FEBS Letters 584 (2010) 1760–1770

FEBS Letters
journal homepage: www.FEBSLetters.org

Review

On the biogenesis of myelin membranes: Sorting, trafficking and cell polarity

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ARTICLE INFO

Article history:
Received 8 September 2009
Revised 29 October 2009
Accepted 30 October 2009
Available online 5 November 2009

Edited by Sandro Sonnino

Keywords:
Myelin
Trafficking
Microdomain
Proteolipid protein
Myelin basic protein
Sorting

ABSTRACT

In the central nervous system, a multilayered membrane layer known as the myelin sheath enwraps axons, and is required for optimal saltatory signal conductance. The sheath develops from membrane processes that extend from the plasma membrane of oligodendrocytes and displays a unique lipid and protein composition. Myelin biogenesis is carefully regulated, and multiple transport pathways involving a variety of endosomal compartments are involved. Here we briefly summarize how the major myelin proteins proteolipid protein and myelin basic protein reach the sheath, and highlight potential mechanisms involved, including the role of myelin specific lipids and cell polarity related transport pathways.

1. Introduction

The myelin sheath is a multilayered membrane system that is produced by and extends from oligodendrocytes (OLG) in the central nervous system. The sheath is highly enriched in lipids (approx. 70% of its dry weight), in particular cholesterol and the glycosphingolipids galactosylceramide and its sulfated analogue sulfatide [1]. In addition, several hundreds of proteins have been detected, as revealed by gel-based proteome analysis [2]. However, most prominently present are the two major proteins (approx. 80% of the total protein fraction) myelin basic protein (MBP, 30%) and proteolipid protein (PLP, 50%). Both proteins are instrumental in the process of myelinating axons, i.e., the overall process that leads to the wrapping of the myelin membrane around these neuronal extensions, thus culminating in a multilayered organization, necessary for rapid salutatory nerve impulse conduction. Perturbation of the sheath ('demyelination') leads to several severe brain diseases, including multiple sclerosis (MS).

Before myelination is initiated, OLG precursor cells first differentiate into pre-myelinating oligodendrocytes and then into myelin-producing cells. This transition is marked by the sequential appearance of myelin specific proteins, their localization shifting during these differentiation stages from cell body to plasma membrane extensions known as processes, which further extend and develop into myelin sheaths. An intact cytoskeleton is necessary for OLG process extension [3], while eventual compaction of myelin involves adhesion between cytosolic and extracellular surfaces, mediated by MBP and PLP, respectively, depolymerization of the cytoskeleton and subsequent elimination of cytosol.

One OLG can myelinate several axons, but since not all axons are myelinated simultaneously, myelin biogenesis appears a highly regulated event. The presence of axons, the natural target of the myelin membrane and regulating actual myelination in vivo by secreting axon-derived signals, is however not crucial as myelin sheath biogenesis also occurs in vitro, i.e., in neuron-devoid OLG cultures. In vitro, the myelin *sheath* is usually referred to as 'myelin *sheet*', as wrapping only occurs in the presence of axons as in mixed brain cultures. During active myelination in rat, it has been estimated that the myelin-membrane surface area expands at a rate of $5–50 \times 10^3$ μm^2/cell/day, compared with a cell body surface area (i.e., the plasma membrane) of approx. 300 μm^2. Thus a strict control of intracellular transport of myelin proteins and lipids to each process is essential in the assembly and maintenance of myelin.

Abbreviations: ARE, apical recycling endosome; CHAPS, 3-[3-chloramidopropyl)-dimethylammonio]-1-propane-sulfonate; CNS, central nervous system; ECM, extracellular matrix; ER, endoplasmic reticulum; GalCer, galactosylceramide; LE, late endosome; MBP, myelin basic protein; MDCK, Madin–Darby canine kidney; MS, multiple sclerosis; NF-155, neurofascin 155; OLG, oligodendrocyte(s); PLP, proteolipid protein; PKC, protein kinase C; SAC, subapical compartment; VSV G, G protein of vesicular stomatitis virus; UTR, untranslated region

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.10.085

In the following, we will briefly summarize recent advances in the biogenesis of myelin sheaths/sheets with a particular focus on molecular mechanisms underlying sorting and transport of the major myelin proteins MBP and PLP, including the role of lipids, axons and other 'exogenous' factors like the extracellular matrix (ECM) in regulating these events. However, to properly appreciate trafficking pathways in OLG, it is particularly relevant to take into account the polarized nature of these cells, containing two distinct membrane surfaces, i.e., the plasma membrane surrounding the cell body and the myelin membrane.

2. Oligodendrocytes express polarized membrane surface domains

Although extending from the plasma membrane of the OLG cell body, the myelin-membrane composition differs vastly from that of the plasma membrane, emphasizing the polarized OLG phenotype. Furthermore, the myelin sheath itself is characterized by compact and non-compact membrane regions that also differ in (protein) composition. Obviously, this polarized nature sets particular requirements for a timely de novo biogenesis of myelin components and their subsequent sorting and trafficking to the sheath. Indeed, several studies have drawn analogies between features of and underlying mechanisms in the biogenesis and maintenance of apical and basolateral membranes in polarized epithelial cells on the one hand, and myelin sheath and cell body plasma membrane on the other (summarized in [4]). In terms of molecular composition, like the enrichment of GPI-anchored proteins, glycosphingolipids (in particular galactosylceramide (GalCer) and sulfatide) and cholesterol, the myelin sheath resembles the apical membrane domain, whereas the cell body plasma membrane matches the basolateral surface [5,6]. However, quite intriguingly, upon expression of the viral model proteins HA of influenza virus and the G protein of vesicular stomatitis virus (VSV G) in cultured OLG, HA localizes largely to the plasma membrane, whereas G VSV G localizes to the myelin sheet [7]. Both these proteins are well-established classical markers for vesicle-mediated protein transport to apical (HA) and basolateral (VSV G) membrane surfaces in epithelial cells, involving the tSNARES syntaxins 3 and 4 as docking sites, respectively [8]. Quite unexpectedly, based on these criteria for polarized transport, this would imply therefore that the OLG plasma membrane is reached by an apical-like and the myelin sheet by a basolateral-like mechanism. Interestingly, preliminary work in our laboratory has revealed that in OLG syntaxin 3 is enriched at the plasma membrane while syntaxin 4 is predominantly found in the myelin sheet, analogous to their enrichment at apical and basolateral membranes in epithelial cells, respectively (Fig. 1). Moreover, a tyrosine motif dictating basolateral sorting via its association with the AP1B adaptor molecule [9] is also essential for myelin-directed targeting. Tyrosine substitution directs VSV G to the plasma membrane instead of the sheet, while insertion of tyrosine in HA results in the opposite effect and causes HA to be transported into the sheet [10]. Together these data suggest (for a more detailed account see [4]) that a basolateral-like transport mechanism dictates molecular translocation to the myelin membrane, whereas plasma membrane directed trafficking is mediated by an apical membrane-like mechanism. However, like in hepatocytes, the major epithelial cell type in the liver, polarized transport in OLG seems more complex than the presence of direct transport pathways to myelin sheath and plasma membrane [11]. Thus like in hepatocytes, also in OLG indirect transport pathways involving transcytosis seem to operate, as revealed by the processing of PLP (see below), following its biosynthesis at the endoplasmic reticulum (ER). Moreover, although likely, a direct pathway of protein transport from trans-Golgi network to plasma membrane in OLG has yet to be demonstrated.

