Universidade de Aveiro Departamento de Biologia
Ano 2017

Bárbara Catarina Gonçalves Correia

Determining how membrane phospholipids regulate CNS myelination and myelin composition

Determinar como os fosfolípidos membranares regulam a mielinização e a composição da mielina no SNC

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Virgília Sofia Almeida de Azevedo e Silva, Professora Auxiliar convidada do Departamento de Biologia da Universidade de Aveiro, e do Doutor Pedro Miguel Teixeira Brites investigador principal do Instituto de Investigação e Inovação em Saúde da Universidade do Porto.

“Never give up on a dream just because of the time it will take to accomplish it.
The time will pass anyway.”

- Earl Nightingale

o júri

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palavras-chave

1-O-tetradecil-glicerol, co-culturas *in vitro,* condrodisplasia rizomélica punctata, Gnpat KO, oligodendrócitos, proteína básica de mielina.

resumo

Condrodisplasia rizomélica puncata é uma doença autossómica recessiva caracterizada por um defeito em enzimas peroxisomais responsáveis pela biossíntese de plasmalogénios. Estes defeitos causam uma deficiência na produção deste fosfolípido e os doentes apresentam anormalidades ósseas e um atraso mental. Esta doença pode ser causada por mutações no gene que codifica a enzima GNPAT que é a primeira enzima necessária para a síntese de plasmalogénios. Alguns estudos demonstraram que a mielinização é afetada pela perda de plasmlalogéneos no sistema nervoso central e periférico. Contudo, ainda não se sabe como é que este defeito se processa a nível celular e molecular.

Com o presente trabalho, demonstramos que é possível usar co-culturas *in vitro* de neurónios corticais e de oligodendrócitos, provenientes de ratinhos *wildtype* (WT) e *knockout* (KO), possibilitando a investigação da relação entre os dois tipos de células e o que origina a desmielinização observada *in vivo*. Este método *in vitro* também pode ser usado para testar diferentes candidatos para eventuais terapias. Os alquil-glicerois já foram indicados como um percursor alternativo de plasmalogénios, mas a sua eficácia nunca foi comprovada em tecidos neuronais. Neste trabalho usámos um tipo de alquil-glicerol, denominado 1-O-tetradecil-glicerol, capaz de restaurar os defeitos da mielina nas co-culturas de ratinhos Gnpat KO. A mielina é também rica em proteínas e algumas têm funções importantes na compactação e manutenção da mielina. Por esse motivo, é importante estudar como é que as proteínas da mielina são afetadas pela deficiência em plasmlalogéneos. O western blot foi usado para analisar as diferentes proteínas presentes tanto na mielina compacta como na mielina não compacta. Os resultados demonstraram um decréscimo significativo da proteína básica de mielina (MBP) em mielina isolada do cérebro e espinal medula de ratinhos KO. Esta proteína é essencial para manter os segmentos de mielina compactos e funcionais. A proteína promotora de polimerização de tubulina (TPPP) não demonstrou diferenças, quer em mielina do cérebro quer da espinal medula. Esta proteína é responsável por regular a diferenciação dos oligodendrócitos e os nossos resultados mostram que um defeito em plasmalogénios não afeta a diferenciação dos oligodendrócitos, mas especificamente a produção de mielina por estas células.

keywords

1-O-tetradecyl-glycerol, Gnpat KO, *in vitro* co-cultures, myelin basic protein, oligodentdrocytes, Rhizomelic chondrodysplasia punctata.

abstract

Rhizomelic chondrodysplasia punctata (RCDP) is an autosomal recessive disorder characterized by a defect in peroxisomal enzymes responsible for the biosynthesis of plasmalogens. This leads to a deficiency of this type of glycerophospholipid, and patients present bone abnormalities, and mental retardation. This disorder can be caused by mutations in the GNPAT gene, which encodes the first enzyme necessary for plasmalogen synthesis. Some studies have shown that myelination in the peripheral and central nervous system is affected by the loss of plasmalogens. However, it is unknown how this defect in myelination is orchestrated in a cellular and molecular scenario. With this work, we showed that it is possible to use *in vitro* co-cultures of cortical neurons and oligodendrocytes, from WT and Gnpat KO mice, to investigate the relation between both cells and what leads to demyelination. This strategy can also help to test different candidates for therapies. Alkyl-glycerols have been indicated as an alternative precursor of plasmalogens, but until now their efficiency to restore plasmalogen levels is still not proven in nervous tissue. We used one type of alkyl-glycerol named 1-O-tetradecyl-glycerol, and it was able to restore the defective myelination in Gnpat KO co-cultures. Myelin is also rich in proteins, and some have important functions for compaction and maintenance of the myelin sheaths. Therefore, it is important to analyze how the proteins are affected by the lack of plasmalogens. We used Western blot to analyze different proteins present in compact and non-compact myelin. We observed a significant decrease in the amount of myelin basic proteins (MBP) in myelin isolated from brain and spinal cord of Gnpat KO mice. MBP is essential to keep the myelin sheaths compact and functional. Other proteins like tubulin polymerization-promoting protein (TPPP) showed no differences in brain or in spinal cord myelin, since this protein is known to regulate the differentiation of oligodendrocytes, and our previous results did not evidence any problems during differentiation.

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CHAPTER I – GENERAL INTRODUCTION

THE CENTRAL NERVOUS SYSTEM

Neuronal network

Neurons are the most important cells in the brain with the ability to process and transfer information throughout the nervous system. Neurons are excitable cells that can be divided into three distinct organizatory parts: dendrites, soma and axon. The soma (cell body) contains many organelles, like the nucleus, mitochondria, and others also present in other types of cells. The cell body has a special domain known as axon hillock from which the axon originates, and has a membrane rich in voltage-gated ion channels which are important for the generation of action potentials. Dendrites arise from the cell body, and are highly branched, forming a dense network called dendritic tree, which is characterized by the presence of synapses. These features provide neurons with the ability to receive and integrate information from other neurons and glial cells (Levitan and Kaczmarek, 2015). The axon is the part capable of transmitting large amounts of information using electrical signals, called action potentials, generated by the flow of ions through the plasma membrane (Purves et al., 2001a; Laughlin and Sejnowski, 2003; Kevenaar and Hoogenraad, 2015). Some axons need to reach long distances, and therefore it is important to ensure an efficient transmission along the axon, which is accomplished by the wrapping of axons with myelin. Myelin is also important in maintaining the functional integrity of the axon (Podbielska et al., 2011; Saab et al., 2013).

Neuroglial cells

The development and maintenance of the nervous system is ensured by the glial cell, which are divided into two different types of cells, macroglial and microglial cells. In the central nervous system, macroglia include astrocytes and oligodendrocytes (Purves et al., 2001b; Frühbeis et al., 2013). Astrocytes have many functions like ion homeostasis, formation and stabilization of neuronal signals and providing neuronal support. These cells are part of the blood brain barrier (BBB) and are important after a brain injury, participating in the scarring process and repair (Frühbeis et al., 2013; Olabarria and Goldman, 2017). Mature astrocytes have several processes extending from the cell body, giving the cell its star-like (e.g. “astro”) shape. This phenotype can suffer some alterations in a pathological scenario like neurodegeneration, trauma or infection in order to protect the CNS. When astrocytes become

reactive, they change their phenotype, leading to astrogliosis (Zamanian et al., 2012; Olabarria and Goldman, 2017). Astrogliosis, also called reactive astrocytosis, is characterized by cellular hypertrophy, overexpression of glial fibrillary acidic protein (GFAP, an astrogliosis marker), astrocyte proliferation and scar formation in severe cases (Eng and Ghirnikar, 1994; Sofroniew and Vinters, 2010).

Oligodendrocytes are another type of macroglial cells capable of wrapping the axons with a lipid-rich structure called myelin sheaths. Myelin is important in conducting electrical impulses along the axon and accelerating the propagation of action potentials given that one of its functions is to act as an insulator (Pang et al., 2011; Rose and Kirchhof, 2015). Further details about oligodendrocytes and myelination will be presented below.

Microglia cells were first characterized by del Río Hortega (1932) and are macrophages-lineage cells able to rapidly respond to signaling molecules, representing a state of vigilance to any extracellular alterations. Microglia plays an important role in several pathological conditions (Kreutzberg, 1996). The activation of microglial cells leads to a morphological change, from a short branched cell to an amoeboid form which become active phagocytes (Kettenmann et al., 2012). Microglial cells are able to secrete molecules capable of inducing reactive astrogliosis (Zamanian et al., 2012).

Myelin: morphology and assembly

As referred above, oligodendrocytes are responsible for wrapping axons with membrane sheaths called myelin. High numbers of oligodendrocytes in the white matter and their position during myelination were some of the observations that supported the role of oligodendrocytes in the generation of myelin segments. The term myelin was first introduced by Virchow in 1858. Myelin sheaths are extensions of the plasma membrane of oligodendrocyte which wrap several times around the axons, originating compact sheaths highly enriched in lipids, containing a low amount of water, and having only a well-defined number of very specific proteins (Baumann and Pham-Dinh, 2001; Simons and Nave, 2016). Myelination is a complex process, initiated by proliferation and migration of oligodendrocyte precursor cells (OPCs) in white matter. OPCs migrate to different areas of the CNS during development to make contact with several axons. These precursor cells differentiate into premyelinating oligodendrocytes and lastly into mature oligodendrocytes, capable of myelinating several axons simultaneously, unlike Schwann cells (i.e., the glial cells responsible for myelination in the peripheral nervous system (PNS)) that can only myelinate a given axon (Baumann and Pham-Dinh, 2001; Fields, 2015). During

differentiation, components of myelin are synthesized leading to an outgrowth of the membrane for axonal wrapping, myelin compaction and formation of nodes (Simons and Nave, 2016). Compact myelin segments, or internodes, are interrupted by small gaps named nodes of Ranvier (FIGURE 1).

