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ORIGINAL PAPER

The Role of 3-*O*-Sulfogalactosylceramide, Sulfatide, in the Lateral Organization of Myelin Membrane

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Abstract Sulfatide (3-*O*-sulfogalactosylceramide, SM4s) was isolated by Thudichum from the human brain in 1884. Together with galactosylceramide, its direct metabolic precursor in the biosynthetic pathway, sulfatide is highly enriched in myelin in the central and peripheral nervous system, and it has been implicated in several aspects of the biology of myelin-forming cells. Studies obtained using galactolipid-deficient mice strongly support the notion that sulfatide plays critical roles in the correct structure and function of myelin membrane. A number of papers are suggesting that these roles are mediated by a specific function of sulfatide in the lateral organization of myelin membrane, thus affecting the sorting, lateral assembly, membrane dynamics and also the function of specific myelin proteins in different substructures of the myelin sheath. The consequences of altered sulfatide metabolism and sulfatide-mediated myelin organization with respect to myelin diseases are still poorly understood, but it's very likely that sulfatide might represent not only a critical player in the pathogenesis of several diseases, including multiple sclerosis and Alzheimer's disease, but also a potentially promising therapeutic target.

Keywords Sulfatide · Lipid rafts · Lipid membrane domains · Sphingolipids · Multiple sclerosis

Abbreviations

AD	Alzheimer's disease
CGT	UDP-galactose ceramide galactosyltransferase
CNS	Central nervous system
CST	Cerebroside sulfotransferase
DRM	Detergent-resistant membrane
ECM	Extracellular matrix
GalCer	Galactosylceramide
GlcCer	Glucosylceramide
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MOG	Myelin/oligodendrocyte glycoprotein
MS	Multiple sclerosis
NCAM	Neural cell adhesion molecule
NF155	Neurofascin 155
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PLP	Proteolipid protein
PNS	Peripheral nervous system
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
SM	Sphingomyelin
Sulfatide	3-*O*-sulfogalactosylceramide (SM4s)
UDP-Gal	UDP-galactose

Introduction

The vertebrate nervous system is characterized by the presence of myelin, a multilamellar extension of the plasma membranes of oligodendrocytes (in the central nervous system) and Schwann cells (in the peripheral nervous system), which wraps around axons acting as an

Ganglioside and glycosphingolipid nomenclature is in accordance with the IUPAC–IUBMB recommendations [1].

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insulator, thus facilitating transmission of nerve impulse and saltatory conduction, and providing metabolic support to axon functions.

Myelin is an incredibly complex membrane structure, organized in highly heterogeneous structural and functional domains [2]. Surprisingly, the molecular organization of these functional domains is supported by a relatively small number of myelin-specific proteins. On the other hand, the myelin membrane is characterized by a very high lipid content, and by a unique lipid composition. The total lipid content of myelin ranges between 73 and 81 % of the total dry weight [3, 4], probably the highest in the human body if we exclude the adipose tissue. Whereas all major lipid classes are present in myelin as in other membranes, myelin still has its characteristic composition. The myelin membrane contains a high level of cholesterol, at least 26 % by weight, and is also substantially enriched in glycolipids (31 vs 7 % in liver cell plasma membranes) [3, 5–7]. In particular, two galactosphingolipids, galactosylceramide (GalCer) and 3-*O*-sulfogalactosylceramide (sulfatide), account for about 20 and 5 % of myelin lipids respectively [8, 9], with minor differences across species. The synthesis of these galactosphingolipids involves two sequential steps (Fig. 1), the addition of galactose from UDP-galactose (UDP-Gal) to ceramide, catalyzed by the UDP-galactose:ceramide galactosyltransferase (CGT) and the subsequent addition of the sulfate group by the enzyme 3′-phosphoadenosine-5′-phosphosulfate:cerebroside sulfotransferase (CST) (for a recent review on sulfatide metabolism see [10]).

The high lipid content in myelin is intuitively consistent with its role as axon insulator. Nevertheless, abundant literature demonstrates that myelin galactolipids have important structural and functional roles in the regulation of proliferation and differentiation of myelin-producing cells, as well as in the organization and stability of mature myelin. At least in part, these roles seem to be linked to the ability of GalCer and sulfatide to organize specific lateral domains in different substructures of the membrane of myelin forming-cells and in the myelin sheath.

This overview will summarize the current knowledge on the role of sulfatide in driving the formation of specific substructures in myelin, and shall attract the attention of the Reader to the possible role of the derangement of sulfatide-driven myelin lateral organization in nervous system pathology.