3. Sorting and trafficking of the major myelin proteins MBP and PLP

The establishment of oligodendrocyte polarity, featured by the appearance of two distinct membrane surfaces, i.e., the plasma membrane and the multilayered myelin sheath/sheet, requires sorting and targeting of membrane components to either surface, and hence a tight regulation of the intracellular trafficking machinery. Moreover, myelin biogenesis is a rather complex event as

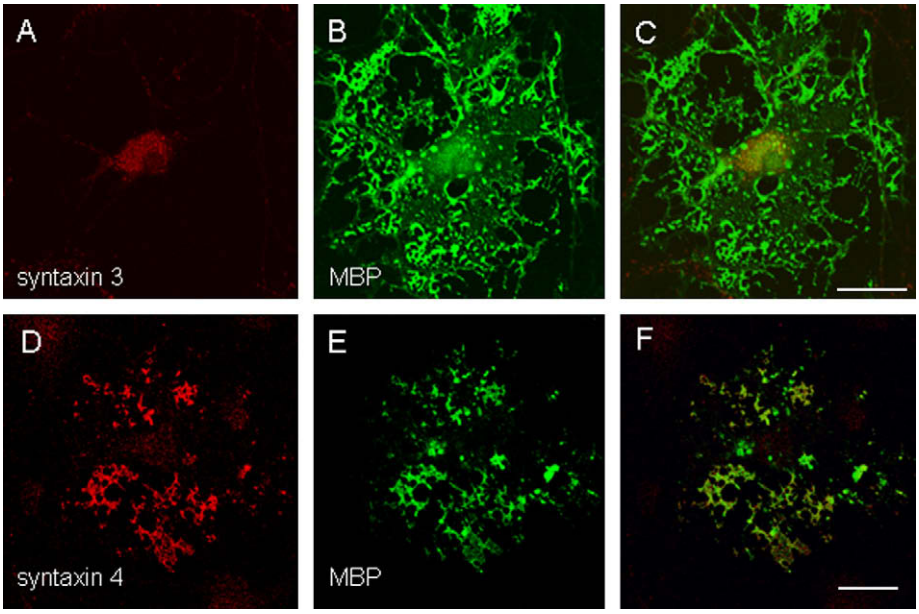

Fig. 1. Localization of syntaxin 3 (A–C) and syntaxin 4 (D–F) in mature oligodendrocytes. OLG were fixed, permeabilized and co-stained for syntaxin 3 (A) and MBP (B), and syntaxin 4 (D) and MBP (E). Merged image of A and B and D and E are shown in C and F, respectively. Syntaxin localization was analyzed by confocal immunofluorescence microscopy. Bar is 20 μm. Note that endogenous syntaxin 3 localizes to the cell body and primary processes and not to myelin-like membranes, whereas endogenous syntaxin 4 primarily localizes to myelin-like membranes.

individual myelin components are expressed in a timely fashion, and often sorted and transported to myelin by different mechanisms. For example, following its biosynthesis at the rough ER, the myelin-specific protein PLP is initially sorted and transported in transport vesicles to the plasma membrane, prior to reaching the myelin sheath. In marked contrast, MBP is synthesised and inserted within the myelin membrane 'on site', involving microtubule-dependent transport of MBP mRNA granules, rather than the native protein, to the sheath. MBP is a basic, highly adhesive peripheral myelin protein that faces the cytosolic membrane surface, where it is involved in compaction of the myelin membranes, while the major membrane-spanning central nervous system (CNS) myelin protein PLP fulfills a similar function by compacting membranes at the exoplasmic side of the myelin that are apposed to one another during the wrapping process (Fig. 2). It can thus be readily rationalized why MBP and not PLP requires transport as its mRNA, rather than that of the intact protein, as premature MBP-induced membrane clustering in myelin biogenesis is precluded in this manner. Furthermore, as both MBP and PLP stabilize apposed myelin membranes in compact myelin, it is of particular importance that their transport and insertion to myelin membranes are segregated and tightly regulated, both in time and space.

3.1. MBP: trafficking, sorting and myelinogenesis

The MBP gene encodes two MBP families, classic MBP and golli-proteins. Classic MBPs are exclusively expressed in myelin membranes, and consist of different isoforms, mainly distinguished by the inclusion or exclusion of exon 2. Strikingly, MBP is the only myelin-specific protein known to be vital and indispensable for myelin biogenesis [12]. Thus, MBP is not just an inert 'glue' that mediates static interactions between intracellular leaflets of membranes, but rather, is imperative in driving myelin assembly. The different isoforms of MBP, which appear to localize to different microdomains in the myelin membrane as reflected by a distinct, isoform-dependent distribution in isolated detergent-resistant microdomains (see also below and [13]) may reflect the multifunctionality of this protein family in myelin maintenance. Indeed, additional roles for MBP have been gradually recognized, including roles in signaling, plasma membrane reorganisation and cytoskeletal interactions (see below and [12,14]).

To preclude MBP, given its strong adhesive properties, from inappropriate and deleterious adhesive interactions with membranes other than the cytoplasmic surface of the myelin membrane, (exon 2 minus) MBP mRNA rather than MBP itself is transported and targeted into OLG processes, thereby enabling protein synthesis 'on site' [15]. In addition, the coupling of translational activity to mRNA localization not only prevents premature ectopic expression of MBP, but also a rapid and selective insertion of MBP into myelin membranes. In addition to MBP mRNA, several other mRNAs, including those for MOPB, CAII and tau are also (co)-transported to the myelin membranes, prior to expression of the proteins [15,16]. However, intracellular trafficking of mRNAs is not exclusive to OLG, and has been demonstrated in a variety of cells, particularly in the context of cell polarity establishment. For example, in neurons a subset of mRNAs is localised to dendrites, whereas others are targeted to axons [17].

Targeting of MBP mRNA via the processes to the myelin sheath is a multistep process that involves nuclear export, assembly into transport particles, trafficking and anchoring to the target site, and finally activation of translation. The first step, i.e., nuclear export of MBP mRNAs is likely regulated by QKI RNA binding proteins, which bind directly to the 3′ untranslated region (UTR) of MBP mRNA. OLG from *quaking viable* mice, an animal model for demyelination, are unable to translocate MBP mRNA into the cytoplasm [18]. The underlying mechanism appears to rely on a failure in switching from the expression of nuclear QKI-5 binding proteins, which retain MBP mRNA in the nucleus, to QKI-6 and QKI-7 RNA binding proteins, which facilitate nucleo-cytoplasmic shuttling.