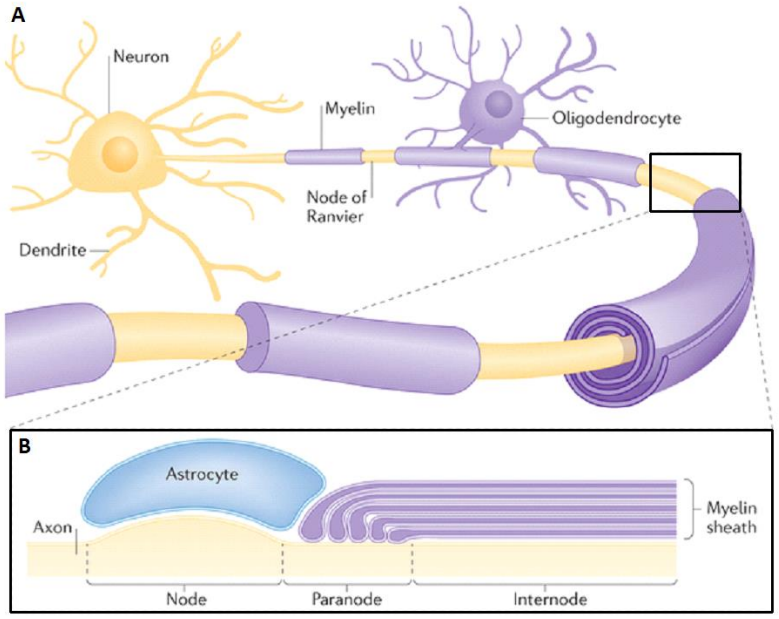

FIGURE 1 – MYELIN STRUCTURE AND ORGANIZATION. **a)** Myelin sheaths are formed by continuously wrapping of the oligodendrocytes' membrane around the axon (internode) leaving small gaps between them termed Nodes of Ranvier. **b)** Longitudinal section of a myelinated axon. The generation of action potentials are confined to the nodes, which contact with perinodal astrocytes, since compact myelin serve as an insulator. Adapted from Fields, 2015.

This distinctive structure, makes it possible to conduct electrical impulses (action potentials) through a saltatory fashion, i.e., from node to node, due to the high number of sodium channels present in the nodes (Baumann and Pham-Dinh, 2001; Seidl, 2014). Saltatory conduction is only possible due to the function of myelin as an insulator, enhancing and accelerating the transmission of electrical signals along the axon. In unmyelinated axons, action potentials are transmitted continuously along the axon with successive depolarization and repolarization of the membrane (Fields, 2015). To trigger an action potential, it is necessary that enough depolarization can bring the membrane potential to or above its threshold (around -53 mV), starting at a resting potential (between -85 mV and -60 mV) (Bean, 2007). Depolarization is caused by the opening of voltage-gated sodium channels leading to a rapid influx of

sodium. This influx reaches a peak potential that will lead to the opening of voltage-gated potassium channels, and consequent efflux of potassium, increasing the membrane potential, also known as repolarization. This process is time and energy consuming, so by confining this process to the nodes of Ranvier, conduction velocity increases up to 100 times comparing to unmyelinated axons (Purves et al., 2001c; Fields, 2014). Nodes of Ranvier act as repeaters, and the velocity of the transmission depends on the length of the node, and the distance between them, as well as the thickness of the myelin sheaths.

Myelin is rich in lipids, between 70% - 80% by dry weight, and only 30% represent proteins present in compact myelin. The most abundant group of lipids are glycosphingolipids (27%), cholesterol (26%) and plasmalogens, which are a type of ether-phospholipid (Aggarwal et al., 2001; Simons and Nave, 2016). Myelin can appear as compact myelin (tight layers of membranes almost lacking cytoplasm between them), and non-compact myelin (regions of myelin within the internode or at the paranode, that contain cytoplasm and organelles). Myelin basic proteins (MBP) and proteolipid protein (PLP) are the most abundant proteins in the compact myelin. In the non-compact myelin proteins like Myelin-associated glycoproteins (MAG), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and myelin oligodendrocyte glycoprotein (MOG) are more abundant. The compaction of myelin is ensured by MBP, due to its strong adhesive properties, which compacts the membranes by a dual action of bringing membranes closer together and the extrusion of cytosolic components (FIGURE 2). PLP is a hydrophobic protein and corresponds to approximately half of the total proteins. This protein also plays a role in compaction (FIGURE 2), given that PLP is known for increasing "adhesion" between two opposing membrane sheaths (this is mediated by PLP-PLP interactions). Whereas MBP is responsible for the generation of the cytoplasmic apposition (also known as major dense line), PLP is partially responsible for the generation of the extracellular apposition (also known as intraperiod line). However, the function of PLP is not essential. Klugmann et al. (1997) demonstrated that PLP-deficient mice were able to myelinate axons, despite the observation that PLP deficiency interfered with the stability of the sheaths. PLP has two isoforms, the most common is called PLP (25 kDa) and the second is called DM-20 (20 kDa) and makes around 10 to 20% in the adult (Baumann and Pham-Dinh, 2001). MAG is another type of proteins present almost exclusively found at the myelin-axon interface, to ensure the maintain the organization of the periaxonal space (Li et al., 1994). Another protein found in myelin is 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP, representing up to 4% of total myelin proteins). These proteins are present in the inner mesaxon or paranodal loop, so instead of a specific function, it might have a

wide-ranging cellular function. MOG can be found on the surface of myelin sheaths, and oligodendrocytes, and might be an intermediate between the interior of the oligodendrocyte and the outside (Baumann and Pham-Dinh, 2001; Lappe-Siefke et al., 2003).

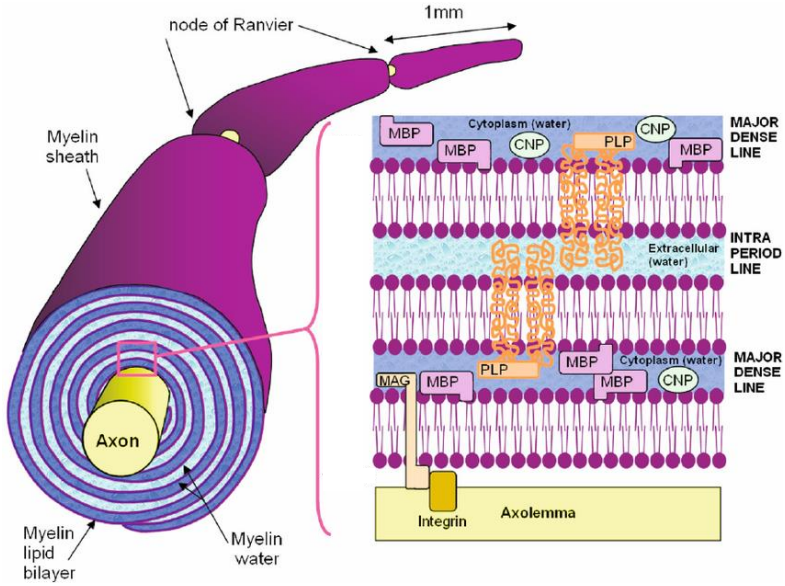

FIGURE 2 - MYELIN STRUCTURE. Compact myelin has only 30% of proteins. PLP and MBP are the most abundant proteins in compact myelin. Other proteins are present in non-compact myelin, like myelin oligodendrocyte glycoprotein (MOG), MAG and CNP. Adapted from Laule et al., 2007.

PEROXISOMES

Peroxisomes are small (0.2 – 1 µm in diameter), single membrane bound organelles and are found in most eukaryotes (Braverman et al, 2013). These organelles are responsible for different physiological functions, with multiple biochemical reactions and are involved in catabolic and anabolic pathways, such as biosynthesis of plasmalogens, α-oxidation of phytanic acid and β-oxidation of very long chain fatty acids (Michels et al., 2005; Bams-Mengerink et al., 2009; Thoms et al., 2009). Peroxisomes can be formed by growth and fission of pre-existing organelles, however, they can also be formed *de novo* through special compartments of the endoplasmic reticulum (ER) (Agrawal and Subramani, 2013; Braverman et al., 2013). Peroxisomes are present in all types of cells in the brain, and

defects in these organelles, either through defects during formation or loss of peroxisomal proteins, can lead to neurological disorders, affecting axon integrity and myelination (Wanders and Waterham, 2005; Kou et al., 2011; Berger et al., 2016).

The biosynthesis of peroxisomes involves a large number of proteins called peroxins (PEX). These proteins are important for the membrane formation, peroxisome inheritance and peroxisome proliferation (Wanders and Waterham, 2005). Two types of peroxisome targeting signals (PTS1 and PTS2) are responsible for the entrance of peroxisomal matrix proteins, which are synthesized in the cytosol. PTS1 is present in the majority of the matrix proteins. This signal is a tripeptide present at the C-terminal with the consensus sequence (S/A/C)-(K/R/H)-L (Gould et al, 1989; Moyersoen et al., 2004; Wanders and Waterham, 2006; Michels et al., 2005). PTS2 is at the N-terminal consisting of a octapeptide (R/K)-(L/V/I)-(X)5-(H/Q)-(L/A). This is a rare signal present in a few proteins like alkylglycerone phosphate synthase (AGPS) and phytanoyl-CoA hydroxylase. Both motifs may differ between species in terms of the length or even the specificity of the signal (Michels et al., 2005; Wanders and Waterham, 2005). PTS are recognized by receptors in the cytosol, PTS1 by PEX5 and PTS2 by PEX7. Due to alternative splicing, two isoforms of PEX5 can be produced: a short form (PEX5S) and a long form (PEX5L). It has been shown that PEX7 interacts only with the long form of PEX5, and not with PEX5S, i.e., PEX5L might mediate the docking of PTS2 to the membrane (Moyersoen et al., 2004; Michels et al., 2005). PEX5 and PEX7 recognize PTS1 and PTS2 containing cargo, respectively. Even though FIGURE 3 only represents the import of PTS1 through PEX5, the docking and the following steps are similar for PEX7. These two complexes dock at the peroxisome membrane formed by PEX13, PEX14 and PEX17. The binding of PEX5 with PEX14 forms a large dynamic membrane pore, which allows the translocation of either PST1 or PTS2 into the peroxisomal lumen. Ubiquitylation of PEX5 serves as a signal for the ATP-dependent dislocation of PEX5 back to the cytosol via PEX1 and PEX6, which are bound to PEX 15, and so anchored to the membrane. Once PEX5 is released, it is able to perform a new cycle of import (Michels et al., 2005; Thoms et al., 2009; Grimm et al., 2012).

Plasmalogens

Plasmalogens are a special type of glycerophospholipids, characterized by their vinyl ether linkage at the sn-1 position of the glycerol backbone. The aliphatic moieties at the sn-1 position consist of palmitic acid (C16:0), stearic acid (C18:0) or oleic acid (C18:1), while the sn-2 position is occupied by polyunsaturated fatty acids, and the head group is either ethanolamine (PE-plasmalogen) or choline (PC-plasmalogen) (FIGURE 4). In humans, plasmalogens represent up to 18% of total phospholipid mass. The highest content of PE-plasmalogen can be found in brain myelin, while PC-plasmalogens can be mostly found in the heart muscle. Plasmalogens can also be found in kidney, spleen, blood cells and skeletal muscle, although in moderate amounts. The organ with the lowest amount is the liver (Brites et al., 2004; Wanders and Waterham, 2006; Braverman et al., 2013).

