per 3 Ns). Assessments for mTOR and UPR activity were performed by measuring the integrated density of pS6, pmTOR and peIF2α in different conditions using FIJI software, as described above.

2.3.6 Assessment on lysosomal activity using Lysotracker

Lysotracker Deep Red (ThermoFisher Scientific, L12492, ON, Canada) was used to assess changes in autophagy. OPCs grown on glass multi-chamber slides were subjected to the treatments: control, rapamycin and TNFα/IFNγ for 48 hrs and 7 days. Then, the cells were treated with Lysotracker Deep Red at a final concentration of 50 nM in phenol red free media for 30 min at 37°C. Cells were then washed twice with PBS, fixed with 3% PFA in PBS, and immunostained with LAMP1 antibody and DAPI to mark all lysosomes and cell nuclei, respectively. Fluorescent images were obtained by an epi-fluorescent microscope at a 60X magnification. Quantification was performed by assessing co-localization of LAMP1+ and Lysotracker structures using the plugin JaCOP from the software FIJI. This plugin calculates the Manders' Colocalization Coefficients (MCC), which directly measures the ratio of co-localization between two signals, reported as coefficients M1 and M2. The coefficients show the estimation of the fraction of one particle that colocalizes with another and vice versa. M1 represents the ratio of LAMP1 co-localized with Lysotracker and M2 represents the ratio of Lysotracker co-localized with LAMP1.

2.3.7 Statistical analysis

Statistical analysis was conducted for all *in vitro* and *in vivo* assessments using GraphPad Prism Software (version 8.4.0, California, USA). *In vitro*, statistical differences between two groups were evaluated by Student's T test. For the morphology assessment, Two-Way analysis of variance (ANOVA) was used. In CPZ mice, statistical differences across cell populations were evaluated by One-Way analysis of variance (ANOVA) followed by multiple comparisons. All data are expressed as mean ± standard error of the mean (S.E.M). Significance was noted statistically if $p < 0.05$.

3. Results

3.1 mTOR activity increases during both acute and chronic phases of demyelination and remyelination in CPZ mice.

Previous reports show that mTOR signaling regulates OPC metabolism in a CPZ model and promotes efficient remyelination in the brain[114]. Here, using CPZ mice, we characterized the activity of mTORC1 in OPCs and OLs in corpus callosum (CC) during acute and chronic demyelination and subsequent recovery period. PDGFRα-CreER:Rosa26mGFP (mT/mG) (in short, PD) mice were treated with CPZ diet for acute and chronic time points. mTOR activity was assessed by immunohistochemical detection of phosphorylated mTOR (pmTOR) and phosphorylated S6 (pS6) for downstream mTORC1 activity (Fig. 4). Oligodendroglial lineage cells were tracked by their GFP+ signal and by immunostaining for OPCs (PDGFRα+) and OLs (CC1+) to differentiate both cell populations. Expression of pmTOR and pS6 was quantified in PDGFRα+ and CC1+ cells in CC. Cell analysis criteria are described in table 2.

Table 2. Criteria set for cell populations using mTOR markers.

	Cell populations				
pmTOR activity	pmTOR+/PDGFRα+/GFP+	pmTOR-/PDGFRα+/GFP+	pmTOR+/PDGFRα+/GFP-	pmTOR-/PDGFRα+/GFP-	OPCs
	pmTOR+/CC1+/GFP+	pmTOR-/CC1+/GFP+	pmTOR+/CC1+/GFP-	pmTOR-/CC1+/GFP-	OLs
pS6 activity	pS6+/PDGFRα+/GFP+	pS6-/PDGFRα+/GFP+	pS6+/PDGFRα+/GFP-	pS6-/PDGFRα+/GFP-	OPCs
	pS6+/CC1+/GFP+	pS6-/CC1+/GFP+	pS6+/CC1+/GFP-	pS6-/CC1+/GFP-	OLs

mTOR phosphorylation by the PI3K/Akt pathway happens at the serine site Ser2448[106], hence we used an antibody to detect phosphorylated mTOR at the Ser2448 site to assess mTOR activity. We found that pmTOR expression significantly increased in GFP+ OPCs after both acute demyelination and remyelination as compared to the control **(Fig 4A-G)** with a higher percentage of pmTOR expressing GFP+ OPCs during the acute remyelination as compared to the demyelination phase **(Fig. 4G).** No significant differences were found in the population of GFP- OPCs that expressed pmTOR in acute CPZ demyelination or remyelination stages compared to the control healthy mice **(Fig. 4H).** The percentage of pmTOR+/GFP+ OPCs also significantly increased during the chronic demyelination stage as compared to the control. This expression was sustained during the short-term (2 weeks) and long-term (4 weeks) remyelination stages and was significantly higher than the control **(Fig. 4I).** However, no significant differences were found between the percentage of pmTOR+/GFP+ OPCs present in the CC of the demyelinating and remyelinating chronic phases. No significant differences were found between the population of GFP- OPCs that expressed pmTOR in chronic demyelination or remyelination conditions compared to control healthy mice (**Fig. 4D-F).** Altogether, these results indicate that pmTOR phosphorylation happens in OPCs during demyelination and is sustained during the recovery period, suggesting that mTOR activation potentially supports remyelination.

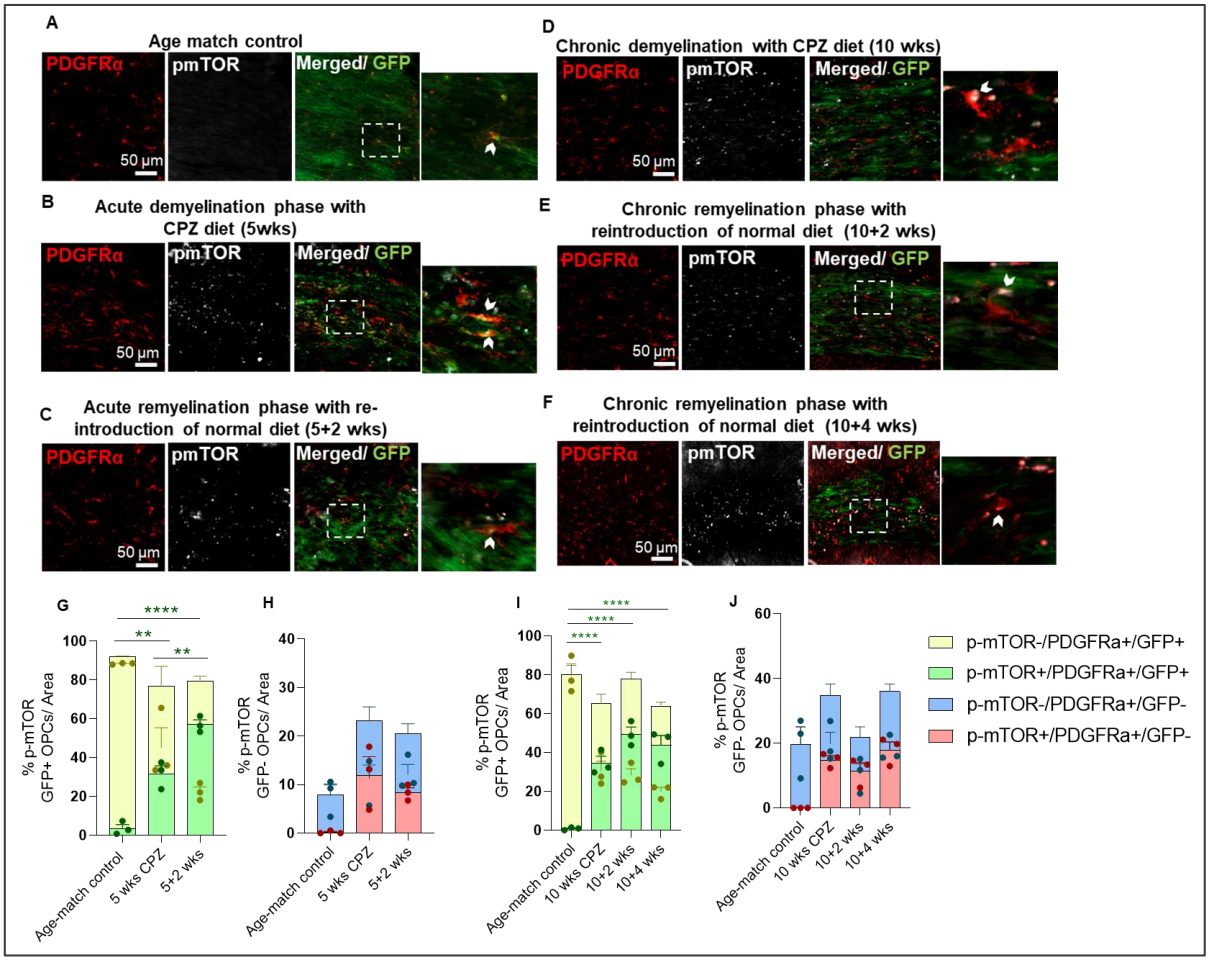


Figure 4. Expression of phosphorylated mTOR increased in OPCs in the corpus callosum (CC) of CPZ mice during acute and chronic phases of demyelination and remyelination. A-F) Representative images of corpus callosum (CC) of PD-mice under control normal diet and mice treated with CPZ diet for 5 and 10 weeks for acute and chronic demyelination, respectively, as well as 2 and 4 weeks remyelinating stages, respectively. **G)** Quantitative analysis of p-mTOR expression in GFP+ OPCs show a significantly higher percentage of new OPCs expressing pmTOR during the demyelinating and remyelinating phases when compared to the control. There was also a significant increase in GFP+/pmTOR+ OPCs during remyelination as compared to the demyelination phase. **H)** Analysis of pmTOR expression in GFP- OPCs during acute demyelination and remyelination showed no significant differences across cell populations and time points. **I)** In chronic CPZ mice, there were significantly more GFP+/pmTOR+ OPCs in the demyelinating and remyelinating phases when compared to the control, while the number of pmTOR- OPCs significantly decreased. However, there were no significant differences in GFP+/pmTOR+ OPCs during injury and repair. **J)** Expression of pmTOR in GFP- OPCs during chronic demyelination and remyelination showed no difference. **P <0.01, ****P <0.0001. Data present Mean ± SEM. One-way ANOVA followed by Sidak's multiple comparisons test, N= 3.

We also assessed mTOR activity in mature OLs by immunostaining for CC1 (**Fig. 5A-J)**. The percentage of pmTOR expressing GFP+ OLs significantly increased during the acute demyelination and remyelination as compared to the healthy control with a significantly higher number during the remyelination stage in relation to the demyelination phase **(Fig. 5G).** No significant differences were found between the population of GFP- OLs that expressed pmTOR during control, demyelination, or remyelination stages **(Fig. 5H).** Similar results were found after the chronic treatment with CPZ (**Fig. 5D-F).** The percentage of pmTOR+/GFP+ OLs significantly increased during demyelination as compared to the control (**Fig. 5I).** This expression was sustained during the short-term (10+2 weeks) and long-term (10+4 weeks) remyelination stages and was significantly higher than the control. No significant differences were found between the percentage of pmTOR+/GFP+ OLs present in the CC of the demyelinating and remyelinating phases **(Fig. 5I).** While significant differences were found between the population of GFP-/pmTOR+ OLs during the demyelination stage comparing to the control, there was no significant differences in this cell population between the demyelination and remyelination stages (short and long term) **(Fig. 5J)**. Similar to the acute phase, these results indicate that mTOR phosphorylation happens in OLs during chronic demyelination and is sustained during the remyelination stages.

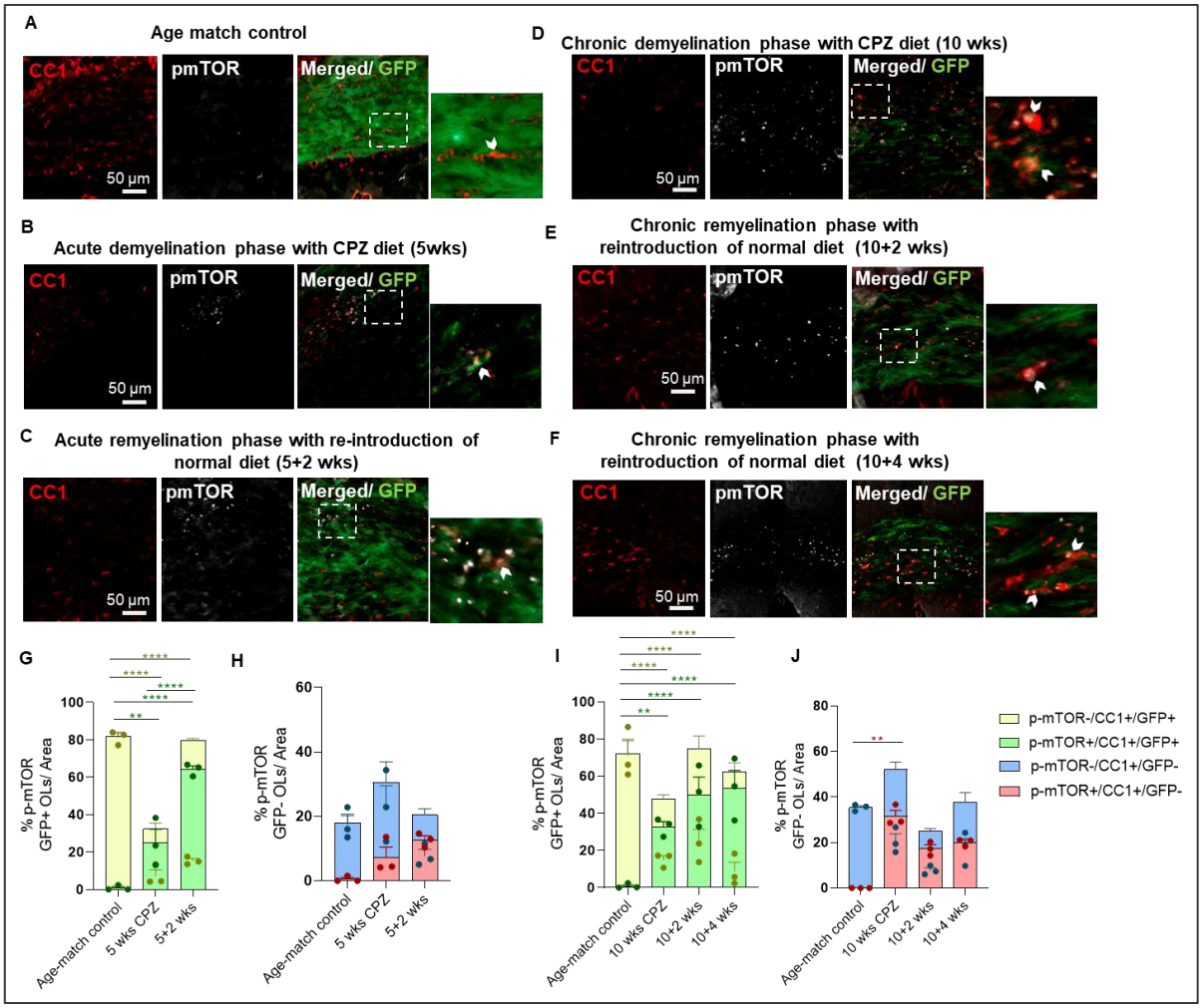


Figure 5. Expression of phosphorylated mTOR increased in mature OLs in the corpus callosum (CC) of CPZ mice during acute and chronic phases of demyelination and remyelination. A-F) Representative images of corpus callosum (CC) of PD-mice under control normal diet and mice treated with CPZ diet for 5 and 10 weeks for acute and chronic demyelination, respectively, as well as 2 and 4 weeks remyelinating stages. OLs were labelled with CC1 and pmTOR. **G)** Quantitative analysis of p-mTOR expression in GFP+ OLs showed a significantly higher percentage of new OLs expressing pmTOR during the demyelinating and remyelinating phases when compared to the control mice with a significantly higher number of GFP+/pmTOR+ OLs during remyelination as compared to the demyelination phase. **H)** Analysis of pmTOR expression in GFP- OLs that during acute demyelination and remyelination showed no significant differences across cell populations and time points. **I)** In chronic CPZ mice, there were significantly more GFP+/pmTOR+ OLs in the demyelinating and remyelinating phases when compared to the control, while the number of pmTOR- OLs significantly decreased. There were no significant differences of GFP+/pmTOR+ OLs among the 10 wks, 10+2 and 10+4 wks groups, suggesting that mTOR activity is sustained during injury and repair. **J)** There were significantly more GFP-/pmTOR+ OLs during the demyelination phase when compared to the control, with no differences among other cell populations or time points.

**P <0.01, ****P <0.0001. Data present Mean ± SEM. One-way ANOVA followed by Sidak's multiple comparisons test, N= 3.

3.2 mTORC1 activity increases during acute and chronic phases of demyelination and remyelination.

