Chapter 1

Myelin Biogenesis in Health and Disease; Involvement of Major Myelin Lipids and Proteins

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Introduction

1. Myelin Biogenesis

In the central nervous system (CNS), oligodendrocytes (OLGs) produce sheet-like extensions at their plasma membrane, the myelin membranes, which ensheath axons in a multilamellar fashion and consist of myelin specific proteins and a distinct set of galactolipids [1–3]. Myelination is essential for proper saltatory nerve conduction, i.e., rapid transmission of nerve impulses, a process which is irreversibly interrupted in neurological diseases such as multiple sclerosis (MS). Myelin biogenesis in vivo requires tightly regulated sequential events in space and time [1–3]. In this context, OLGs need to go through series of sequential events such as proliferation, migration, differentiation and maturation to synthesize the myelin sheaths. In order to produce the unique ultrastructure of myelin, OLGs require carefully organized myelination machinery, involving a tight regulation of sorting and trafficking of major myelin lipids and proteins in space and time.

1.1. From oligodendrocyte progenitor cells to myelin forming oligodendrocytes

Unlike other cell types, OLGs only have proliferative and migratory properties during early development, i.e., when they are still defined as 'early oligodendrocyte progenitor cells' (OPCs) [2]. OPCs migrate actively from multiple origins in the CNS to reach newly forming white matter where they will stop proliferating and start differentiating by synthesizing myelin specific components (Fig. 1, [2,4]). In consideration of sequential expression of myelin lipids and proteins, the proliferation and differentiation time-line has been well characterized in cultured OLGs by identifying and defining stage-specific lipid markers at the cell surface, followed by the expression of mature myelin proteins myelin basic protein (MBP) and proteolipid protein (PLP) [4,5]. Notably, OLGs are able synthesize myelin-like membranes in vitro called 'myelin sheets' [6] in the absence of neurons, following a similar proliferation and differentiation time-line. For example, the migratory early OPCs are characterized by surface expression of specific gangliosides, like GD3, and ganglisides that are recognized by antibody A2B5 [4]. Upon further differentiation, early OPCs transform into late OPCs, morphologically featuring a few primary processes extending from the cell body, and are further characterized by the surface expression of another lipid marker, the galactolipid sulfatide (recognized by the O4 antibody). Late OPCs are still proliferative, but not migratory. Upon further differentiation, the gangliosides

disappear from the surface, and another galactolipid, galactosylceramide (GalC), appears (recognized by the O1 antibody). These immature OLGs have lost their proliferative capacity, and undergo dramatic morphological changes, displaying an appearance of secondary and branched processes. Immature OLGs also start to express the myelin specific protein 2', 3'-cyclic-nucleotide 3'-phosphodiesterase (CNP). Immature OLGs eventually reach the fully differentiated stage, where they synthesize 'myelin sheets' in vitro, which are membranous extensions that are continuous with the primary processes. These mature OLGs synthesize myelin-specific proteins, including PLP and MBP, neurofascin155 (NF155), myelin associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG) [2,3]. In *vivo*, the growing edge of the myelin membrane, called 'inner tongue', makes contact with the axons which are then further enwrapped by myelin membranes in a multilamellar fashion. When the enwrapping is complete, which depends among others on the diameter of the axon, compaction of the myelin membranes will take place starting from the outer tongue going through the inner tongues of the myelin membranes, leading to the formation of compact internodes and non-compact adaxonal, abaxonal and paranoal subdomains ([7], described in more detail in chapter 2).