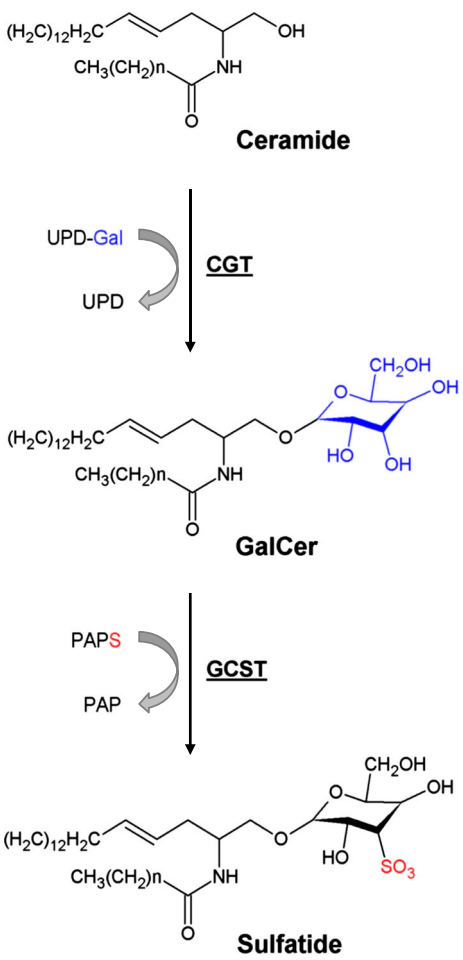

Fig. 1 Structure and biosynthetic pathway of sulfatide, the major sulfoglycolipid in the nervous system. 3-*O*-sulfogalactosylceramide, the major sulfoglycolipid in the nervous system, is highly heterogeneous in its fatty acid composition. The main fatty acids found in mature CNS myelin are long chain fatty acids (24:0 and 24:1), including a significant amount of 2-hydroxylated fatty acids. Sulfatide species with shorter-chain fatty acids are more abundant during the early stages of CNS development and have been found in neurons and astrocytes [100]. Sulfatide synthesis requires the addition of galactose from UDP-galactose (UDP-Gal) to ceramide, catalyzed by the UDP-galactose:ceramide galactosyltransferase (CGT, EC 2.4.1.45, encoded by the *ugt8* gene), and the subsequent addition of the sulfate group by the enzyme 3′-phosphoadenosine-5′-phosphosulfate:cerebroside sulfotransferase (CST, EC 2.8.2.11, encoded by the *gal3st1* gene)

Sulfatide in Model Membranes

Lipid-driven phenomena within biological membranes (such as the formation of multiple phases in the membrane lipid environment, and the local clustering of specific lipid components within the fluid glycerophospholipid bilayer) are supposedly very relevant for the lateral organization of membrane components, leading to the formation of lipid membrane domains with specialized molecular composition, architecture and biological functions. Due to their peculiar structural and geometrical features, sphingolipids, especially glycosphingolipids, have been described as important players in this sense. Phase separation of gangliosides (the acidic glycosphingolipids typical of neuronal cells) in glycerophospholipid bilayers has been studied extensively using membrane model systems, of different complexity, with heterogeneous experimental approaches, providing the conceptual background supporting the

development of the lipid raft hypothesis (reviewed in [11]). Phase separation/lateral segregation of gangliosides in biomembrane-mimetic lipid mixtures is driven by three major factors [12]: (1) the formation of a thick network of hydrogen bonds at the water–lipid interface, due to the presence in the hydrophobic ceramide moiety of acceptor and donor groups for hydrogen bonds; (2) the bulky oligosaccharide chain, which dictates the geometrical properties of the whole molecule; (3) the high enrichment in saturated fatty acids typical of brain gangliosides. Sulfatide is a ceramide-based lipid, however, its galactose sulfate head group is smaller than the average oligosaccharide chain typical of brain gangliosides (even if the presence of the negatively charged polar group results in a cross-sectional area larger than that of GalCer in phosphatidylcholine (PC) bilayers [13]) (Fig. 2), and the fatty acid composition of sulfatide from nervous tissues is much more heterogeneous than that of brain gangliosides, with a significant proportion of long-chain and hydroxylated fatty acids. Only a few papers (compared with the abundant literature on gangliosides) describe the thermotropic phase behavior of sulfatide/phospholipid mixtures with defined fatty acid composition [14–16]. These studies indicate that the miscibility of mixtures of symmetric PC or phosphatidylethanolamine (PE) species, containing saturated fatty acids, with sulfatides is strongly dependent on sulfatide concentration and on the chemical structure of the fatty acid linked to ceramide in sulfatide (with a phase separation dependent on the fatty acid chain length in sulfatide), thus suggesting that the effect of sulfatide on the lateral order of biological membranes could be very different in different biological systems, and that the physiological regulation of the composition of sulfatide hydrophobic moiety might play a very important role in this sense. Interestingly, these studies also suggested that, under certain experimental conditions, long chain asymmetric sulfatide and symmetric PC formed mixed interdigitated phases [15, 16]. Interdigitation of the fatty acyl chains of lipids belonging to opposite bilayers has potentially important consequences on the lateral and transverse organization of membrane components. Neutron diffraction studies in stratum corneum model membranes suggested that the presence of long chain free fatty acids resulted in a significant reduction of membrane thickness due to a partial interdigitation of the free fatty acyl chains [17]. On the other hand, it has been demonstrated that in artificial bilayers the C24 fatty acyl chain in lactosylceramide can interdigitate with the acyl chains of lipids in the opposite bilayer [18]. However, possible role of interdigitation in the case of sulfatide-driven membrane dynamics is still controversial, since it has been reported that C26 sulfatide in a bilayer of shorter chain symmetric phospholipids, in the presence of cholesterol and at physiological temperature, is not able to interdigitate [19].