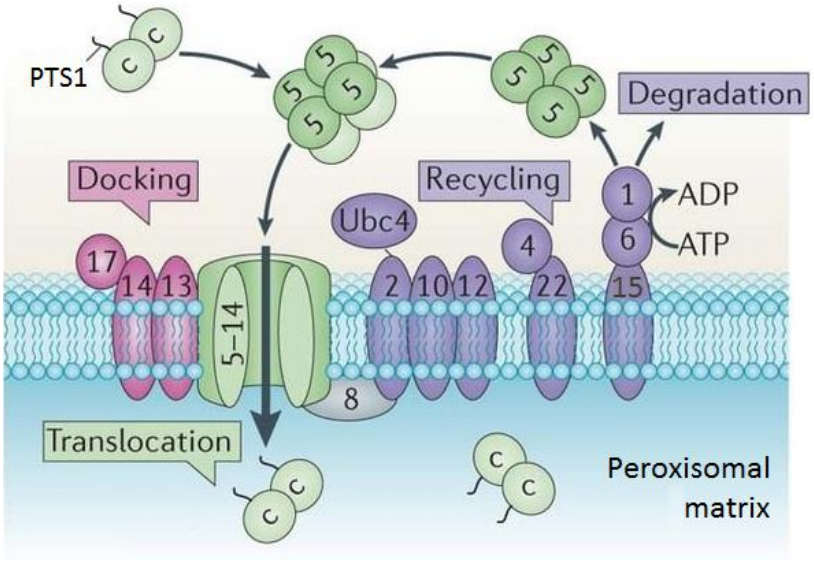

FIGURE 3 – IMPORT OF PEROXISOMAL MATRIX PROTEINS IN *S. CEREVISIAE*. Translocation of PTS is possible through the action of several peroxins like PEX5, PEX14, PEX13 and PEX17. Ubiquitin modification allows PEX5 to go back to the cytosol through PEX1 and PEX6, were it can start a new cycle of import. Adapted from Smith and Aitchison, 2013.

Plasmalogens have several biological functions: they serve as antioxidants, are able to mediate membrane dynamics and storages of polyunsaturated fatty acid (PUFAs), lipid mediators and as

reservoirs for second messengers. Their ability to function as an antioxidant comes from their unique vinyl-ether bond, which is susceptible to oxidative stress (Brosche and Platt, 1998). This special bond is easily oxidized due to its low dissociation energy, therefore, single oxygens react faster with plasmalogens than with other types of lipids or proteins. The distinct structure of plasmalogens confers unique features to the membranes, affecting membrane fluidity and membrane fusion. Some studies have shown that plasmalogens are also able to regulate cell differentiation and impacting cellular signaling (da Silva et al., 2014; Malheiro et al., 2015; Dean and Lodhi, 2016). PE-plasmalogen and choline PC-plasmalogen are found in large amount in macrophages and neutrophils and serve to produce platelet activation factors important for antimicrobial defense and during inflammation (Wanders and Waterham, 2006).

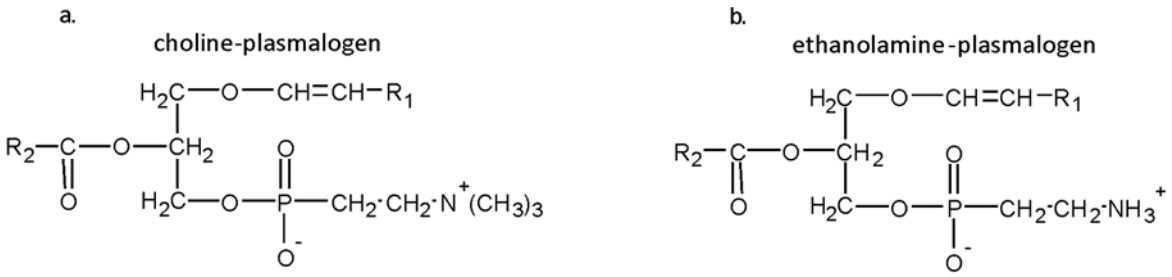

FIGURE 4 - STRUCTURE OF PLASMALOGENS. Plasmalogens are unique glycerophospholipids due to the presence of a vinyl ether bond at the sn-1 position. **a.** PC-plasmalogens can be mostly found in the heart muscle. **b.** PE-plasmalogen can be found in brain myelin. At the position R1 a medium fatty acid is usually present. At the position R2 a polyunsaturated fatty acid is usually present. Adapted from Acar et al., 2012.

The biosynthesis of plasmalogens is initiated in the peroxisome and finalized at the endoplasmic reticulum (ER). The first step (FIGURE 5) consists of the esterification of dihydroxyacetone phosphate (DHAP) with a long-chain acyl-CoA ester, accomplished by glyceronephosphate O-acyltransferase (GNPAT). Alkylglycerone phosphate synthase (AGPS) catalyzes the replacement of the sn-1 fatty acid (acyl group) with a long-chain fatty alcohol (alkyl group), resulting in 1-0-alkyl-DHAP as a product. These fatty alcohols can be originated by the reduction of long-chain acyl-CoA through acyl-CoA reductase 1 and 2 (FAR1 or FAR2, respectively). Acyl/alkyl-dihydroxyacetone phosphate reductase (AADHAPR) catalyzes the reduction of the ketone group at the sn-2 position of 1-alkyl-DHAP, originating 1-0-alkyl-2-hydroxy-sn-glycerophosphate (GPA). At this point, further reactions are confined to the ER,

which will then form the mature plasmalogens. Alkyl/acyl-GPA acyltransferase places an acyl group at the sn-2 position. Phosphatidic acid phosphatase is responsible for removing the phosphate group, and forms the 1-0-alkyl-2-acyl-sn-glycerol. Cytidine-diphosphate-ethanolamine (CDP-ethanolamine) is incorporated with help of ethanolamine phosphotransferase (E-PT), in the presence of magnesium, resulting in 1-0-alkyl-2-acyl-sn-GPEtn. This molecule is desaturated into 1-0-(1Z-Alkenyl)-2-acyl-sn-GPEtn (PE-plasmalogens) by a cytochrome b5-dependent microsomal electron transport system and plasmanylethanolamine desaturase. The formation of PC-plasmalogens can be either from 1-0-alkyl-2-acyl-sn-glycerol using choline phosphotransferase, or through PE-plasmalogens hydrolysis originating 1-0-(1Z-alkenyl)-2-acyl-sn-glycerol, which can form PC-plasmalogens after modification (Itzkovitz et al., 2012; Malheiro et al., 2015; Dean and Lodhi, 2017).

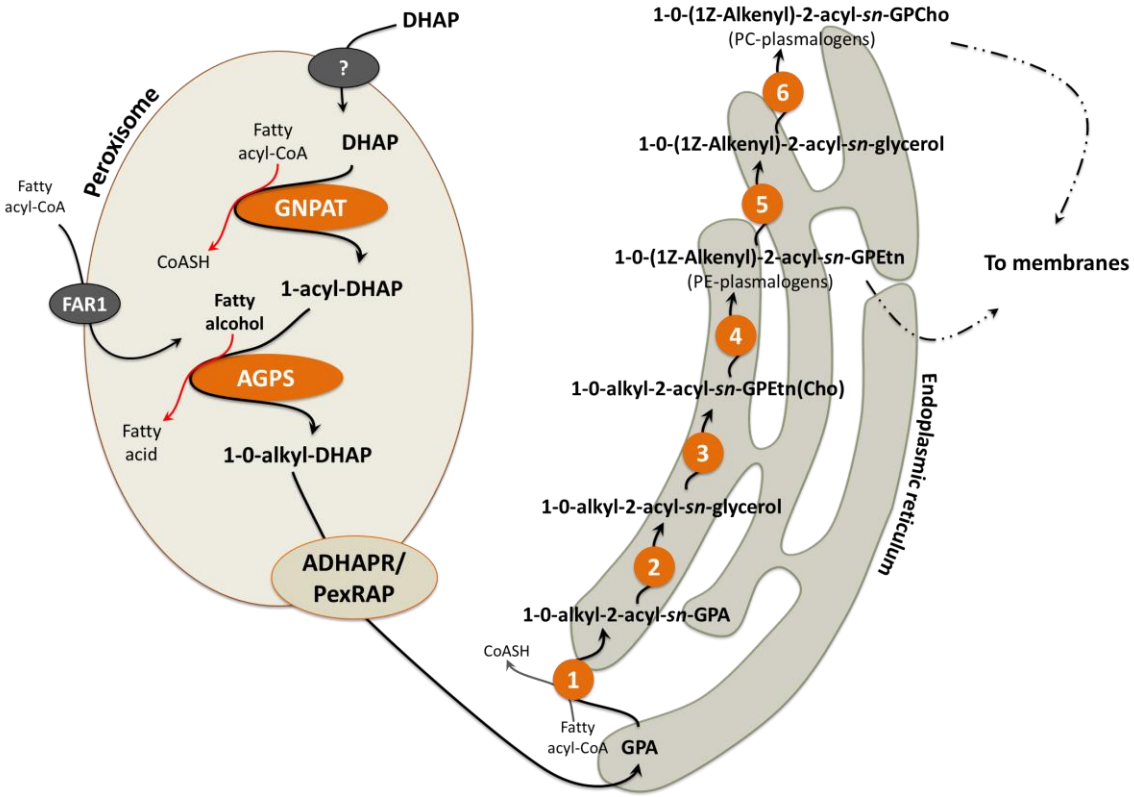

FIGURE 5 - BIOSYNTHESIS OF PLASMALOGENS. The synthesis of plasmalogens initiates in the peroxisome where a reaction cascade originates 1-0-alkyl-2-hydroxy-sn-glycerophosphate (GPA) that will be exported to the endoplasmic reticulum (ER) for maturation and generation of the two types of plasmalogens, PE-plasmalogen and/or PC-plasmalogen. Adapted from Malheiro et al., 2015.

Plasmalogens and neurological disorders

Many neurological disorders occur due to incorrect formation of plasmalogens or deficiency of its synthesis. As stated before, plasmalogens have many biological functions, therefore, a deficiency of this phospholipid can lead to several tissue defects. Many studies have been able to relate plasmalogen with several disorders like ischemic injury, Alzheimer's disease, malnutrition and spinal cord injury (Farooqui and Horrocks, 2001; Nagan and Zoeller, 2001; Dean and Lodhi, 2017). Peroxisome disorders can be caused by single enzyme deficiency or by an incorrect peroxisomal assembly (Peroxisome biogenesis disorders), causing defects in several enzymes (Wanders and Waterham, 2005; Thoms et al., 2009). Rhizomelic chondrodysplasia punctata (RCDP) and Zellweger spectrum are two types of peroxisome biogenesis disorders (PBS) caused by a deficiency in plasmalogen (Braverman and Moser, 2012). The Zellweger spectrum consists of three disorders: Zellweger syndrome, neonatal adrenoleukodystrophy (NALD) and infantile Refsum's disease (IRD). Patients within the Zellweger spectrum are characterized as having psychomotor retardation, retinal degeneration and hypotonia, seizures (Wanders and Waterham, 2005; Dean and Lodhi, 2017).