In the cytoplasm, MBP mRNA assembles into ribonucleoprotein complexes termed 'granules', representing translational transport units that, besides RNA, contain the machinery for mRNA translation and protein synthesis [19]. This kind of assembly into granules is not specific, as non-transported mRNAs display a similar aggregation behaviour when microinjected into the cytoplasm [20]. However, specificity is conveyed at an earlier stage in the nucleus when pre-mRNAs are selectively sorted upon association with a subset of heterogeneous nuclear ribonucleoproteins (hnRNPs), which determine the fate of the mRNA, i.e., its cytoplasmic location, translation efficiency and mRNA turnover. This association involves *cis*-acting location elements in the 3′UTR of the mRNA, which binds to a specific *trans*-acting factor involved in transport and translational control. The 3′UTR of MBP mRNA contains the hnRNP A2 response element (A2RE), which is recognized by hnRNP A2 and CBF-A, both of which mediate the intracellular trafficking of MBP mRNA to the myelin membrane [21,22]. mRNA localization requires translational control, i.e., translational suppression during transport until its arrival at the final destination. In OLG, this is accomplished through binding of the translational inhibitor hnRNP E1 to hnRNP A2 [23]. Interestingly, MBP mRNA containing granules lack Staufen [24], a well-characterized factor that generally participates in mRNA transport and localization, as characterized in

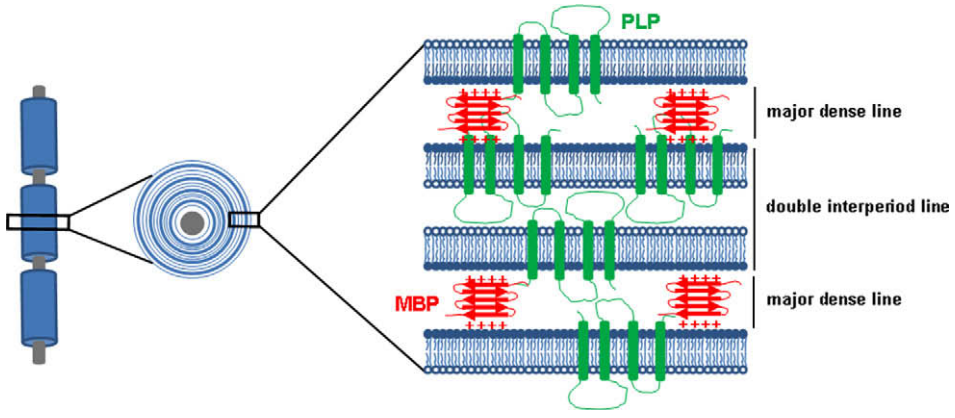

Fig. 2. Schematic illustration of the structure of the myelin sheath. Compact myelin is formed by the PLP-mediated apposition of the external leaflets of the bilayer membrane (forming the 'double intraperiod line'), which will further facilitate MBP-mediated apposition of the internal leaflets and the extrusion of the cytoplasm (forming the 'major dense line'). Adapted from [96].

several other cell types. This could imply the likelihood of the existence of a different mRNA transport machinery in OLGs. The need for an additional location element in the 3′UTR, required to target MBP mRNA to the final designated location in the membrane, is supportive of this suggestion [20].

The alternatively spliced isoforms of MBP are developmentally regulated and targeted to different subcellular locations in OLG, depending on the presence or absence of exon 2. These isoforms differ in their coding sequence, but not in the untranslated regions of their mRNAs. However, MBP mRNAs lacking exon 2 are transported into OLG processes, whereas exon 2 plus isoforms localize to the nucleus and cell body cytoplasm [25,26]. What prevents transport of exon 2 containing MBP isoforms is still obscure, but it might be associated with the secondary structure of exon 2, which could prevent or induce binding of a protein involved in MBP mRNA transport. However, it is very likely that the activation of the MBP mRNA transport machinery is regulated by other means than the mere presence or absence of exon 2, as impaired isoprenylation also prevents transport of MBP mRNA [27]. Thus a role for a developmentally expressed isoprenylated protein involved in regulation of MBP mRNA could also be envisioned.

mRNA transport granules move to their site of destination along microtubules in a kinesin-dependent manner [28] but their docking is still poorly understood. Thus, the actin cytoskeleton might be important for anchoring and localizing mRNA, as observed in other cells, but the identity of proteins involved in target recognition and docking is still largely obscure. It has been suggested that the kinesin motor protein Kif1b, which is associated with a susceptibility to MS, is specifically required for ectopic expression of myelin by anchoring MBP mRNA to myelin membranes [29]. TOG appears to be necessary for efficient translation of MBP mRNA [30] while at the target site Fyn activation triggers hnRNP A2 phosphorylation, causing the release of hnRNP E1, thereby allowing local protein synthesis to occur [31].

An interesting issue is obviously the question as to whether the mechanism of mRNA transport shares common features with the mechanism of vesicular transport. In fact, prevailing evidence suggests a closer resemblance in both events than previously thought. Next to a role of microtubules in mediating both vesicular and mRNA transport, as noted above, genetic screens in *Drosophila* revealed that the ESCRT (Endosomal Sorting Complex Required for Transport) complex, known to be involved in endosomal sorting, is directly participating in the localization of *bicoid* mRNA [32]. Intriguingly, in zebrafish it has been shown that the ATPase N-ethylmaleimide sensitive factor (NSF), a cofactor in SNARE-mediated membrane fusion, is essential for the organization of myelinated axons, and for correct expression of MBP [33]. Similarly, although a role in mRNA transport has not been examined yet, sec8, an exocyst component and a central player in OLG vesicular transport, is essential for MBP expression [34]. Also in yeast, a correlation between vesicle transport and the integrity of the actin cytoskeleton, and subsequent mRNA transport and its localization to the growing bud has been reported [35]. Finally, for the proper localization of (oskar) mRNA in *Drosophila* a 'tethering and anchoring' role for the small GTPase rab11, which traffics vesicles from recycling endosomes to the plasma membrane, has been described [36]. However, it should be noted that the exact mechanism and the quantitative contribution of recycling pathways in myelin sheath biogenesis and maintenance are still largely unknown.

The use of similar molecular entities to direct trafficking of mRNA granules and membranous vesicles is surprising since mRNA granules are protein–RNA complexes, and not membrane bounded systems. Moreover, the estimated size of the MBP mRNA granules is 600–800 nm [15], a diameter which is almost an order of magnitude larger than commonly observed transport vesicles. Of further interest in this regard, our unpublished findings suggest that the SNARE machinery – instrumental in vesicular docking – is also involved in MBP mRNA transport, emphasizing a causal relationship between the vesicular transport machinery and mRNA transport in OLG. Monitoring MBP mRNA transport from the site of initial granule assembly to its arrival and translation at its ultimate destination as a function of manipulation of vesicular trafficking routes would be important to further clarify and define the connection between vesicular and mRNA transport.