Given mTOR phosphorylation is detected in OPCs and OLs during demyelination and is sustained in remyelination in acute and chronic phases, we next determined if this phosphorylation was accompanied by the downstream activity of mTORC1. Immunostaining for pS6 (**Fig. 6A-J)** showed a significant increase in pS6 expression in GFP+ OPCs in both acute demyelination and remyelination phases as compared to the control **(Fig. 6G).** We also found significant differences among the population of GFP- OPCs that expressed pS6 during acute remyelination as compared to the control, but no differences were observed when compared to the demyelinating phase (**Fig. 6H).** Similar results were found in chronic CPZ lesions (**Fig. 6D-J).** The percentage of pS6+/GFP+ OPCs significantly increased during the chronic demyelination stage as compared to the control (**Fig. 6I).** This expression was sustained during the short-term and long-term remyelination stages and was significantly higher than the control **(Fig. 6I).** However, no significant differences were found between the percentage of pmTOR+/GFP+ OPCs present in the CC during chronic demyelination and remyelination**.** Additionally, there were significantly more GFP-/pS6+ OPCs after the 10, 10+2 and 10+4 wks time points when compared to the control, while there were no differences in the percentage of GFP-/pS6- OPCs among the groups. These results indicate that there is downstream mTORC1 activity in OPCs after the initial phosphorylation of mTOR during demyelination and remyelination stages.

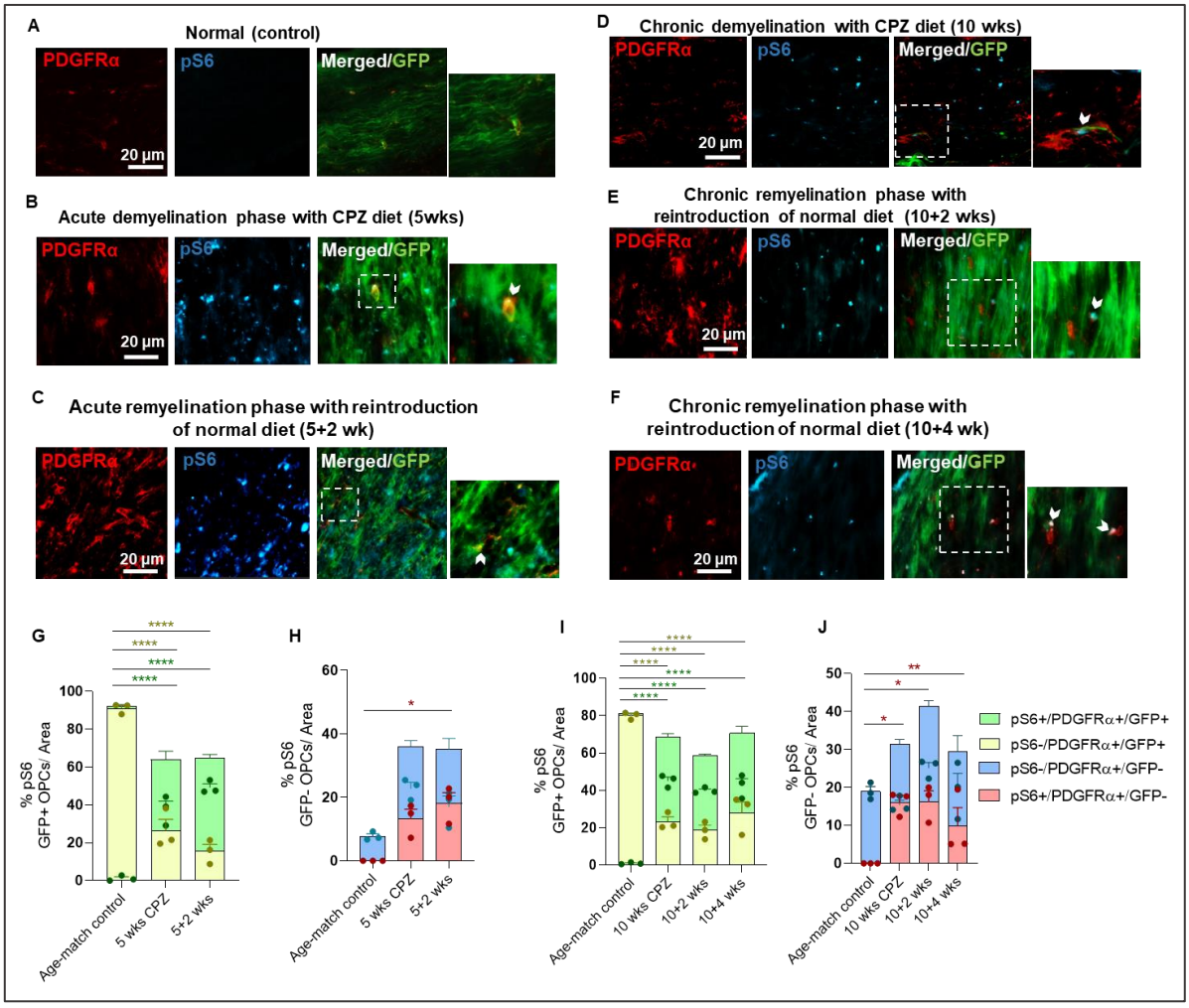


Figure 6. Expression of phosphorylated S6 increased in OPCs in the corpus callosum (CC) of CPZ mice during acute and chronic phases of demyelination and remyelination. A-F) Representative images of corpus callosum (CC) of PD-mice under control normal diet and mice treated with CPZ diet for 5 and 10 weeks for acute and chronic demyelination, respectively, as well as 2 and 4 weeks remyelinating stages. OPCs were labelled with PDGFRα and pS6.**G)** Quantitative analysis of pS6 expression in GFP+ OPCs showed a significantly higher percentage of OPCs expressing pS6 during the acute demyelinating and remyelinating phases when compared to the control mice, with a significant decrease in GFP+/pS6- OPCs. **H)** Quantitative analysis of pS6 expression in GFP- OPCs showed a significant increase in the percentage of GFP- OPCs that expressed pS6 during the acute remyelination phase when compared to the control, there were no significant differences among the other cell populations and timepoints. **I)** In the chronic CPZ mice, there was a significant increase in the percentage of GFP+/pS6+ OPCs during the demyelinating and remyelinating phases when compared to the control, while the percentage of pS6- OPCs significantly decreased. There were no significant differences of GFP+/pmTOR+ OLs between the groups 10 wks, 10+2 and 10+4 wks, suggesting that mTOR activity is sustained during injury and repair.

J) There were significantly more GFP-/pS6+ OPCs during the demyelination and remyelination phases when compared to the control, no other differences were found for these cell populations in other time points. *<0.05, **P <0.01, ****P <0.0001. Data present Mean ± SEM. One-way ANOVA followed by Sidak's multiple comparisons test, N= 3.

Immunostaining for pS6 was also performed in CC1+ mature OLs (**Fig. 7 A-J)**. The percentage of pS6+/GFP+ OLs increased significantly during the acute phases of demyelination and remyelination in comparison to the control, but no differences were found between demyelination and remyelination phases indicating a sustained mTORC1 activity (**Fig. 7G).** No significant differences were found in the GFP- OLs that expressed pS6 at any of the time points (**Fig. 7H**). Similar results were found in chronic CPZ. The percentage of pS6+/GFP+ OLs significantly increased during the chronic demyelination stage as compared to the control (**Fig. 7I).** This expression was sustained during the short-term (10+2 weeks) and long-term (10+4 weeks) remyelination stages and was significantly higher than the control, although, no significant differences were found between the demyelinating and remyelinating phases. We also observed a significantly higher percentage of GFP-/pS6+ OLs during the long-term remyelination phase when compared to the control **(Fig. 7J).** These findings show evidence of sustained TOR phosphorylation in OLs within CPZ lesions during demyelination and remyelination stages.

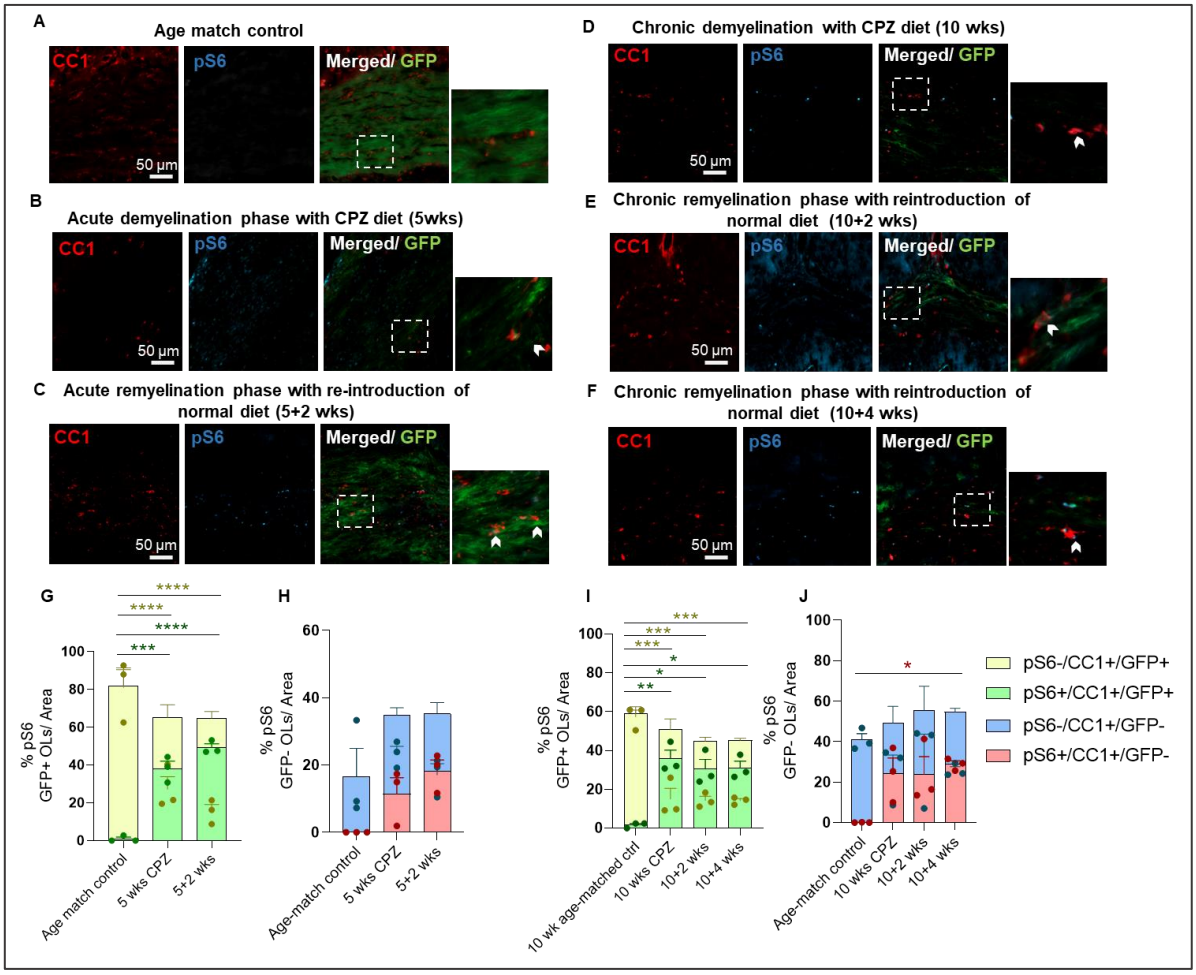


Figure 7. Expression of phosphorylated S6 increased in OLs in the corpus callosum (CC) of CPZ mice during acute and chronic phases of demyelination and remyelination. A-F) Representative images of corpus callosum (CC) of PD-mice under control normal diet and mice treated with CPZ diet for 5 and 10 weeks for acute and chronic demyelination, respectively, as well as 2 and 4 weeks remyelinating stages. OLs were labelled with CC1 and pS6. **G)** Quantitative analysis of pS6 expression in GFP+ OLs showed a significantly higher percentage of OLs expressing pS6 during the acute demyelinating and remyelinating phases when compared to the control mice, with a significant decrease in GFP+/pS6- OLs. No significant differences were found between the other timepoints. **H)** Quantitative analysis of pS6 expression in GFP- OPCs showed no significant differences among cell populations and timepoints. **I)** In the chronic CPZ mice, there was a significant increase in the percentage of GFP+/pS6+ OLs during the demyelinating and remyelinating phases when compared to the control, while the percentage of pS6- OLs significantly decreased. There were no significant differences of GFP+/pmTOR+ OLs between the groups 10 wks, 10+2 and 10+4 wks, suggesting that mTOR activity is sustained during injury and repair. **J)** There were significantly more GFP-/pS6+ OPCs during the demyelination and remyelination phases when compared to the control, no other differences were found for these cell populations in other time points.

*P <0.05, **P <0.01, ***P <0.001, ****P <0.0001. Data present Mean ± SEM. One-way ANOVA followed by Sidak's multiple comparisons test, N= 3.

3.3 Phosphorylation of eIF2α increases during both acute and chronic phases of demyelination and remyelination.

The ISR is an evolutionarily conserved signaling pathway that helps maintain protein homeostasis (proteostasis) in eukaryotic cells. It is activated in response to viral infection, oxidative stress, ER stress, nutrient deprivation or proteostasis defects[89]. Previous reports have demonstrated that the ISR protects OLs from the cytotoxic impact of CNS inflammation[94]. Furthermore, prolonging the ISR provides further protection in an EAE mouse model by delaying the onset of clinical symptoms, reducing OL and axonal loss, and decreasing the presence of T cells in the CNS[95]. We aimed to study the expression of phosphorylated eIF2α in CPZ model and follow its changes in OPCs and mature OLs during acute and chronic demyelination and remyelination in PD mice. To assess the initiation of the ISR we used a marker to detect peIF2α in OPCs and OLs. Oligodendroglial lineage cells were tracked by their GFP+ signal and by immunostaining for OPCs (PDGFRα+) and OLs (CC1+). Cell populations were considered according to the criteria described in table 3.

Table 3. Criteria set for cell populations using an ISR marker.

	Cell populations				
peIF2α activity	peIF2α+/PDGFRα+/GFP+	peIF2α-/PDGFRα+/GFP+	peIF2α+/PDGFRα+/GFP-	peIF2α-/PDGFRα+/GFP-	OPCs
	peIF2α+/CC1+/GFP+	peIF2α-/CC1+/GFP+	peIF2α+/CC1+/GFP-	peIF2α-/CC1+/GFP-	OLs

In OPCs, we observed initial expression of phosphorylated eIF2 α (peIF2α) after acute demyelination, although it was not significantly different from the control (**Fig. 8G**). During the acute remyelination period, there was a significant increase in the percentage of GFP+ OPCs that expressed peIF2α when compared to the control, however, there were no significant differences between the demyelinating phase and the other timepoints (control and remyelinating phase). Although there were no significant changes, we observed a trend in the expression of peIF2α, which initiated during the demyelination phase and was sustained during repair. There were no significant differences between the population of GFP- cells during the acute time points (**Fig. 8H**). Similar results were found in the chronic stages (**Fig. 8I),** where we observed a significant increase in the percentage of peIF2α+/GFP+ OPCs during the demyelinating and remyelinating stages when compared to the control, although no significant differences were found among other cell populations and timepoints, this could suggest that ISR activity is initiated during demyelination and is sustained during remyelination. No significant differences were found between the GFP- OPC cell populations and chronic timepoints. Altogether, these results indicate that the ISR is initiated in OPCs during demyelination and is sustained during the remyelination stages.

