On the cause of multiple sclerosis

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Chapter 1

Trafficking and sorting in oligodendrocytes; the role of galactolipids, MAL and syntaxins, in establishing and maintaining the myelin sheath

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ABSTRACT

Oligodendrocytes (OLGs) are myelinating cells, present in the central nervous system. Myelination entails the biogenesis of a membrane system that extends from the OLG plasma membrane, and eventually enwraps axons to optimize saltatory signal conductance. Failure of (re)myelination leads to neurodegenerative diseases such as multiple sclerosis (MS), the etiology of which is still unknown. Clarifying underlying mechanisms of myelin biogenesis, assembly and its maintenance, will be crucial in the rational development of an effective therapy in diseases such as MS. Important in this context is the gradual awareness that OLGs should be considered as polarized cells, exposing two distinct surface membrane domains, i.e., the cell body plasma membrane and the myelin membrane. It is therefore anticipated that valuable mechanistic insight into the polarized nature of OLGs in general, and myelin biogenesis in particular, might be derived from previous studies on a similarly polarized nature of epithelial cells. Here we will discuss the extent to which membrane polarity of OLGs in terms of trafficking and sorting mirrors that of epithelial cells. We will highlight current knowledge of the trafficking of several myelin specific proteins, including proteolipid protein and myelin basic protein, and the role of myelin enriched galactolipids as sorting platforms. The role of additional molecular entities in sorting and trafficking such as MAL and SNAREs, and their polarity-specific features, will also be discussed. Particular attention will be given to the potential involvement of a 'common recycling endosomal system' in OLGs, which has been well-characterized in regulating polarized trafficking and membrane maintenance in epithelial cells.

INTRODUCTION

The myelin sheath is a specialized membrane structure that enwraps axons of neurons, which is crucial for efficient conduction of impulses along the nerves in the central nervous system (CNS) of vertebrates. Myelin can be pictured as an onion-ring like structure, showing a tightly wound multilayered membrane sheath around the axon (Fig. 1). In this manner an effective insulating layer is created, which is virtually devoid of cytosol. The electrical resistance of myelin allows for saltatory conduction of nerve impulses, driven by a membrane potential, arising from the presence of sodium channels that are localized in small, myelin-devoid gaps (Fig. 1A). These gaps, the so-called nodes of Ranvier, are formed in between consecutive myelin coated regions, known as internodes, along the axon. When myelin is damaged or disappears, and when remyelination fails, axons can suffer long term damage and as a consequence, nerve impulse conduction is severely hampered. Ultimately, axons may degenerate (as reviewed in Ref. 23). In the CNS, oligodendrocytes (OLGs) are the cells responsible for myelin production, whereas in the peripheral nervous system (PNS) Schwann cells display this capacity. During development, as monitored in cultured oligodendrocyte progenitor cells (OPCs), extensions protrude from the cell's plasma membrane that spontaneously mature into large myelin membrane 'sheets', the *in vitro* equivalent of myelin 'sheaths' (Fig. 1C). *In vivo*, the process is more rigorously regulated and depends among others on axon-glia recognition [9,10,24,25].

The molecular composition of the myelin membrane differs from that of the plasma membrane [26]. This implies that mature OLGs are polarized cells and, like epithelial cells, express different surface membrane domains, i.e., the cell body plasma membrane and the myelin membrane (as summarized in [9,27–29]). Upon wrapping of the latter around the axon, most of the cytosol and its contents are locally displaced and the opposed bilayers become closely apposed in order to form a compact multilayered membrane structure (Fig. 1B, D). Myelin membranes are relatively enriched in lipids (70% of the cell's dry weight) and contain proteins unique to myelin. The two major proteins are the multi-membrane spanning proteolipid protein (PLP) and the peripherally membrane associated myelin basic protein (MBP), which, among others, are required for promoting myelin compaction by tightly linking outer- and inner membrane surfaces, respectively [30–32]. Mechanisms underlying the biogenesis and maintenance of myelin membranes are gradually emerging [9,10,16,27,33,34]. Given the polarized nature of OLGs, such insight particularly benefits from detailed studies carried out previously on polarized trafficking in epithelial cells, which expose apical and basolateral surface membrane domains of different composition. Interestingly, the myelin membrane is further segregated into distinct subdomains with differences in the lateral distribution of proteins in the radial and longitudinal dimension, implying a further sophistication in terms of protein and, presumably, lipid sorting [9,16,26,28,33,35]. Hence, studies on molecular trafficking and sorting in polarized OLGs will similarly contribute to improving our insight into fundamental cell biological principles in general. In addition, this insight will also be most useful in potentially remedying defects in myelination, as occurs in a variety of neurological disorders, including multiple sclerosis (MS).

Here, we will summarize and discuss recent progress in clarifying the regulation of polarized sorting and trafficking in developing and mature OLGs. In particular we will focus on the sorting and vesicle-mediated trafficking of PLP on the one hand, and the processing of MBP, on the other, the latter being transported as mRNA in granules via microtubule-dependent transport and translated 'on-site', i.e., at the myelin membrane [12–14]. Where relevant, the processing of additional myelin-specific proteins will be included. We will discuss the intracellular processing of these proteins in the context of known regulators of