3.2. PLP trafficking: vesicular transport in a polarized cell

PLP is an integral membrane protein, composed of 276 amino acids and is predominantly expressed in OLG of the CNS. The PLP gene is alternatively spliced during development to encode DM20 and PLP. Both proteins consist of four transmembrane domains with one intracellular loop and two extracellular ones. The N- and C terminal are facing the cytoplasm (Fig. 2). DM20 differs from PLP by a hydrophilic internal 35 amino acid segment deletion in the intracellular loop. As a structural protein PLP plays a major role in the correct apposition of the extracellular leaflets of the membrane (double intraperiod line; Fig. 2), thereby stabilizing the multilayered myelin membrane structure upon compaction. Other functions of PLP have been suggested as well, including a role in OLG survival and adhesion in relation to migration. Whereas MBP is crucial for myelination to occur, PLP appears dispensable and in its absence myelination of axons still occurs, albeit that in PLP null mice myelin stability diminishes as a function of time [37].

PLP is synthesized in the ER and subsequently transported via vesicles to the Golgi, followed by transport to the myelin sheath. The underlying sorting machinery as to how PLP reaches its final destination is unclear, but insight of the overall transport pathway is gradually emerging and suggests indirect transport of PLP to the sheath, i.e., via the plasma membrane of the cell body rather than its direct transport from the Golgi to the sheath. Palmitoylation at three cysteine residues in the N-terminal region of PLP is required for myelin-membrane localization [38], but other sorting determinants are likely playing a role as well. Indeed, DM20 seems to assist the trafficking of PLP [39], and a role for both GalCer and sulfatide in PLP transport has been suggested, although the exact role of these lipids in PLP transport is still a matter of controversy. In isolated brain slices transport of PLP into myelin was reduced by approx. 50% upon inhibition of overall glycosphingolipid synthesis, whereas neither the insertion of MBP nor protein synthesis as such were affected. In addition, a correlation between PLP and sulfatide was suggested based on vesicular co-transport in brains of 20 day old rats [40]. Furthermore, PLP association with cholesterol and GalCer-enriched membrane domains during biosynthetic transport in cultured oligodendrocytes appears critical [41] (see below). In contrast, other studies demonstrated that the sorting and trafficking of PLP to the processes and myelin sheet is not affected upon inhibition of sulfatide synthesis [42], while PLP is transported to the plasma membrane in GalCer- and sulfatide deficient CHO cells [5]. These latter observations are in line with in vivo data, showing that in mice, unable to synthesize GalCer and/or sulfatide, transport of PLP to myelin sheaths was uninterrupted [43]. These apparent contradictory findings may originate from the different experimental conditions. In this regard it should be particularly taken into account that multiple pathways seem to operate in overall PLP transport, implying that distinct stages could involve distinct transport vehicles involving potential localization of PLP in different microdomains in a transient manner. In the context of the polarized nature of the OLG, it is of interest that early after biosynthesis, PLP localizes at the surface of OLG in similar domains as the apical viral model protein, HA, i.e., the cell body plasma membrane [41]. This could suggest that like in polarized epithelial cells, PLP might reach the myelin sheet via an indirect, i.e., transcytotic

pathway. Consistent with such a notion are observations that in Oli-neu cells, an in vitro cell model for OLG, PLP, rather than by direct transport from Golgi to myelin membranes, is first transported to the cell body plasma membrane. In the absence of neuronal signals, PLP is subsequently internalized by a clathrin-independent but cholesterol-dependent mechanism and stored into late endosome (LE)/lysosomal compartments [44]. Neuronal signals cause the release of PLP from these compartments and its return to the plasma membrane, which is presumably instrumental in subsequent transport of PLP to the sheath, thus promoting myelin biogenesis and stability. Quite intriguingly, in the same Oli-neu cell line and the human oligodendroglioma derived cell line HOG, a MAL-2 containing compartment has been identified which might be reminiscent of the apical recycling endosome (ARE) or subapical compartment (SAC) [45]. This compartment is also localized in epithelial cells, including liver cells, where it acts as a sorting and trafficking center in transcytotic transport, in which MAL2 functions as an essential transport regulator (see [11]). It will therefore be of great interest to investigate the exact involvement of this compartment in OLG as well, including its (functional) relationship with the endosomal/lysosomal storage compartment, if any.

In line with this polarity-based transport mechanism of PLP are further observations that when stably expressed in polarized Madin–Darby canine kidney (MDCK) cells, PLP is transported to the apical (mechanistically reminiscent of the plasma membrane in OLG) rather the basolateral domain (reminiscent of the myelin sheet) [46]. Similarly, upon transient expression in polarized HepG2 cells, PLP is localised exclusively at the apical membrane. However, quite intriguingly, when stably expressed in HepG2 cells, localization of PLP at the apical (bile canalicular) membrane is followed by transcytosis to the basolateral membranes (our unpublished observations). Interestingly, although extrapolation of findings obtained in different cell types should be done but with great caution, it is very tempting to emphasize here that in mature OLG PLP reaches its final destination via an indirect, transcytotic pathway, very reminiscent of that seen for many apical resident proteins in hepatocytes, traveling via the basolateral surface [11]. Moreover, in both HepG2 and MDCK cells, (fluorescently-tagged) GalCer but not GlcCer is specifically transported to the basolateral membrane [47], paralleling mechanistic features of the myelin membrane in OLG [12].

Thus far knowledge concerning docking and final insertion of PLP into the sheath/sheet is scanty. A variety of syntaxins have been detected in OLG [6,48], our unpublished observations], but their localization, let alone their involvement in docking of transport vesicles, is largely unknown. Our preliminary data suggest a role of syntaxin 3 in PLP transport, most likely in plasma membrane docking after transport from the Golgi, following de novo biosynthesis. Quite interestingly, OLG express SNAP-29 during myelination [48,49] and this tSNARE contains in its N-terminal region a binding domain for rab3A. Moreover, overexpression of PLP and rab3A in HEK293 cells promoted surface directed trafficking of PLP, thus suggesting that rab3A may be involved in membrane fusion of PLP-containing transport vesicles, docking at SNAP-29 target sites. It will be of particular interest to reveal in OLG whether and in which complex transport step the rab3/SNAP-29 machinery is operating.

The dynamic integration of PLP into distinct membrane domains in a time dependent manner, following its biosynthesis and ensuing transport to the sheet, is reflected by its detergent-solubility behaviour, usually applied as a criterion for localization in a distinct membrane microdomain [1]. Thus, after biosynthesis PLP acquires significant 3-[3-chloramidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) detergent resistance only after 30–60 min, i.e., the time likely required for PLP-containing transport vesicles to reach the plasma membrane and subsequently LE/lysosomal compartments [41]. PLP's redistribution from a CHAPS-soluble to a CHAPS-insoluble domain presumably reflects a sorting step, but where this transition takes place is an important, but as yet unresolved issue. Viral infection based pulse chase experiments suggest that PLP is largely CHAPS-soluble in the Golgi and at the plasma membrane, and that PLP becomes CHAPS-insoluble upon its accumulation with cholesterol in LE/lysosomal compartments [50]. Of interest in this context is the observation that *rsh* PLP, a natural mutation occurring in mice, fails to sequester to CHAPS-insoluble domains due to an impaired cholesterol binding [51]. However, *rsh* PLP traverses the Golgi to the cell surface and then enters the endocytic pathway for transport to LE/lysosomal compartments, but does not reach myelin membranes. Together, these findings support the view that PLP's localization in a cholesterol-enriched microdomain is crucial for final transport to the myelin sheet, but is not required for transport through the Golgi and to the plasma membrane. Whether the cholesterol dependence of PLP's internalization (possibly in conjunction with the presence of sulfatide, see below) at the plasma membrane relates to the need of PLP's localization into such a cholesterol-enriched domain for internalization or is merely a matter of cholesterol-dependent invagination per se, merits further investigation. Another intriguing matter is how PLP-mediated adhesion of opposing membranes in the ER is prevented. Conformational changes forced upon PLP by its segregation into different lipid environments along its trafficking route, is an appealing option. Recently, we have extensively discussed the evidence and structure–function relationship of rafts in OLG [1]. Here some comments are warranted as to the role of GalCer and sulfatide in myelin biogenesis.