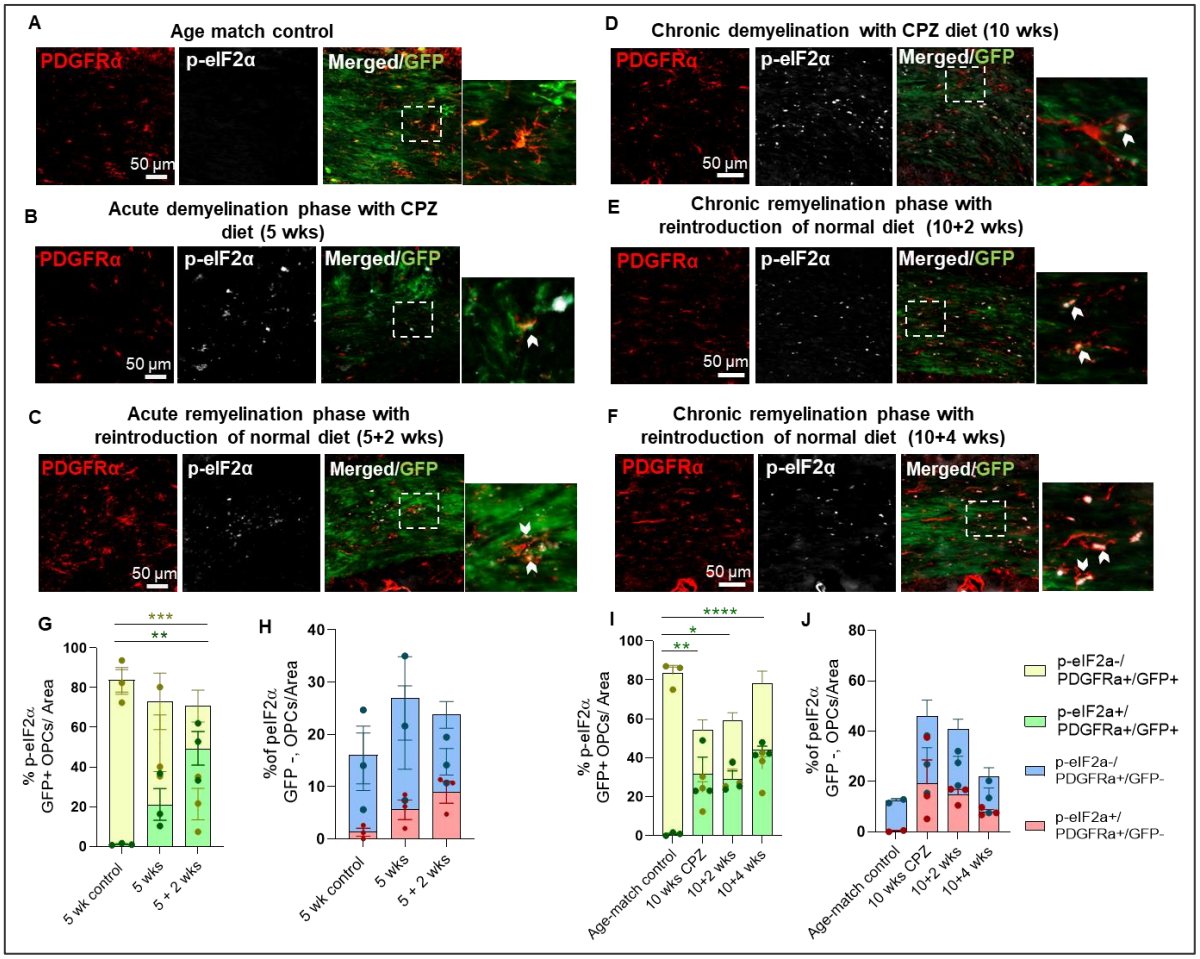


Figure 8. Expression of phosphorylated eIF2α increased in OPCs in the corpus callosum (CC) of CPZ mice during acute and chronic phases of demyelination and remyelination. A-F) Representative images of corpus callosum (CC) of PD-mice under control normal diet and mice treated with CPZ diet for 5 and 10 weeks for acute and chronic demyelination, respectively, as well as 2- and 4-weeks remyelinating stages. OPCs were labelled with PDGFRα and peIF2α. **G)** Quantitative analysis of peIF2α expression in GFP+ OPCs showed a significantly higher percentage of OPCs expressing peIF2α during the acute remyelinating phases when compared to the control mice. No significant differences were found between the other timepoints. **H)** Quantitative analysis of peIF2α expression in GFP-/OPCs showed no significant differences among cell populations and timepoints. **I)** Quantification of the percentage of GFP+ OPCs during the chronic demyelination and remyelination stages showed that there was a significant increase of peIF2α+/GFP+ OPCs in the demyelinating and remyelinating phases when compared to the control, no significant differences were found among other cell populations and timepoints. **J)** There were no significant differences in the percentage of GFP- OPCs that expressed or not peIF2α during chronic demyelination and remyelination. points. *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001. Data present Mean ± SEM. One-way ANOVA followed by Sidak's multiple comparisons test, N= 3.

We further studied eIF2α phosphorylation in mature OLs. The percentage of peIF2α-expressing GFP+ OLs, marked by CC1, significantly increased during the acute demyelination and remyelination phases compared to the control (**Fig. 9A-G**), and the same increase was also observed in GFP- OLs (**Fig. 9H).** Interestingly, eIF2α phosphorylation was further increased during remyelination phase (**Fig. 9H).** There was also a significant decrease in the percentage of peIF2α- OLs after CPZ treatment and during the recovery period (**Fig. 9H)**. Percentage of peIF2α+/GFP+ OLs increased in chronic demyelination when compared to the control and remained elevated during the remyelination period (**Fig. 9I)**. As for the GFP- population, the expression of peIF2α was also significantly increased in OLs after chronic demyelination and short-term remyelination (**Fig. 9J).** Altogether, this data shows increased expression of peIF2α in OLs during demyelination and remyelination, which may suggest that eIF2α phosphorylation and the ISR have a role in both injury and repair of OLs.

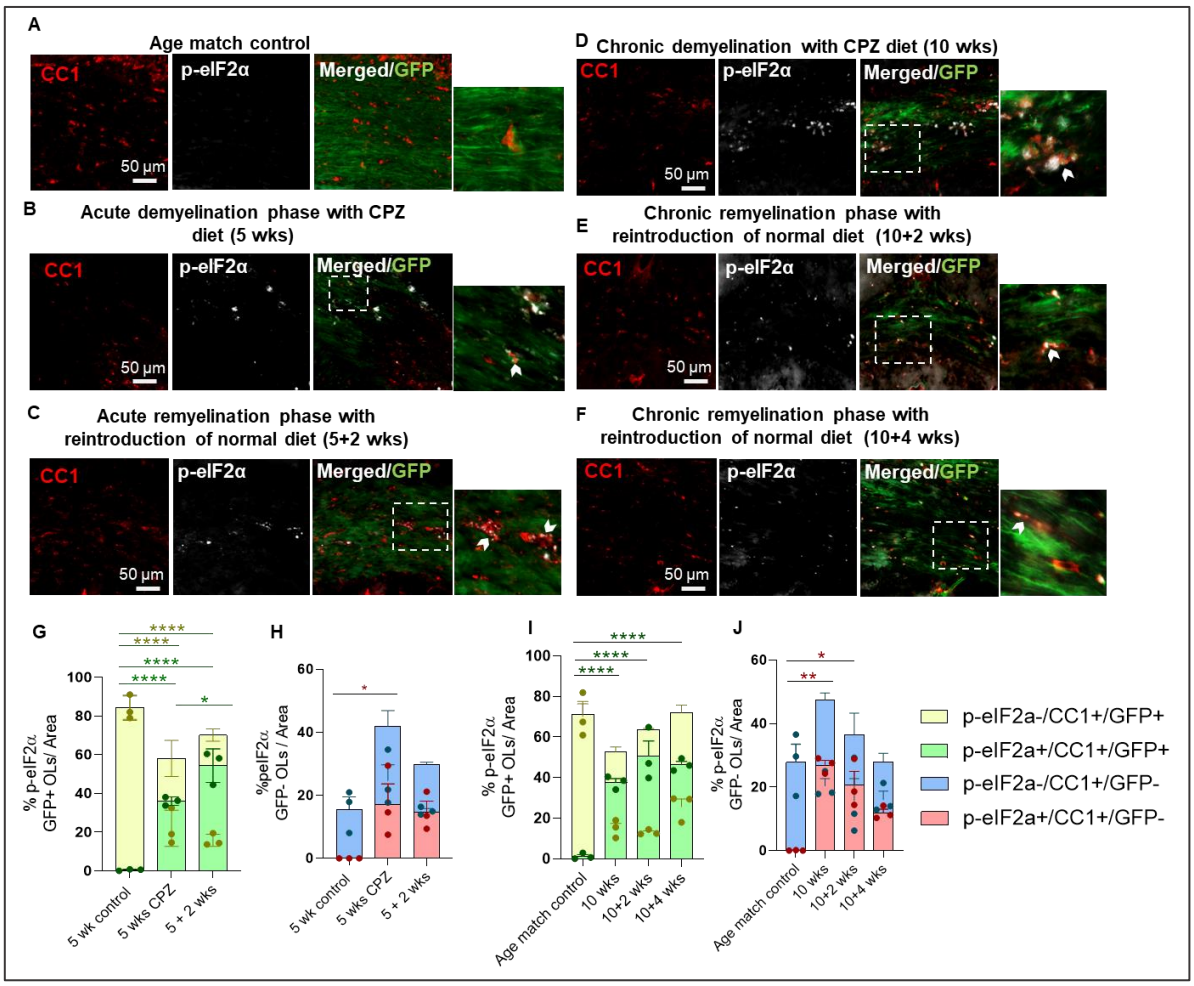


Figure 9. Expression of phosphorylated eIF2α increased in OLs in the corpus callosum (CC) of CPZ mice during acute and chronic phases of demyelination and remyelination. **A-F)** Representative images of corpus callosum (CC) of PD-mice under control normal diet and mice treated with CPZ diet for 5 and 10 weeks for acute and chronic demyelination, respectively, as well as 2- and 4-weeks remyelinating stages. OLs were labelled with CC1 and peIF2α. **G)** Quantitative analysis of the acute timepoints showed that there was a significant increase in the percentage of GFP+ OLs that expressed peIF2α during the demyelinating and remyelinating phases when compared to the control, furthermore, the expression was increased significantly during the remyelination phase as compared to the demyelinating phase. We also found a significant decrease in GFP+/peIF2α- OLs, indicating that most OLs were undergoing ISR. **H)** There was a significant increase in the GFP-/ peIF2α+ OLs during the demyelination phase when compared to the control, no significant differences were found among other cell populations and timepoints. **I)** Quantification of the chronic timepoints showed that there was a significant increase in the percentage of GFP+/ peIF2α+ OLs in the demyelinating and remyelinating stages when compared to the control, no significant differences were found among other cell populations and timepoints. **J)** After quantitative analysis, we found a significant increase in the percentage of GFP-/ peIF2α+ OLs during chronic demyelination and short-term remyelination when compared to the control.

*P <0.05, **P <0.01, ****P <0.0001. Data present Mean ± SEM. One-way ANOVA followed by Sidak's multiple comparisons test, N= 3.

3.4 Rapamycin-inhibition of mTORC1 reduces OPC proliferation but does not affect their survival.

We next studied the role of mTOR on OPC proliferation and maturation through direct *in vitro* experiments using rapamycin that is well-known to inhibit mTORC1 and its downstream mechanisms. Rapamycin is a specific allosteric inhibitor of mTORC1 that selectively binds to the protein FKBP12 that induces conformational changes in mTORC1 and thus prevents its normal downstream activity[107]. Primary mouse OPCs were placed under 10 nM rapamycin treatment [75,108] for 48 hours or 7 days to determine the effects on survival and proliferation (**Fig. 10**). Cortical OPCs were characterized before the experiments, and a purity of ~83.5% in the cultures was confirmed (**Fig. 10A**).

To confirm the efficacy of rapamycin in inhibition of mTORC1 in OPCs, we studied pS6 expression via immunocytochemistry. Rapamycin-treated OPC showed significantly reduction in pS6 expression after 48 hours and 7 days compared to control condition (**Fig. 10C-E**). Assessment of OPC survival by LIVE/DEAD assay did not show any significant cell death in the rapamycin-treated group (**Fig. 10F-G**), confirming that the dose used for these experiments was not toxic. Rapamycin treatment significantly reduced OPC proliferation (EdU+/DAPI+) after 48 hours compared to control condition (**Fig. 10H-I**), indicating the importance of mTORC1 for OPC expansion. After 7 days in culture, overall proliferation activity of OPCs declined under control condition and there was no significant changes in OPC proliferation between rapamycin treated and control cultures (**Fig. 10H-J**).

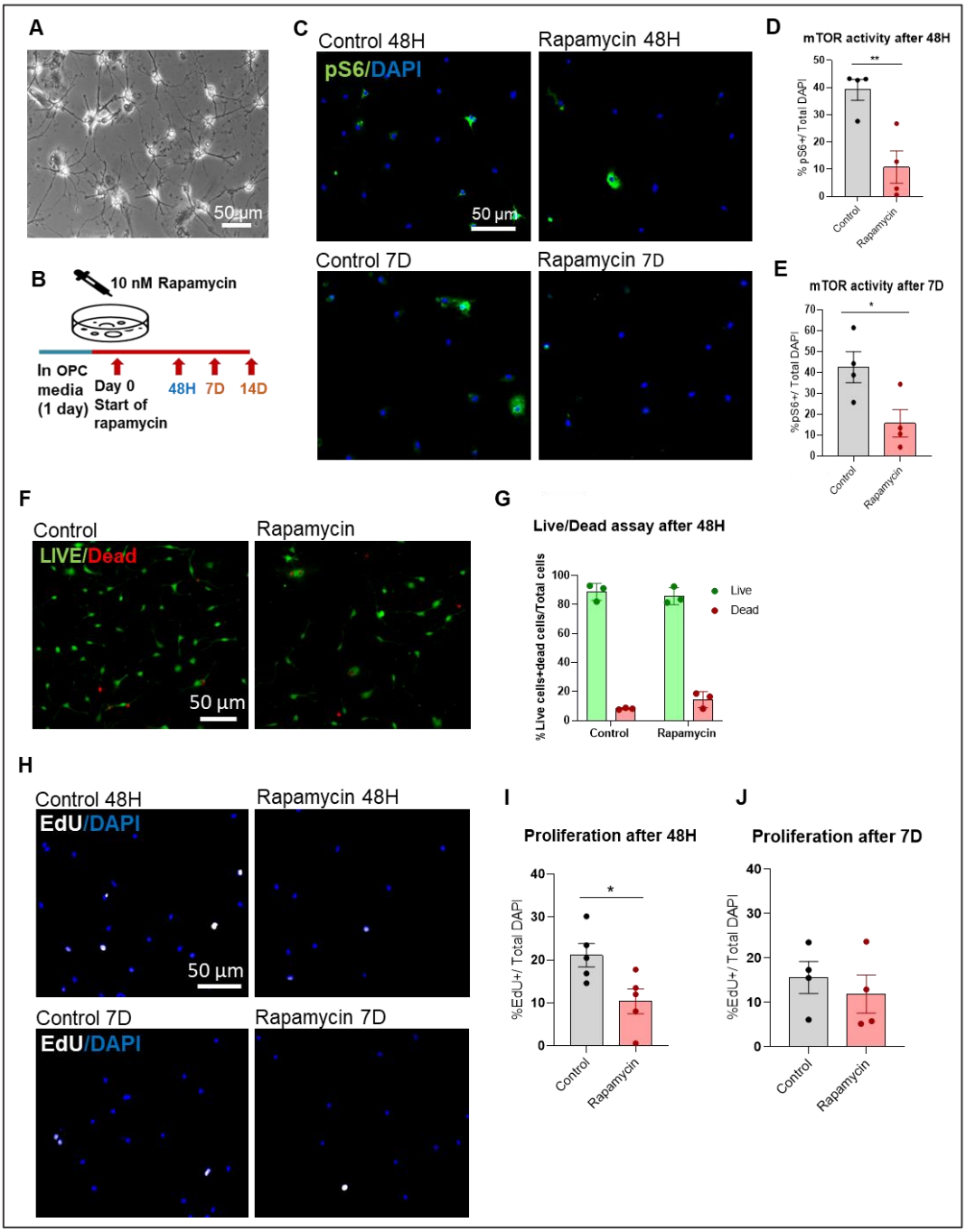


Figure 10. Rapamycin-inhibition of mTORC1 reduces OPC proliferation *in vitro* while preserving their survival. A-B) Postnatal cortical OPC cultures were treated under rapamycin and control conditions. Proliferation assessments were done at 48h and 7 days (7D). **C-E** Assessment of mTOR activity in OPCs after 48h and 7D by immunocytochemistry for pS6 showed a significant reduction. **F-G)**. LIVE/DEAD assay shows no significant effects of rapamycin on cell death. **H-J)** OPCs proliferation with EdU assay showed a significant decrease after 48h of rapamycin treatment with no significant differences at 7 days. Unpaired Student t test. *P <0.05; Data represents mean ± SEM. N=4-5 cultures.

3.5 Rapamycin-inhibition of mTORC1 reduces OPC differentiation and MBP expression.

OPC proliferation and maturation are distinct separate processes, each of them regulated by different factors[109]. Proper OPC differentiation into mature myelinating OLs is essential for myelination. Given that factors that inhibit proliferation may not affect differentiation, we next studied the impact of mTORC1 on differentiation and maturation of OPCs to oligodendrocytes capable of expressing myelin. OPCs were treated with rapamycin for 7 and 14 days. Degree of OL maturation was assessed immunocytochemically by studying the expression of NG2 for OPCs, O4 for immature oligodendrocytes, and MBP for mature oligodendrocytes (**Fig. 11)**. Percentage of each oligodendroglia population was quantified after 7D and 14D. While rapamycin treatment did not affect the number of mature cells as compared to the control condition (**Fig. 11A-B & E-F**), it significantly inhibited the expression of O4 and MBP (i.e. integrated density) at both 7 days and 14 days (**Fig. 11C-D & G-H**). Under control condition, MBP expression was gradually increased as OLs matured in culture after 7D and 14D. However, maturation of rapamycin treated OLs was inhibited (**Fig. 11I**). Altogether, these assessments showed that mTORC1 positively regulates differentiation and maturation of OLs.