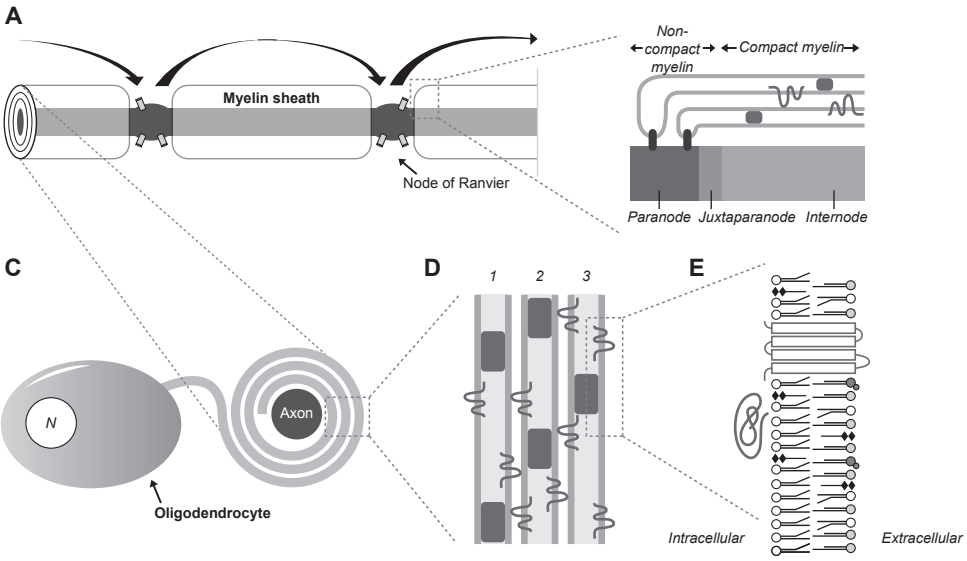


Figure 1. Myelin and the oligodendrocyte

A) The myelin sheath is wrapped around the axon, leaving small segments, the nodes of Ranvier, exposed to allow salutatory (jumping) signal transduction via sodium channels. **B)** The myelin consists of tightly wrapped compacted myelin at the internode. Towards the node of Ranvier, at the paranode, myelin is non-compact and interacts via oligodendroglial NF155 with receptors on the axonal membrane. **C)** The myelin sheath is formed by the oligodendrocytes in the central nervous system, extensions from the plasma membrane wrap several times around the axon and expel their cytoplasm and extracellular space during compaction. **D)** The compaction of myelin is facilitated by the two major myelin specific proteins, MBP and PLP, on the intracellular and extracellular side of the lipid bilayer, respectively. This results in consecutive layering of plasma membranes virtually devoid of cytoplasm and extracellular space of which 3 layers are shown. **E)** The intracellular leaflet of the membrane lipid bilayer contains phospholipids and cholesterol, the extracellular leaflet contains in addition the galactolipids galactosylceramide and sulfatide, which are enriched in myelin. The peripheral protein MBP resides at the intracellular leaflet via electrostatic interaction, while PLP is a multi-membrane spanning protein.

intracellular polarized transport, in particular N-ethylmaleimide-sensitive factor attachment protein receptor proteins (SNAREs) and myelin and lymphocyte protein (MAL), which are also expressed in OLGs [9,36–39]. In addition, we will take into account the role of the glycosphingolipids galactosylceramide (GalC) and its sulfated form, sulfatide, which are specifically enriched in the membrane of the myelin sheath. Together with cholesterol and phospholipids with saturated acyl chains, glycosphingolipids separate into distinct membrane microdomains, which play an important role in sequestering and trafficking of proteins as well as provide signaling scaffolds in polarized cells. A both timely and spatially correct expression of all these components appears crucial for a proper development of OLGs and their myelinating capacity.

DYNAMICS OF GALACTOSYLCERAMIDE AND SULFATIDE: INVOLVEMENT IN MYELINATION

Although the net lipid content of the myelin membrane is relatively high compared to that of a regular plasma membrane, their qualitative overall composition is similar and consists of the major lipid classes commonly found in membranes, i.e., phospholipids, cholesterol, and glycosphingolipids [16,40]. However, relative to its content in the plasma membrane of the OLG cell body, the glycosphingolipid content in the myelin membrane is considerably enhanced, accounting for approximately 25% of the total lipid pool versus 5% in the plasma membrane [41,42]. This membrane domain-dependent difference in glycosphingolipid distribution is reminiscent of such an enrichment of glycolipids in the apical versus basolateral membrane domain in polarized epithelial cells [43–45]. Interestingly, the pool of glycosphingolipids essentially consists of GalC and its sulfated derivative sulfatide, which are present at a molar ratio of 6:1 [46], and both lipids are expressed prior to the expression of the major myelin proteins PLP and MBP [47–49]. GalC is synthesized at the luminal side of the endoplasmic reticulum (ER) by linkage of activated galactose to a ceramide molecule, mediated by the enzyme UDP-galactosylceramide galacatosyl transferase (CGT). A vesicular transport pathway subsequently carries the lipid to the Golgi apparatus where it is partly converted into sulfatide. This occurs by attachment of a sulfate group to the 3'-OH moiety of the galactose moiety by the enzyme 3'-phosphoadenylylsulfate:galactosylceramide 3' sulfotransferase (CST) present in the lumen of the Golgi, employing 3'-phosphoadenosyl-5'phosphosulfate as a substrate [50,51]. From the Golgi, the galactolipids subsequently reach the cell surface by means of vesicular transport. In addition, it cannot be excluded that a fraction of the newly synthesized GalC may have reached the surface membranes in a direct manner by non-vesicular mechanisms, such as via lipid transfer proteins and/or lipid droplet-mediated transport [9,52–54]. In these glycolipids, the carbohydrate residue is attached to a ceramide backbone structure to which two long hydrocarbon tails are attached, one mono-unsaturated alkyl and one fatty acyl chain, that may vary in lenght from 16-26 carbon atoms [55–57]. Together with cholesterol, glycosphingolipids frequently localize in specific membrane microdomains, operationally defined by their resistance to solubilization in distinct non-ionic detergents. These microdomains, also known as 'rafts', are considered highly dynamic structures, providing platforms that are instrumental in a variety of processes, ranging from sorting and transport of myelin proteins to harboring signaling entities that regulate OLG behavior (Chapter 4 and [7,11,15,58–65]). Of interest, GalC and sulfatide may partition into distinct domains and may contribute in this manner to protein sorting and/or regulate the lateral distribution of myelin proteins [15]. For example, PLP and MBP reside in compact myelin with GalC, whereas sulfatide seems a driving force for the localization of NF155 (neurofascin, Mw 155 kDa) in non-compact myelin [9,60,66]. Whether cholesterol levels also play a governing role in establishing a preferred myelin protein/galactolipid environment, given that PLP is closely linked to cholesterol biosynthesis and transport [11,67,68] and hence the potential assembly of cholesterol/GalC enriched microdomains, remains to be determined. In this context it is of interest to note that the biosynthesis of cholesterol is rate limiting for myelin biogenesis, i.e., when cholesterol synthesis diminishes, the synthesis of myelin protein mRNA is downregulated and its transport to the sheet becomes perturbed [11,69,70]. Furthermore sulfatide versus GalC-enriched domains

display differences in detergent resistance, likely reflecting differences in molecular domain composition. As discussed elsewhere (Ozgen et al., in revision), fatty acyl hydroxylation plays an important role as well, increased levels perturbing the formation of membrane microdomains [71,72]. In this context, hydroxylation levels of sulfatide are remarkably increased in brain material of MS patients [57], which thus could affect distinct raft-associated functions such as signaling and membrane transport, including the formation of paranodes [7,15,61,65,73–76]. A further level of complexity in the functioning of galactolipids may thus arise when taking into account fatty acyl species-dependent differences in their lateral distribution, and hence their extent of partitioning in distinct membrane microdomains to express domain-specific functions. Systematic studies in that regard have not been undertaken so far.