4. Membrane microdomains in OLG: role of GalCer, sulfatide and cholesterol

It is now widely accepted that membranes contain heterogeneities, implying that different domains of different composition and physical properties may coexist within the lateral plane of the bilayer. Indeed, like from virtually every cell type, also from OLG membrane fractions can be isolated, that are resistant toward extraction with or soluble in non-ionic detergents, findings that are interpreted of reflecting the presence of liquid-ordered membrane microdomains, also know as 'rafts', and fluid liquid domains, respectively. These findings are consistent with the abundant presence of galactolipids and cholesterol in the myelin sheath, comprising approx. 90% of the outer leaflet of the myelin membrane [5] and lipid-modified myelin proteins [by fatty acylation (PLP) or GPI anchoring (NCAM120, contactin)] that display a preference of interacting with such domains. Moreover, it has been well-established that interference with cholesterol synthesis results in defects of myelination [52] and that the potential beneficial therapeutic use of statins to suppress inflammation in demyelinating diseases is highly frustrated by its ability to inhibit at the same time squalene synthase, a crucial intermediate step in cholesterol biosynthesis. Interestingly, for myelination in the peripheral nervous system (PNS) it has been reported that neurons exploit a cholesterol homeostatic mechanism that forces Schwann cells to produce new membranes for the myelin sheath [53]. This is accomplished by neuronal secretion of neuregulin and subsequent activation of neuregulin receptors, which eventually increases the transcription of 3-hydroxy-3-methylglutarylcoenzyme A reductase, the rate-limiting enzyme for cholesterol biosynthesis. Also a strong phylogenetic correlation between myelination and cholesterol biosynthesis was revealed. Moreover, to further emphasize the essential role of cholesterol in myelin biogenesis, quantitative analysis of the cholesterol pool in white matter of control and MS patients revealed that cholesterol is depleted in white matter

during both active-MS and disease inactivity [54]. Consequently, these notions further emphasize the importance of maintaining a proper pool of membrane cholesterol and its critical role in myelin biogenesis, in all likelihood particularly related to the assembly of membrane microdomains. Given the involvement of such domains in sorting and trafficking as well as distinct functional aspects, like intercellular interactions and signaling, the role of these microdomains in OLG functioning are warranted and recent reviews have focused on several of these aspects [1,4,55]

Although lipids, as an inherent part of the membranes of transport vesicles, can be moved in this manner to their sites of destination, non-vesicle-mediated transport pathways, involving lipid-specific transport proteins have also been reported, as observed for a variety of lipids, including ceramide [56], glucosylceramide [57], and cholesterol [58]. Current evidence supports predominant vesicle-mediated transport of the major glycosphingolipids GalCer and its sulfated analogue, sulfatide, to the sheath, following biosynthesis of GalCer at the ER lumen, and sulfation of part of this fraction in the Golgi. However, a significant fraction of GalCer may circumvent the Golgi, and has been suggested to be directly targeted, together with cholesterol, from the ER to endosomal compartments and/or myelin membranes in sigma-1 receptor positive lipid droplets [59]. Interestingly, whatever their mechanism of transport, both lipids do not colocalize in the sheath, but rather are distributed to distinct domains, thereby presumably reflecting differences in their sorting and trafficking (i.e., localization in distinct membrane domains and perhaps transport vesicles) and, likely, their function. Thus, in vivo, GalCer is found in compact myelin, where PLP localizes, whereas sulfatide localizes to non-compact myelin [4]. In cultured OLG sulfatide is strongly enriched in the cell body plasma membrane and primary and secondary processes, but is largely excluded from the sheet, where GalCer primarily localizes [4]. Thus, whereas the association of PLP with GalCer and cholesterol in LE/lysosomal compartments appears to be a crucial step in trafficking of PLP to the sheet, a role for sulfatide is less clear. Our recent data suggest that PLP and sulfatide are not co-transported to the plasma membrane, but rather that sulfatide has a regulatory role in the cholesterol-dependent and clathrin-independent internalization of PLP, and its association to CHAPS-resistant membrane microdomains. Therefore, distinct roles of GalCer and sulfatide at different stages in trafficking can be envisioned. Indeed, in mice that lack GalCer and sulfatide, PLP is CHAPS soluble, and fails to segregate to CHAPS-resistant domains [41]. However, in these mice PLP trafficking is apparently not timely regulated and traffics to the myelin membranes by bulk flow or an unknown compensatory mechanism that allows CHAPS-soluble PLP to insert into myelin membranes. Indeed, downregulation of the genes for ceramide galactosyl transferase and cerebroside sulfotransferase, key enzymes in the synthesis of GalCer and sulfatide, respectively, does not dramatically perturb overall myelin structure [43,60], but rather appears restricted to perturbations of the formation and maintenance of the paranodal structure, possibly related to aberrant trafficking of neurofascin 155 (NF-155) and contactin-associated proteins (Caspr-1 and 2) [85], and/or their exclusion from detergent-resistant microdomains, which seems of high relevance for the trafficking and localization of NF-155 at the paranodal loop [61]. Indeed, occasionally the myelin wraps lose contact at the extracellular leaflets of the membranes, which may well be related to the observation that impaired synthesis of galactosphingolipids inhibits efficient trafficking of PLP to the myelin sheath, as noted in the previous section, but does not eliminate it.

Sulfatide localizes particularly in the plasma membrane of mature OLG, where its preferential internalization over other simple glycosphingolipids by endocytosis has been reported [62]. Here, it may also play a role in the sorting of PLP and its internalization via a raft-mediated non-clathrin-mediated endocytic pathway [44] as part of a specific transcytotic pathway to the sheet membrane, after having reached the plasma membrane following biosynthesis. Importantly, compact and non-compact myelin may thus be reached by distinct transport pathways as reflected by differences in the distribution of compact and non-compact membrane proteins. Indeed, proteins localizing in these different domains have been noted to follow distinct endocytic and recycling pathways [63].