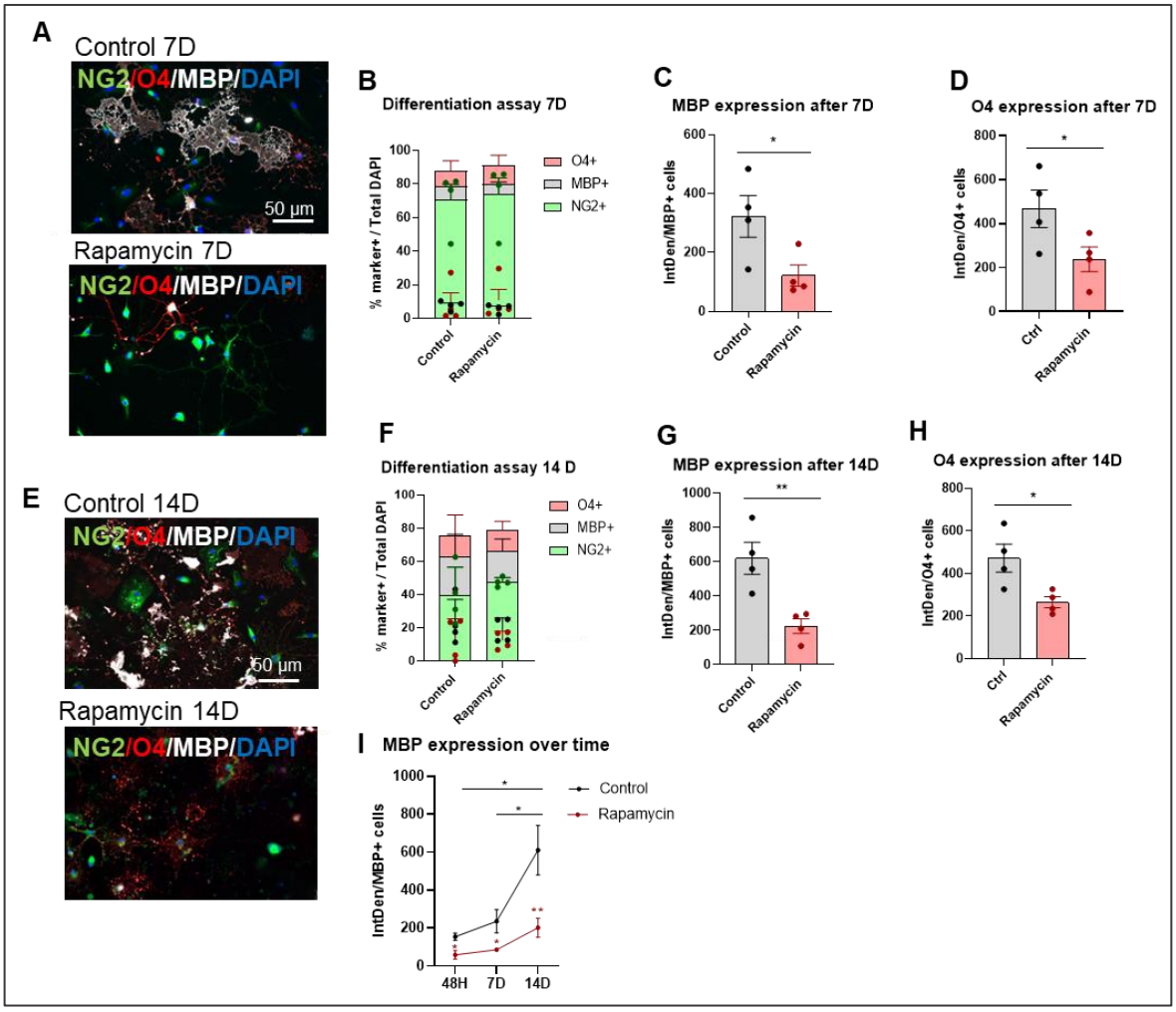


Figure 11. Rapamycin-inhibition of mTORC1 reduces OPC differentiation and MBP expression. We used 3 markers: NG2 for OPCs, O4 for immature OLs, and MBP for mature OLs. Some cells were in transition, and those O4+ cells that also expressed MBP were considered MBP+ cells. Quantification is expressed as % of cells expressing one of the three markers normalized to the total number of cells marked with DAPI. **A-B)** While No significant differences were found at 7D, (C-D) the expression level of O4 and MBP (integrated density) was significantly lower in the rapamycin treated cells. **E and F)** Similar results were found at 14D timepoint, with no significant differences between the percentage of cells. **G-H)** However, there was a significant reduction in O4 and MBP protein expression level. I) MBP expression increased progressively in the control condition over time, and rapamycin treatment significantly inhibited MBP expression as compared to the control. Data represent mean ± SEM. N=3 for 48H, N= 4 for 7D and 14D. For MBP expression: Unpaired Student t-test and multiple t-tests. *P <0.05; **P <0.01. Data represent mean ± SEM. N=4 for 7D and 14D.

3.6 Rapamycin-inhibition of mTORC1 reduces the morphological complexity of oligodendrocytes.

OLs go through distinct phases of cellular development. The differentiation phase has unique characteristics, one of them is the rapid increase in morphological complexity or "branching" of oligodendrocytes[41]. OLs are characterized by their ability to myelinate several axons at the same time due to their high number of branches in one single cell. Thus, we next study the effect of rapamycin on OLs morphology by Sholl analysis on O4+ OLs (**Fig. 12A**). After 7 days of differentiation, rapamycin-treated OLs were significantly less elaborated morphologically than the control condition, and had significantly fewer branches, junctions, and primary paths (**Fig. 12B-F)**. Additionally, the rapamycin-treated cells showed significantly fewer intersections at distances 20-90 μm from the OL soma (**Fig. 12H**). Interestingly, while the overall cellular branching was reduced by rapamycin, the extension of the longest branches was not significantly different from the control (**Fig. 12G**). These results show that mTOR is important for preserving the morphological complexity of OLs, and that inhibiting the mTORC1 complex hindered OL maturation.

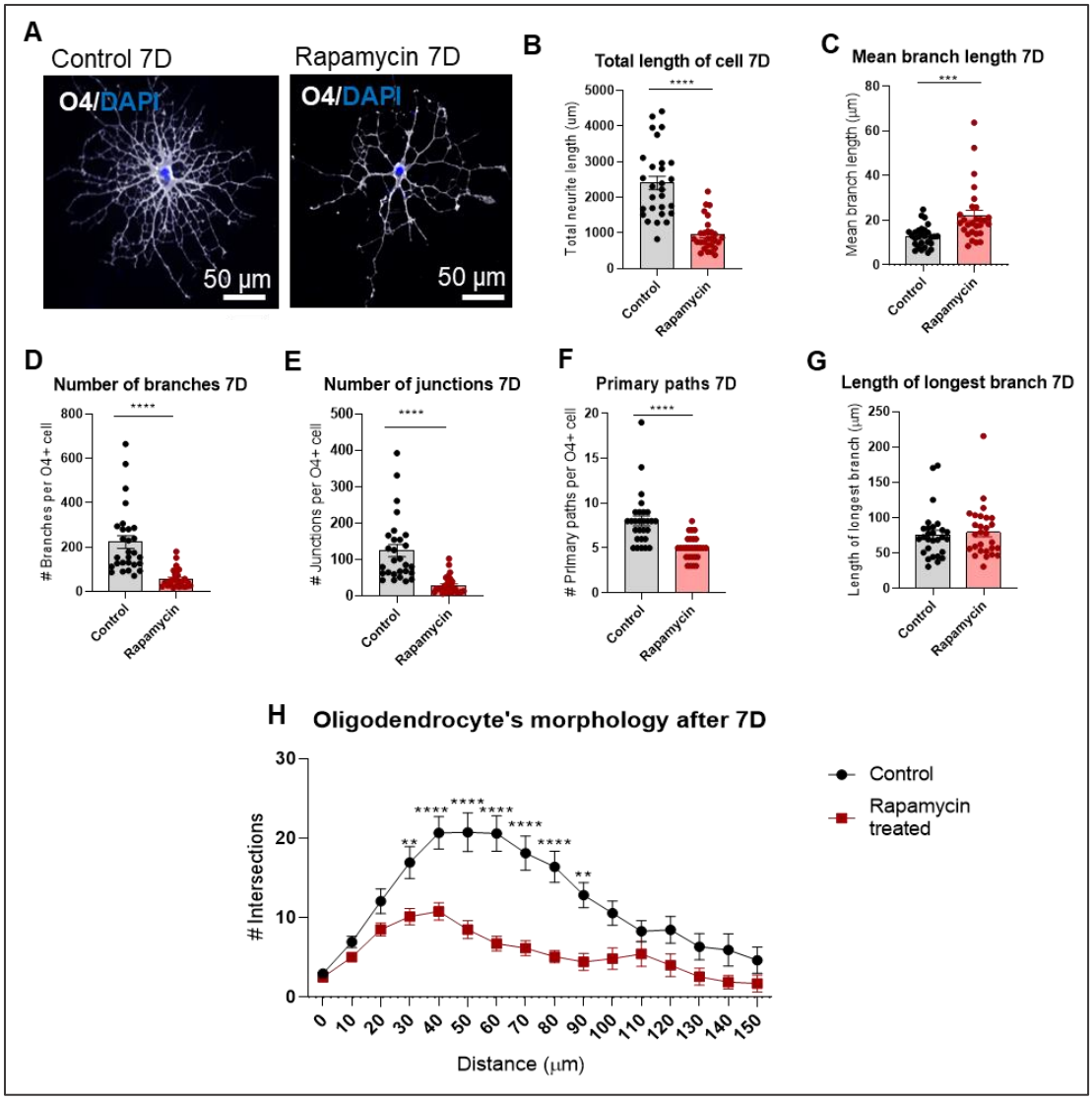


Figure 12. Rapamycin-inhibition of mTORC1 reduces morphological complexity of oligodendrocytes. Sholl analysis was performed to assess arborization and morphology of OLs. **A)** Rapamycin-treated cells showed less complexity as compared to the control after 7 days of treatment. **B-D)** The total cell length and cell ramification of the Rapamycin-treated OLs was significantly lower than the control. **E-F)** Additionally, the number of junctions and primary paths were significantly lower in the Rapamycin condition. **G)** The length of the longest branch showed no significant changes between control and rapamycin**. H)** Sholl analysis showed a significantly less complex morphology in the Rapamycin condition, with significantly less intersections as compared to the Control. N= 3, 10 cells each. Unpaired t-Student test was performed for B-G. ***P ≤ 0.001; ****P ≤ 0.0001. Sholl was analyzed by two-way ANOVA followed by multiple comparisons. ** P ≤ 0.01. Data represent mean ± SEM.

3.7 MS-relevant pro-inflammatory cytokines delays OPC proliferation but it does not affect their survival.

In MS lesions, OPCs are exposed to disease associated pro-inflammatory cytokines. Next, we studied how pro-inflammatory cytokines, TNFα and IFNγ, affect OPC properties in MS lesions using an *in vitro* system. TNFα and IFNγ are two key cytokines that are upregulated in MS lesions by infiltrating leukocytes and reactive glia[110]. To mimic MS pro-inflammatory environment *in vitro*, OPCs were co-treated with TNFα and IFNγ (50 ng/ml and 40 ng/ml respectively) as used routinely in our laboratory for other glial cultures (**Fig. 3A**). Quantitative analysis of LIVE/DEAD assay showed no significant differences between control and TNFα and IFNγ treated OPCs at 48h and 7D time points (**Fig. 14B-E**), which confirmed that these concentrations is not toxic for OPCs. Then, we assessed OPC proliferation under cytokine treatment by EdU incorporation *in vitro*. We found a significant decrease in proliferation of cytokine-treated OPCs after 48h (**Fig. 13F-G)**, while there was no significant differences in the percentage of EdU+ OPCs under TNFα and IFNγ after 7D treatment (**Fig. 14H-I)**. This suggests that OPC proliferation of is transiently suppressed *by* these cytokines *in vitro* and their proliferation recovers after long-term treatment.

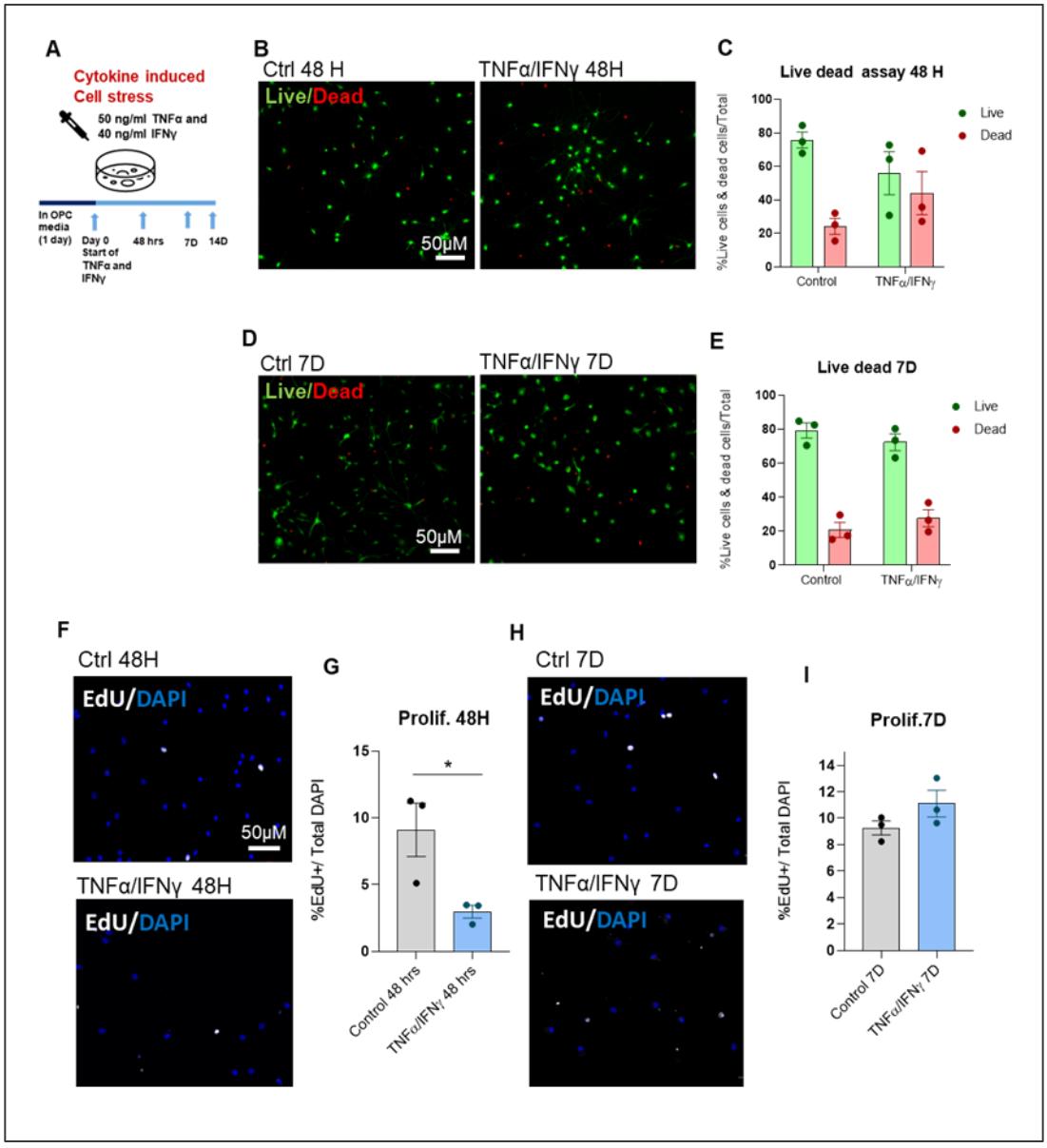


Figure 13. TNFα/IFNγ treatment is not toxic for OPC but delays their proliferation. A) TNFα and IFNγ were added to OPC cultures in concentrations of 50 ng/ml and 40ng/ml respectively. OPC proliferation and survival assessments were done under control or cytokine treatment for 48h and 7D.. **B-E)** LIVE/DEAD assay shows that TNFα and IFNγ, do not affect OPC survival at 48h or 7D. **F-G)** However, proliferation assessment using EdU showed a significant reduction in the number of EdU+/ DAPI+ OPCs after 48h of cytokine treatment as compared to its control. **H and I)** Proliferation assessment after 7D showed no significant difference indicating that cytokines acutely reduce proliferation, but over time OPC proliferation recovers to the level of control. *P < 0.5, Unpaired t-Student test. Data represents mean ± SEM, N=3 independent cultures.