The role of membrane microdomains, serving as a platform in a variety of signaling events, is well recognized. Similarly, GalC/sulfatide containing microdomains may transduce signals that are crucial for OLG development. For example, it has long been established that antibodies directed against GalC and/or sulfatide reversibly inhibit differentiation of OPCs into mature OLGs (Chapter 4 and [62,77–81]). In addition, *in vitro* and *in vivo* studies have shown that sulfatide is a negative regulator of OLG differentiation (Chapter 4 and [62,82–85]). Not only may such signaling arise from lateral interactions between glycolipids and/or proteins, residing in rafts, it has also been proposed that GalC and sulfatide may engage in transmembrane interactions thereby giving rise to the formation of glycosynapses, involving trans carbohydrate-carbohydrate interactions [86–88]. These interactions may lead to a perturbation of the cytoskeleton and result in membrane domain clustering, thus initiating transmembrane signaling [86,89,90]. Evidently, these latter types of interactions likely play a role in actual myelination, for example in wrapping the myelin sheath around the axon and/or in regulating compaction, given the need for closely apposed membranes in order to allow such interactions to occur. It is of particular interest in this regard that MBP dynamics is mainly governed by GalC, in spite of the fact that the protein and lipid are asymmetrically distributed across the bilayer [15,87,89,91,92]. Specifically, the presence of GalC promotes MBP's association with detergent-insoluble microdomains, suggesting that a lateral redistribution of the lipid may affect MBP's functioning in myelin compaction [15,80].

Sulfatide has been implicated in adhesive processes, and in the myelin membrane the lipid can interact with the extracellular matrix (ECM) molecule tenascin R [93], which suggests a function in cell recognition and adhesion. This galactolipid may also interact with the ECM molecule laminin-2 [94–98], present on the surface of developing axons [99] implying a potential role in axon-glia cell interaction [7]. Disruption of laminin-2/sulfatide interactions impedes OLG differentiation and myelin-like membrane formation (Chapter 4[62]). Furthermore, laminin-2 facilitates the interaction between integrin $\alpha 6\beta 1$ and PDGFRα, initially localizing in separate membrane microdomains [63]. Sulfatide resides in these microdomains and serves as a receptor for laminin-2 (Chapter 4[62]), while the extent of its expression determines the presence of PDGFRα in membrane microdomains [100]. In cells grown on laminin-2, sulfatide laterally associates with integrin $\alpha 6$ in membrane microdomains, a necessary event for integrin $\alpha 6\beta 1$/PDGFRα interactions. Anti-sulfatide antibodies disrupt this interaction on laminin-2 and cause demyelination in myelinated spheroid cultures (Chapter 4[62]). Taken together, these

findings highlight the importance of laminin-sulfatide interactions in the formation of membrane microdomain-associated signalling platforms, essential for myelination, triggered upon axonal demand.

Thus, both GalC and sulfatide play distinct roles in myelin biogenesis and maintenance, thereby determining the overall stability and proper functioning of the myelin membrane. In this regard, observations in CGT-knock-out mice, i.e., lacking both GalC and sulfatide, revealed severe alterations in the myelin structure, displaying myelin sheaths that are thin, incompletely compacted and unstable, with structural abnormalities in the nodal and paranodal regions, including disrupted axo-glial junctions [73,84,101–105]. The underlying cause of these defects has been related to a perturbed adhesion between neuron and glial cells at the paranodal region [73,84,106]. The functional relevance of sulfatide containing microdomains in OPC proliferation and differentiation is further emphasized upon manipulation of its pool size. *In vivo*, sulfatide homeostasis is regulated by CST and arylsulfatase A (ASA), which is responsible for the lysosomal hydrolysis of sulfatides. ASA deficiency, as observed in the genetic disorder metachromatic leukodystrophy (MLD), causes accumulation of sulfatide in the brain and, importantly, demyelination occurs [107–109]. A misbalance in sulfatide homeostasis appears to influence the production of OLGs as a result of deregulating PDGFRα levels [100], a membrane-bound receptor for platelet-derived growth factor (PDGF) A that is essential for controlling OPC proliferation, survival and differentiation [110,111]. In fact, this particular function of the growth factor depends on its presence in membrane microdomains [63,112,113], apparently requiring a specific composition, since in ASA-deficient OPCs the association of PDGFRα with detergent-resistant membranes becomes severely impaired, although sulfatide levels in the cells were significantly increased [100]. However, upon rescue with ASA, PDGFRα levels and its localization to detergent-resistant membranes is restored, as well as the differentiation of OLGs [100], highlighting a distinct role of sulfatide-containing microdomains in this event.

Next to serving as platforms for cell adhesion and receptor signalling, GalC/sulfatide microdomains also engage in intracellular protein trafficking during myelin biogenesis and maintenance.