Rhizomelic chondrodysplasia punctata (RCDP)

RCDP is an autosomal recessive disorder and patients show symmetrical shortening of the proximal limbs, cataracts, mental retardation, dwarfism and bone abnormalities. In general, most patients die within the first decade of life (Brites et al., 2004; Wanders and Waterham, 2005; Braverman and Moser, 2012). RCDP can be caused by mutations in at least three distinct genes: PEX7, GNPAT and AGPS. RCDP type 1 is the most common form, caused by mutations in PEX7, which recognize peroxisomal targeting signal 2 (PTS2), and leads to a deficiency in the synthesis of plasmalogens. RCDP type 2 and 3 are caused by mutations in GNPAT and AGPS (Braverman et al., 1997; Bams-Mengerink et al., 2009). As described previously, GNPAT is one of the first enzymes necessary for the correct synthesis of plasmalogens, which means that any alteration or depletion of this enzyme will lead to a decrease in plasmalogens. As stated before, the highest content of PE-plasmalogen can be found in brain myelin, this means that a problem with the production in plasmalogens might lead to defect in myelination. This was shown by Sztriha et al (1997), that observed using magnetic resonance imaging a delay in the formation of myelin, and also dysmyelination, in RCDP type 2 patients.

Animal model

Currently there are 2 mouse models for rhizomelic chondrodysplasia punctata with complete defects in the levels of plasmalogens, i.e., the Pex7 knockout (KO) mice for RCDP type 1, and Gnpat KO mice for RCDP type 2. These mice present early lethality, but their life span can vary between animals. Gnpat KO mice were generated by removing exon 5 to 7 by homologous gene recombination (Rodemer et al., 2003). The absence of Gnpat in Gnpat KO mice causes the complete lack of plasmalogens in all tissues and cells (da Silva et al., 2012). In homozygous mutants, the animals are viable but they have defects in reproduction, the males are infertile and females are sub-fertile. Other deficiencies are present in KO mice, like defects in eye development, and growth (Liegel et al., 2011; Rodemer et al., 2003). These observations validate the Gnpat KO mouse as a good model for RCDP.

Objectives

Plasmalogens are a special type of glycerophospholipids and are one of the most abundant lipids in the central nervous system, specially in myelin. Previous *in vivo* studies performed at the Neurolipid Biology lab have shown that a deficiency of plasmalogens has a great impact on CNS myelination, and showed that demyelination is more severe and has an earlier onset when compared to the PNS (da Silva et al., 2014). However, it is still not known how exactly a loss of plasmalogens would lead to defects in myelination, and until now there are no *in vitro* experiments that show the same defect in myelination. Using Gnpat KO mice, we aimed to develop an *in vitro* co-culture that could validate the *in vivo* results, and helps to understand the effects of plasmalogen deficiency in oligodendrocytes and myelination. After validating the *in vitro* assay, we wanted to teste alternative plasmalogen precursors and determined if they can serve as a candidate therapy. We also wanted to study myelin isolated from WT and plasmalogen-deficient mice in order to characterize and determine how a deficiency in plasmalogens affects the proteins needed for proper myelination.

CHAPTER II – MATERIAL AND METHODS

Animal model

All procedures made in mice were done according to the European Directive 2010/63/EU established by the European Parliament and of the Council, as well as the National legislation (Decreto-Lei 113/2013). Mice were used only after approval of the Portuguese General Veterinarian Board.

Gnpat Knockout mice

Gnpat KO mice and WT littermates were obtained from mating Gnpat heterozygous mice (on a Swiss-Webster background). All animals were maintained with ad libitum access to rodent food and water, and were kept in a 12:12h light and dark cycle facility. Genotyping was performed by the IBMC CCGen facility using previously developed strategies (Rodemer et al., 2003).

Mixed cortical neuron-glial culture

Cortical neurons and glial cells were isolated from mouse embryos, at embryonic day 16-18 (E16-18). Brains were dissected from individual embryos of wild type (WT) and Gnpat knockout (KO), and were cleaned from meningeal shreds and superficial blood vessels to allow the cortices to be further dissected and collected in HBSS. The cortices were then washed with DMEM:F12 before incubation with 0.05% Trypsin + 0.2 mg/ml EDTA for 8min at 37^{o}C. Digestion was stopped by adding DMEM:F12 + 10% FBS, centrifuged at 1700 rpm (5min at room temperature (RT)). The supernatant was removed and the pellet of cortices was resuspend and dissociated in DMEM:F12. The cell suspension was passed through a 70 µm cell strainer and centrifuged at 1700 rpm (5min at RT). The supernatant was removed and replaced with neuron culture medium (Neurobasal medium with 2x B27 supplement, 0.1 mM L-glutamine and 1% penicillin-streptomycin). The number of cells present in the suspension was counted using trypan blue-exclusion, and cells were plated at a density of 1.5x10^{5} cells/well onto glass coverslips within 24-well plates, previously coated with Poly-L-Lysine (20 µg/ml in PBS, P2636 Sigma) and laminin (2 µg/ml in PBS, L2020-1MG Sigma). Cells were allowed to settle for 30min at 37^{o}C before removing the complete medium and adding a new slightly different plating medium (Neurobasal medium with 1x N21 supplement, 2 mM L-glutamine, 1% penicillin-streptomycin). New medium was added every 2 days, and neurons were able to adhere, mature and extend axons for 5 days. At 6 days *in vitro* (DIV), half of the plating medium in each well was removed and the same amount of myelination

medium (MyM; Watkins et al, 2008) was added. The cultures were maintained at 37°C until DIV 20 by adding new myelination medium every 2 days.

Treatments

Some cultures were treated with either SC79 (an AKT activator) or 1-O-Tetradecyl-rac-glycerol (1-O-TDG; Biosynth). For the cultures treated with SC79, the medium was either enriched with 0.01% v/v DMSO as a control or 0.8 µg/ml SC79 (Millipore) in DMSO. For the treatment with TDG, medium was enriched with 0.01% absolute ethanol (control) or with 7 µM TDG in ethanol. For both approaches, new medium with fresh agents (either SC79 or TDG) was added to the cells at DIV 10, 14 and 19. At DIV 20, cells were fixed for further processing.

Immunocytochemistry

The coverslips containing the cultured cells were fixed either at DIV 15 or 20 with 4% paraformaldehyde (PFA) for 10min at RT. After washing the coverslips with PBS, cells were permeabilized with 100% methanol at -20°C for 10min and rinsed with PBS. Coverslips are incubated in 0.2M NH_4Cl in H_2O for 10min at RT for quenching autofluorescence. This solution was removed, and blocking buffer (5% normal donkey serum (NDS) in PBS) was added to the cells for 1h at 37°C. Cells were then incubated with primary antibody against β-III tubulin (1:500, Synaptic Systems) and anti-MBP (1:250, Milipore) overnight at 4°C, which was followed by 3 washes in PBS and the incubation with secondary antibodies (anti-rabbit AF488, 1:500; anti-rat AF594, 1:250, respectively). At the end, coverslips were washed with PBS and were mounted with Fluoroshield with DAPI (F6057, Sigma). Stained cells were viewed using an epifluorescence microscope - AxioImager Z1 (Carl Zeiss, Germany).

Myelin isolation and purification

Myelin was isolated using two rounds of a discontinuous sucrose gradient, adapted from a previous protocol developed by Norton and Poduslo (1973). For these experiments we used brains and the thoracic portion of the spinal cord from 18-month old WT and KO mice. It was important that all solutions and materials were kept on ice. The brains and thoracic spinal cord were homogenized separately in ice cold 0.32 M sucrose using a mechanical overhead stirrer (Heidolph, Fisher Scientific,

North America) followed by a centrifugation to clean the homogenate from tissue debris. In a tube, the supernatant was carefully layered over a layer of 0.85 M sucrose, and centrifuged at 75,000g, using a swingout rotor (SW 31 TI, Beckman ultracentrifuge, North America), for 45min at 4°C. A layer of crude myelin was formed at the interface of the two sucrose solutions. Myelin was removed using a glass pipette, transferred into an ice cold 26.3 ml tube and filled with ice cold distilled water to purify the crude myelin by osmotic shock. Afterwards, the tubes were centrifuged at 20,000g in a fixed angle rotor (70.0 TI, Beckman ultracentrifuge, North America) for 15min at 4°C. Supernatant was discarded, and the pellet was resuspend in 0.32 M sucrose, and again layered on a new layer of 0.85 M sucrose and centrifuged as described above. A second osmotic shock was performed, after centrifugation, the pellet was resuspend in distilled water and transferred to a 1.5 ml tube and centrifuged at 21,000g at 4°C for 15min. The myelin pellet was kept at -80°C with only a drop or two of water covering the pellet to prevent lyophilization.

Western blot

Isolated myelin from brain and spinal cord was sonicated with PBS containing 1.5% SDS, 0.5% sodium carbonate (Na_2CO_3) and protease inhibitor cocktail (Roche). The samples (corresponding to 2 µg of total protein) were separated by SDS-PAGE gels using TGX Stain-Free™ FastCast™ 12% Acrylamide (Bio-Rad) and analyzed using a ChemiDoc imaging system before transferring the proteins onto nitrocellulose membranes. Before incubation with antibodies, the membranes were blocked with 5% skim milk (Fluka) in Tris-buffered saline with 1% Tween 20 (TBS-T) for 1h at RT. After washing with TBS-T, the membranes are probed with different primary antibodies (as shown in TABLE 1) diluted in 5% BSA + TBS-T (overnight at 4°C). HRP-labeled secondary antibodies were diluted 1:5000 in 5% skim milk in TBS-T and incubated for 1h at RT. Membranes were developed using ECL (Luminata Crescendo Western HRP Substrate, Milipore). Using Quantity One 4.6.9 software it was possible to scan, using a Molecular Imager GS800, and quantify the blots for further analysis.

TABLE 1 - LIST OF ANTIBODIES USED FOR WESTERN BLOT.

Antigen	Dilution	Host	Company
MBP	1:10,000	Goat	Santa Cruz
TPPP	1:10,000	Rabbit	Abcam
SEPT7	1:2000	Rabbit	Proteintch
14-3-3	1:1000	Rabbit	Cell Signaling
MAG	1:1000	Rabbit	Santa Cruz
PLP/DM20	1:800	Rabbit	Abcam

Statistical analysis

All data were analyzed using GraphPad Prism software, and results are expressed as mean ± S.E.M. To compare two different groups, the student's T-test was used and *P < 0.05, ** P < 0.005, *** P < 0.001 were considered as significant different.