3.8 TNFα and IFNγ treatment reduces OPC maturation and MBP expression.

Next, we assessed OPC differentiation under TNFα and IFNγ cytokines for 7D and 14D *in vitro*. Media and cytokines were refreshed every two days (**Fig. 14)**. At 7D and 14D the percentage of NG2+, O4+ and MBP+ subpopulations of OLs remained unchanged between the control and cytokine-treated conditions (**Fig. 15A-B & E-F)**. However, we did find a significant increase in the percentage of NG2+ OPCs after 14D under differentiation media and cytokines suggesting inhibition of OL maturation under prolonged exposure to TNFα and IFNγ cytokines (**Fig. 14F**). We also studied the expression level of O4 and MBP in each OL by integrated density analysis to assess the degree of cellular differentiation and maturity more precisely. While we did not find any significant differences in the expression of O4 or MBP between the two conditions at 7D (**Fig. 14C-D**), there was a significant reduction in the expression of O4 and MBP after 14D of differentiation in the cytokine-treated cells compared to the control media (**Fig. 14G-H**). Altogether we found that although TNFα and IFNγ co-treatment do not affect the percentage of immature and mature oligodendrocytes, they significantly reduce the expression levels of maturation and myelinating proteins, suggesting that pro-inflammatory cytokines have a negative role in OPC differentiation and maturation in MS inflammatory lesions.

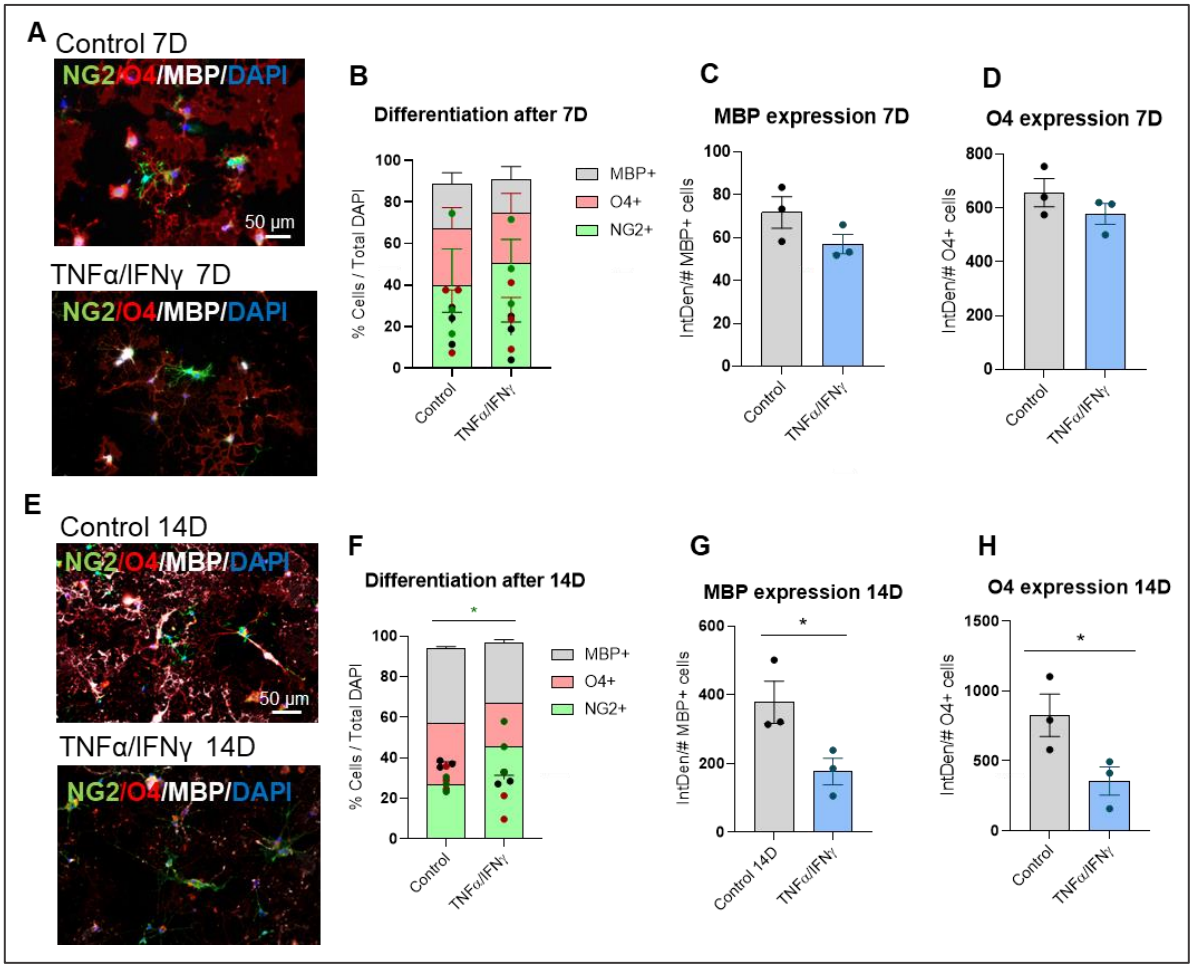


Figure 14. TNFα/IFNγ co-treatment hinders OL differentiation and maturation. Cultures were immunolabeled with NG2 (for OPCs), O4 (for immature OLs) and MBP (for mature OLs). Cells expressing each marker and DAPI were counted and normalized to the total number of cells. **A-D)** At 7D, there was no significant differences found in the number of cells expressing O4 and MBP markers and the cellular expression of these OL mature markers between cytokine-treated and control conditions. **E-F**) At 14D post-differentiation, there was also no significant differences in the number of cells expressing O4 and MBP markers, although a significant increase was found in the percentage of NG2+ cells in the cytokine-treated group after 14D. **G-H)** However, at 14D, a progressive increase in the cellular expression level of MBP and O4 expression was observed in OLs under the control condition, which was significantly decreased in the cytokine-treated cells. Data represent mean ± SEM. N=3 for. Multiple t-tests and Unpaired t-Student test. *P <0.05;. Data represent mean ± SEM.

3.9 TNFα and IFNγ treatment significantly decreases the morphological complexity of OLs.

We also studied the effect of TNFα and IFNγ co-treatment on the morphological complexity of OLs as described in earlier sections. After 7D under differentiation media in either control or cytokine conditions, Sholl analysis on O4+ cells showed that cytokine-treated OLs had a less complex morphology than the control (**Fig. 15A**) with a significant reduction in total cell length (**Fig. 15B**), and fewer branches and junctions (**Fig. 16D-E**). Mean branch length, length of longest branch and primary paths were not significantly different between both groups (**Fig. 15C, F & G)**. Additionally, the cytokine-treated group showed significantly fewer intersections at distances 50-70 μm from the OL soma (**Fig. 16H**). These results show that TNFα and IFNγ directly affect OLs and impede proper OL differentiation and their morphological complexity which is critical for myelination.

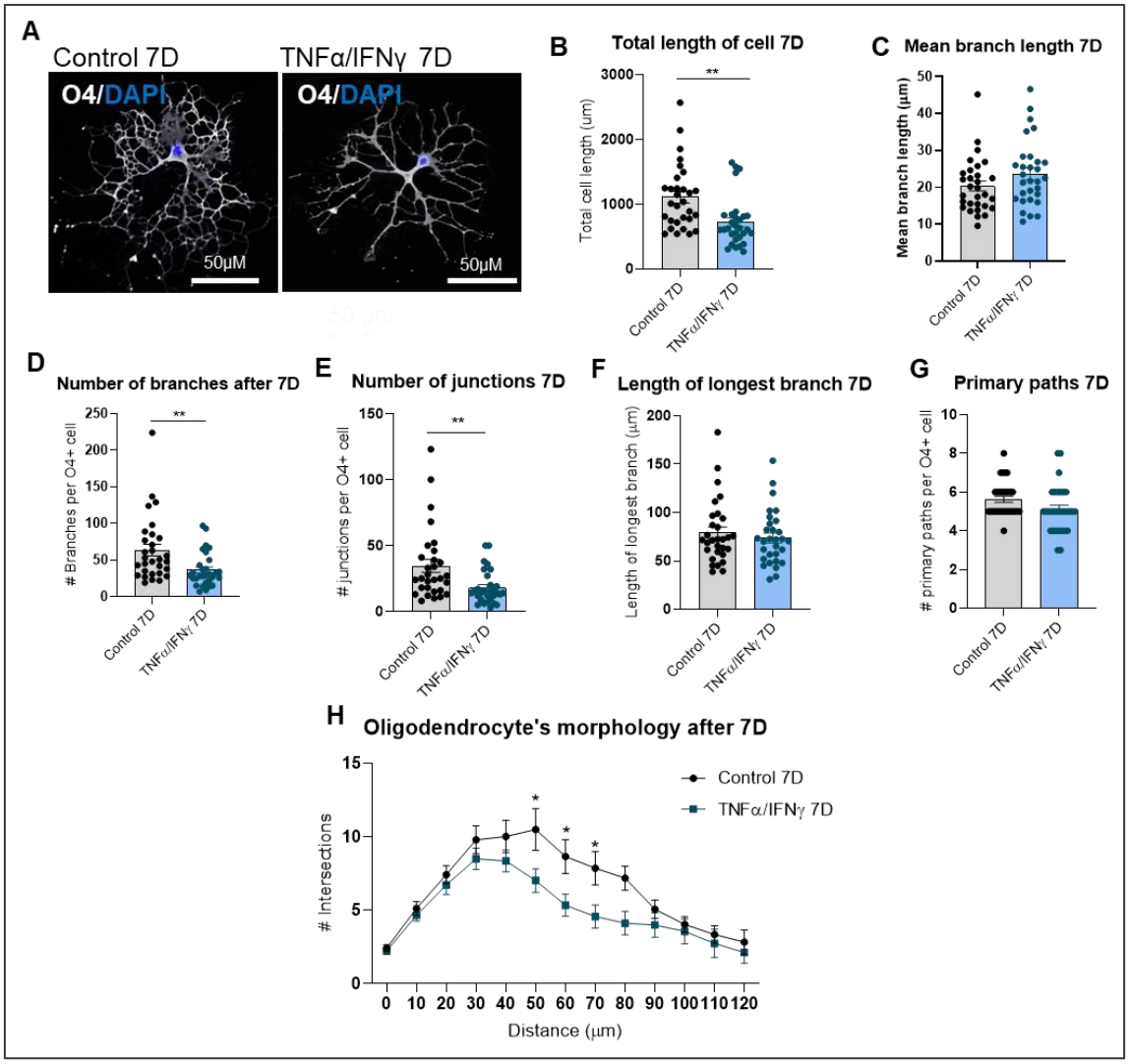


Figure 15. TNFα and IFNγ co-treatment significantly reduces the morphological complexity of OLs. Changes in the morphology of OLs after cytokine treatment were assessed by Sholl analysis. **A)** Cytokine-treated OLs showed less complexity as compared to the control after 7D of treatment. **B-C)** The total length of cytokine-treated cells was significantly lower in cytokine condition than the control, while the mean branch length showed no significant changes between the two conditions. **D-E)** The number of branches and junctions were significantly lower in the cytokine-treated cells. **F- G)** No significant changes were found in the primary paths nor the length of the longest branch**. H)** Sholl analysis showed a significantly less number of intersections in OLs branches under cytokine condition as compared to the control. N= 3, 10 cells each. Unpaired t-Student test was performed for B-G. **P ≤ 0.01. Sholl was analyzed by two-way ANOVA. **P ≤ 0.05. Data represent mean ± SEM.

3.10 Lysosomal activity significantly increases after both Rapamycin and cytokine treatments.

We next aimed to assess lysosomal activity of OPCs under rapamycin and TNFα/IFNγ treatment. LysoTracker Deep Red Dye is a fluorophore that is protonated in the presence of the acidic pH of active lysosomes[111]. We assessed lysosomal activity after the proliferation and differentiation of OPCs for 48h and 7 days, respectively. Cells were immunostained with LAMP1, a general lysosomal marker (**Fig. 16)**. To assess the ratio of active lysosomes, we performed a co-localization assessment by using the plugin JaCOP tool in the Fiji software. Co-localization was measured by Mander's coefficient. Active lysosomes were considered as the LAMP1-positive lysosomes that co-localize with LysoTracker signal in both timepoints (**Fig.16C&F**). Specificity was assessed with the M1 coefficient, which represents the LysoTracker vesicles that co-localize with LAMP1. It is expected that all LysoTracker vesicles are LAMP1+ (**Fig.16C&F**). These results showed a basal lysosomal activity in OPCs under the control proliferation condition. Under rapamycin- and TNFα/IFNγ treatments, at 48h OPCs showed a higher degree of overlapping between LAMP1 and lysotracker-positive vesicles as compared to the control (**Fig. 16B**), but no significant differences were observed between the two treatments. After 7 days under differentiation media, we found a basal lysosomal activity in OLs under the control condition; however, we did not find any significant changes between the control and both rapamycin- and TNFα/IFNγ treatments (**Fig. 16E**). Taken together, these data indicate that elevated lysosomal activity in OPCs occurs downstream of mTOR inhibition and cytokine activation under proliferation conditions but there are no major changed under differentiation.

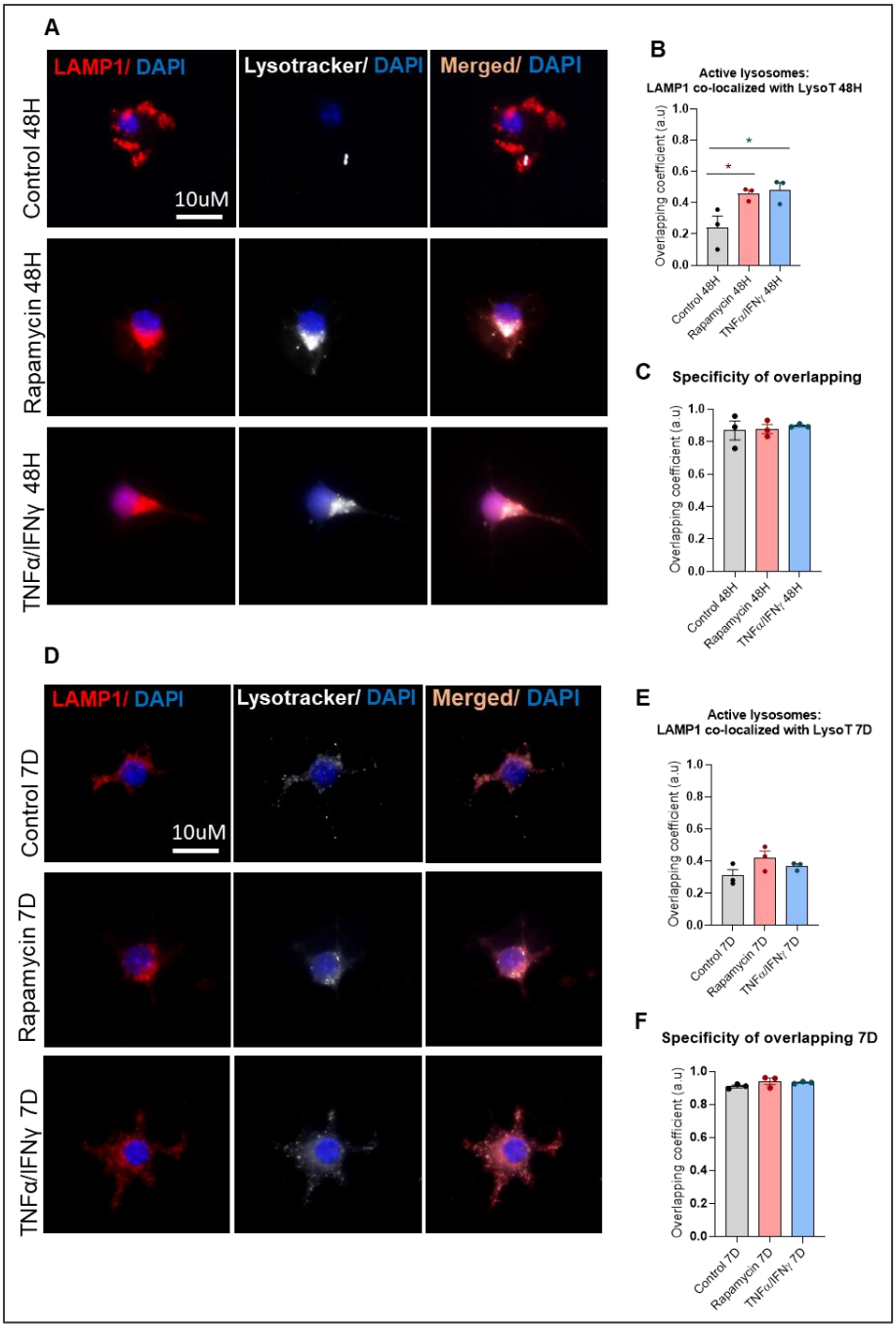


Figure 16. Lysosomal activity significantly increases after rapamycin and cytokine treatment. A&D) Lysosomal activity was assessed by using LAMP1 (a lysosomal marker) and Lysotracker (a marker for active lysosomes) after 48H and 7D. Overlapping coefficient of active lysosomes was determined by assessing co-localization between LAMP1 and Lysotracker under control, rapamycin and TNFα/IFNγ conditions. Fifty (50) cells were quantified per each condition per biological replicate. **B)** Significant differences were found between rapamycin and cytokine-treated conditions as compared to the control, while there was no difference between rapamycin and cytokine-treated cells. **C)** Mander's coefficient was used to assess the specificity of the quantification between LAMP1 and lysotracker.