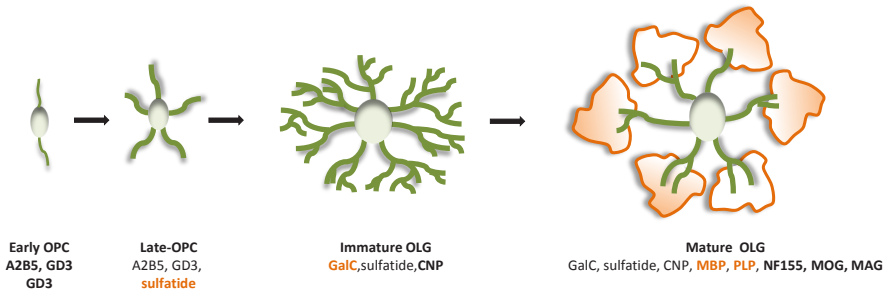

Figure 1: A schematic illustration of in vitro oligodendrocyte development.
Oligodendrocyte (OLG) progenitor cells (OPCs) are proliferative (early and late OPCs) and migratory (late OPCs) displaying a few processes, and stage-specific lipid surface markers. As OPCs mature, they elaborate more and branched processes (depicted in green) reaching the immature OLG stage. Immature OLGs express in addition to sulfatide also GalC at their surface, and myelin proteins such as CNP. When they reach the fully mature form (mature OLGs), they synthesize myelin sheets (depicted in orange), the in vitro equivalent of myelin sheaths, and myelin specific proteins such as PLP, MBP, NF155, MAG and MOG.

1.2. Trafficking of major myelin proteins in oligodendrocytes

Similar to epithelial cells, OLGs display a polarized nature, containing two distinct membrane surfaces, the plasma membrane surrounding the cell body, target of apical-like trafficking and the myelin membrane, enriched in cholesterol and galactolipids, which is targeted by basolateral-like trafficking [1,8,9]. In addition, myelin lipids

and proteins are organized in a distinct way, i.e., proteins and lipids are sorted and transported to compact and/or non-compact myelin, which implies that myelin membranes as such also display 'polarized' properties [1]. The exact transport mechanisms of major myelin proteins are just emerging [8,9], and suggest direct and indirect (transcytotic) transport routes, the latter reflecting polarized apico-basolateral trafficking.

The peripheral membrane protein MBP is the only known structural protein that is imperative for myelin biogenesis. MBP consists of several prenatal (embryonic) and postnatal isoforms, which are expressed in a species dependent manner, i.e., 4 major postnatal MBP isoforms exist in rat, 6 in mouse and 4 in human [10,11]. These MBP isoforms are derived from a gene complex called 'Golli ' (gene in the oligodendrocyte lineage) which is comprised of eleven exons from which the seven downstream ones give rise to a single MBP transcript. The alternative splicing of this single transcript produces different prenatal and postnatal MBP isoforms. Among these isoforms, the ones containing exon-II, i.e., 17 and 21.5 kDa, are localized within the nucleus and cytoplasm, whereas the other two, 14 and 18.5 kDa, localize to (compact) myelin membranes. MBP isoforms are expressed in a developmentally regulated way; exon-II negative MBPs are expressed in late development during active myelination [6], whereas exon-II containing MBP isoforms are predominantly expressed at the onset of myelination [12–15]. The exon-II containing or exon-II devoid MBPs have different transport machineries (Fig. 2), suggesting that the presence of exon-II might regulate the different trafficking of MBP. Exon-II negative MBPs have a special translation machinery called 'translation on site', which means that they are not transported to the myelin membranes as a protein but rather as mRNA, assembled in granules (Fig. 2) [10]. MBP is a highly basic, i.e., positively charged protein, capable of interacting with the negatively charged cytoplasmic leaflet myelin lipids like phosphatidylserine and phosphatidylinositol [10,11,16]. Therefore, MBP is translated on site, because if MBP is transported as a protein rather than mRNA, it might 'glue' the internal membranes and inner cytoplasmic leaflet lipids before it actually reaches its proper place. Recent findings revealed that MBP also acts as a molecular barrier by preventing that proteins with a large cytoplasmic tail enter myelin (Fig. 2, [6]), therefore also contributing to OLG polarization [17]. In contrast, exon-II containing MBPs are not expressed in myelin membranes, but instead shuttle between nucleus and cytoplasm at different conditions (Fig. 2, [14,18,19]). However, thus far most attention has been focused on myelin localized MBPs, which is why the function of exon-II positive MBPs is only just emerging (see chapter 6).