CHAPTER III – *IN VITRO* ASSAY OF CNS MYELINATION

Background

Previous studies using co-cultures of dorsal root ganglia (DRG) neurons and Schwann cells observed fewer and smaller myelin segments of Schwann cells in Gnpat KO (KO) compared with WT mice (da Silva et al., 2014). Some other studies performed in our group have shown that plasmalogen deficiency compromises myelination in spinal cord and leads to a late onset demyelination. They observed a decrease in the number of myelinated fibers (FIGURE 6A), as well as, a severe demyelination with drastic reduction in myelin thickness in spinal cord from 1.5 year old KO mice when compared with WT mice. The results also show a decrease in the number of myelinated fibers (FIGURE 6B), and an increase in the number of demyelinating fibers in KO mice. Knowing this, we wanted to observe this phenotype *in vitro* to understand the relation between the neurons and the oligodendrocytes, and how a deficiency in plasmalogens affects the differentiation of oligodendrocytes. Oligodendrocytes differentiation starts from precursor cells, then progenitors and mature cells that lead to myelinating oligodendrocytes (Baumann and Pham-Dinh, 2001). Using MBP as a marker, it is possible to identify the final stages from oligodendrocyte differentiation, allowing us to compare their morphology and evolution in WT and KO mice.

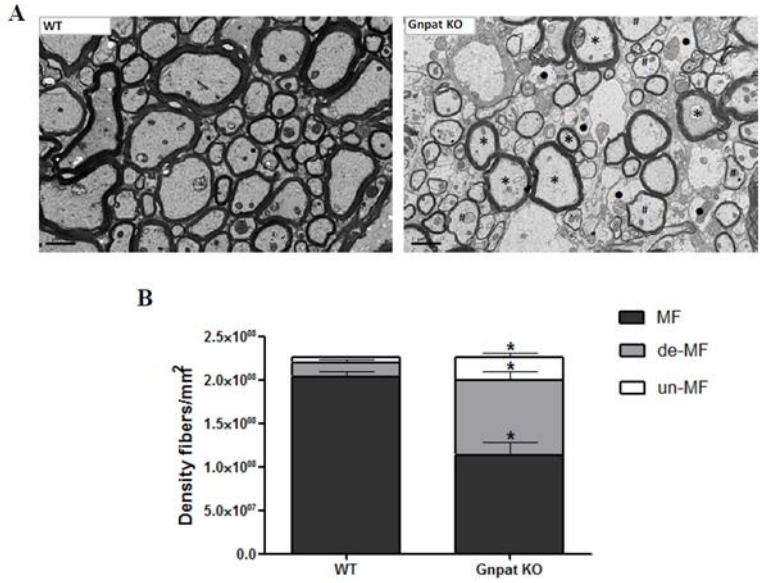

FIGURE 6 - ULTRASTRUCTURAL ANALYSIS OF SPINAL CORDS FROM 1.5 YEAR OLD GNPAT KO MICE. A| Electron micrographs of spinal cords of aged mice, revealed a decrease in the number of myelinated fibers, as well as a severe demyelination with drastic reduction in myelin thickness. B |morphometric analysis revealed a decrease in the number of myelinated fibers (MF), and an increase in the number of demyelinating fibers in KO mice (de-MF), and an increase in fibers lacking myelin (un-MF). Bars: 2µm. *$P < 0.05$. Adapted from Malheiro, 2012.

Results

To setup an *in vitro* myelination assay we used a co-culture system of neurons and glial cells isolated from the cortex of embryonic mice at E17 (17 days of gestation) (Watkins et al., 2008; Lariosa-Willingham et al., 2016). As described in the material and methods section, the isolated cells from cortices of either WT or Gnpat KO mice were plated and initially cultured for 5 days *in vitro* (DIV5). During this period, neurons extend their axons and dendrites, and glial cells divide and populate the culture. At DIV6 we started the induction of oligodendrocyte differentiation by changing the culture medium to myelination medium, which lacks platelet-derived growth factor (PDFG) and fibroblast growth factors (FGF) to induce differentiation. The co-culture was maintained in myelination medium for an additional 15DIV.

To evaluate the extent of myelination in the co-cultures from WT and Gnpat KO mice, cells were fixed at 20DIV and cortical neurons and oligodendrocytes were immune-stained using antibodies against β-III tubulin (to visualize neurons) and MBP (to visualize differentiated oligodendrocytes). Under the microscope we observed that it was possible to clearly identify four types of oligodendrocytes, i.e., oligodendrocytes with low MBP expression, oligodendrocytes with high MBP expression, myelinating oligodendrocytes and degenerating oligodendrocytes (FIGURE 7). Cells with low MBP were highly branched and still immature, while cells presenting high MBP start to lose most of the extensions and prepare to myelinate the axons. Myelinating cells, as the name implies, already showed some myelin segments and fragmented cells with abnormal morphology were considered degenerating cells.

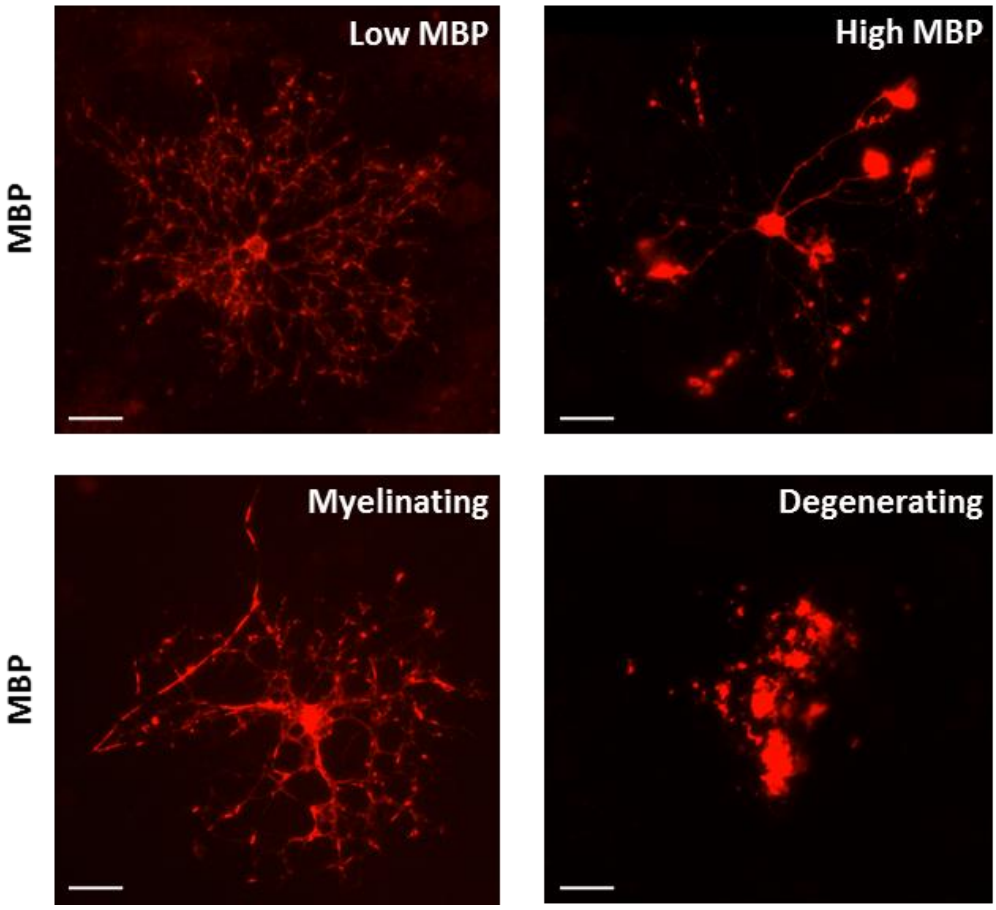

FIGURE 7 - DIFFERENT TYPES OF OLIGODENDROCYTES. Oligodendrocytes were divided into four categories depending on their morphology, the intensity of MBP expression and the presence of myelin segments. Cells with low MBP are highly branched and are starting to produce MBP. Cells with high MBP start to lose some extensions and the presence of MBP is higher, enabling the formation of myelin sheaths that can be observed in myelinating cells. Degenerating cells are fragmented and have abnormal morphology. Scale bar: 20µm.

The analysis of immunostained co-cultures from WT and Gnpat KO evidenced that the co-culture of Gnpat KO cells had a higher number of oligodendrocytes with high levels of MBP after 20 days *in vitro* (DIV20) when compared to WT. In KO co-cultures, the number of oligodendrocytes that presented myelin sheaths was very low when compared with the WT co-culture. To show that there really were differences in the amount of high MBP cells and cells with myelin segments, we proceeded to quantify the number of cells for each category, for both WT and Gnpat KO co-cultures (FIGURE 8). The quantification revealed a significant increase of oligodendrocytes with high levels of MBP cells in Gnpat KO cultures when compared to WT. Importantly, we observed a decrease in the percentage of

myelinating oligodendrocytes in Gnpat KO cultures, which indicates the importance of plasmalogens for the process of myelination. No significant differences were observed in oligodendrocytes expressing low levels of MBP or in degenerating oligodendrocytes. In sum, these results confirm previous *ex vivo* studies where a loss of myelinating fibers were observed in spinal cord, and provide the supporting evidence that plasmalogens are important for the formation of myelin sheaths. In addition, these results indicate that plasmalogens are not necessary for the initial stages of oligodendrocyte differentiation.

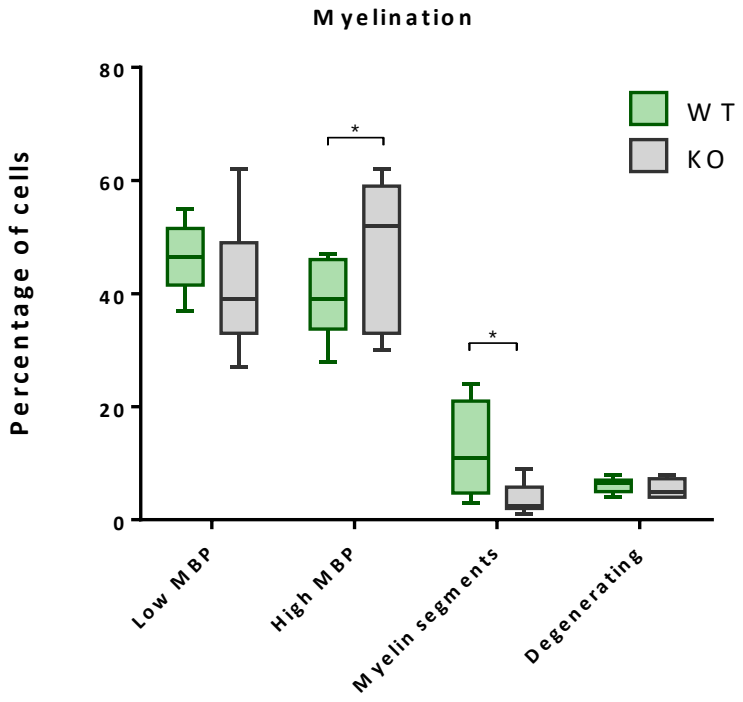

FIGURE 8 – QUANTIFICATION OF EACH TYPE OF OLIGODENDROCYTE ON CO-CULTURES FROM WT AND GNPAT KO MICE. The percentage of cells for each category for WT and Gnpat KO, evidence a significant increase in cells with high MBP. On the other hand, there is a significant decrease in cells with myelin segments for Gnpat KO mice. Low MBP and degenerating cells show no significant differences. Graphs are presented as mean + minimum and maximum of all data. *$P < 0.05$.