POLARIZED TRAFFICKING IN OLIGODENDROCYTES

As observed in polarized epithelial cells, mature OLGs display distinct, polarized surface membrane regions, reflected by differences in the molecular composition of plasma membrane and myelin membrane (as reviewed in Ref. 9,27–29). Intriguingly, several mechanistic studies on the nature of the trafficking pathways towards either OLG surface suggest that the plasma membrane domain is being served by an apical-like mechanism, while the sphingolipid-enriched myelin membrane, in terms of composition reminiscent of the epithelial apical surface, is the target of a basolateral-type trafficking mechanism (Chapter 2 and [9,29,114–117]). Of further interest in this context are recent observations that the basal polarity complex protein Scribble is expressed in OLGs and up-regulated in mature cells, in which it localizes towards the (basolateral-like) myelin sheath. Elimination of the expression of Scribble in mice resulted in a disrupted initiation of myelination and sheath extension [118]. Together, this kind of knowledge is of relevance in appreciating the nature of underlying mechanisms, pertinent to the biogenesis of both plasma membrane and myelin sheath in OLGs, and the compatibility with previously obtained principles in polarized trafficking in epithelial cells. Thus throughout myelin biogenesis and its maintenance, OLGs sort and transport different cargos from the cell body via primary processes to the growing myelin sheath, and available data indicate that trafficking to the OLG plasma membrane and myelin membrane are driven by an apical and basolateral mechanism, respectively [7–9,19,27,39,114,119–121].

Overall, following *de novo* biosynthesis of core structures at the ER; (membrane) proteins, ceramide and GalC are transported to the Golgi apparatus for posttranslational modification (proteins) or complex biosynthesis (glycolipids), and are subsequently segregated into distinct microdomains in the trans-Golgi network for targeted delivery via vesicular transport to the apical or basolateral surface. To maintain a polarized distribution in either domain in the face of continuous endocytosis, polarized epithelial cells also employ a so-called 'common recycling endosomal system' for sorting and recycling of internalized apical and basolateral proteins and lipids (Fig. 2A [122–125]). In addition, not all apical resident proteins in epithelial cells are sorted and transported directly to their final destination. Rather, as prominently apparent in the major epithelial cells in the liver, hepatocytes, distinct apical (bile canaliculus) resident proteins are first transported to the basolateral surface prior to their sorting and trafficking to the apical membrane domain, a mechanism known as transcytosis [126–128]. Interestingly, among others these different pathways were found to be related to the nature of the membrane anchor of transported apical proteins and to the presence of cholesterol [126]. Specifically, the distinct apical resident proteins become integrated into distinct raft domains, characterized by differences in detergent solubility, which subsequently partitioned into transport vesicles, mediating direct (i.e., transport from the Golgi to the apical surface) or indirect 'apical' transport, i.e. transport from the Golgi to the basolateral surface prior to their transport to the apical surface. Thus, multi-membrane spanning proteins were found to engage in the direct route, whereas single membrane spanning proteins, including GPI-anchored proteins, participate in the indirect, transcytotic pathway [126]. Whether similar mechanistic features and a similar common recycling

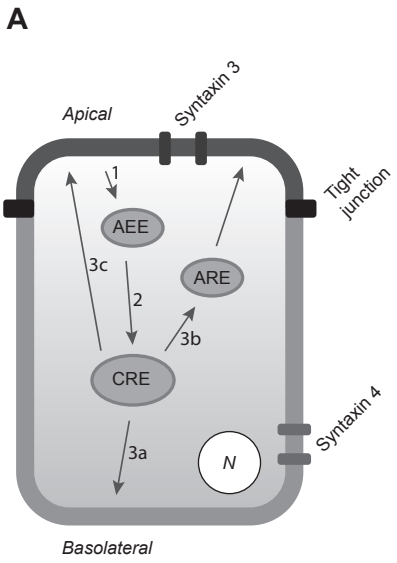
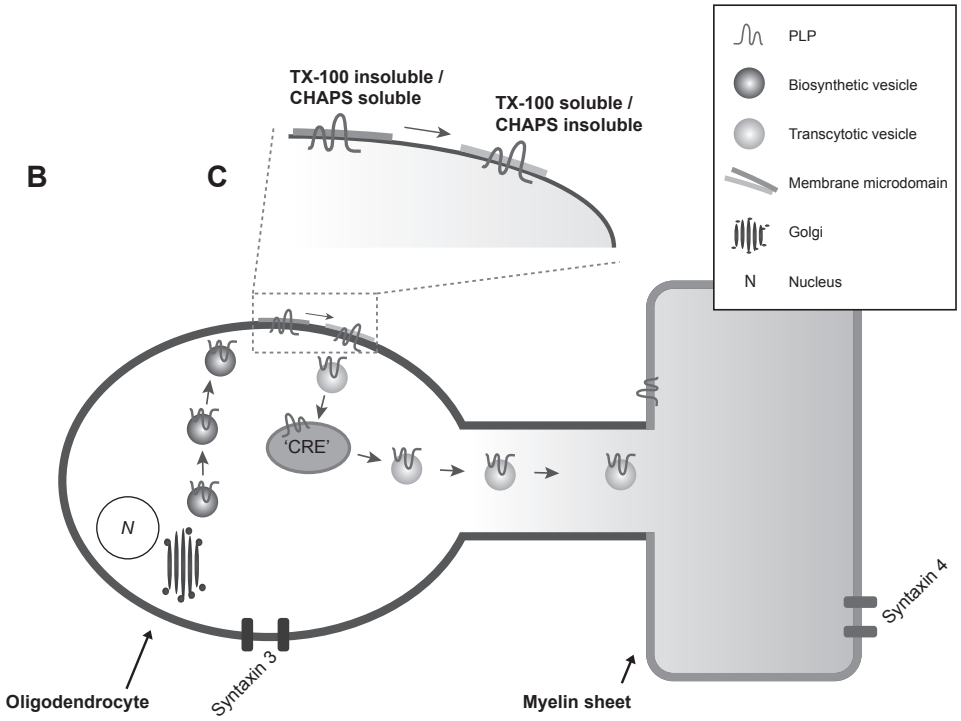


Figure 2. A model for transport of PLP in oligodendrocytes via a common recycling endosome