The integral membrane myelin protein PLP has 2 different isoforms, i.e., PLP and a spliced isoform called DM20 [20]. PLP is synthesized at the ER, passed through

the Golgi apparatus, and then transported to the myelin membrane via a vesicular transport pathway [21]. PLP plays a role in the stabilization of the intraperiod line, i.e., where the extracellular myelin leaflets oppose each other, thus contributing to myelin compaction [22]. Importantly, to prevent premature compaction, PLP has to reach the myelin membrane after MBP [5]. The transport mechanism of PLP to its final destination is not yet known in detail. Given the polarized nature of cultured OLGs, the transient accumulation of PLP in a LAMP-1 positive late endosomal compartment suggests that PLP is transported to myelin membranes via transcytosis [21]. Further evidence suggested that the SNARE machinery, which mediates the final docking of the vesicles by specific pairing of vesicle (v) and target (t) membrane SNAREs, is involved in PLP trafficking. Functional removal of the v-SNAREs VAMP3 and VAMP7 prevents PLP transport to the surface and PLP's integration into myelin-like membranes [23]. Additionally, VAMP7 knockout mouse revealed decreased PLP levels in CNS myelin. These results further suggest that VAMP3 and VAMP7 might be involved in PLP trafficking. Moreover, recent studies revealed that PLP acts as a cholesterol transporter, thereby regulating cholesterol levels in myelin [24]. Also myelin galactolipids might play a role in PLP trafficking; i.e., the association of PLP with GalC-enriched membrane microdomains upon transport towards myelin membranes, and the role of sulfatide in vesicular PLP transport [25,26]. The latter is still controversial, as the plasma membrane localization of PLP is independent of galactolipids [27,28]. Therefore, the transport pathway of PLP requires further investigation (see chapter 3).

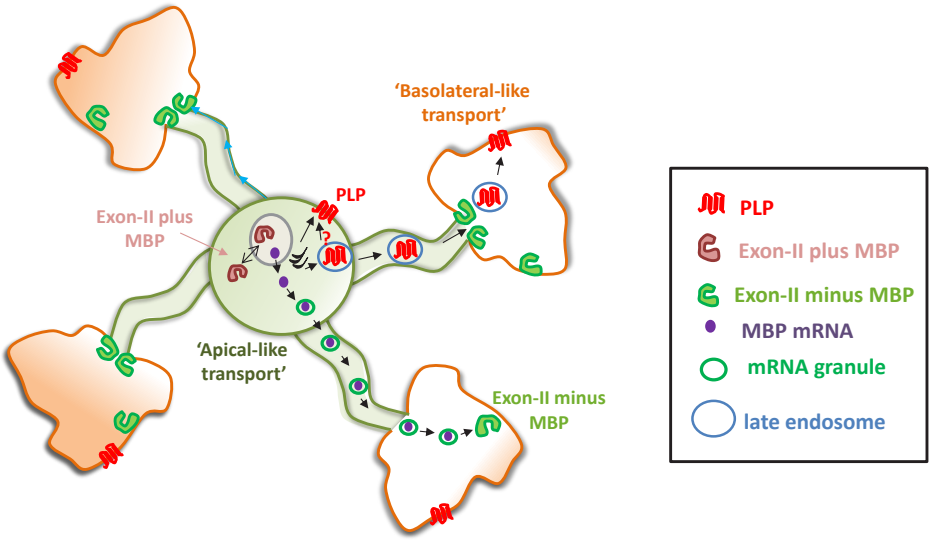

Figure 2: Trafficking routes of MBP and PLP.
Oligodendrocytes are polarized cells, i.e., the cell body plasma membrane is target of an 'apical-like trafficking' pathway (green) and the myelin sheet of 'basolateral-like trafficking' route (orange). The major transmembrane myelin protein PLP (depicted in red) reach the myelin membrane via an indirect vesicular transport pathway, involving a late endosomal compartment (depicted in blue) (see the text for more details). The other major myelin protein, peripheral membrane protein MBP has different isoforms, their localization being dependent on the presence and absence of exon-II. Exon-II-containing MBPs (depicted in pink) shuttle between nucleus and cytoplasm, whereas exon-II negative MBP isoforms are transported to the myelin sheets in mRNA granules (granules depicted in green, mRNA depicted in purple), followed by MBP protein (depicted in green) translation 'on site' , i.e., myelin membranes. Exon-II containing MBPs also localize at the end of the primary processes where it acts as a port for the entry into myelin, i.e., proteins with a large cytoplasmic tail that reach the myelin membrane via lateral diffusion (blue arrows) are excluded. Note that PLP and MBP trafficking are here depicted in a separate OLG processes, all the trafficking routes apply for all processes.