Other studies suggest that the distribution and dynamics of sulfatide in phospholipid bilayers can be affected by external factors, such as the presence of mono- or divalent cations [20–22], pH (the effect of pH being at least in part mediated by the different hydration of the galactose sulfate head group as a function of its protonation [23, 24]), or the addition of soluble sulfatide-binding proteins (such as calcitonin) [25].

The ability of sulfatide to form laterally ordered domains in a fluid 1-palmitoyl, 2-oleoyl PC (POPC) bilayer

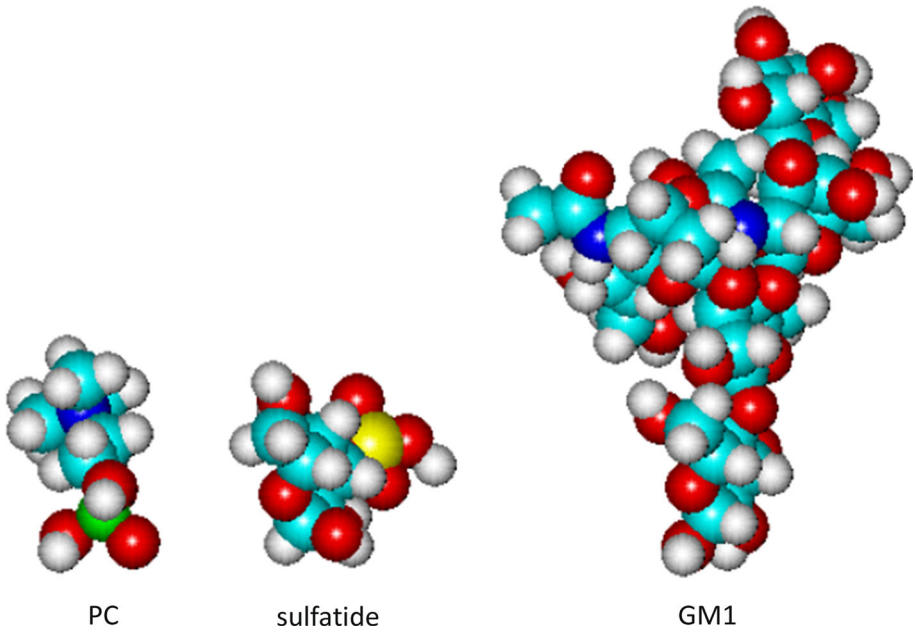

Fig. 2 Schematic representation of the volume occupied by phosphocholine, the headgroup of PC, by galactose sulfate, the headgroup of sulfatide, and by the oligosaccharide chain of GM1, a monosialoganglioside of the gangliotetraose series

in the presence of cholesterol, sphingomyelin (SM) and GalCer has been studied using phase-selective fluorescent probes [26]. In this study, the complexity derived by the heterogeneity of the fatty acid chain in ceramide has been avoided by comparing exclusively *N*-palmitoyl-sphingolipids. *N*-palmitoyl-sulfatide alone was able to segregate from the POPC-rich phase; however, cholesterol was excluded from the sulfatide-rich domain. Apparently this finding is in contrast with previous observations indicating a relevant interaction between sulfatide and cholesterol involved in lateral domain formation (obtained in mixed monolayers at the water/air interface) [27]. However, in sulfatide/SM/POPC mixtures, sulfatide was preferentially associated with the SM-rich ordered (gel-like) domain rather than with the POPC-rich fluid phase, and when present, cholesterol was also included in the SM/sulfatide domain. Sulfatide also formed laterally ordered domains when mixed with GalCer, or with GalCer and SM, and the resulting sphingolipid-rich domains were able to accommodate cholesterol. This study suggests that sulfatide is able to form phase-separated domains alone and with other typical myelin sphingolipids and cholesterol, and it is particularly relevant since the above mentioned observations have been recorded using sulfatide concentrations and molar ratios with the other lipids in the mixture that fall within the expected range for myelin lipids.