A) The apical endocytic system in polarized epithelial cells employs transcytotic trafficking to serve the basolateral membrane (light grey). Proteins that have been transported to the apical membrane (dark grey) after biosynthesis, traffick via an apical early endosome (AEE), route 1, to a common recycling endosome (CRE), route 2, after which several paths are possible; proteins are transported to the basolateral surface (route 3a) or are returned to the apical membrane directly (route 3c) or via an apical recycling endosome (ARE) (route 3b). **B)** Likely, in oligodendrocytes a similar transcytotic route is in place for PLP transport to myelin membranes. After biosynthesis in the Golgi, PLP is transported to the apical-like cell body plasma membrane (dark grey). Here, under influence of a.o. sulfatide, PLP localization changes from microdomains that are TX-100-insoluble, CHAPS-soluble (dark grey) to microdomains that are TX-100-soluble, CHAPS-insoluble (light grey) **(C)**, coinciding with a conformational change of PLP. Upon internalization PLP is transported to an 'CRE' from which it is further trafficked to the basolateral-like myelin sheet (light grey).



endosomal system also regulate polarized trafficking in OLGs is of major interest. So far evidence in support of such a system in these cells remains to be provided. However, some suggestions might favour this possibility, as further discussed in the following. The (transient) association of the major myelin protein PLP with distinct membrane microdomains, defined by differences in detergent resistance, appears instrumental in its sorting and trafficking in OLGs [7]. As discussed above, MBP is not transported as the native protein, but rather as mRNA, which does not depend on vesicular transport. Nevertheless, within myelin, the peripheral membrane protein MBP (partly) associates with detergent-resistant microdomains that contain GalC, cholesterol and GM1 as well as typical raft proteins like flotillin and caveolin [129–135]. Critical for the sorting and trafficking of the multi-membrane spanning protein PLP and its subsequent assembly into myelin is its transient partitioning into distinct galactolipid-enriched rafts [7,11,15]. Interestingly, in a variety of cell model systems, including primary OLGs, it has become apparent that following *de novo* biosynthesis, PLP is trafficked along an indirect, i.e., transcytotic pathway towards the basolateral-like myelin sheet (Fig. 2B [7,8,11,136]). This pathway involves initial, 'direct' transport from the ER via the Golgi to the apical-like cell body plasma membrane. This is consistent with the direct, apical directed pathway of similar multi-spanning membrane proteins in epithelial cells, followed by internalization at the plasma membrane and subsequent vesicular transport to the developing myelin sheet (Fig. 2B). These different steps can be biochemically discerned by PLP's dynamic partitioning into different microdomains, initially in TX-100-resistant domains and after arrival at the cell body plasma membrane of the OLG its lateral transfer into CHAPS-resistant domains. This implies integration of PLP, possibly triggered by an induced conformational change in the protein [7], in sulfatide-enriched domains (Fig. 2C). These domains, which do not assemble upon inhibition of sulfatide and cholesterol biosynthesis [7,11,68], are subsequently internalized, presumably by an endocytic mechanism, followed by vesicular transport of PLP to its final destination, i.e., the basolateral-like myelin sheet [7,11,137]. In this regard, a distinct role of cholesterol [11,68,136] in conjunction with a preferred endocytic capacity of sulfatide [138] facilitating subsequent transport of PLP from the cell body plasma membrane, is of particular interest. In support of this notion, *rsh* PLP, a natural mutation occurring in mice, traverses the Golgi and is transported to the plasma membrane, but fails to sequester the protein to CHAPS-insoluble domains due to impaired cholesterol binding [136], suggesting a role for cholesterol in the localization and trafficking route of multi-membrane spanning PLP. The exact route of the latter part of the overall transport pathway of PLP, i.e., following its internalization from the plasma membrane surface, has not been entirely resolved yet. An initial accumulation of endocytosed PLP in a late endosomal/lysosomal compartment, positive for the presence of LAMP1, has been identified as an intracellular storage and sorting site, prior to PLP's transport to the myelin sheet [20,136,139]. However, in line with the discussion above, it would be of particular interest to examine whether this endocytic compartment in OLGs bears any (functional) similarity to the common recycling endosomal system, as identified in polarized transport in epithelial cells [122–125,140]. In fact, in epithelial cells the common recycling endosomal system is also connected to the late endosomal/lysosomal degradation pathway, identified by LAMP1, and it cannot be excluded that this pathway may become particularly prominent in studies in which trafficking is monitored of overexpressed proteins (cf. Ref. 20). Yet, it should also be noted that the common endosome in these cells may receive cargo from both

apical and basolateral surfaces, which may traverse the compartment and/or recycle to the membrane domain of departure. In this context, although the myelin protein PLP may travel via a common endosome from the apical-like plasma membrane to the basolateral-like myelin sheet, it remains to be seen whether the compartment may also receive cargo from the myelin sheet for degradation, for example during myelin sheet maintenance. Nevertheless, it seems fully justifiable to further investigate a role of a common recycling endosomal system in the regulation of protein trafficking in OLG, also given the presence of known markers of this system, including a Rab11 compartment that lacks internalized transferrin in these cells, and its potential involvement in transcytotic trafficking of PLP [38,121]. Moreover, it has also been reported that Rab3, a family of regulatory small GTPases in the transcytotic pathway, the presence of which in apical recycling endosomes as part of the common recycling endosomal system has been reported [141], can associate with PLP, and is likely involved in PLP trafficking to the plasma membrane [142]. Indeed, Rab3 associates with SNAP29 in OLGs [143], which does not interact with the t-SNARE syntaxin 4 in OLGs [39], but as shown in other cells with the t-SNARE syntaxin 3 [144], which is specifically expressed at the (apical-like) cell body plasma membrane (Chapter 2 [9,116]).

Finally, it is apparent that sulfatide plays a prominent role in myelination at various levels, including axon-myelin attachment, as discussed above, and in specific myelin-directed trafficking events. However, in spite of this involvement, myelin synthesis is not inhibited when the synthesis of galactolipids is inhibited by deleting CGT, although irregularities are apparent in myelin folding around axons [73,84,101–104]. Interestingly, since a very similar phenotype is seen when only CST is deleted, a major role of sulfatide is implied in this case. Thus although galactolipids are not crucial for synthesis of myelin *per se*, a specific role of sulfatide for myelin maintenance and stability has been noted. GalC is prominently present in compact myelin, whereas sulfatide is mainly found in paranodal regions [27]. Specifically, sulfatide is indispensable for the axo-glial adhesion at the paranodes [66,74,75,145]. This role appears to correlate with the sulfatide-dependent localization of the OLG membrane protein NF155 at the paranodes, which allows the protein to associate with axon-localized contactin and contactin associated protein (Caspr), jointly constituting the junctional complex that mediates myelin-axon adhesion [59,99,145–147]. Indeed, a diminished association of NF155 to membrane microdomains is accompanied by the disassembly of the paranodal junction in MS [60,148], and a redistribution is seen of both sulfatide and GalC in distinct membrane microdomains [60].