Discussion

Myelin is highly enriched in plasmalogens, suggesting that defects of this ether-glycerophospholipids may lead to an abnormal formation of myelin. Until now several studies have used animal models for RCDP, enabling the investigation on how a deficiency in plasmalogens affects the CNS and the formation of myelin sheaths. Our group showed a decrease in myelinated nerve fibers in KO mice and a late onset of demyelination.

Many studies have been done *in vivo*, but almost none were done *in vitro*, which would facilitate the determination on how plasmalogens affect oligodendrocytes and myelin assembly. Co-cultures of neurons and oligodendrocytes were performed and myelination was induced by adding medium which lacks platelet-derived growth factor (PDFG) and fibroblast growth factors (FGF) to induce differentiation. We were able to make this *in vitro* co-culture assay work, which is better than the *in vitro* assay using only oligodendrocytes plated onto a dish (Barateiro and Fernandes, 2014). The results obtained with this co-culture assay enabled us to validate the *in vivo* results previously obtained in our group, and clearly demonstrate that a deficiency in plasmalogens affects myelination. After quantification, we verified that the deficiency in plasmalogens does not cause a defect in oligodendrocyte differentiation, what we observed was that the rate of maturation seems to be halted during the final stage of differentiation, i.e., the production of myelin sheaths. Therefore, a higher number of oligodendrocytes with high MBP expression is observed in KO, i.e., they are able to differentiate into mature oligodendrocytes, but myelination is halted. We also showed that a defect in plasmalogens does not seem to affect oligodendrocyte survival, which corroborates the *in vivo* results. In summary, plasmalogen deficiency seems to specifically impair the final stages in myelin formation in differentiated oligodendrocytes.

CHAPTER IV – CANDIDATE THERAPIES

Background

As shown in the previous chapter (chapter III), we observed a decrease in the ability of oligodendrocytes devoid of plasmalogen to myelinate axons of cortical neurons. The development and validation of this *in vitro* assay allows us to develop and test potential therapeutic agents capable of restoring myelination. Previous studies have shown that it is possible to increase plasmalogen concentrations by adding an alkyl-glycerol (AG) *in vivo* and *in vitro* (Zoeller et al., 2002; Brites et al., 2011). Alkyl-glycerols serve as alternative precursors in the biosynthesis of plasmalogens since they already contain an alkyl-bond and they enter the biosynthesis at the first ER step (see FIGURE 5). Previous studies used either 1-O-hexadecylglycerol or 1-O-octadecylrac-glycerol, as alkyl-glycerols. However, the *in vivo* studies using 1-O-octadecylrac-glycerol, showed that this alkyl-glycerols could not rescue plasmalogen levels in nervous tissue (Brites et al., 2011). The development of an *in vitro* system for myelination allowed us to test other alkyl-glycerols. In the present study, we used 1-O-tetradecyl-glycerol (TDG) in our cell cultures, to determine if this alkyl-glycerol would be able to rescue myelination in co-cultures from Gnpat KO mice.

Previous studies showed defects in AKT-mediated signaling due to a deficiency in plasmalogens (da Silva et al., 2014), and therefore, we also aimed to test compounds that could restore AKT-mediated signaling and rescue cellular defects. Following this aim, we tried to use SC79 in our *in vitro* co-cultures. SC79 is an activator of AKT, that bypasses the need of plasmalogens (da Silva et al., 2014). However, we were not able to have enough samples to accurately analyze the efficiency of this compound.

Results

Using our validated *in vitro* system, we assayed the ability of TDG in rescuing the myelination defect of Gnpat KO oligodendrocytes. We followed the same protocol and strategy, and at DIV6 with the change to myelination medium we also added 7 μM TDG to medium to treat WT and Gnpat KO cultures with this alkyl-glycerol. To WT and Gnpat KO control-cultures we added ethanol, which was the vehicle used to dissolve TDG.

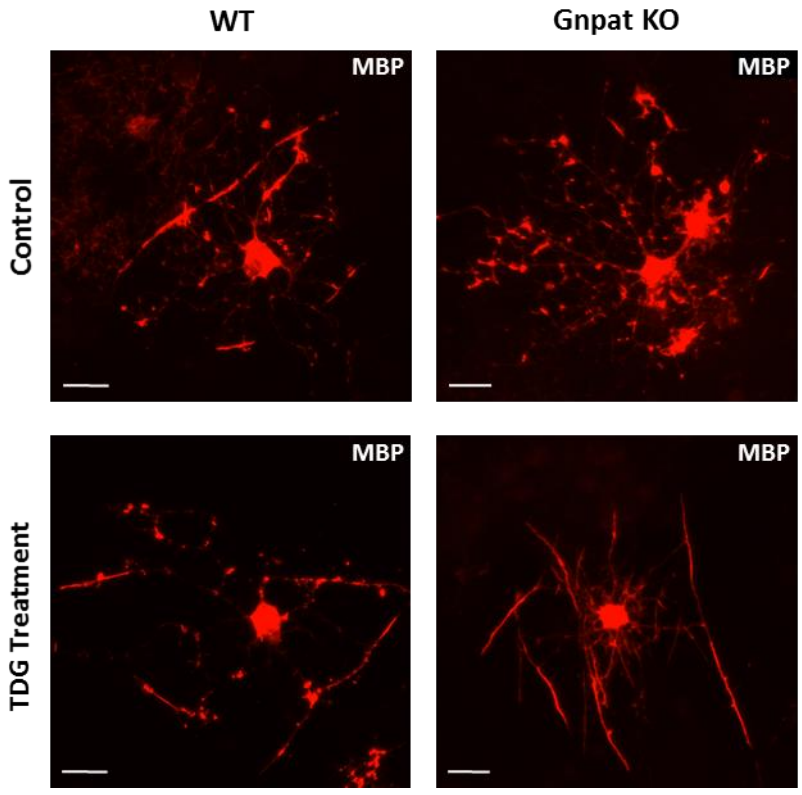

FIGURE 9 - MORPHOLOGICAL COMPARISON OF MYELINATING OLIGODENDROCYTES AFER TREATMENT WITH 1-O-TETRADECYL-GLYCEROL (TDG). Analysis of the myelin segments formed in the different cells (WT and KO) in not treated and treated cells with TDG, evidence a decrease in the length of the myelin segments in KO mice in non-treated cells compared to WT. After treatment, the length and number of myelin segments are visibly increase in KO, and no difference was seen in the number of myelin segments in WT cells treated with TDG. Scale bar: 20μm.

After DIV20, cells were fixed and processed for immunostaining (FIGURE 9) and we quantified the number of oligodendrocytes in each category (FIGURE 10), for both control *versus* TDG, and WT *versus* Gnpat KO co-cultures. From analysis of the immunostaining with an antibody against MBP, it was clear that the lack of plasmalogens in Gnpat KO co-cultures impaired myelination, as mutant

oligodendrocytes differentiated (high levels of MBP), but were unable to form normal myelin segments. However, the treatment with TDG was beneficial. In Gnpat KO co-cultures, we could easily identify oligodendrocytes with myelin segments that were normal in terms of number and size (FIGURE 9). These results indicate that TDG could be used to rescue myelination in Gnpat KO mice.

Observing our results (FIGURE 9), we can see a difference in the number of myelin sheaths and in their length in non-treated cells, i.e., KO oligodendrocytes present a decreased number of segments, comparing with WT cells. Treatment with TDG was able to increase the number and length of the segments compared to the control KO.

To further validate the beneficial effects of TDG we quantified 4 different oligodendrocyte categories (FIGURE 10). In control-treated co-cultures from Gnpat KO mice (FIGURE 10A) we observed a decreased number of oligodendrocytes with low MBP levels, an increase in the number of oligodendrocytes with high MBP levels, a decreased number of myelinating oligodendrocytes and increased number of degenerating oligodendrocytes. After the TDG treatment (FIGURE 10B), the number of oligodendrocytes with low and high MBP were similar and showed no significant differences when compared to oligodendrocytes from WT co-cultures.