The coefficient shows the estimation of the fraction of one particle that colocalizes with another, in this case, it measures the Lysotracker signal overlapping with LAMP1. Graph C represents all the LysoTracker signal that overlaps with LAMP1. It is expected that the coefficient would be close to 1 across all conditions, as all Lysotracker should overlap with LAMP1, otherwise, the Lysotracker signal would be considered as non-specific particles. There were no significant differences across the conditions, which indicates the specificity of the quantification. This data shows that OPCs under rapamycin and cytokine stress have an increased lysosomal activity. **E)** Lysosomal activity in OLs did not show significant differences across conditions under differentiation media. **F)** Graph showing specificity of overlapping, all conditions are close to 1. N= 3 cultures, 50 cells each. Unpaired t-Student test was performed. *$P \leq 0.05$. Data represent mean ± SEM.

3.11 Effect of TNFα/IFNγ treatment on mTOR and eIF2α phosphorylation in OPCs *in vitro*.

Given our immunohistochemical expression data in CPZ mice showed increased expression of pmTOR and peIF2α in OPCs, we next asked whether exposure to cytokines can activate the ISR and mTOR pathway in OPC. **(Fig. 17A-F)** Immunostaining of OPCs for pmTOR under TNFα/IFNγ treatment showed no difference in the number of cells that expressed pS6 at 48h and 7D in culture compared to the control condition, although at 48h cellular expression of pmTOR was higher in cytokine-treated OPCs suggesting increased expression levels of pmTOR (**Fig. 17D**). I also attempted to perform Western blotting to study the expression of pS6. The WB did not show any differences in the expression of pS6 between the control and the cytokine-treated group (**Fig. 17G-J**), However, the results were inconsistent and inconclusive mostly due to high variation in my technique. Overall, these initial immunohistochemical data suggest that mTOR activity is initiated in OPCs after exposure to pro-inflammatory cytokines, although it is not significantly different from the control conditions. However, it should be taken into consideration that Western blotting is a more suitable method to assess protein expression.

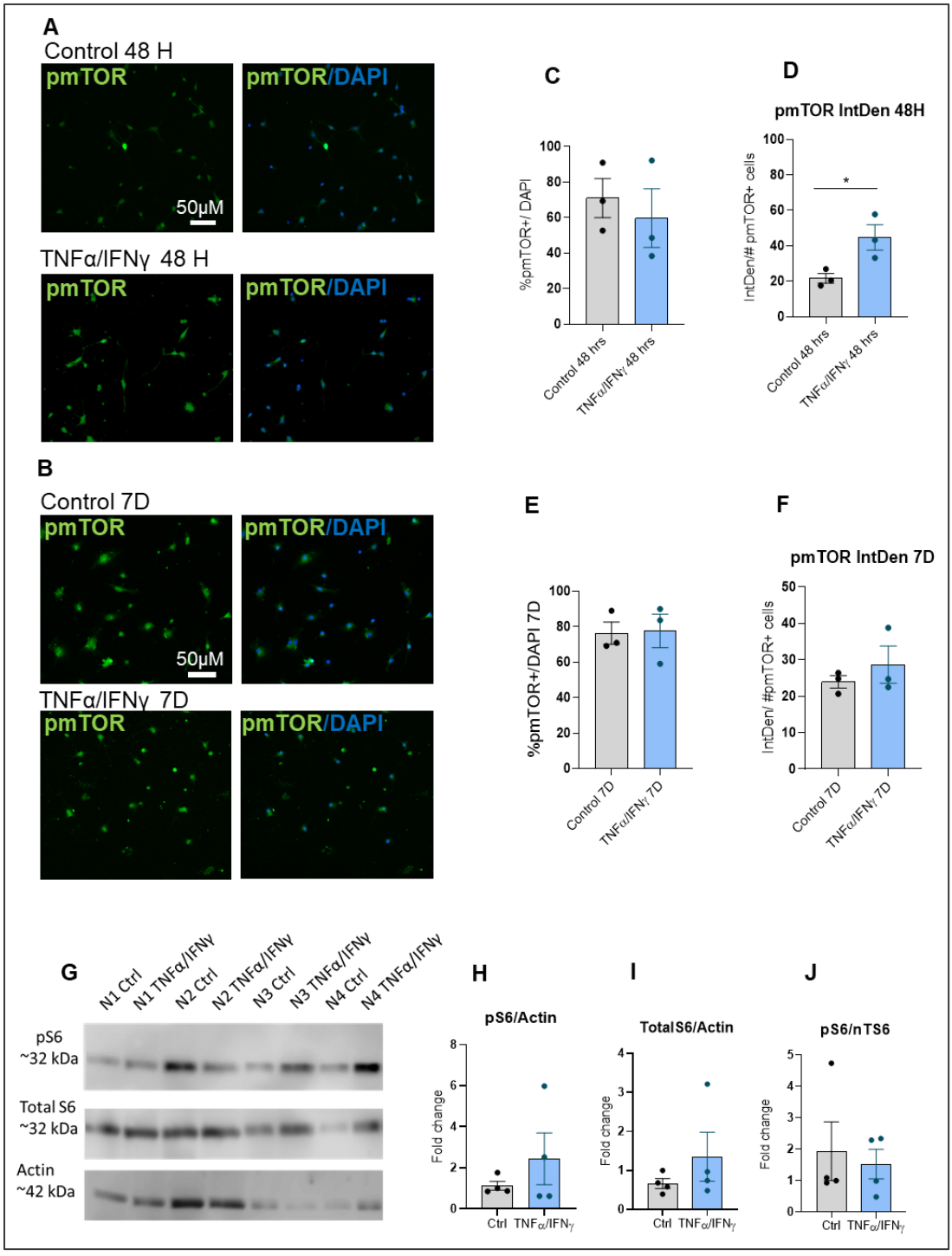


Figure 17. pmTOR expression significantly increases in OPCs under acute cytokine treatment, but it is not affected after long-term exposure. OPCs were cultured under control and TNFα/IFNγ conditions for 48h and 7D. A) Expression of pmTOR was assessed by ICC by counting the number of p-mTOR+/DAPI+ cells and quantifying its cellular expression by density measurement. **A-D)** At 48h, there was no significant difference between the percentage of pmTOR+/DAPI+ cells after 48 hours or 7D.

However, after 48h, the integrated density was significantly higher in the cytokine-treated group. **B-F)** No significant differences were found after 7 days. **G-J)** Expression of pS6 and total S6 was assessed by Western blot. pS6 and total S6 were normalized to the actin and ration of pS6/total S6 was also quantified. These assessments showed no significant differences between the control and cytokine conditions. However, due to high technical variations (example blot is provided), Western blot assessment was inconclusive. *P <0.05; T-student test. Data represent mean ± SEM. N=4 for WB, N=3 for ICC.

Next, we assessed expression of p-eIF2α in OPCs under TNFα/IFNγ condition **(Fig. 18).** In the cytokine-treated condition, there were significantly higher number of p-eIF2α+/DAPI+ cells compared to the control condition, while no significant differences were found in the cellular expression of p-eIF2α (integrated density analysis) between the two conditions (Fig. 18 B-C).

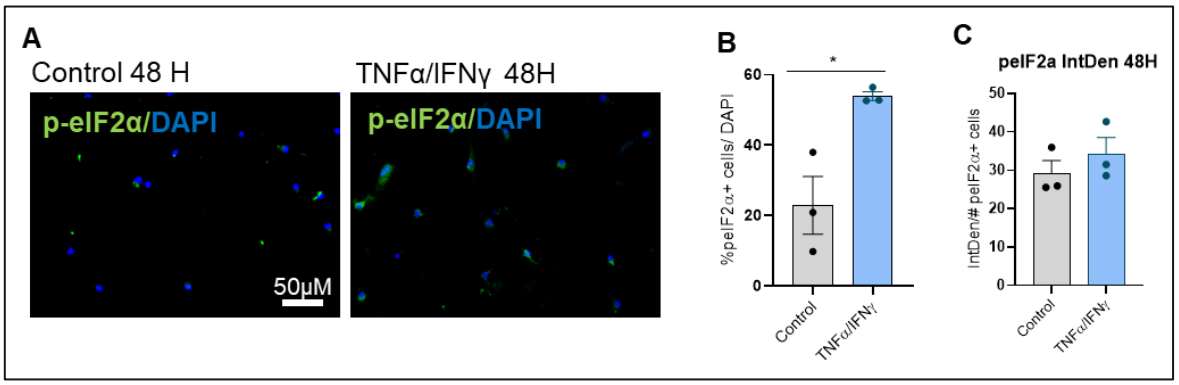


Figure 18. TNFα/IFNγ treatment promotes the number of p-eIF2α expressing OPCs while it does not affect the degree of cellular expression. A) ICC was performed on OPCs under control or cytokine treatment after 48h *in vitro*. **B)** In the cytokine-treated condition, there were significantly higher number of p-eIF2α+/DAPI+ cells compared to the control condition. **C)** No significant differences were found in the cellular expression of p-eIF2α (integrated density analysis) between TNFα/IFNγ treated and control conditions. Unpaired t-Student test; Data represents mean ± SEM N=3

3.12 Trials for inducing the UPR *in vitro.*

One of our initial aims was to induce UPR *in vitro* and then assess the changes in the proliferation and maturation of OPCs. For this purpose, it was initially decided to use Tunicamycin, a common UPR inducer. Tunicamycin acts by blocking the N-linked glycosylation in cells, arresting the G1 phase of the cell cycle and causing ER stress[112]. I performed a gradient study to identify the most efficient concentration for our study. Based on the literature, the tunicamycin concentrations included in the pilot study were 0.1, 0.25, 0.5, 0.75, 1, 1.5 and 2 μg/ml. OPCs were treated with control and various tunicamycin concentration for 16 hours at 37°C. First, I determined cell survival under tunicamycin gradient using LIVE/DEAD assay and found a significant cell death at 1.5 and 2 μg/ml concentration(**Fig. 19A-B**). Next, I determined the efficacy of tunicamycin in induction of UPR by immunostaining for the downstream marker of UPR, CHOP (**Fig. 20**). The results showed that even the lowest concentration (0.1 μg/ml) could induce CHOP expression compared to OPCs under the control condition with no detectable CHOP signal (**Fig. 20A-B**). We selected 0.25 μg/ml concentration for further investigation, as it showed slightly more effect than 0.1 μg/ml. Next, OPCs were treated with 0.25 μg/ml of Tunicamycin for 48h to assess OPC proliferation. However, it was found that Tunicamycin was toxic for OPCs after this time point, as it resulted in 90% cell death evidenced by LIVE/DEAD assay (**Fig. 21A-B**) and completely arrested proliferation in the surviving OPCs (**Fig. 21C-D**). With this observation, we did not pursue tunicamycin experiments due to associated toxicity for our target long term experiments for proliferation (48h) and differentiation (7D) experiments.

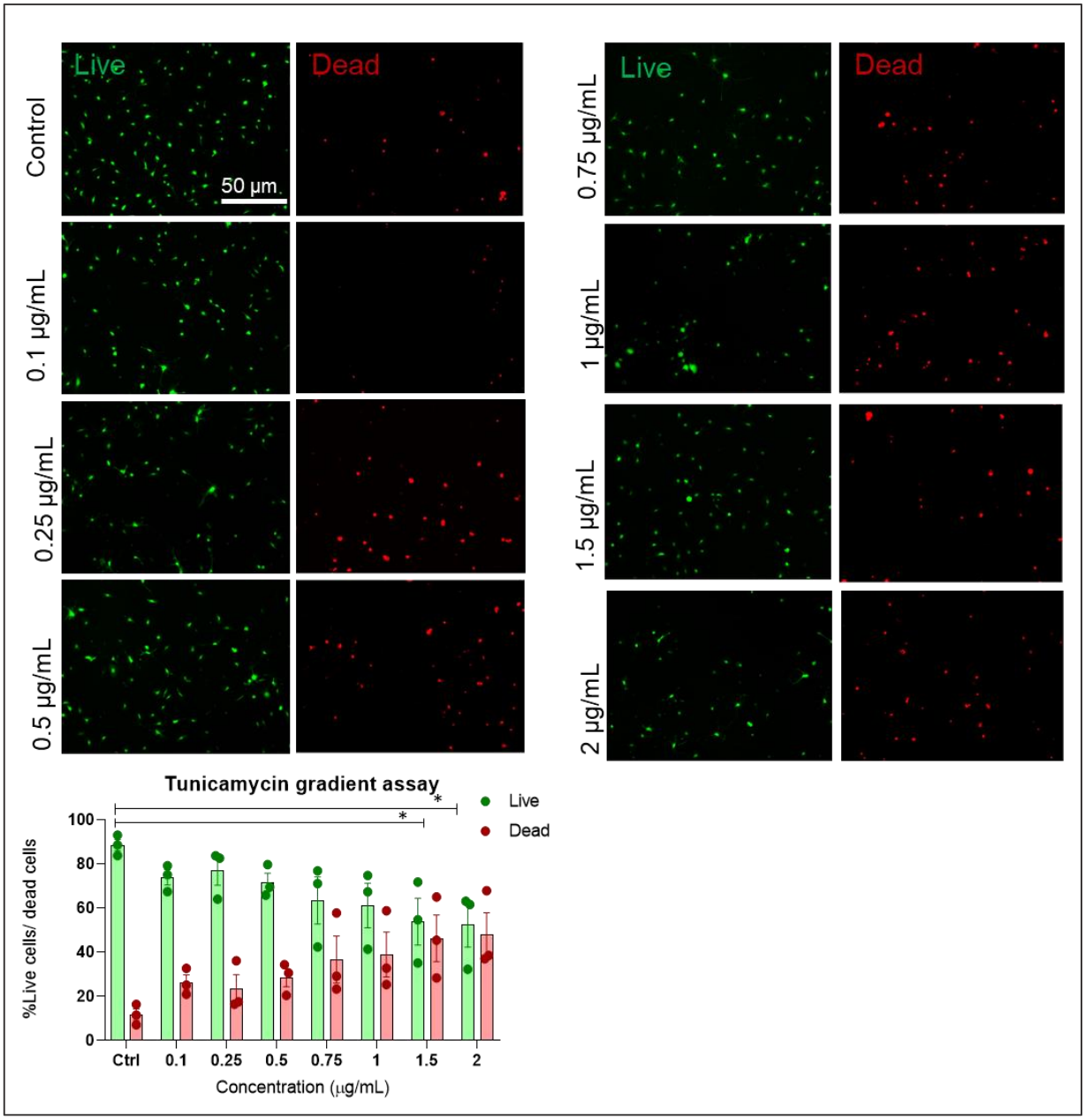


Figure 19. Gradient assay to identify a non-toxic concentration for tunicamycin treatment of OPCs. A) A gradient assay was performed using different concentrations of Tunicamycin (0.1, 0.25, 0.5, 0.75, 1, 1.5 and 2 µg/mL) in OPC culture for 16 hours. **B)** We found a significant increase in the number of dead cells at 1.5 and 2 µg/mL tunicamycin compared to the control condition. No significant differences were found among tunicamycin treated conditions. Multiple comparisons. *P <0.05; Data represents mean ± SEM. N=3 culture