In general, transport of molecular cargo by membrane vesicles relies, among others, on its proper sorting into membrane domains and its subsequent packaging into distinct membrane vesicles, depending on the site of destination. In addition the intracellular flow of the transport vesicles needs to be carefully controlled, and requires effective delivery of vesicular contents at the desired site of destination. Distinct machineries appear available for such purposes. Here we will focus on two of such 'traffic' regulators, which have been well-characterized in polarized epithelial cells, i.e., the proteolipid MAL, which plays a prominent role in apical targeting of cargo, and SNAREs, which direct vesicular targeting and finalize biosynthetic transport by mediating the merging of the transport vesicle with the target membrane.

MAL AS A REGULATOR OF POLARIZED TRAFFICKING IN OLIGODENDROCYTES

MAL is a 17 kDa proteolipid protein with a hydrophobicity pattern that indicates a four transmembrane domain structure, similar to PLP [149,150]. MAL has been identified as a component in regulating apical trafficking. In polarized epithelial cells, apical proteins are clustered in rafts and packaged into vesicles destined for the apical membrane. Such vesicles also contain MAL, which appears important for both the assembly and targeting of the apical transport vesicles [151–154]. Thus, blocking the expression of MAL, using antisense constructs, inhibits the trafficking of apical-directed proteins in polarized MDCK cells, while its overexpression results in elaboration of the apical membrane surface [153]. More specifically, MAL binds tightly to glycosphingolipids, in particular sulfatide [155,156], and promotes and stabilizes the formation of detergent-resistant raft domains in the Golgi [157]. In these domains, single membrane spanning and GPI-anchored apical resident proteins are recruited. Upon their arrival at the apical membrane, the MAL-induced sorting platforms disassemble, and the proteins redistribute by lateral diffusion [157,158]. Thus in this case, MAL is a key component in organizing lipid membrane sorting platforms that are instrumental in the recruitment of apical proteins, crucial to the assembly of the apical membrane in the biosynthetic pathway.

Myelinating cells, i.e., both OLGs (CNS) and Schwann cells (PNS), express MAL [36,159,160]. In immature, non-myelinating Schwann cells, MAL has a function in differentiation, and is present prior to myelin formation [159], whereas in OLGs MAL is upregulated during later stages of myelination [36,37], i.e., after PLP and MBP are expressed. MAL overexpression in Schwann cells impedes peripheral myelinogenesis, as evidenced by a delay in the onset of myelination and a reduced expression of the myelin proteins [131]. Moreover, at these conditions both the cytoskeletal organization and the plasma membrane dynamics are impaired, possibly related to changes in the genetic expression of *S100a4*, *RhoU* and *Krt23*, which frustrate Schwann cell differentiation and myelination [161]. In the CNS, MAL is predominantly localized in compact myelin and is tightly associated with GalC and sulfatide [155,156]. In this context, a regulatory link appears to exist between MAL expression and galactolipid levels. Thus, MAL is expressed in tissues with relatively high amounts of GalC and sulfatide, such as in myelin and in epithelial cells, derived from kidney and stomach [156]. Upon sulfatide accumulation in lysosomes, as is the case in ASA-deficient adult mice, a reduction of MAL expression in OLGs, but not of other myelin specific proteins is detected [162,163]. In addition, in these mice, mistargeting of MAL to a late endosomal compartment in kidney cells is observed [163]. However, in CGT-overexpressing mice, MAL expression is specifically enhanced [164]. Given that GalC levels in ASA-deficient mice are dramatically decreased, whereas sulfatide levels are only marginally affected upon CGT overexpression, the expression level of MAL is likely linked to GalC levels and/or CGT activity. It is tempting to suggest that this potential correlation may be chemically related to the extent of hydroxylation or the length of the fatty acyl chain in GalC. Of interest in this regard is that CGT overexpression particularly results in altered ratios of non-hydroxylated versus hydroxylated galactolipids [164], while also upon development these ratios are subject to changes [165].

In transgenic mice with increased MAL gene dosage, controlled under its own promoter, mild hypomyelination, aberrant myelin formation and disturbed axon-glia interactions are observed [166], indicating that MAL plays a role in the stabilization and maintenance of myelin. Indeed, MAL gene disruption studies emphasized a critical role for MAL in myelin maintenance and assembly of the paranodes in CNS [131]. Thus, while the onset of myelination is not affected, major alterations at the structural level are seen, including aberrant inclusions of cytoplasm within compact CNS myelin. Furthermore, paranodal loops at the node of Ranvier are detached and face away from the axon. Since MAL is absent from the paranodal region, the protein likely interferes in an indirect manner with paranode formation. In fact, MAL interacts with the cytoskeleton protein septin 6, a member of a protein family that is involved in membrane compartmentalization and vesicular transport [167]. Biochemical analysis of myelin and myelin-derived CHAPS-insoluble membrane microdomain fractions obtained from MAL-deficient mice revealed reduced levels of myelin-associated glycoprotein (MAG), MBP and NF155, but not of galactolipid and cholesterol levels [131]. This demonstrates that maintenance of axon-glia interactions in the CNS is dependent on a balanced expression of MAL. Because MAL is exclusively expressed by OLGs in the brain, it is likely that a lack and/or defect of MAL expression is causing altered axon-glia interactions, and malfunctioning of the paranodal NF155, which would be a very likely candidate in causing impaired axon-glia interactions (see above). Given that MAL and NF155 do not physically interact, MAL may well control the proper sorting of NF155 into distinct membrane microdomains, along a mechanism that mirrors its regulatory role in apical membrane assembly in the biosynthetic pathway, as discussed above. Specifically, in OLGs and purified myelin, MAL is localized to CHAPS-insoluble and TX-100-insoluble microdomains [37,155,163,168]. As noted, MAL displays the ability to interact with glycosphingolipids, including GalC and sulfatide, thereby facilitating a lateral segregation of lipids in distinct membrane microdomains. Since the localization of NF155 in paranodes depends on its proper integration in sulfatide-enriched membrane microdomains [66,145], a role of MAL in sorting of this protein can thus be readily envisioned. Given that NF155 is initially clustered at the paranodes in MAL-deficient mice [131], MAL is likely involved in stabilizing NF155-containing membrane microdomains in paranodes rather than in NF155 trafficking towards the paranodal region. Like a stabilizing effect on NF155 localization, transcytotic trafficking of PLP also depends on its proper and timely incorporation into galactolipid-enriched membrane microdomains (as discussed above). In this context, it is plausible that the timely expression of MAL, dictated by the galactolipid expression levels, is accompanied by changes in the protein sorting machinery of PLP, once myelin biogenesis is triggered, which in turn may be of importance in the maintenance of compact myelin and paranodes. Taken together, the timely expression of MAL in OLGs may relate to altered intracellular sorting mechanisms, once OLG differentiation is initiated. This might suggest an additional role, next to MAL's ability in regulating the organisation and stabilisation of membrane microdomains in myelin membranes. In this manner it may also facilitate the formation of glycosynapses and support galactosphingolipid-mediated signalling events (see above).