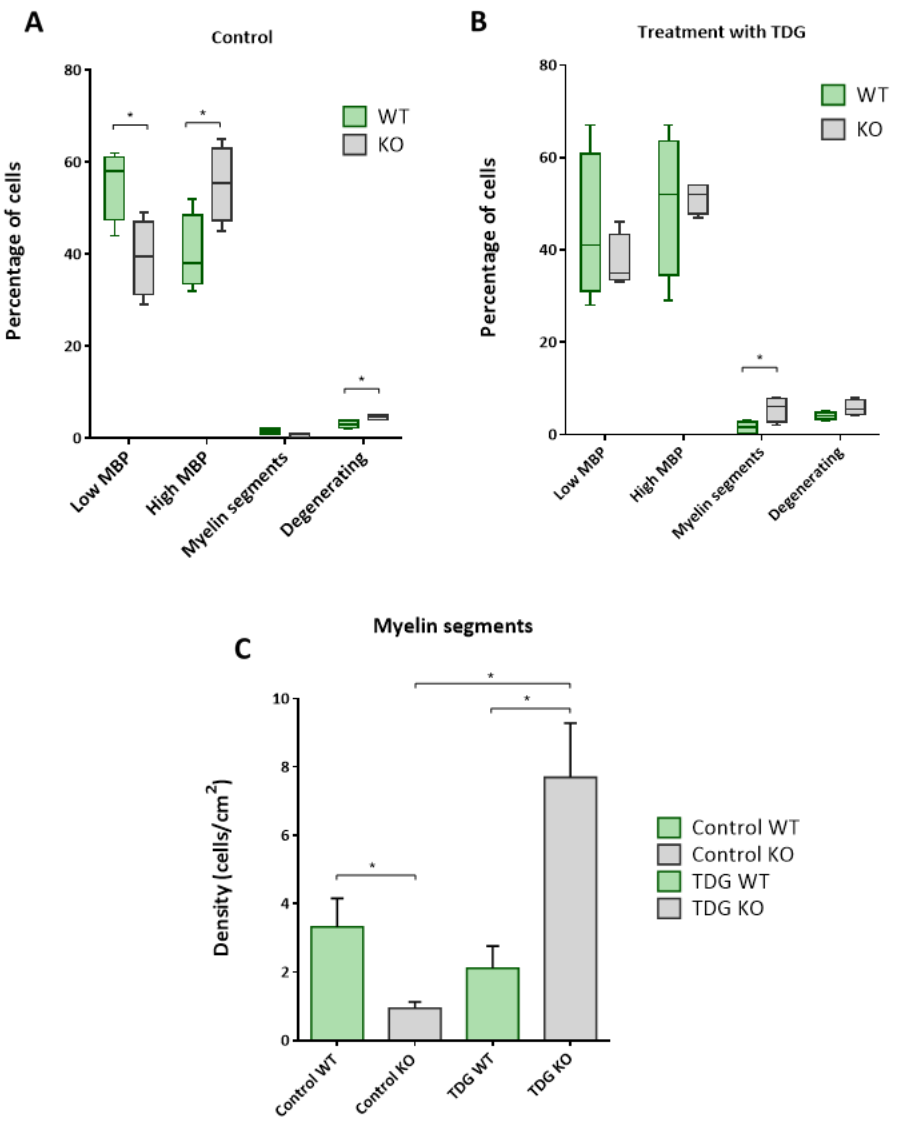

FIGURE 10 - QUANTIFICATION OF OLIGODENDROCYTES IN WT AND KO CELLS WITH AND WITHOUT TDG TREATMENT. A and **B|** Percentage of cells for each category of oligodendrocytes. **A|** Results for control (not treated) cells show a decrease in the percentage of oligodendrocytes with low MBP, however, for high MBP there is an increase in KO cells. A significant increase was also observed for degenerating cells. **B|** After treatment, the number of cells with low and high MBP is similar and show no significant differences. The same is seen for degenerating cells. On the other hand, there is a significant increase in KO cells with myelin segments. **C|**Comparing the density of cells with myelin segments in the different conditions, we observed that there is a significant increase of cells with myelin segments after treating KO cells (TDG KO) compared to control KO. We also showed a significant decrease in cells with myelin segments in control KO when compared with control WT. Graphs **A** and **B** are presented as mean + minimum and maximum of all data. Graph C is presented as mean + SEM. **P* < 0.05.

Validating the beneficial effect of TDG, the number of oligodendrocytes with myelin segments increased in TDG-treated Gnpat KO co-cultures, indicating that TDG could induce and restore myelination (FIGURE 10B). Similar results were observed if the analysis did not take into consideration the entire population of oligodendrocytes (the global quantification of all oligodendrocyte types, expressed as percentage), but only the quantification of oligodendrocytes with myelin segment (FIGURE 10C).

The treatment with TDG was able to restore the defect in myelination in Gnpat KO co-cultures, but it did not affect myelination in WT co-cultures (FIGURE 10). However, in Gnpat KO co-cultures the density of oligodendrocytes with myelin segment was greater than that of WT co-cultures. This may be explained by the fact that WT cells may not utilize TDG, given that they follow the normal plasmalogen biosynthesis from the endogenous precursor DHAP. As such, supplying other alternative precursor may not cause an additive effect. In KO cells, the complete lack of plasmalogens causes a massive usage of the alternative precursor with stimulation of the biosynthesis. The arrest in oligodendrocyte maturation at the level of formation of myelin, must be released upon the treatment with TDG, causing the observed increase in the number of oligodendrocytes with myelin segments.

Discussion

Previous results (Brites et al., 2011) showed that 1-0-octadecylglycerol was not effective in the nervous system, presenting a minimal increase in plasmalogen levels in cerebrum of PEX7 KO mice, and no increase was observed in spinal cord. The results observed in the central nervous system, might be due to the blood-brain barrier that can lead to the inability of 1-0-octadecylglycerol to normalize plasmalogen levels. Zoeller et al. (2002) was able to increase cellular levels of plasmalogens in cultures human pulmonary arterial endothelial cells (PAEC) by adding sn-1-O-hexadecylglycerol to the medium. However, it is still not proven that supplementing cultures with an alkyl-glycerol is able to increase the levels of plasmalogens in the central nervous system, and therefore restore myelination.

The development of an *in vitro* myelination assay allows a faster testing of potential therapeutic approaches, which are time consuming to do *in vivo* and have higher costs. For our experiments, we used 1-O-tetradecyl-glycerol (TDG) as an alternative precursor for plasmalogens. After treating the cells, a significant increase was observed in the percentage of cells with myelin segments. In the previous chapter, we observed that oligodendrocytes from KO mice have a defect in the formation of myelin sheaths. The same was observed in control-treated cells, but after treatment no differences were observed for cells with high MBP between WT and KO, meaning that TDG was able to induce mature oligodendrocytes to produce myelin, and showed for the first time that myelination could be restored in the brain using an alternative plasmalogen precursor. The percentage of cells with myelin segments in KO co-cultures without treatment, showed again the impact that plasmalogen deficiency has on myelination, but the treatment with TDG improved the extent of myelination showing that this treatment could be a good option for a possible therapy. However, these are preliminary results and it is now possible to move to *in vivo* tests using TDG. Our data also showed that the addition of alternative precursors has no effect in normal cells, i.e., WT co-cultures are not able to take in TDG, since they have a normal production of plasmalogens from the endogenous precursor, i.e., DHAP.

CHAPTER V – CHARACTERIZATION OF MYELIN

Background

Jhan et al. (2009) was able to identify more than 300 proteins in purified CNS myelin using mass spectrometry (MS). Many of these proteins are present in low abundance and are involved in cell adhesion, vesicular trafficking and the cytoskeleton. Other studies presented some examples of proteins present in isolated myelin from human and mice brains. They identified proteins like MBP, PLP/DM20, MAG, MOG and CNP (Ishii et al., 2009; Thakurela et al., 2016). Our work verified that there is a defect in myelination caused by the deficiency in plasmalogens, so our next step was to see how myelin is affected by the lack of plasmalogens in terms of protein composition. Therefore, we analyzed using western blot the expression of myelin proteins in myelin isolated from aged WT and Gnpat KO mice. This analysis could give an inside view of the protein regulation in KO mice during the active demyelination that is observed in aged KO mice. In this experiment we quantified myelin associated glycoprotein (MAG), myelin basic protein (MBP), septin 7 (SEPT7), tubulin polymerization-promoting protein (TPPP), and 14-3-3 (a family of conserved regulatory molecules) in myelin isolated from brain samples, and proteolipid proteins (PLP/DM20), MBP and TPPP in myelin isolated from spinal cord samples.

Results

Myelin was isolated from brain and spinal cord of old mice. Western blots were performed using TGX Stain-Free™ FastCast™ 12% Acrylamide gels, which allow stain-free visualization and identification of total proteins present in the samples, with stain-free enabled imaging systems like ChemiDoc (FIGURE 11A and FIGURE 12A). This enables us to normalize the results obtained for individual proteins. Total protein was chosen to normalize the results, since no differences were observed in the gel. Our results for the brain samples show a decrease in the amounts of MAG, MBP and SEPT7 and no differences in TPPP and 14-3-3 (FIGURE 11B). After normalizing the quantifications of these proteins with total protein (FIGURE 11C), we can see that for MAG, MBP and SEPT7 there is a significant decrease in KO mice. This can be compared to the decrease observed in the number of myelinating cells presented in the previous chapters, showing that deficiency in some myelin proteins lead to a defect in myelin assembly. MBP is the protein responsible for myelin compaction, and the results show that this protein has a more significant decrease in KO compared to the other proteins. TPPP is expressed in myelinating oligodendrocytes and was shown to be necessary during differentiation of oligodendrocytes. We evidenced in chapter III that the differentiation of the oligodendrocytes is not affected, what we saw was a slower maturation of oligodendrocytes. This would explain why the quantity of TPPP does not differ from WT and KO mice. In spinal cord, we observed a decrease in the amount of MBP, but PLP/DM20 and TPPP do not evidence differences between WT and KO (FIGURE 12B). After normalizing with total protein (FIGURE 12C), MBP shows a significant decrease for KO mice. PLP/DM20 show a tendency to decrease in KO mice, although, this decrease is not significant. In brain myelin we showed that TPPP did not change between WT and KO, and the same was observed in myelin from spinal cord samples.

Overall, these results suggest that a differential deficiency in different types of myelin proteins may cause or contribute to the demyelination. In this study we only presented a small number of proteins identified in purified myelin, and it would be interesting to analyze other proteins and see how they are affected by a deficiency in plasmalogens. This could help in determining if a specific dysregulation in expression of a myelin protein contributes or causes the cascade of myelin loss that causes demyelination. Nevertheless, the results obtain may suggest that the loss of MBP, may modulate the progression of demyelination given that MBP is important for the compaction and maintenance of myelin.