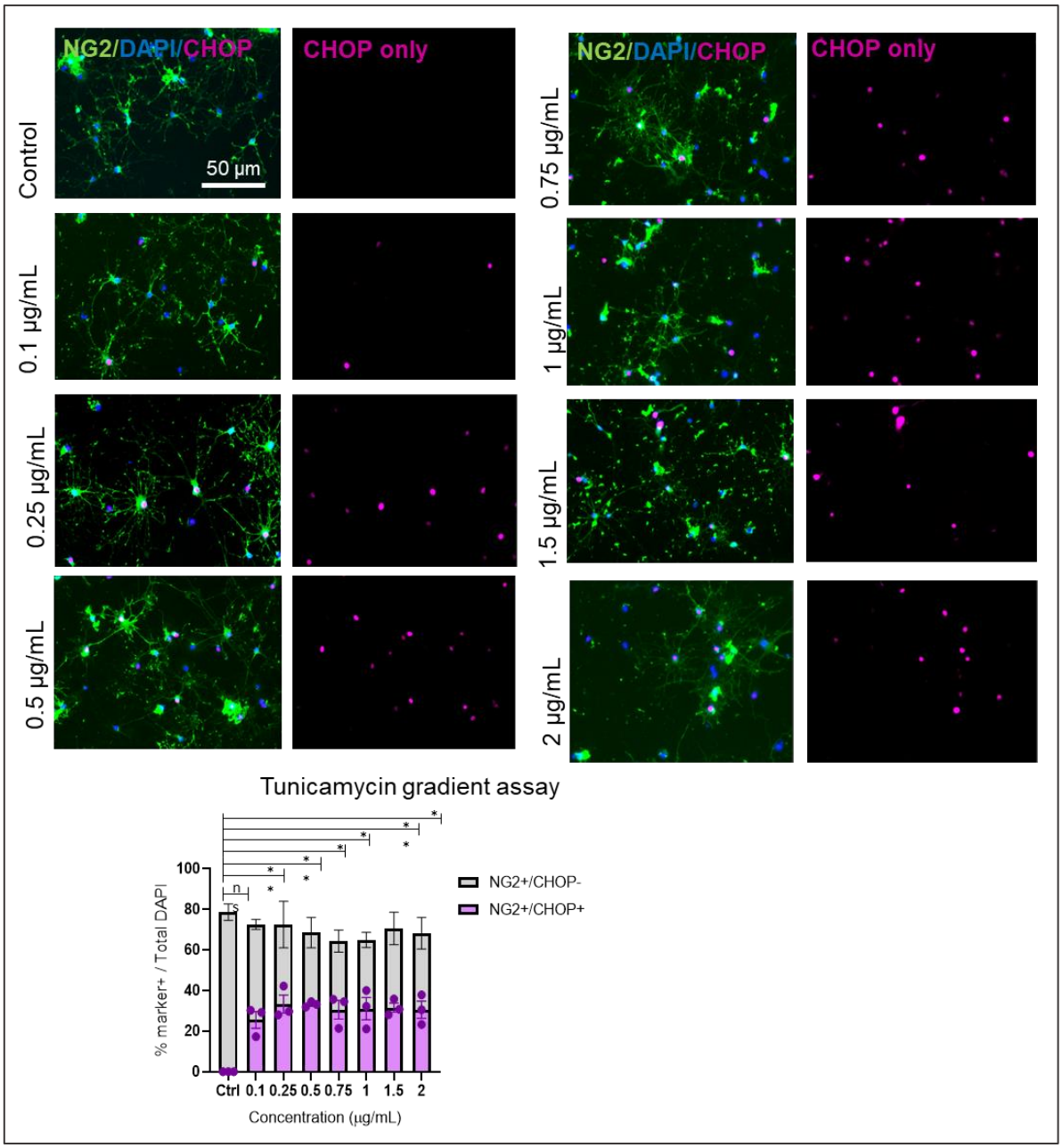


Figure 20. Gradient study to determine an efficient concentration of Tunicamycin in inducing UPR in OPC culture. A) Activity assessment was performed in tunicamycin-treated OPCs by studying the expression of CHOP after 16 hours. **B)** As little as 0.25 µg/ mL of Tunicamycin was enough to significantly induce CHOP expression in OPCs compared to the negligible CHOP expression under baseline control condition. Hence, 0.25 µg/mL was identified as an effective concentration without toxicity for further experiments. One-way ANOVA. *P <0.05; ** P ≤ 0.01. Data represent mean ± SEM. N=3 culture.

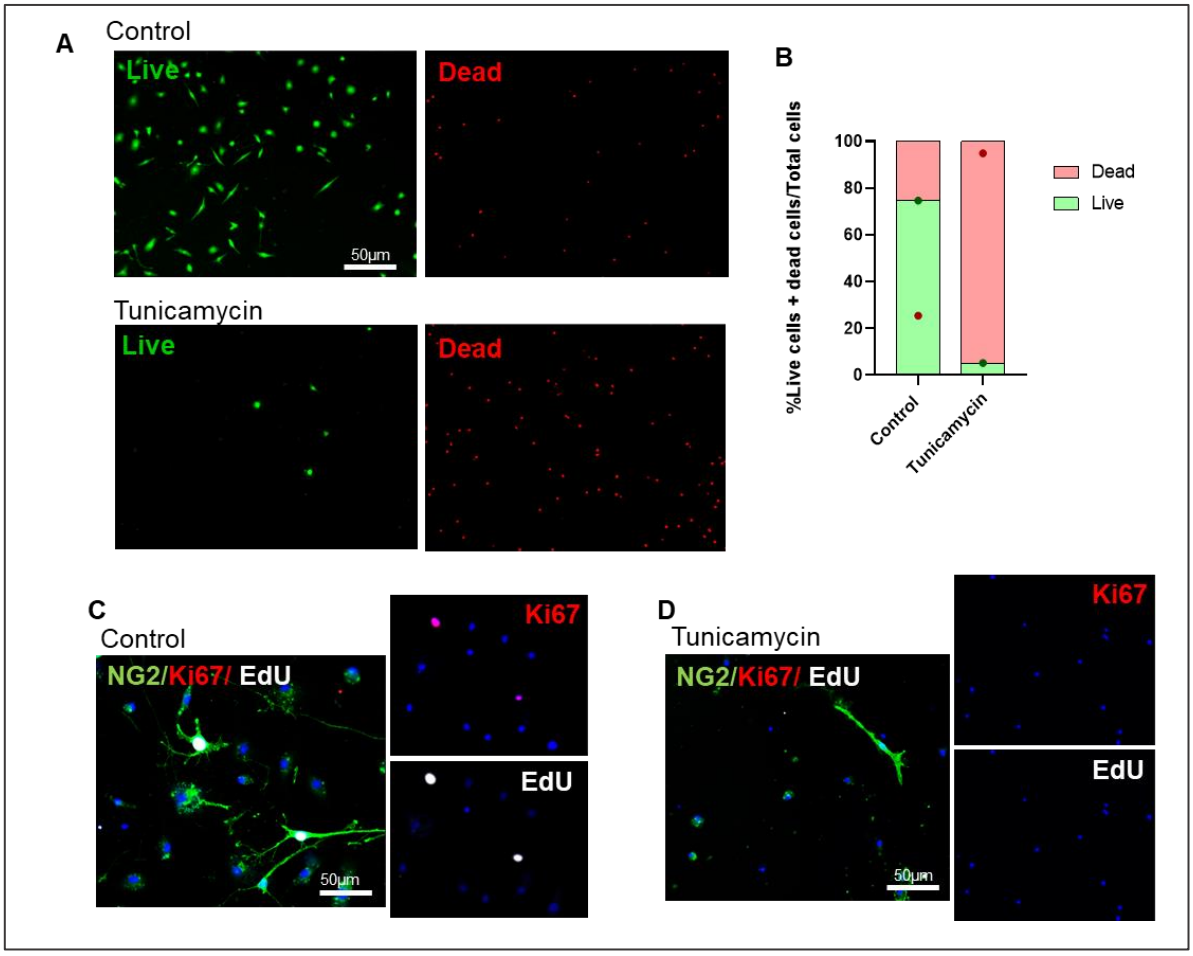


Figure 21. Tunicamycin is toxic for OPCs after 48 hours of treatment and arrest proliferation of surviving cells. **A-B)** Our pilot with tunicamycin treatment of OPCs at 48 hours timepoint (our target experimental timepoint for OPC proliferation) showed ~90% cell loss after tunicamycin treatment (0.25 µg/mL). **C-D)** OPC proliferation assay using EdU and Ki67 showed arrest in proliferation after tunicamycin, which reflects cell loss as we also confirmed smaller/dense nuclei with DAPI staining.

4. Discussion

In this study, we show that the mTOR and eIF2α are phosphorylated in OPCs and OLs in cuprizone-induced demyelinating lesions in mice, indicating the activation of the mTOR and ISR pathways. This activity was sustained during acute and chronic remyelination. This provided initial evidence that both pathways are involved in cellular response by oligodendrocyte lineage in demyelinating lesions. These processes could include OPC proliferation, differentiation, and /or OL maturation and myelination and that the activity of these pathways could be either beneficial or detrimental for remyelination. Interestingly, in another mouse model of MS, experimental autoimmune encephalomyelitis (EAE), we also observe induction of mTOR activity by elevated levels of pS6 at the peak of the disease. To begin understanding the impact of mTOR on OPC properties, we designed *in vitro* studies to disrupt mTORC1 using rapamycin in primary cortical OPC cultures. These studies indicated a supportive role for mTOR in proliferation, differentiation, MBP expression, and morphological complexity of OLs, as rapamycin reduced all these properties. We also found that rapamycin increased OL lysosomal activity. Interestingly, when OPCs were exposed to TNFα and IFNγ, two key pro-inflammatory cytokines involved in MS pathogenesis, they showed similar response as observed under rapamycin treatment, suggesting a potential link between MS condition. However, a relationship between cytokine-induced changes in OPCs and mTOR and/or eIF2α activity could not be established in this study, due to the lack of consistent technicality, which was one of the main limitations of this thesis to allow further characterization.

Taken together, this study has provided initial data that suggests the involvement of mTOR and ISR in oligodendrocyte behavior in demyelinating lesions, which need further investigation to unravel the nature and molecular basis of this involvement using molecular biology techniques. I have discussed major findings of this thesis as well as limitations.

4.1 OPC proliferation, differentiation, and lysosomal activity change under pharmacological inhibition of mTORC1 and under cytokine treatment.

The demyelinated CNS relies on OPCs for myelin repair, hence proper proliferation and migration of OPCs to the site of injury, and their subsequent differentiation and maturation into myelinating OLs, are essential for remyelination of MS lesions. These cellular processes are not entirely independent from each other as it is shown that proliferation is a requirement for OPC differentiation during remyelination[113]. The role of mTOR signaling on OPCs started to gain relevance nearly 15 years ago after identifying that mTOR activity was essential for OPC differentiation *in vitro*[59]. Given our initial observation that showed activation of mTOR in OPCs and OLs in cuprizone lesions, investigating these processes *in vitro* was key to obtaining insights into the role of mTOR on OPC properties in particular OL maturation and lysosomal activity. Here, we confirmed that inhibiting mTOR activity, most specifically, the mTORC1 complex with rapamycin resulted in decreased proliferation and differentiation, followed by a decrease in O4 and MBP expression and morphological complexity, all of which are essential for the proper development of myelinating OLs.

Our experiments were performed by using OPCs from primary cultures of neonatal mice, which represent the developmental population of OPCs. Since culturing and maintenance of OPCs from adult CNS is difficult, neonatal OPCs are used commonly. Our *in vitro* results showed a response similar to the ones described in the literature[58,102,114]. However, mice studies *in vivo* have demonstrated that mTOR signaling is, in fact, dispensable for OPC differentiation during developmental myelination in the brain, but it may be important for spinal cord myelination[117,115]. A different study investigated the role of mTOR in remyelination by conditional knockout of mTOR from NG2+ cells (i.e. OPCs), and their results also showed that mTOR deletion in both CPZ and LPC models had no impact on the proliferation or differentiation of adult OPCs during remyelination in the brain and spinal cord. These findings suggest that adult OPCs may not require mTOR to differentiate, as opposed to developmental OPCs, as the two populations seem to be intrinsically distinct from each other[75]. As such, future *in vivo* studies are required to more closely study the impact of mTOR on adult OPC population.

Regarding the specificity of rapamycin in inhibiting mTORC1, while rapamycin has been used to selectively inhibit only mTORC1, early studies showed that it can also inhibit mTORC2 when used in long-term treatments[116]. Additionally, mTORC2 is well-known for regulating the cytoskeleton of cells[78]. Hence, with rapamycin application on primary OPCs for 7D in our assessments, it is plausible that some effects of rapamycin on morphological complexity and maturation of OLs may be related to mTORC1 and/or mTORC2 complexes.

Proinflammatory cytokines like TNFα and IFNγ are released by immune cells and astrocytes in MS lesions[12], and are known to regulate both demyelination and remyelination process. Hence, we were interested in understanding how these cytokines affect OPCs and whether their effects are associated with changes in mTOR and eIF2α activity in these cells. Interestingly, we found that there was a significant decrease in OPC proliferation after 48 hours of treatment, however, after 7 days there were no differences between the cytokine-treated group and the control. Our findings are in agreement with some previous reports. For example, *in vitro* studies have shown that TNFα decreases OPC proliferation *in vitro*[117]. However, there are also other contrasting reports on the role of TNFα in the literature. According to some early studies, TNFα promotes OPC proliferation through TNFR2 signaling *in vivo*[118] and *in vitro*[119]. Although TNFα is a well-known proapoptotic factor, a recent study demonstrated that this cytokine promoted oligodendrogenesis in an organotypic cerebellar slice culture[120]. Altogether, current evidence suggests a dual role for TNFα in OL regulation, which could be related to the different effects of TNF receptors, with TNFR1 being inhibitory and TNFR2 being pro-regenerative[121]. IFNγ is another key cytokine that drives MS pathology. This cytokine has been shown to be generally inhibitory for OPC proliferation[122]. Similar to TNFα, it is shown that IFNγ directly induces apoptosis in OL lineage cells[123,124]. In our study, OPC survival was not significantly affected after 48 hours or 7 days of TNFα/ IFNγ co-treatment; however, it decreased OPC proliferation after 48h followed by a recovery after 7D. This could suggest that the combination of the TNFα/ IFNγ may result in a delayed proliferation, which is then recovered due to increased proliferation through TNFR2 activation or other pathways, and/or there is apoptotic cell death but was not identified by the live/dead assessment that we performed.

Cytokines also decreased differentiation with significantly less MBP expression and reduced morphological complexity. Other studies show that TNFα impairs OPC differentiation *in vitro*[125,121,126] and changes the morphology of OLs[120]. Furthermore, TNFα significantly reduces transcript expression of both MBP and PLP[121]. IFNγ has also been shown to significantly reduce the differentiation of primary rat OPCs into OLs and attenuate MBP expression[127] and decrease the morphological complexity of OLs[120]. Furthermore, a more recent study reported that IFNγ significantly decreased the differentiation of human OPCs into mature OLs[116]. Altogether, our findings are in agreement with the previous literature, although these reports mainly focused on one of the cytokines. In this project, we used TNFα and IFNγ together to simulate an MS relevant microenvironment, where several cytokines participate in the inflammatory and demyelinating processes. However, it is important to note that the effects observed in our experiments and the reports from the literature are dependent on the composition of the cellular media and the concentrations used for each cytokine. Thus, it would be more relevant to expose OPCs to conditioned media from activated microglia, to more closely mimic an MS-like environment *in vitro*. This will be pursued in future experiments in our laboratory.

The lysosomal activity of OPCs under either rapamycin or cytokine treatment was assessed by measuring the overlapping coefficient LysoTracker with LAMP1. We found that lysosomal activity was increased under proliferation conditions in both rapamycin and cytokine conditions after 48h. The control had a basal lysosomal activity, which indicates that lysosomes have a role during OPC proliferation.

It is expected to observe an increase in lysosomal activity under rapamycin treatment, as it is well known to induce autophagy and consequently promotes lysosomal activity. We did not find any significant differences in lysosomal activity across conditions after 7 days under differentiation media. It is noteworthy that increase in lysosomal activity may or may not be related to autophagy as a marker for autophagosomes, such as LC3, is needed to discriminate between lysosomes and autolysosomes and assess a relationship with autophagy[128]. A better approach to measure autophagy is by detecting the LC3 conversion (LC3-I to LC3-II), along with other autophagic markers like Atg8, Atg5 and p62 via western blot analysis[129]. Western blot would have been the best approach, however, due to inconsistent technicality this could not be achieved. Increased lysosomal activity in the cytokine-treated condition could be related to autophagy or to lysosomal permeabilization. OPCs exposed to TNFα treatment showed increased levels of LC3, indicating an increase in autophagy[123]. Additionally, TNFα has been found to induce autophagy in necrotizing enterocolitis (NEC) model[130], in melanoma cells[131]. Early reports suggest that TNFα induces lysosomal permeabilization in a cathepsin B-dependent manner in hepatocytes[132]. TNFα induces lysosomal permeability by producing mitochondrial reactive oxygen species (ROS) in different cell lines[133]. IFNγ also induces lysosomal stress and subsequent permeabilization in conventional dendritic cells[134]. Altogether, our results showed an increased lysosomal activity, however, further investigation is required to determine whether this is related to autophagy or lysosomal permeabilization.

4.2 Identifying the involvement of the ISR and mTOR pathway in cytokine-induced effects on OLs.

I attempted to identify whether the effects of TNFα/ IFNγ on OPC proliferation and differentiation were related to the changes in the activity of mTOR and eIF2α. I performed several Western blotting for pS6 and peIF2α on OPCs and OLs after cytokine treatments. However, there was a high degree of inconsistency and technical issues in my Western blotting that made the results inconclusive. Based on preliminary Western blot data, there was no significant change in pS6 expression in cytokine-treated OPCs, indicating that mTORC1 activity was not changed, while immunostaining suggested an increase in pmTOR. Overall, further investigations with complementary molecular biology approaches are required to elucidate the effects of cytokines on mTOR pathway in OPCs. Currently, this information is scarce in the literature.

The trials for identifying the involvement of the ISR using peIF2α Western blot were also unsuccessful due to high degree of variations among samples and blots. With immunocytochemistry, initial data suggested a higher number of peIF2α expressing OPCs under cytokine condition. However, Western blot approach was required to perform a series of assessments using various ISR and UPR markers such as peIF2α, total eIF2α, ATF4, CHOP, Xbp1s as well as EIF4EBP and SESN2 that are shown to link the ISR to the mTOR pathway[135]. Furthermore, with these markers, we could have established if the expression of peIF2α was related to ISR or UPR. Based on the available literature, ER stress and the ISR in rat OPCs are related to IFNγ, as this cytokine has shown to mildly induce the ISR[87,94,136].