On the other hand, the importance of the lipid environment in determining the surface topology of sulfatide has been suggested by the analysis of the reactivity of different anti-sulfatide antibodies toward sulfatide in a sphingomyelin/cholesterol environment versus a dipalmitoyl-PC/cholesterol environment [28]. Distinct populations of anti-sulfatide antibodies were able to react preferentially with sulfatide/cholesterol/PC or sulfatide/cholesterol/SM domains, suggesting that a specific laterally ordered multimolecular complex is required for antigen recognition.

Finally, the phase separation of sulfatide in PC bilayers is deeply affected by the presence in the model membrane of proteins that are supposed to be physiologically relevant partners of sulfatide, such as myelin basic protein (MBP). MBP induced the separation of sulfatide- and protein-rich domains in PC bilayers, with relatively large variations in the proportion of the different phases in response to small changes in MBP concentration [13, 29].

Sulfatide in Oligodendrocyte Maturation, Myelin Formation and Myelin Stability

Oligodendrocytes in the central nervous system (CNS) [30] and Schwann cells in the peripheral nervous system (PNS) [31] produce a highly specialized membrane, the myelin sheath, whose most striking feature is probably represented by the high enrichment in lipids, and in particular in galactolipids (as detailed in the Introduction). Even though GalCer and sulfatide are not myelin-specific lipids, their enrichment in myelin is much higher than in any other tissue. In addition, in the CNS the amount of GalCer and sulfatide expressed in neurons and astrocytes is not neglectable, although it is much lower than in oligodendrocytes [32–34]. The high enrichment in GalCer and sulfatide is a common feature of both CNS and PNS across species (even if quali- and quantitative differences are present), and, within the CNS, it is clearly emerging that there are heterogeneous distribution patterns for different sulfatide molecular species in different CNS regions [35–37]). It has been estimated that a single oligodendrocyte during the active myelination period produces up to 50,000 μm^2 of myelin membrane [38]. Synthesis of GalCer and sulfatide is maximal in rat brain at the developmental stage of most rapid myelination [39]. Mature, myelin-producing cells are the final stage of a precise developmental lineage, which is well defined for oligodendrocytes and, at lesser extent, for Schwann cells. In glial cultures, the synthesis of GalCer and sulfatide is switched on at the onset of the terminal differentiation (and remains constant in mature, myelin-producing oligodendrocytes) and their transport to the cell surface hallmark the transition from the pro-oligodendroblast/late progenitor stage to the immature oligodendrocyte stage [40–42]. In fact, anti-galactosphingolipid antibodies (R-mAb, that reacts with GalCer and sulfatide; O1, that recognizes GalCer; O4, that reacts with sulfatide in oligodendrocytes and with the sulfated pro-oligodendroblast antigen in the late progenitors) have been consistently used as markers for oligodendrocyte identification [38, 43].

Thus, it is clear that the synthesis, transport and assembly of GalCer and sulfatide represent key events in myelin formation, suggesting that GalCer and sulfatide might represent not only structural components of the myelin membrane but also active players in the regulation of myelin formation and maintenance. This notion has been reinforced by several lines of evidence, summarized in the next paragraphs, and further confirmed by the phenotypic analysis of mice lacking CGT or cerebroside sulfotransferase.

Functional Effects of Anti-galactolipids Antibodies

Treatment of cultured oligodendrocytes to different antibodies reacting with galactolipids was able to alter oligodendrocyte differentiation deeply. Exposure of murine oligodendroglia in culture to anti-GalCer IgG caused marked alterations in the structure of membrane sheets without altering GalCer turnover [44]. This effect was at least partly mediated by changes in the organization of