Studies of protein–lipid and protein–membrane interactions carried out in lipid monolayer systems have revealed that membrane proteins may act as fundamental structuring factors, driving lateral heterogeneity. Thus proteins are known to mediate the formation of domains through electrostatic and hydrophobic lipid–protein interactions [64], and exclusion or partitioning into cholesterol-enriched phases [65]. Both PLP and MBP constitute major structuring factors in that their integration in or adsorption to the myelin membrane leads to a local lateral reorganization of the lipid molecules, forming microdomains around the proteins and accounting for the myelin-membrane's surface topography, including membrane rafts [66]. Other proteins could act in concert with the formation of these domains and thereby provide the platforms for intracellular sorting and trafficking (PLP) to their site of destination and/or functioning otherwise, i.e., in stabilizing the sheath (MBP and PLP), among others via reduction of electrostatic dipolar repulsion of the lipids and strengthening of Coulomb interaction [66]. Seemingly subtle structural differences within a myelin protein, based upon the presence of distinct isoforms of some of them, provide an additional parameter for driving a protein's distinct distribution in raft and non-raft fractions. This is observed for MBP, showing that phosphorylated MBP rather than non-phosphorylated MBP is localized in rafts [67]. It has been similarly suggested that structural differences of the protein on the level of the carbohydrate groups could be important factors in the protein's lateral membrane distribution in myelin [68], and possibly thereby their ability of organizing different microdomains and/or targeting, which may include, for example, lectin-like molecules. Thus different structural isoforms of the proteins may exist which in this manner might thus dictate their distinct lateral membrane localization, which in turn determines the protein's fate, in terms of recruitment in distinct vesicles and ensuing transport. In this context, it has been shown that in the PLP gene mutant shp (shaking pup) the mutated PLP selectively accumulates in the rough ER, i.e., its trafficking is perturbed [69], as has been reported to occur for many other mutations in PLP [51,70]. The shp mutation involves a substitution of the conserved amino acid His by Pro (an alpha helix breaker) in the first extracellular loop (Fig. 2). Also in this case structural aberrations, which are not directly related to the transmembrane domains, presumably underlie the failure of the protein to be recruited for exiting from the ER and its transport to the Golgi for subsequent posttranslational modifications. Simultaneously, the presence of other major proteins (including MBP) in shp myelin was reduced, causing severe myelin deficiency and suggesting the coordinated regulation of overall myelin protein recruitment. Part of this diminishment was due to enhanced apoptosis of OLG, but the extent to which de novo synthesis or transport efficiency were affected, remains to be resolved. The intriguing possibility may exist that by recruiting distinct lipid environments, integration of other proteins in such distinct domains may be facilitated as well. Such a co-dependence in transport of a distinct protein has been shown [2] in a PLP*null* mutant where a virtual absence in the sheath of sirtuin 2 (SIRT2), a nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase of unknown function, was noted as well. This could suggest that PLP/DM20 is required for the transport of distinct proteins into the myelin compartment, possibly involving a mechanism that by interacting with

specific lipids, PLP may create domains that facilitate integration of certain proteins in such domains followed by their co-transport to the sheath. Also DM20 seems to assist in the trafficking of PLP [52,53].

In this context it is finally relevant to note that in model membranes, sulfatide readily engages in domain formation with other sphingolipids, including GalCer, and mixes with cholesterol in phosphatidylcholine bilayers where liquid-ordered and liquid-disordered phases coexist [71]. Interestingly however, depending on its fatty acid composition, sulfatide (like other glycolipids) may associate with cholesterol to a different extent [72], thereby likely affecting the physicochemical properties of the microdomain and hence its cargo.

Apart from their role in the lateral organization of the membrane, transmembrane interactions between GalCer and sulfatide, i.e., between apposed membranes, may also occur and contribute to myelin biogenesis and stability. It has thus been proposed (for review see [73]) that upon wrapping around the axon, the lipids in apposed membranes might interact and initiate GalCer/sulfatide-enriched microdomains that are thought to participate in trans carbohydrate–carbohydrate interactions, acting across apposed OLG membranes, when they face each other in the multilayered myelin sheath. This event subsequently triggers transmembrane signaling that leads to a depolymerization of the cytoskeleton and clustering of membrane constituents. This may result in squeezing out the cytosol and promote increased adhesion of the apposed surfaces, thereby producing and stabilizing compact myelin. Thus, it has been suggested [73] that in this way, i.e., in a transient manner as a result of membrane undulation, a so-called 'glycosynapse' can be formed, which might be instrumental in myelin–axon communication.

5. Myelin biogenesis: exogenous regulation

Although in vitro OLG produce myelin-like membranes in the absence of axons, in vivo myelin is only produced upon axonal 'request'. Thus, axonal cues, either adhesive or secreted, influence OLG development, which includes initiation and regulation of sorting and trafficking of myelin-specific proteins and lipids. Astrocyte-mediated signals also contribute to myelination, but likely not in the early phase of this process.

It is well documented that OLG in culture develop a polarized phenotype when grown on a 'non-specific' adhesive substrate and in the presence of an overwhelming cue of signals, such as growth factors, present in the culture medium. However, in vivo the situation is obviously more complex. During CNS development, each OLG process is in contact with a distinct temporally and spatially changing environment. Thus myelin formation is initiated when the axonal membrane and that of the OLG process meet, i.e., actual myelination commences when several processes interact with axons, rather than as a result of a single process–axon interaction [74].

5.1. Adhesive interactions

Several cell–cell and cell–ECM adhesions contribute to induction and maintenance of myelin. Other parameters, triggering and/or facilitating the onset of myelination include the axonal diameter, and developmentally regulated axonal signals. Indeed, a plethora of signals and molecular interactions seem to drive the early onset of myelination, but how these various processes are orchestrated is still largely unknown. Among others, the initiation of myelination relies on contacts at the paranodal regions and signals involving a trans-mode type of interaction between OLG NF-155 and axonal Caspr [75], which may mutually affect their recruitment and distribution at the contact side, presumably localizing to specific membrane domains, acting as interaction platforms. Other key players in the initial interaction events in CNS myelination seem to include the cell-adhesion molecule Nectin-like 1 which is exclusively expressed by neurons in the CNS, its disruption resulting in a developmental delay of myelination [76]. Furthermore, also the developmentally upregulated axonal adhesive protein L1, and the ECM protein laminin-2 [77], have been implicated in the initiation of myelination. Interestingly, other axonal proteins such as PSA-NCAM and LINGO-1 that are developmentally downregulated may delay the rate of myelination [78,79], thus reflecting the tight balance of regulation of myelination by axonal protein expression per se.

ECM molecules and their major receptors, integrins, are involved in governing and orienting apical–basal polarity in a variety of cells. The onset of polarity development in epithelial cells requires basal ECM attachment, a situation which might also be valid in OLG polarity development. OLG, cultured on laminin-2, show specific myelin sheet-directed vesicular traffic of the viral basolateral model protein VSV G [80] while formation of myelin sheets is promoted, relative to the sheet formation of cells grown on the inert substratum poly-L-lysine. Given its expression in developing axonal tracts [77], and the observed myelination defects in mice and humans upon its deficiency, laminin-2 is an ideal candidate as a spatial cue to orient OLG polarization, i.e., by facilitating basolateral-directed trafficking to myelin membranes, thereby initiating myelination. Interestingly, when OLG are grown on fibronectin, another ECM molecule that is abundantly expressed in MS lesions, myelin sheet-directed vesicular traffic of VSV G and PLP is significantly impaired [80], coinciding with a frustrated polarity development of the cells. Interestingly, MBP is expressed in OLG grown on fibronectin, and although myelin formation is strongly reduced, some cells were able to produce myelin sheets. These myelin sheets contain MBP, but lack PLP and VSV G, suggesting that transport of MBP-containing mRNA granules and its translation is less strictly ECM-dependent than sheet-directed vesicular trafficking, which is required for PLP delivery into the sheet.