2. Myelin biogenesis in multiple sclerosis

MS is a chronic inflammatory neurodegenerative disease of the central nervous system which is characterized by the presence of demyelinated areas, blood brain barrier disruption, axonal loss and reactive gliosis [29]. The extensive demyelination of CNS causes impairment of nerve impulse conduction, leading to neurological disabilities. The main cause of MS and the localization of its onset are still not known. Earlier studies suggested that MS is an autoimmune disease, whereby the adaptive immune system attacks the myelin, leading to OLG death. In contrast, more recent studies indicate that extensive OLG apoptosis accompanied by axonal degeneration can be the starting point in the etiology of MS [30,31]. However, both views merge at the point of impairment of (re)myelination, a process in which the myelination machinery should (re)activate adult OPCs, recruited to the demyelinated regions in order to restore myelin sheath biosynthesis, thereby providing functional recovery and axonal support [32]. Especially in the later stages of MS, this myelination machinery fails and remyelination is permanently impaired, leading to disease progression and secondary axonal loss.

The exact cause for remyelination failure in MS lesions is not fully understood. However, different studies suggest that an altered extracellular signaling microenvironment might be an underlying reason [33,34]. During development, OLGs follow the above mentioned proliferation-differentiation time-line to reach a myelin-producing stage with a functional myelination machinery. Along this time-line OLGs are in close contact with other glial cells such as astrocytes and microglia, which provide an efficient signaling environment for OLG migration, proliferation and differentiation [35]. Also neurons contribute to this signaling environment, i.e., regulating OLG survival and the timing of myelination. This signaling environment

has to be tightly regulated in time and space. For instance, astrocytes produce growth factors such as platelet derived growth factor (PDGF), whereas microglia produce insulin like growth factor 1 (IGF-1) and fibroblast growth factor (FGF) for OPC proliferation, differentiation, and survival during myelin biogenesis [35]. Upon CNS injury, astrocytes and microglia secrete in addition other signals, i.e., those related to injury and local inflammation, to restore (re)myelination or to maintain already synthesized myelin membranes. These signals, i.e., the upregulation of some extracellular matrix (ECM) proteins or pro-inflammatory cytokines, are normally transient and after guiding the injury repair and OPC recruitment, they have to disappear to allow OLGs to maturate and synthesize myelin [36,37]. However, in MS lesions, some of these transient signals persists and become permanent which impairs remyelination (see below). Additionally, these changes in MS lesions not only interfere with the de novo myelin biosynthesis but might also interfere with the maintenance of existing myelin membranes (see chapter 5).

2.1. Pro-inflammatory cytokines in multiple sclerosis lesions

Pro-inflammatory cytokines such as TNFα, IFNγ, and IL1β are upregulated in MS lesions [38]. Due to these secreted cytokines, OLGs might undergo apoptosis and/or necrosis resulting in extensive OLG loss [39]. However, the effect of some pro-inflammatory cytokines is puzzling, as positive roles for these cytokines have also been described [40]. IL1β, for example, enhances OLG differentiation, whereas TNFα increases OPC proliferation [36,37]. Along differentiation OLGs express two different TNF receptors with distinct functions in a developmentally regulated way; TNFR2 is mainly expressed by OPCs and connected to cell proliferation and remyelination [42], whereas TNFR1 is expressed by mature OLGs and is connected to the apoptosis pathway [40]. On the other hand, studies performed in OLG monocultures revealed that TNFα rather than inducing apoptosis impairs OLG differentiation [43]. Therefore, in MS, the persistent upregulation of TNFα might affect the survival and maturation of recruited OPCs at the lesion site, whereas its presence is necessary for OPC proliferation. Hence, the physiological function of pro-inflammatory cytokines upon demyelination, and their potential pathological effect(s) in MS lesions with regard to myelin biogenesis, is not very well understood and requires further investigations (see also chapter 5). For instance, TNFα is known to change the polarization of epithelial cells [44], which might be also the case for OLGs, thereby potentially interfering with myelin membrane directed trafficking of myelin proteins and lipids.