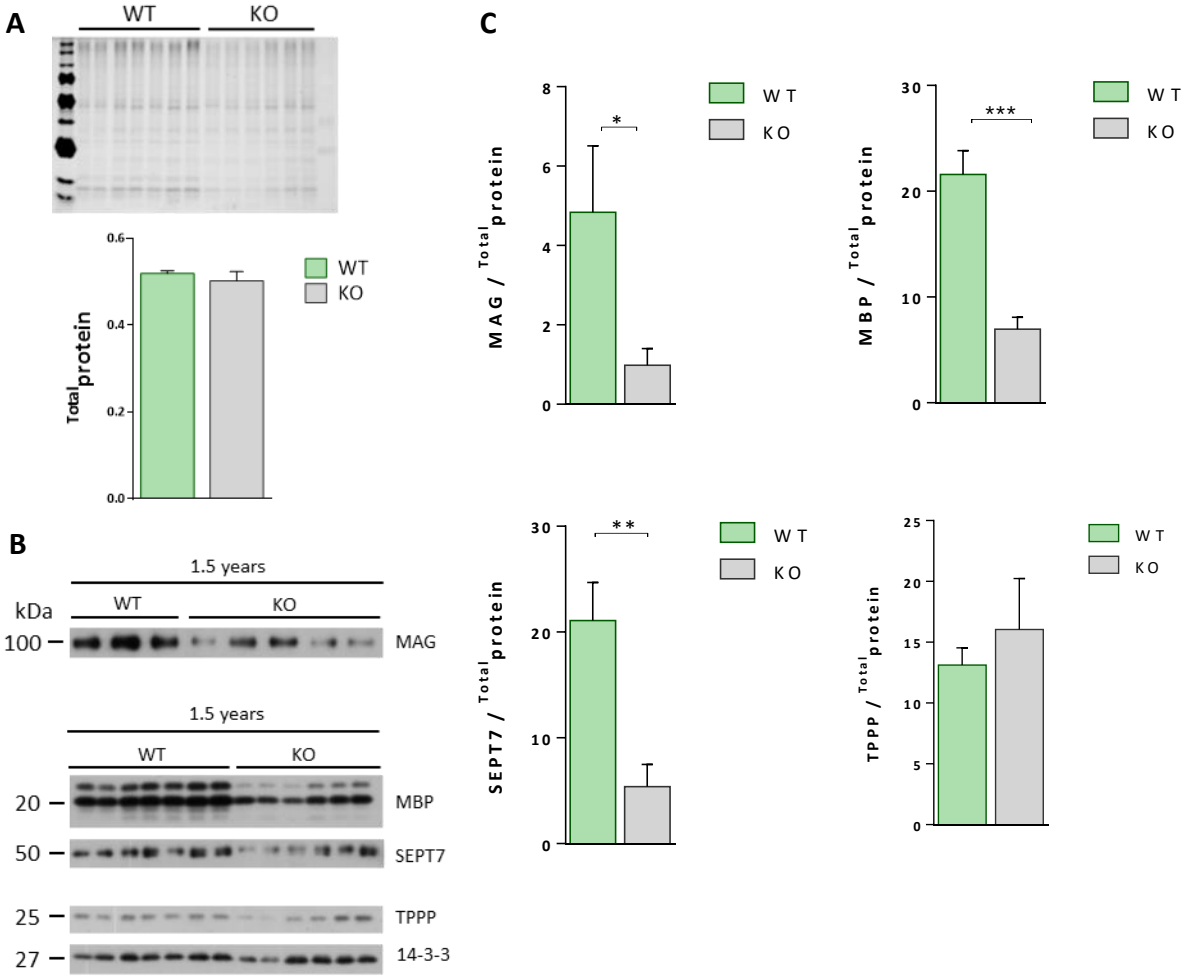

FIGURE 11 - QUANTIFICATION OF MYELIN PROTEINS IN BRAIN SAMPLES. A| Image and quantification of total protein from myelin isolated from brains of old mice (WT and KO) using ChemiDoc and ImageJ. No differences were observed in the amount of total myelin protein between WT and KO mice. **B|** Western blot analysis of brain-myelin from WT and Gnpat KO mice. Some of the proteins analyzed were MAG, MBP, SEPT7, TPPP and 14-3-3. **C|** Quantification of protein levels normalized to total myelin protein amount. A significant decrease was observed for MAG, MBP and SEPT7, and no differences were observed for TPPP. Graphs are presented as mean + SEM. *$P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

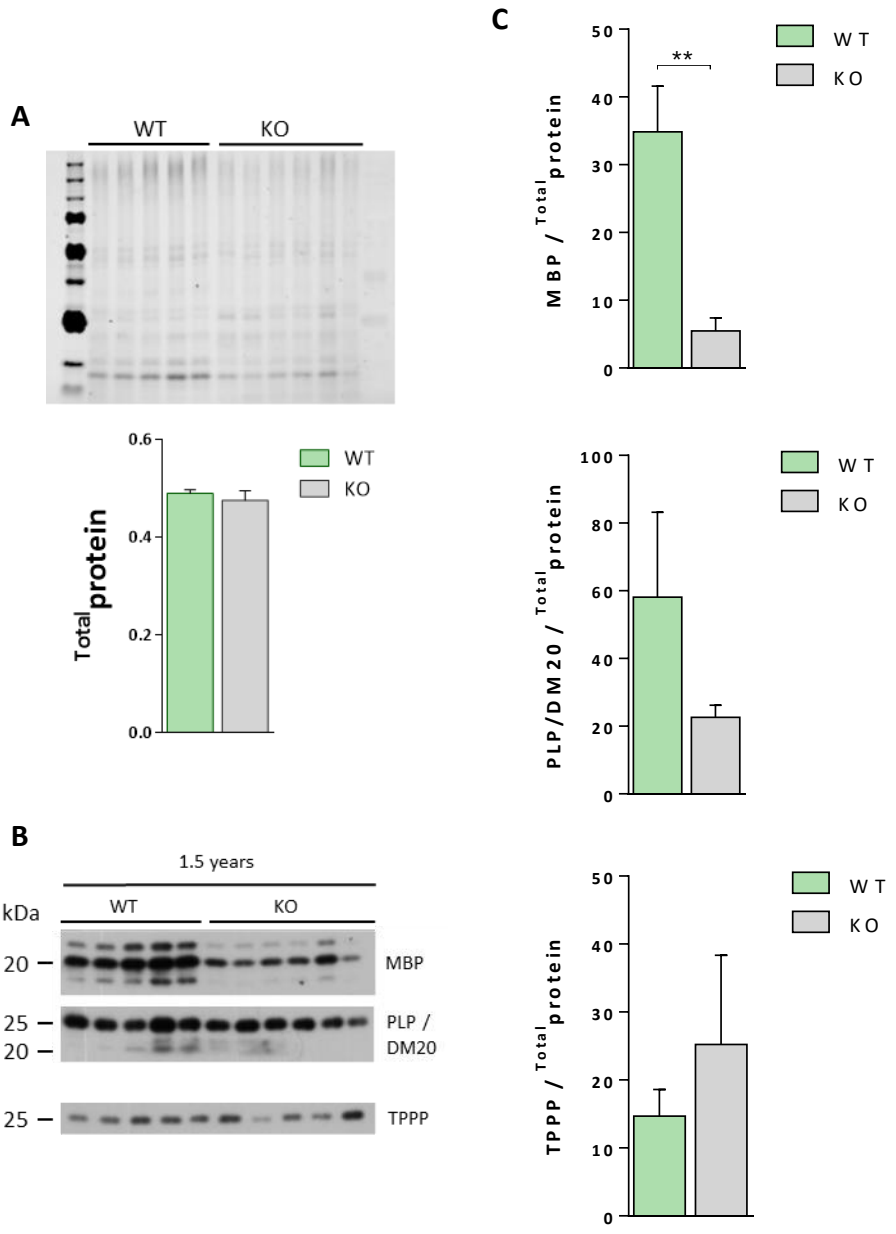

FIGURE 12 - QUANTIFICATION OF MYELIN PROTEINS IN SPINAL CORD SAMPLES. A| Image and quantification of total protein in spinal cord from brains of old mice (WT and KO) using ChemiDoc and ImageJ. No differences were observed in the amount of total myelin protein between WT and KO mice. **B|** Western blot analysis of spinal cord myelin for WT and Gnpat KO mice. Some of the proteins analyzed were PLP/DM22, MBP and TPPP. **C|** Quantification of protein levels normalized to total myelin protein amount A significant decrease was observed only for MBP, and no differences were observed for PLP/DM22 and TPPP. Graphs are presented as mean + SEM. $^{**}P < 0.005$.

Discussion

The demyelination observed in the previous chapters, caused by the lack of plasmalogens, was studied further to determine if a specific loss of myelin component(s) could explain the onset of demyelination ant its progression. Until now, nothing is known about how a deficiency in plasmalogens affects myelin proteins. This could give an important insight of why the cells cannot form myelin sheaths, or why once assembled myelin undergoes a process of degeneration causing a generalized demyelination throughout the CNS. There are some studies that validated the presence of hundreds of proteins in purified myelin in human and mouse brain (Jhan et al., 2009). We examined the expression of 12 proteins known to be present in myelin. The group of proteins studied contained myelin-specific proteins (e.g. MBP, MAG, PLP/DM20 and CNP) as well as, proteins expressed by myelinating oligodendrocytes (e.g. 14-3-3, TPPP, Septins 2 and 7, ATP1A1, ADAM22, SIRT2 and, tubulin β-IVA). This last group of proteins besides being expressed in myelin they are also expressed in other cells, and this is the reason we isolated myelin from WT and Gnapt KO mice. This approach allowed us to specifically measure expression differences in myelin. We present results for MAG, MBP, TPPP, PLP/DM20, SEPT7 and 14-3-3, which validate that a deficiency in plasmalogens only affects a subset of myelin proteins. The preliminary results show normal levels of TPPP and PLP/DM20, and decreased levels of MBP, MAG and SEPT7, which may indicate that their loss could trigger demyelination. MBP is the most abundant protein in myelin sheaths in normal CNS, and functions as a strong adhesion molecule to allow compaction of the myelin layers. Some studies have shown that a decrease of MBP triggers demyelination due to the loss of its interaction with the myelin membrane (Gendelman et al., 1985; Weil et al., 2016). MAG is important for communication between the oligodendrocyte and the neuron, more specifically the axon. Previous studies have shown that for some diseases, like multiple sclerosis, ischemia and progressive multifocal leukoencephalopathy, demyelination is characterized by the loss of MAG (Gendelman et al., 1985; Aboul-Enein et al., 2003). TPPP was one of the proteins that showed no differences between WT and KO. This protein is important for the differentiation of oligodendrocytes, and as we showed in our co-cultures, no differences were observed during differentiation. PLP/DM20 is the second most abundant protein in compact myelin and even though it has a role in myelin compaction, Klugmann et al., (1997) showed that in the absence of PLP, oligodendrocytes are still able to myelinate. Our results show that in spinal cord, no significant

differences in PLP were observed between WT and KO mice, although there is a tendency for a decrease in KO mice. We compared myelin isolated from brain and spinal cord. These two regions of the CNS show loss of myelin in KO mice. The results indicate a common mechanism for myelin loss, given a similar pattern of loss of myelin proteins and normal expression of the remaining proteins.

CHAPTER VI – FUTURE PROSPECTS

We were able to develop an *in vitro* myelination assay, which allowed us to show for the first time a rescue of the levels of plasmalogens, and therefore restore myelination. With these results it is possible to start testing 1-O-tetradecyl-glycerol *in vivo*. Given our results, it is still not known how exactly a lack of plasmalogens affect myelination. Lee et al. (2012) developed a neuron-free culture using polystyrene nanofiber plates that can be produced with different diameters (4 µm for non-aligned fibers, and 2 µm for aligned fibers; produced by The Electrospinning Company Ltd), which work as an artificial scaffold for myelination. The use of these nanofibers could simplify the *in vitro* system, given that the nanofibers resemble axons, and there is no need to keep neurons alive for long periods of time. Preliminary results already indicate that this approach will be advantageous.

To characterize myelin, we analyzed a low number of myelin proteins, thus the number of proteins should be increased to get a better idea of which myelin components are preferentially lost or degraded, leading to myelin instability and consequently demyelination.

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