Finally, MAL2 is a 19 kDa protein with a similar structure as MAL [169–171]. However, in contrast to MAL, MAL2 is glycosylated. MAL2 has been shown to be a key component in the transport

machinery to the apical, bile canalicular membrane in HepG2 cells [170]. Interestingly, MAL2 localizes specifically to apical recycling endosomes [38,172], thought to represent an inherent part of the common recycling endosomal system, as discussed in the previous section. At least in several respects, different properties of MAL2, compared to those of MAL, can be distinguished. For example, in some cells, MAL2 is not associated with membrane microdomains [171,173], indicating that specific MAL2-binding partners are likely involved in regulating apical membrane directed protein trafficking. In this context, in an OLG cell line, MAL2 has been shown to colocalize and interact with (endogenous) PLP after its internalization from the plasma membrane [121]. Most interestingly, this finding suggests that PLP enters an endosomal recycling compartment, a feature in this (non-polarized) cell line that may mimic the subsequent step in PLP's transport to the myelin sheet, as occurs in polarized OLGs. Accordingly, these considerations fully warrant a careful analysis of the dynamics and localization of MAL2 in maturing OLGs and its role in transcytotic PLP transport. As such these observations will bear particular relevance to further exploring the potential functioning of a common endosomal recycling system, involved in polarized transport in OLG.

As noted, like in other mammalian cells, the delivery of vesicular cargo in OLGs will depend on a proper machinery that regulates the targeting of transport vesicles to the desired site of destination. Here we will finally briefly discuss the role of SNAREs in the transfer of PLP and expression of MBP in the myelin sheath.

FUNCTIONAL ROLE OF SNARES IN THE POLARIZED DISTRIBUTION OF PLP AND MBP

Key players in the correct delivery of vesicular cargo to target membranes are SNAREs, whose mechanism of action relies on pairing of specific vesicle and target membrane SNAREs. This key-lock mechanism represents the onset of a complex series of events, involving a host of additional proteins, that leads to membrane fusion between vesicle and target membrane and the integration of *de novo* synthesized membrane proteins into the target membrane [119,174]. Originally, SNAREs were classified as v- (vesicle-associated) or t- (target-membrane) SNAREs on the basis of their location and functional role in a typical trafficking pathway. However, a more recent classification scheme takes into account structural features of SNAREs, dividing them into R-SNAREs and Q-SNAREs on the basis of whether the central functional residue in their SNARE motif is arginine (R) or glutamine (Q). Often, R-SNAREs act as v-SNAREs and Q-SNAREs as t-SNAREs [175].

Consistent with a polarized distribution of t-SNAREs in epithelial cells, syntaxin 3 localizes primarily at the apical and syntaxin 4 at the basolateral membrane surface [176–178]. In primary OLGs the t-SNARE syntaxin 3 localizes at and near the plasma membrane of the cell body, whereas basolaterally localized syntaxin 4 localizes to the myelin sheet (Fig. 2B, Chapter 2 [9,116]). Moreover, during OLG maturation, the expression level of syntaxin 4, relative to that of syntaxin 3, increases rapidly upon the initiation of myelination (Chapter 2 [39,116,179]), supporting its specific role in the biogenesis of the myelin sheet. Apart from target membrane SNAREs, also specific vesicular SNAREs have been detected in OLGs, in particular VAMP3 and VAMP7, their expression being increased upon OLG differentiation [39]. In epithelial cells and OLGs, VAMP3 pairs with syntaxin 4 at the basolateral surface, i.e., the equivalent of the myelin membrane in OLGs, whereas VAMP7 binds to syntaxin 3, required for delivery of vesicular cargo at the apical surface, i.e., the plasma membrane in OLGs [39,178,180]. Thus, it is tempting to suggest that in OLGs VAMP7 might be involved in vesicle-mediated transport of PLP toward the plasma membrane, whereas one would predict VAMP3 to be engaged particularly in myelin sheet directed trafficking, pairing with syntaxin 4. In fact, in PLP trafficking studies in the oligodendroglial precursor cell line Oli-neu, co-localization of (over-expressed) PLP and VAMP3 in recycling endosomes, and PLP and VAMP7 in late endosomes/lysosomes, was observed. VAMP3 is thought to mediate fusion of recycling endosome-derived vesicles with the oligodendroglial plasma membrane [8,10], and its colocalization with PLP in vesicles derived from recycling endosomes has been suggested to relate to the biosynthetic route from the Golgi to the plasma membrane. However, as discussed above, an alternative explanation could be that the VAMP3/PLP containing vesicles are derived from a common recycling endosomal system, representing an intermediate sorting station in the overall vesicular transport pathway leading to PLP's integration into the myelin membrane, where syntaxin 4 is localized. VAMP7 has been proposed [8,10] to regulate exocytosis of PLP, after internalization following its initial delivery to the plasma membrane, from late endosomal/lysosomal organelles as part of a transcytotic pathway from the plasma membrane to the myelin membrane. However, as noted, since the common endosomal recycling system is also connected with the lysosomal degradation pathway, it remains to be determined whether this appearance of