oligodendrocyte cytoskeleton and of domains enriched in MBP [45], which, in turn, could be mediated by the influx of Ca^{2+} consequent to the opening of Ca^{2+} channels on the oligodendrocyte surface elicited by the anti-GalCer [46]. Treatment of mixed primary cultures from fetal rat telencephala with the O4 IgM antibody, reacting against sulfogalactolipids, caused a marked up-regulation of sulfatide biosynthesis and of the expression of myelin protein markers, without affecting the number of O4-positive cells. These data suggested that O4 treatment was able to influence the extent of oligodendrocyte differentiation in culture [47]; on the other hand, treatment of cultures enriched in oligodendrocyte progenitors with R-mAb antibody, reacting against both GalCer and sulfatide, or with O4, reacting against sulfatide, resulted in the reversible inhibition of oligodendrocyte progenitor differentiation [48, 49]. Antibody treatments reversibly blocked oligodendrocyte terminal differentiation into myelin-producing cells without altering the normal sequence of events involved in this progression. Since this effect was not obtained by treating cultures with the anti-GalCer O1 antibody [49], the crucial galactolipid involved in the negative regulation of terminal oligodendrocyte differentiation should be sulfatide. Interestingly, treatment of cultured Schwann cells with R-mAb caused a depletion of cell surface galactolipids and a blockade of the progression of myelin formation, confirming a role for galactolipids in PNS myelination [50]. On the other hand, exposure of an in vitro model of peripheral myelin (Schwann cell myelin formed in culture around the axons of dorsal root ganglion cells) to hybridoma cells producing either O4 or O1 resulted in a marked de-compaction of already formed myelin [51].

Alteration of Myelin Lipids and Appearance of Anti-sulfatide Antibodies in Pathology

The levels of myelin lipids, and in particular of sulfatide, are altered in humans during aging [52], and dysregulation of myelin sulfatide synthesis probably represents a risk factor for cognitive decline associated with age [53]. A substantial reduction of sulfatide has been reported in brain tissue samples from Alzheimer's disease (AD) patients with mild cognitive impairment and mild dementia, as well as in individuals with pre-clinical AD, in agreement with reports indicating that demyelination occurs in the cerebral cortex of AD patients [54, 55]. Sulfatide levels are dramatically reduced in plaque tissues and in the normal appearing white matter in brains from patients with multiple sclerosis (MS), the most common demyelinating disease in the CNS [56, 57]. However, the link between sulfatide and MS is quite complex. Increased sulfatide levels are found in serum (with stage-specific accumulation of different sulfatide molecular species) [58] and cerebrospinal fluid from patients with MS [59] and in their healthy siblings [60]. The latter result strongly suggests that the appearance of sulfatide in biological fluids is not the consequence of myelin lesions but rather represents a significant risk factor and prognostic element for the onset and the progression of MS. On the other hand, sulfatide plays crucial roles in the modulation of immune system cells within the CNS, underlying the inflammatory autoimmune response associated with MS and other diseases, [61–64], and the appearance of anti-sulfatide antibodies in serum is frequently associated with MS [59, 60, 65, 66]. Considering the effects of anti-sulfatide antibodies discussed in the previous paragraph, this raises the possibility that the appearance of these antibodies could be responsible for further functional alterations in myelin-producing cells in this disease. Remarkably, alterations in the levels of myelin lipids, including sulfatide, were also reported in other neurological diseases, suggesting that dysregulation of myelin lipid metabolism, leading to altered myelin organization, might be a common trait involved in the pathogenesis of several diseases [67].

Phenotypic Alterations in CGT-Null and CST-Null Mice

To gain a better understanding of the role of GalCer and sulfatide, genetically altered models have been established and analyzed. In this paragraph, we will outline the features of the animal models lacking the enzymes responsible for the two sequential steps leading to the synthesis of GalCer and sulfatide, CGT knock-out mice and CST knock-out mice.

The microsomal enzyme UDP-galactose:ceramide galactosyltransferase catalyzes the transfer of a galactose from UDP-galactose to ceramide to form GalCer and is abundantly expressed in the actively myelinating CNS and PNS [68, 69]. CGT knock-out mice cannot synthesize GalCer or sulfatide, however they are able to form myelin with an apparently normal structure. This could be due to a partial compensation of the loss of these galactolipids by synthesizing 2-hydroxylated glucosylceramide (GlcCer), usually not present in myelin. Hydroxy-fatty acid-containing sphingomyelin (HFA-SM) is also higher in CGT (−/−) mice myelin suggesting that, in this model, the α-hydroxy ceramide normally destined for HFA-GalCer production is most likely used for the synthesis of HFA-GlcCer and HFA-SM [70]. However, it has been shown that the deletion of glucosylceramide synthase activity in oligodendrocytes from CGT (−/−) mice had no effects on the severity of the myelin defects, thus confirming that synthesis of GlcCer and of GlcCer-based glycolipids is not required for proper myelination, and indicating that the increased HFA-GlcCer levels observed in the CGT (−/−) mice are not sufficient to compensate for the absence of