However, it is also important to note that using just one marker does not provide enough information about the downstream activity of a pathway under an experimental condition. Thus, manipulation of mTOR and ISR (activation or inhibition) in OPCs would be important to determine whether cytokine induced changes in OPCs is mediated through these pathways.

4.3 Trials for inducing the UPR *in vitro:* Tunicamycin is not suitable for assessments longer than 48 hours.

With our initial trials with Tunicamycin, we could identify that concentrations as low as 0.25µg/ml are sufficient to induce CHOP expression in OPCs without significant cell loss after 16h according to our immunocytochemistry and Live/dead assay. However, after 48h of tunicamycin treatment, there was a highly significant cell loss in OPC culture that did not allow to assess OPC cellular response that required long-term assessments in particular maturation and myelin protein expression. CHOP is a transcription factor downstream of the PERK branch that mediates apoptosis[137], thus, it was logical to observe cell death with induction of CHOP under tunicamycin treatment. Previous findings have also reported that tunicamycin is lethal for OPCs after 24 hours[138]. For future assessments, the ER stressor thapsigargin could be used to induce an initial stress response in OPCs, followed by the addition of Sephin1 to prolong the ISR. This method would allow to perform differentiation assessments with a minimized risk of cell death as described by other groups[95,133].

4.4 mTORC1 activity in OPCs and OLs in acute and chronic phases of demyelination and remyelination in cuprizone mice.

In EAE mice, a more physiologically relevant MS model, we found that pS6 expression significantly increased during the peak of the disease and it was sustained after the recovery period, which indicated that mTORC1 was activated during and after the injury, perhaps having a supportive role. However, reports in the literature suggest that mTORC1 activity in EAE is, in fact, disease-promoting as rapamycin ameliorated EAE symptoms and decreased the expression of inflammatory cytokines like IL-17 and IFNγ[139]. mTOR is shown to regulate T-cell differentiation in EAE, hence rapamycin's beneficial effects are most likely related to a decrease in inflammatory response[140,141]. After our initial findings with the EAE samples, we were interested to determine OL specific expression of mTOR signaling in demyelinating and remyelinating lesions. The cuprizone model provides an excellent opportunity to focus on oligodendrocytes.

By inducing cuprizone demyelination in PDGFR-GFP mice we were able to track the oligodendroglial population within these lesions. Interestingly, we observed a significant increase in the expression of pmTOR in both OPCs and OLs during acute and chronic demyelination, and this expression was sustained during remyelination. The phosphorylation of mTOR is associated with both mTORC1 and mTORC2 complexes. The antibody we used for detection of pmTOR is selective for the phosphorylation site SER2448[142]. According to the literature, pmTOR SER2448 can be used to confirm mTOR signaling, however, this is not a reliable measure for mTOR kinase activity[143]. Studies have shown that the phosphorylation of mTOR at SER2448 is mediated by pS6, downstream of mTORC1, and not by the canonical AKT/mTOR pathway[144,145].

We found that pS6 expression increased significantly in both OPCs and OLs during acute and chronic demyelination, and this expression was sustained during remyelination. This could suggest that mTORC1 may possibly drive OPC differentiation and maturation into OL to support myelin repair. Knowing that acute remyelination happens successfully after removing the CPZ diet[97], this mTORC1 activity could be beneficial or required for restoring myelin. This observation is supported by the recent findings by Jeffries and colleagues in 2021 that demonstrated mTOR signaling mediates the metabolic function of OPCs and promotes remyelination in a CPZ demyelinating mouse model[74].

These studies also suggested that CPZ suppressed the metabolic function of differentiating OPCs which had a negative impact on remyelination. There were no effects on proliferation or differentiation after deleting mTOR from adult OPCs, although remyelination was delayed. Their data suggest that while mTOR does not impact adult OPC differentiation, their metabolic function is decreased which results in poor remyelination[74]. Our findings in the acute and chronic phases of CPZ show that mTORC1 is active during demyelination and increases significantly during remyelination, but its role in OPCs and OLs would require further investigation. The contrasting results from EAE and CPZ models, as reviewed in the literature[74,136,146] suggest that mTOR seems to regulate several processes in different cell types, and inhibiting mTOR in EAE could be beneficial to decrease inflammation.

We noticed that the cell populations, either OPCs or OLs, that showed the most expression of mTOR-related markers were GFP+. The presence of GFP+ OPCs indicates a successful recombination, and GFP+ OLs represent the OPCs that were initially recombined and matured into OLs.

We also found a population of GFP- cells that also expressed pmTOR and pS6 which could represent either the OPCs that were not recombined or a population non-oligodendroglial cells, likely some of astrocytes, that also express CC1 and PDGFRα. During demyelination, OPCs can proliferate and migrate to the lesion site where they can differentiate into mature OLs[110,117], an early report mentioned the high presence of OPCs during and after CPZ[147]. As cuprizone is known to directly affect mature OLs[148] and not OPCs, it is plausible that these cells are newly generated OLs from the existing GFP+ OPC population, and the presence of pmTOR markers could suggest that this pathway also contributes to OL differentiation and maturation. Studies have suggested that chronic demyelination and remyelination may fail due to the CPZ-induced progressive depletion of OLs and OPCs[149]. However, our findings in PDGFR-GFP mice clearly shows that after removal of CPZ diet in chronic timepoint new OPCs repopulate the corpus callosum.

Thus, the open question is why remyelination happens in acute lesions but fails in chronic while OLs are repopulated in both lesions. Currently, other members of the lab are working towards performing Magnetic-activated cell sorting (MACS) to isolate adult OPCs and OLs from CPZ mice at the different time points during demyelination and remyelination with the goal of performing RNA sequencing that could provide more insight about the profile of these cell populations. To further investigate the relevance of mTOR signaling in OPC differentiation and remyelination, an OL specific conditional knockout mouse model of mTOR would be advantageous.

4.5 Phosphorylation of eIF2α increases in OPCs and OLs during both acute and chronic phases of demyelination and remyelination.

We observed a significant increase in the expression of peIF2α in both GFP+ OPCs and OLs during acute and chronic demyelination, and this expression was sustained during remyelination, which indicates that the ISR was initiated. The ISR has gained attention in recent years in demyelination and remyelination. Studies have demonstrated that prolonging the ISR is beneficial and supports remyelination in inflammatory environments[94,95]. After inflammatory stress, the ISR is triggered in OLs, initiated by the phosphorylation of eIF2α[91,149]. Altogether, current views in the literature suggests that the ISR protective effects are dependent on inflammation. Our observational findings show that the ISR is initiated in oligodendroglial populations, which could suggest that this pathway contributes to OPC differentiation and maturation; however, further work is required to study how the induced ISR is related to remyelination failure in chronic cuprizone lesions. Future work will also include studying downstream effectors of ISR such as ATF4, and GADD34. RNA sequencing could aid to identify the profile of oligodendroglial cells that are undergoing ER stress by any of the aforementioned pathways.

5. Conclusion

In summary, this thesis provided an immunohistochemical characterization of the expression mTOR and ISR markers in OPCs and OLs in acute and chronic demyelinating lesions and in MS-like in vitro condition. We used a transgenic reporter mouse model to specifically study OL lineage cells *in vivo*.

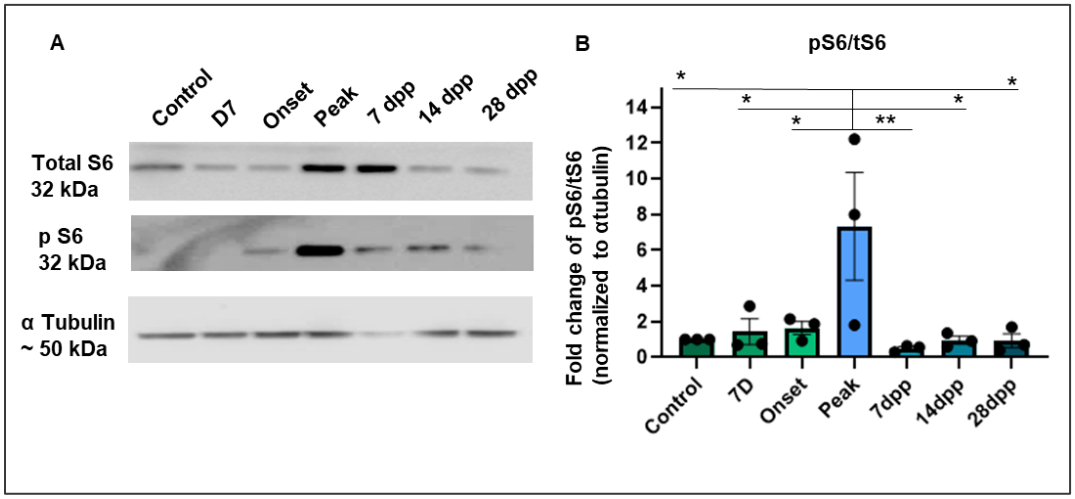
Parallel complementary *in vitro* studies also allowed us to study the direct impact of mTOR inhibition as well as proinflammatory cytokines on the properties of OPCs and OLs. We found that increased lysosomal activity is downstream to both rapamycin and cytokine treatments suggesting a possible relationship, which requires further elucidation using molecular biology approaches. Although this characterization was heavily through immunohisto- and immunocyto-chemical assessments, it provided initial insights on the involvement of mTOR and the ISR in OPC differentiation and OL maturation, which would require further investigation to determine their impact on demyelination and remyelination failure in chronic MS lesions.

5.1 Limitations and future directions

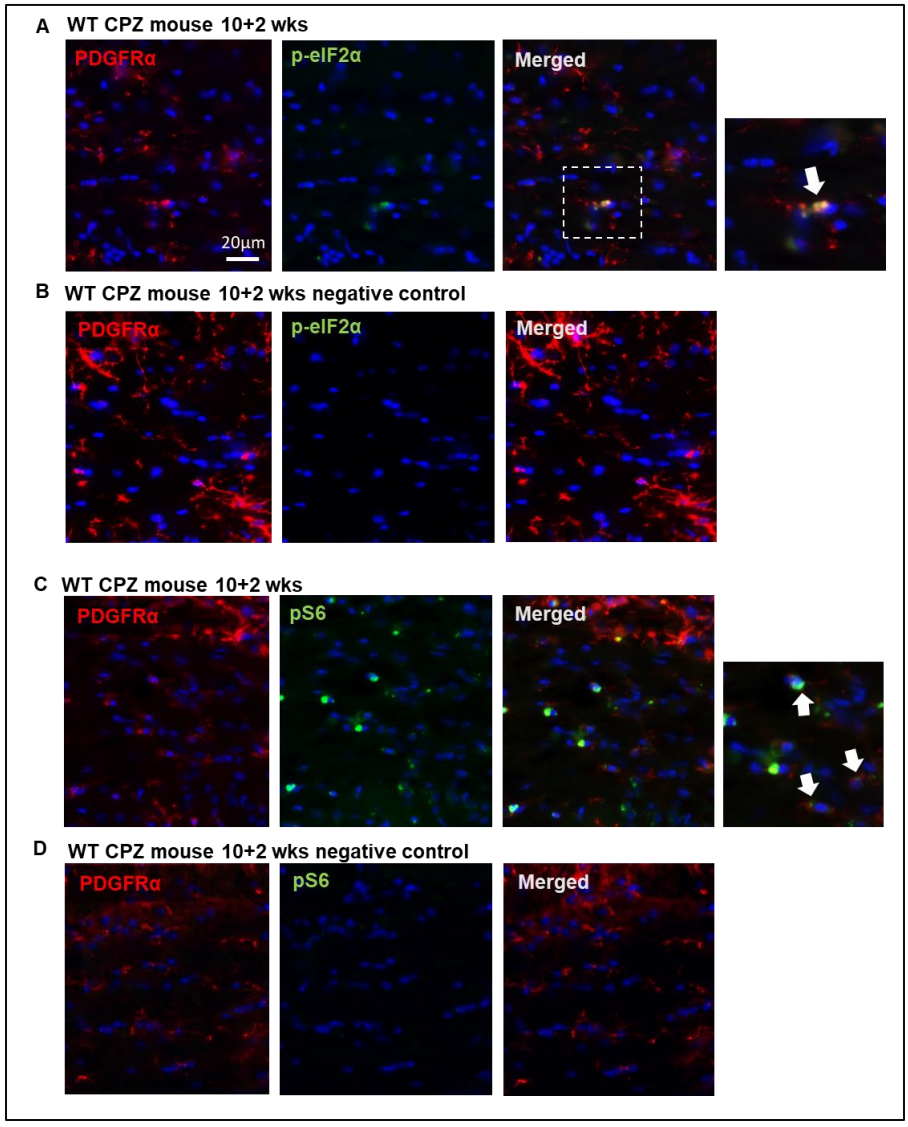
One of the main limitations of this study was the lack of consistency in the technicality. The *in vitro* part of this study could have been extended; however, I encountered several rounds of infections in OPC cultures that delayed experiments for three months. I also lacked consistency in other techniques such as protein extraction and Western blotting, which were essential to identify a relationship between the effects of the treatments on OPCs and the pathways. Aside from the technical limitations and after reading the literature, one limitation to be considered is the use of primary cells from pups. According to the studies mentioned in the discussion, mTOR signaling is required for developmental OPC differentiation but it is dispensable for adult OPCs, which could be due to intrinsic differences between the two populations[150,151]. Our results were similar to the ones reported in the literature where OPCs from pups were used.

In the future, it would be relevant to do studies on adult OPCs which will help to identify if there is a relationship between their differentiation, remyelination failure, and the involvement of mTOR and the ISR. Given the inherited challenges with culturing OPCs from adult rodents, future use of human OL cell lines can be considered. For cell survival assessment, I could use additional assays such as lactate dehydrogenase (LDH) assay on the media of the same cultures that were used for Live/dead assay assessment. Assessing apoptosis by cleaved caspase 3 is another approach that needs to be conducted in this project. However, the challenges that I had with frequent culture infection did not allow me to perform complementary approaches.

In the literature, rat OPCs have been used more frequently and the baseline results are slightly different from mouse OPCs, in particular their baseline proliferation rate or their maturation time. A study reported significant qualitative differences in OPCs from mice and rat, and that the information between both cannot be generalized among species[152]. However, in MS studies, mouse OPCs are mostly used, as the majority of MS animal models including EAE and cuprizone are induced in mice. Additionally, the use of transgenic and genetic mouse models in our research requires mouse-derived cells for parallel *in vitro* studies. As future directions of this project, there are several approaches that can be pursued. For *in vivo* experiments, transcriptomic profiling of OPCs and OLs in acute and chronic lesions is ongoing in our laboratory. mTOR and ISR pathways, need to be manipulated pharmacologically or genetically to assess a relationship between their signaling and their implications on OL differentiation and remyelination.



Supplementary Figure 1. mTORC1 is transiently activated at the peak of EAE a physiologically relevant mouse model of MS. A) The preliminary data was performed by using the available samples collected from the MS mouse model of (EAE) in our laboratory. These initial data suggest that mTORC1 is transiently activated during the peak of the disease, which is reduced at later time points. EAE peak represents the highest pro-inflammatory phase of the disease. High variation at the peak of EAE exhibits the complexity of EAE pathophysiology and heterogeneity in disease development in mice. pS6 was non-detectable in both the control and non-symptomatic phase of EAE (7-days-after EAE induction). **B)** Data represent initial analysis of three EAE mice per time-point including non-EAE control, 7 days post EAE induction (7D), onset of EAE, peak of EAE, 7 days post-peak (7 dpp), 14 days post-peak (14 dpp) and 28 days post-peak (28 dpp). *P < 0.05; Data represents mean ± SEM One-way ANOVA followed by Sidak's multiple comparisons test, N= 3.



Supplementary Figure 2. Specificity of antibodies in IHC. Before performing IHC on PD-CPZ tissue, we determined the specificity of the antibodies by using positive and negative controls with WT-CPZ tissues. A) Positive control for peIF2α shows the corpus callosum of a WT-CPZ mouse that received CPZ for 10 wks and normal chow for another 2 wks (remyelination short term); stained for peIF2α along with PDGFRα+. B) Negative control for peIF2α where the primary antibody was not used, shows the corpus callosum of a WT-CPZ mouse that received CPZ for 10 wks and normal chow for another 2 wks (remyelination short term). C) Positive control for pS6 shows the corpus callosum of a WT-CPZ mouse that received CPZ for 10 wks and normal chow for another 2 wks (remyelination short term); stained for peIF2α along with PDGFRα+. B) Negative control for pS6 where the primary antibody was not used, shows the corpus callosum of a WT-CPZ mouse that received CPZ for 10 wks and normal chow for another 2 wks (remyelination short term).

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