(over-expressed) PLP in the late endosomal/lysosomal compartment could have resulted from a common regulation of myelin homeostasis. Yet, the relevance of VAMP7, pairing specifically with syntaxin 3 [178], which localizes on the (apical-like) OLG plasma membrane where PLP is delivered prior to its (transcytotic) vesicular transport to the sheet (Chapter 2 [7,116]), is emphasized by observations that myelination becomes perturbed upon silencing its expression, whereas a similar silencing of VAMP3 shows no significant effect on myelin integrity [8]. It would thus appear that the cognate VAMP3-syntaxin 4 pairing, but not that of VAMP7-syntaxin 3 is dispensable for PLP trafficking and myelin sheet delivery (Chapter 2 [7,8,116]). The nature of alternative vesicular transport routes to the sheet and mechanisms of their regulation (i.e., possibly different combinations of syntaxins (e.g., syntaxin 2) and v-SNARES (e.g., VAMP2)) remains to be determined (see below).

Interestingly, in primary cell cultures of OLGs, a distinct role of VAMP3 and its binding partner syntaxin 4 has been observed in the overall processing of MBP during myelination (Chapter 2 [116]). As noted, in contrast to PLP, biosynthetic transport of MBP does not occur as the native protein, but rather in its mRNA form. Although hypothesized to prevent in this manner premature MBP-induced membrane sticking, this mode of processing might also be more economical, as it may facilitate a more ready response to externally-triggered signalling, initiating timely MBP translation. Following transcription, MBP mRNA associates in the cytosol with heterogeneous nuclear ribonucleoprotein (hnRNP) proteins to form granules that are transported to the myelin sheath in a microtubule and dynein dependant manner [18,181,182]. In general, the selection of mRNAs is mediated by sequence specific binding of the trans-acting protein heterogeneous nuclear ribonucleoprotein (hnRNP) A2 to cis-acting hnRNP A2 response elements (A2REs) in the mRNAs [183–188]. An intact microtubule network is required for MBP mRNA transport [12,189,190], while translation is suppressed during transport by the translational inhibitor protein hnRNP E1 [183,187]. When the granules have reached the site of destination in the developing myelin sheath, individual mRNAs are released and translated [191,192]. As mRNA granules are devoid of a membrane [14,193], it is not obvious that SNAREs are directly involved in MBP mRNA delivery. Intriguingly, however, silencing of syntaxin 4, and to a lesser extent that of VAMP3, results in an impediment of MBP production, suggesting that SNAREs are essential for proper MBP transcription (Chapter 2 [116]). Specifically, downregulation of syntaxin 4 effectively repressed MBP mRNA transcription in primary OPCs, but not in immature OLGs. Moreover, this OLG developmental-dependent defect in MBP-biosynthesis could be rescued upon substitution of the medium by conditioned medium of developing OLGs, suggesting the involvement of a syntaxin 4 dependent autocrine signalling mechanism in initiating MBP mRNA transcription (Chapter 2 [116]). Intriguingly, in syntaxin 4-downregulated OLGs, delivery of myelin sheath-specific membrane proteins such as PLP, integrin $\alpha 6$ or the viral model protein VSV G was not perturbed (Chapter 2 [116]). Hence, additional work will be needed to better characterize the functional involvement of SNARE complexes in OLGs, including alternatives for syntaxin 4/VAMP3 as noted above, such as for example syntaxin 2/VAMP2 [8,39,179]. Furthermore, the exact localization of VAMP3 in OLGs and myelin is unclear as well, as is its functional role as binding partner for the v-SNARE SNAP23 [8]. Whether the potential presence of several SNARE entities at the OLG plasma membrane is indicative of distinct transport routes, and the processing of different cargo's, remains to be determined.

CONCLUDING REMARKS

Current evidence supports the notion that OLGs are polarized cells, which display different membrane surface domains, analogous to apical and basolateral domains, as defined in polarized epithelial cells. Intriguingly, based on classical definitions in terms of sorting signals, the OLG plasma membrane is reminiscent of the apical domain, while the myelin membrane is representative of a basolateral domain. Consistently, also the polarity-specific distribution of the SNARE machinery entities syntaxin 3 and 4, appears to match this peculiar polarity feature of OLGs. Clearly, in this context, knowledge of OLG polarity will also contribute to a better appreciation and understanding of cell polarity in general. Typical polarity-related trafficking events such as transcytosis evidently play a role in the processing of *de novo* synthesized transmembrane proteins like PLP in OLGs. After its arrival at the plasma membrane of the cell body, subsequent transport of PLP to the myelin sheet occurs and a major challenge in forthcoming work will be to better clarify the nature of this subsequent transport step to the sheet. Of particular interest is the potential involvement of a so-called common recycling endosomal system in this event, and circumstantial evidence justifies further research in this direction.

Finally, the mature myelin sheath consists of a series of tightly packed membranes, which may frustrate and/or impede local vesicular trafficking, including vesicular delivery of *de novo* synthesized myelin proteins. In this regard, a tight physical separation between apical and basolateral membrane surfaces in epithelial cells, constituted by a tight junction, precludes the surface exchange of molecules by lateral diffusion, thereby ensuring maintenance of the proper identity of each surface domain. However, such tight junctions do not exist between plasma membrane and myelin membrane domains in OLGs. Accordingly, it cannot be excluded that as a function of development, i.e., possibly during myelin maintenance, rather than by vesicular transport, the myelin membrane might thus also receive cargo by means of lateral diffusion of specific myelin proteins, like PLP, after their arrival at the plasma membrane. However, knowledge of a potential communication between plasma membrane and myelin membrane via lateral diffusion is still scanty, let alone whether regulatory proteins might operate in such an event (for example MAL, whose expression is only apparent subsequent to the initiation of myelin assembly). Together, these considerations lead us to conclude that further investigations of the polarity properties of oligodendrocytes will greatly benefit our understanding of the cell biology of cell polarity in general, and, specifically, that of molecular mechanisms underlying demyelination in disease, including the potential for therapeutic interference.

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