GalCer or sulfatide [71]. This notion has been further reinforced by the observation that CGT (−/−) mice with an additional deletion of the fatty acid 2-hydroxylase gene, had no significant phenotipyc differences respect to the CGT (−/−) mice, despite the great reduction in the GlcCer levels observed in the double mutants [72]. As mentioned above, CGT (−/−) mice can form apparently normal myelin with normal major dense and intraperiod line periodicity [73]. These animals, however, exhibit a neuropathological phenotype, characterized by tremor, splaying of the hind limbs and ataxic locomotion that worsens progressively, resulting in death of most animals by the third month of age [70, 74]. It is worth to recall that CGT is also expressed in non neuronal tissues and that the loss of function of this enzyme, in these tissues, could contribute to the phenotype of the CGT mutants [75]. The phenotype of CGT (−/−) mice is consistent with the disruption of nerve conduction despite the presence of compact myelin and, in fact, the action potential measured in the spinal cord of these mice is smaller and has a longer latency than in wild type mice [70]. Moreover, a more detailed analysis of the structure of myelin in CGT (−/−) mice revealed a number of ultrastructural abnormalities associated with myelination in the CNS. CNS myelin sheaths are thinner, nodal length is increased and lateral loops are widely spaced. The disorganization of the lateral loops suggests a disruption in the formation of the tight junctions, not surprisingly since sulfatide is a prominent constituent of myelin tight junctions and the formation of these junctions may be dependent on the presence of this lipid. These disorganized loops are not tightly opposed to the axolemma, thus facilitating the entrance of cellular and non cellular material into the paranodal periaxonal space [75]. Myelin sheaths in mutant mice also exhibited an extensive and progressive vacuolation between the sheaths and the axolemma [70, 75]. In addition, about one-third of the myelin processes in oligodendrocytes in CGT (−/−) mice retained oligodendrocyte cytoplasm, an indication of immature myelin, in regions with otherwise structurally mature myelin. Also, profiles of compact myelin often showed more than two oligodendrocytic loops in a single internodal segment [73, 76]. Interestingly enough, despite the presence of these abnormalities in the CNS myelin, the PNS myelin in the knockout mice appears normal suggesting that galactolipids might be less critical in the formation and maintenance of PNS myelin sheath structure [73, 75, 77, 78]. What remains to be elucidated is whether this is related to the fact that galactolipids are less abundant in the PNS respect to the CNS, or, more likely, whether the interacting partners of these lipids are different in CNS and PNS.

Obviously, the use of the CGT (−/−) model does not allow to address a very relevant point, i.e. to discriminate the possible specific functions of GalCer and sulfatide in myelin, since, in these mice, both galactolipids are absent. A major advancement toward the understanding of the specific functions of the two major myelin galactolipids was the development of CST knock-out mice [79]. CST (−/−) mice completely lack sulfatide, while the levels of other glycolipids, including GalCer, are not significantly altered in the brain. Phospholipids homeostasis is also not significantly altered [80]. CST-deficient mice are born healthy but start displaying hind limb weakness around 6 weeks of age, followed by pronounced tremor and progressive ataxia. The phenotype of these mice is similar but milder than that of CGT (−/−) mice in terms of age of onset, life span and severity of symptoms. In fact, CST knock-out can survive for more than 1 year [79].

CST (−/−) mice produce compact myelin, even if it is thinner than that of normal mice and shows alterations in the paranodal structure similar to those of CGT knock-out mice. The myelin sheaths in young mice, however, are relatively stable, the node/paranode structure is only moderately altered and axon size is comparable to that of wild type mice. Nevertheless, with age these mice exhibit a nodal structure deterioration, myelin vacuolar degeneration and also reduction of axon caliber [81]. Moreover, electron microscopy analysis of myelinated nerve fibers revealed disorganized termination of the lateral loops at the node of Ranvier [10]. Clustering of Na^+ and K^+ channels at the node is also deteriorated in CST null mice [82]. In normal CNS axons, Na^+ channels cluster at the nodes of Ranvier and the K^+ channels concentrate in the juxtaparanodal regions. In mutant mice, however, Na^+ channels concentrate in small regions, presumptive nodes of Ranvier, and the lengths of the clusters were occasionally longer than the ones present in the wild type mice. The K^+ channels clusters instead accumulated in regions adjacent to the Na^+ channels clusters in presumptive paranodal regions [82]. Remarkably, the alterations in the localization and clustering of ion channels are present both in CNS and in PNS in mutant mice, and are accompanied, in both cases, by an altered distribution of proteins involved in the structure of nodes, such as Caspr, contactin and neurofascin155 (NF155) [82, 83].