2.2. Extracellular matrix proteins in multiple sclerosis lesions

ECM, a part of extracellular environment, is very important for myelin membrane formation and organization and any alteration can change dramatically myelin assembly, and thereby (re)myelination, a situation that might be valid in MS [37,45]. Upon CNS development, the ECM molecule laminin-2 enhances myelin membrane formation [46,47]. Upon demyelination, other ECM proteins, such as chondroitin sulfate proteoglycans (CSPGs) [48], high molecular weight hyaluronan [49], and fibronectin [36], are transiently expressed to guide injury repair. Thus, fibronectin guides recruitment of OPCs to the demyelinated areas and increases their proliferation [36], but is degraded at the onset of myelin biogenesis to allow for new myelin membrane formation. However, in MS, these myelination-inhibitory ECM proteins, expressed at very high levels, are persistent [35]. For example, in MS the degradation of fibronectin is prevented, as it aggregates [33]. The persistence of fibronectin (aggregates) interferes with myelin biogenesis, since it alters the lateral membrane organization of some myelin proteins and lipids, i.e., their association with 'lipid rafts' [50], nano-sized highly dynamic membrane microdomains, also known for their detergent resistance (described in detail in chapter 2). The presence of fibronectin (aggregates) significantly reduces membrane microdomain association of the paranodal protein NF155 and galactolipid sulfatide [51,52], and internodal protein PLP [43, chapter 3 and 4], as well as myelin-direct vesicular transport [54]. However, further investigations are needed to elucidate the underlying mechanisms as to how fibronectin and other ECM proteins, interfere with myelin biogenesis and thereby contribute to MS pathology.

Scope of the thesis

Myelin membrane biogenesis and maintenance require a tight regulation of complex intracellular trafficking machineries, involving appropriate and sequential transportation of major myelin lipids and proteins to their final destination, where they can exert their function. As the function of major myelin lipids and proteins are closely related with their localization and lateral organization, any alteration in the pattern of these molecules might dramatically change the normal biogenesis of these specialized membranes. For example, in demyelinating diseases such as MS, the localization and organization of myelin specific components are misregulated, leading to (de novo) myelin biogenesis failure, caused, among others, by an altered extracellular environment. The work described in this thesis focuses on molecular mechanisms that regulate the trafficking, localization and lateral organization behavior of the major myelin proteins PLP and MBP (at the membrane) in conjunction

with intrinsic elements, such as the galactolipids GalC and sulfatide, and extrinsic elements, such as extracellular matrix proteins and pro-inflammatory cytokines.

In chapter 1, we outline the specialized proliferation and differentiation time-line and trafficking of major myelin proteins in OLGs. Moreover, we describe how the altered extracellular environment in MS, a demyelinating disease of the CNS, might potentially affect the myelination machinery. In chapter 2, current knowledge of the myelin structure in terms of myelin lipids and proteins, and their interaction(s) and trafficking, is outlined in detail. Furthermore, recent findings on OLG-myelin membrane dynamics with a combination of biochemical and biophysical tools is discussed. Within this context, we first focus on the trafficking of the major myelin protein PLP, since the intracellular flow of this major myelin protein is still not very well understood. Hence, in chapter 3, the biosynthetic transport mechanism of PLP in relation with the t-SNARE machinery and in dependence of the myelin galactolipids GalC and sulfatide, was investigated. Having determined that sulfatide is a key regulator in transcytotic transport and in the lateral organization of PLP, we then set out to obtain further insight into the dynamic behavior of an integral membrane protein as PLP, i.e., its lateral mobility and membrane organization was investigated in relation to GalC and sulfatide (in chapter 4). In addition, the effect of the extracellular microenvironment, such as the ECM proteins fibronectin and laminin-2, was examined. Similar investigations were carried out for the other major protein of compact myelin, the peripheral membrane protein 18.5-kDa MBP. In addition to ECM components, also soluble factors can affect the lateral organization of myelin proteins, also in mature myelinating OLGs, thereby potentially affecting myelin maintenance. Given that the pro-inflammatory cytokine TNFα is upregulated in MS lesions, we examined in chapter 5, the effect of TNFα on the expression and localization of MBP in mature OLGs, using monocultures as well as an an in vitro myelinating culture system. MBP consists of several developmentally regulated isoforms which display distinct localization patterns; i.e., in the cytoplasm, the nucleus or in association with the myelin membrane. Since the function of nucleus-localized MBP isoforms was not known, we unraveled in chapter 6 the function of exon-II containing MBP isoforms, including 21.5-kDa MBP and embryonic 16-kDa MBP, which transiently localize into the nucleus. Finally, in chapter 7 the findings in this thesis are summarized and discussed, and future perspectives are provided.

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