At the molecular level, laminin-2 interferes with the lateral distribution of integrins, allowing integration of integrin and growth factor signaling to direct OLG behaviour, in particular survival and myelination [81]. Integrins, a family of heterodimeric transmembrane receptors, are often critical mediators of cellular interactions with ECM. Of the 20 known integrin species, OLG express a limited repertoire, i.e., $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 8$, and $\alpha 6\beta 1$, and the expression of each integrin receptor is specifically associated with one aspect of OLG behaviour. $\beta 1$ integrin has been implicated in target-dependent myelination, although seemingly different and conflicting results have been published upon loss of function of $\beta 1$ integrin concerning its role in oligodendrocyte survival and/or myelination [82–84]. This might be due to the timing of $\beta 1$ integrin deficiency, i.e., whether it occurs at the progenitor, premyelinating or myelinating stage of OLG development. Furthermore, a loss of $\beta 1$ integrin in OLG can be compensated for by dystroglycan, a laminin receptor present in OLG [85], that organizes cellular polarity in epithelial cells. Nevertheless, since myelin defects, both in vitro and in vivo are observed upon loss of $(\alpha 6)\beta 1$ integrin function in OLG, a poor polarization of the cells upon $\beta 1$ integrin deficiency could be the underlying mechanism.

Intracellular integrin signaling is often mediated by small GTPases, such as Rac, Rho and Cdc42. Cd42 and Rac1 play a critical role during the early stages of myelination, particularly in compaction [86]. Mutant mice show thinner myelin sheaths, but the periodicity of compact myelin and the expression of myelin proteins, including MBP and PLP, is not affected. In addition, large vesicles are sometimes found in the excess cytoplasm, suggesting a role of these small GTPases in vesicular transport and/or OLG polarity

development. Rho inactivation inhibits clathrin-independent endocytosis of PLP, enhances MBP expression, and condenses the plasma membrane in a polarized manner [87]. An unknown factor, secreted by neurons, downregulates Rho activity, and thereby transcytosis of PLP, whereas the effect on MBP expression is likely mediated by adhesive axo-glia interactions. Indeed, axonal LINGO-1 prevents myelination as it decreases fyn kinase activity in OLG, resulting in enhanced Rho activity, with a subsequent decrease in MBP expression [88], possibly related to diminished transport of MBP mRNA granules into the sheet. In contrast, tenascin-R mediated inhibition of Rho activity prevents myelin-membrane formation without altering the number of MBP-expressing cells [89]. Tenascin-R is an ECM molecule, mainly expressed by OLG, which acts as an autocrine factor and binds to OLG via a sulfatide-mediated mechanism. It is tempting to suggest that tenascin-R may 'shield' sulfatide in this manner and thereby preclude sequestration of the lipid into correct membrane microdomains. Alternatively, tenascin-R might modulate Rho activity via a fyn-independent pathway allowing OLG differentiation without immediate myelin formation.

Fyn, a member of the src kinase family, appears to play a central role in OLG differentiation and initial events of myelination. Mice that lack fyn display a myelin deficient phenotype, showing reduced amounts of MBP [90]. As discussed above, the underlying mechanism likely involves fyn-mediated translation of MBP mRNA via phosphorylation of hnRNP A2. Axonal L1 binding to oligodendroglial contactin (F3) appears to be the trigger for fyn activation and subsequent targeted MBP translation [30]. Fyn is however also one of the components of the intracellular signaling pathway of integrins, acting upstream of Rho, Cdc42 and Rac. Assembly of a lateral integrin/contactin complex, which may also include NCAM 120 has been implicated in the differential control of fyn activity [91], mediating OLG survival and myelination, and co-amplifying cell–cell and cell–ECM adhesion. In passing, all these components are known to concentrate in rafts, and their clustering in this manner would thus provide an optimal environment for sequential and timely activation of molecular players in the overall process. In fact, such coordinated adhesion, might be an elegant way to synchronise trafficking of the major myelin components MBP and PLP, as further discussed below.

5.2. Soluble factors

Similar to adhesive interactions, secreted soluble factors could also influence sorting and trafficking of myelin proteins. As already referred to above, a diffusible neuronal signal may inhibit Rho activity and thereby clathrin-independent endocytosis of PLP [87]. In addition, a neuronal cAMP-dependent signaling pathway triggers PLP exocytosis from late endosomal storage compartments to myelin-like membranes [44]. However, the identity of the secreted neuronal signal(s) remains to be determined.

Protein kinase C (PKC) activation prevents OLG differentiation via differential control of cognate polarized trafficking pathways at the onset of myelination [92]. Upon PKC activation, both OLG progenitors and mature cells are no longer capable of regulating actin-membrane interactions [80,92]. As a result of PKC-mediated phosphorylation of MARCKS, actin filaments redistribute to the submembranous or cortical actin cytoskeleton, which impedes plasma membrane directed traffic of 'basolaterally' sorted VSV G [80,92]. Consistent with data obtained for this viral model protein as a marker of sheet-directed transport, PKC activation also inhibits PLP transport to myelin-like membranes, but not the appearance of MBP [10], emphasizing the specificity of this effect on vesicular trafficking, possibly on docking, given the dependence of both MBP mRNA granules and PLP transport vesicles on filament integrity. FGF-2, which is secreted by neurons and astrocytes, shows a similar PKC-dependent actin redistribution in OLG progenitors [93], suggesting that FGF-mediated inhibition of OLG differentiation might be due to impaired polarity development.

6. Myelin biogenesis: the myelin synapse

Taken together, the evidence obtained thus far is consistent with the notion that MBP and PLP trafficking are physically segregated to limit ectopic assembly and compaction of myelin membranes around axons. Regulation of their trafficking is under neuronal control, and in addition, tightly linked and coordinated. The fact that MBP trafficking is mainly regulated by adhesive interactions, and PLP by secreted factors is very intriguing and lead us to suggest the following model for myelin-membrane formation (Fig. 3).