The PNS of CST (−/−) mice also exhibits axonal protrusions at node level. The protrusion often contain either degenerated mitochondria or abnormally large vesicles filled with electron dense material [83]. In addition, the structure of paranodal axo-glial junctions and the nerve conduction velocity were markedly altered in peripheral nerves from CST-null mice. Remarkably, heterozygous mice also exhibited paranodal abnormalities and a reduced nerve conduction velocity, whose extent was proportional to the reduction in sulfatide levels, strongly confirming the importance of the fine regulation of sulfatide levels in myelin for the normal function of myelinated axons [84].

The loss of GalCer and sulfatide affects the proliferation and survival rate of oligodendrocyte precursors. CGT (−/−) mice exhibit a significant increase in cellularity in the spinal cord [78, 85]. In CST (−/−) mice, an increased number of oligodendrocytes is observed [86–88], and oligodendrocytes mature earlier and in greater number [86]. The increase in the oligodendrocyte population seems to be determined by an increased proliferation and by a reduced rate of apoptotic death in the cells belonging to the oligodendrocytic lineage [87], and occurs prior of the onset of myelination [88]. These data seems to be in contrast with observations indicating that in primary cultures from CGT (−/−) mice the increased number of mature oligodendrocytes is not determined by a higher proliferation and/or survival rate, but rather by and increased terminal differentiation [49]. This seems to be supported by experiments showing that the perturbation of GalCer and/or sulfatide membrane organization using anti-galactolipid antibodies leads to a dramatically altered maturation process in myelin-forming cells, as summarized in a previous section of this article. Remarkably, in CST-null optic nerves the increase in cell number involved only mature oligodendrocytes, whereas astrocytes and microglia showed no significant variation in their number, even if their sulfatide content was reduced. In wild type mice, the number of oligodendrocytes is strictly regulated by axons during development, and, in fact, the number of myelinating cells that survive seems to be precisely matched to the number and length of axons requiring myelination. In young CST-null mice, oligodendrocytes have fewer processes indicating that axons might need a greater number of oligodendrocytes to compensate for the fewer processes. However, it is still unknown if the same is true for adult CST-null oligodendrocytes [88]. Thus, while it seems quite convincing that galactolipids, in particular sulfatide, play a role as the physiological gatekeepers, controlling the timely formation of mature, myelin-forming oligodendrocytes at the appropriate stage of CNS development, it is still not clear if the main contribution to this is represented by the control of the proliferation/survival of oligodendrocyte precursors, or by the control of oligodendrocyte terminal differentiation.

Taken together, the data obtained by the study of CST- and CGT-null mice strongly suggest that the functions of GalCer and sulfatide in myelin are only in part overlapping, and that the two galactolipids have several specific functions in myelin. In summary, the available information support the notion that GalCer is primarily involved in myelin formation and maturation, while sulfatide contributes to the long term stability of myelin structure, in particularly affecting the integrity of the nodal and paranodal regions.

Sulfatide Regulates the Function of Myelin Proteins via Lateral Interactions

As mentioned above, the studies on the physico-chemical properties of sulfatide in model membrane systems are suggestive of a structure-specific role of sulfatide in the creation of lateral order within biological membranes, participating to the formation and stabilization of “lipid membrane domains” or “lipid rafts”. This notion is further supported by studies in cultured oligodendrocytes and in myelin [89]. The majority of these studies relied on the preparation and analysis of detergent-resistant membrane fractions, usually regarded as biochemical preparations enriched in lipid raft components, even if the use of detergent-insolubility as a criterion to determine lipid raft association of a cellular component has been fiercely criticized in the past (for a critical overview on the subject, see [11, 90]). Several papers described the behavior of different myelin-associated proteins toward detergent extraction, and the association of these proteins with detergent-resistant (DRM) membrane fractions prepared from cultured oligodendrocyte precursor, mature oligodendrocytes, and myelin purified from animals at different stages of myelination under different experimental conditions (in particular, using different detergents and operational temperatures) [91–99]. These experiments were based on the assumption that DRM fractions are enriched in galactolipids (including sulfatide) and/or cholesterol, but only a few papers actually analyze the lipid composition of the isolated DRM fractions [91, 94, 97]. Only very recently, an ultra-high-pressure liquid chromatography tandem mass spectrometry method specifically aimed at quantitatively analyzing the sulfatide composition of CNS lipid rafts has been developed [100]. Thus, the data presented in these papers accurately describe some properties of oligodendrocyte and myelin proteins, however, their interpretation in terms of describing the association of these proteins with lipid membrane domains or their interaction with myelin lipids should be critically and cautiously considered.