Upon differentiation OLG extend multiple (primary) processes, which branch into secondary and tertiary ones, in search of myelin receptive axons. Upon axonal recognition, secondary and tertiary processes disappear, and axonal L1 and laminin-2 engage their ligands, i.e., oligodendroglial contactin for L1 and integrin $\alpha 6\beta 1$ and/or dystroglycan for laminin-2 (Fig. 3, 1). Likely, these receptor–ligand pairs become spatially organized into a distinct stable signaling entity, e.g. a raft-like structure. The L1–contactin as well as the laminin-2–integrin interaction will differentially control fyn kinase activity, which will induce MBP translation (Fig. 3, 2), orient OLG polarity (Fig. 3, 3a) and initiate myelin-membrane biogenesis (Fig. 3, 3b). These interactions across the apposed membranes very much resemble the interface between an antigen presenting cell and a T-cell, i.e., the immunological synapse, and in the case of myelination can therefore analogously be referred to as a 'myelin synapse'. MBP expression mediates lateral raft clustering, plasma membrane condensation, and cytoskeleton disassembly, thereby narrowing intermembrane distances that enables in concert with the action of (extracellular) PLP multilamellar myelin-membrane growth and stabilization around an axon. In addition, MBP binds to the SH3 domain of fyn [94], and might regulate its activity. At the same time, when OLG polarity development further matures in the presence of laminin, de novo synthesis of PLP will be promoted and stored PLP will be recruited from LE/lysosomal storage compartments for myelin biogenesis and ensuing myelination (Fig. 3, 3a and 4). To this end we suggest that neurons will trigger the secretion of a soluble cAMP-dependent factor, via an as yet unknown mechanism (Fig. 3, 5), which will trigger co-transport of PLP/GalCer/cholesterol from the storage sites via a direct or indirect (e.g. via ARE/SAC) transport pathway to the myelin membranes (Fig. 3, 6). The subsequent integration of PLP into myelin membranes will allow close apposition of the extracellular leaflets of different wraps, thereby providing a possibility of trans-carbohydrate–carbohydrate interactions between GalCer/sulfatide, exposed on extracellular leaflets, across opposing membranes, as discussed above. The formation of these 'glycosynapses' may then trigger a redistribution of MBP in the intracellular leaflet, enabling its association with PLP and reorganisation (depolymerisation) of actin filaments and microtubules [14], events that are imperative for compaction to occur (Fig. 3, schematic step 7).

Obviously, to test this model, further studies at a multidisciplinary level, from biophysics to cell biology, involving model monolayer studies, in vitro cell cultures and animal studies, are warranted to fully understand the overall process of myelin biogenesis, myelin wrapping and compaction, including myelin stability. However, the myelin synapse model does accommodate that MBP initiates the onset of myelination and is therefore indispensable for myelination, whereas PLP is not. This is also reflected by

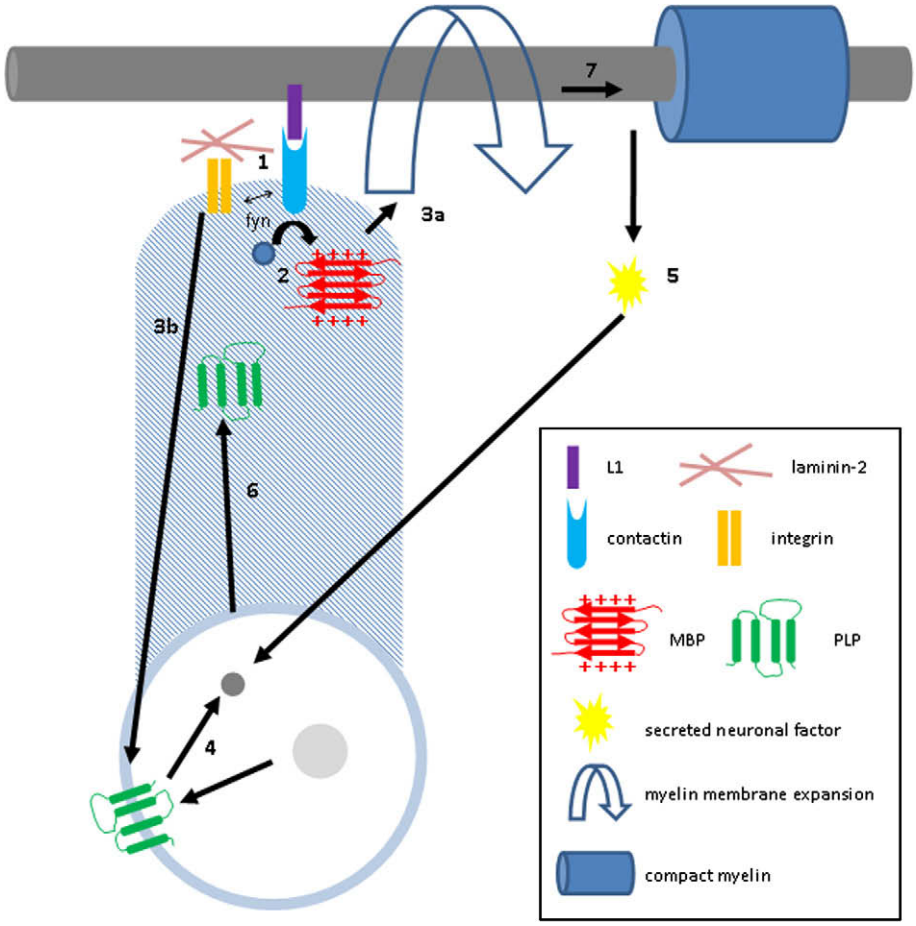

Fig. 3. Schematic illustration of the 'myelin synapse'. Two distinct adhesive signals – L1–contactin and laminin-integrin α61 – between the axon and a primary process of oligodendrocytes will form a complex ('myelin synapse', 1) that will initiate a cascade of reactions ultimately leading to an insulating myelin sheath: synthesis of the myelin-specific proteins MBP (2) and PLP (3b, 4), myelin-membrane extension and axonal enwrapment (3a), transcytotic PLP transport via a direct of indirect pathway (6), directed by a diffusible axonal signaling factor (5), and finally myelin compaction (7, see Fig. 2). See text for further details.

fewer naturally occurring MBP than PLP mutants. Furthermore, *Shiverer* mice, which bear a mutation of MBP, are myelin deficient, and have reduced amounts of PLP. Indeed, it has been reported that PLP expression is initiated upon correct axonal adhesion. This MBP-mediated secondary reduction of PLP is actually very often observed. In addition, in rat-derived myelinating mixed brain cell cultures, MBP appears in myelin membranes prior to GalCer and PLP (our unpublished observations). Furthermore, structurally altered PLP or its overexpression leads to premature death of OLG at the end of the myelination period [95], indicating that myelin-membrane expansion and wrapping is not dependent on PLP.

The model implies that the neuron senses when myelin formation succeeds and it will be important to identify molecular players involved in this process, for example like neuregulin in Schwann cell myelination in the PNS, as described above. By a direct link between signaling and trafficking, such an axon-coordinated, sequential recruitment of MBP and PLP to myelin is warranted, thereby preventing premature and inefficient myelin compaction.

Acknowledgements

Work in our laboratory is supported by grants from the Dutch MS Research Foundation ('Stichting MS Research', W.B., D.H.), and the Netherlands Organization of Scientific Research (NWO, 'VIDI', W.B.). All members of the Hoekstra laboratory, as cited in references, are gratefully acknowledged for their expert assistance in and enthusiastic support and contributions to these studies.

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