Nevertheless, the current literature points out, quite convincingly, that galactolipid-enriched membrane domains are involved in the correct sorting and trafficking of the major myelin proteins, and that GalCer- and sulfatide-rich domains in the oligodendrocyte membranes regulate the lateral organization of several myelin proteins, deeply affecting the survival, proliferation and differentiation of oligodendrocytes [89]. In the early stages of myelin formation, when galactolipid levels are absent or very low, only a few of the typical myelin proteins are associated with lipid rafts (i.e., detergent-insoluble). However, when GalCer and sulfatide are synthesized at detectable levels, during the mid-myelination stage, the myelin proteolipid protein (PLP) and

myelin/oligodendrocyte glycoprotein (MOG) tend to localize in lipid rafts, and subsequently, in the final stages of myelination, myelin-associated glycoprotein (MAG) and MBP are also translocated into lipid rafts [99, 101–103].

As pointed out by the analysis of the phenotypic alterations of CGT-null and CST-null mice, the deficiency of GalCer and/or sulfatide does not simply imply a general derangement of myelin structure, but rather fine and specific defects in myelin's different functional domains, implying that specific myelin proteins might interact with and be modulated by myelin lipids with different modalities. From this point of view, one paper is particularly informative [97]. These Authors applied the classical Triton X-100 extraction method to myelin membranes at the temperature of +20 °C (while most of the preparation of Triton-resistant membrane fractions have been carried out at +4 °C, an experimental condition frequently seen as a source of possible artifacts in term of lipid phase separation), and were able to isolate two different DRM fractions, both describable as glycolipid/cholesterol-enriched membrane fractions, based on the twofold higher GalCer/glycerophospholipid and cholesterol/glycerophospholipid ratios respect to unfractionated myelin. However, only the fraction with slightly higher density was significantly enriched in sulfatide and GM1 ganglioside respect to whole myelin. Moreover, different myelin proteins usually associated with DRM rafts were differently enriched in the two fractions. In particular, the "sulfatide-containing" DRM fraction was enriched in cytoskeletal proteins, caveolin, flotillin-1, and, at lesser extent, in PLP, while it was less enriched in MBP, MAG and NCAM120 respect to the "GalCer-enriched" fraction. This is probably the first convincing evidence that GalCer and sulfatide can form separate domains in myelin, thus regulating the lateral organization of specific myelin proteins. This notion has been recently reinforced by the finding that the detergent-resistant association of NF155 and MAG with the myelin membrane is dependent on sulfatide, but not on GalCer (since the detergent extractability of these proteins is increased in CST-null mice) (Fig. 3), while the association of MOG and MPB is sulfatide-independent [98]. NF155 and MAG are paranodal adhesion proteins. Long-term axon-myelin stability requires *trans* interactions between the gangliosides GD1a and GT1b in specialized domains of the axonal surface, and MAG [104–107], whose correct localization in the myelin membrane is in turn regulated by the association with galactolipid-rich domains [103]. NF155 is usually localized within the paranodal loops where it interacts with a protein complex (Caspr1-F3/contactin) on the axon, stabilizing axon-glial contacts. NF155 is one of the myelin proteins that are recruited into lipid rafts during the final stages of myelin development [102, 103] (see above) and, in CGT (−/−) mice, NF155 is absent from the paranodes leading to instability of these structures [108]. Taken together these results strongly suggest that sulfatide exerts a specific role in maintaining the proper lateral organization of NF155 domains and the integrity of the paranodal structure. Intriguingly, the integrity of the paranodal structure is affected in MS, thus suggesting that the link between sulfatide and this pathology might be represented at least in part by alterations of the paranode ultrastructure as consequence of the above-mentioned alterations in sulfatide metabolism or by the presence of anti-sulfatide antibodies (possibly able to bind sulfatide at the paranode thus affecting the lateral organization of paranodal proteins such as NF155) observed in MS patients.

A differential involvement of GalCer-rich and sulfatide-rich membrane domains in modulating the function of specific myelin proteins has been further supported by a recent study, that analyzes the effect of myelin galactolipids on the lateral organization and membrane dynamics of two myelin proteins, PLP and MBP, combining classical biochemical assays based on detergent solubility with a non-invasive biophysical approach in living cells [99]. The role of sulfatide and GalCer has been studied by separately overexpressing CGT or CST on a galactolipid-null background, using the rat immature oligodendrocyte cell line OLN-93, that does not produce galactolipids. This study revealed that membrane association (as association with detergent-insoluble membrane fractions) and dynamics (as lateral motility mobility in intact cells) of MBP is dictated from GalCer, while those of PLP are regulated by sulfatide (Fig. 4). This clearly indicates the importance of the formation of distinct lipid membrane domains in the myelin membrane for the lateral organization of MBP and PLP. Interestingly enough, GalCer-enriched membrane domains are mainly associated with the myelin membrane of oligodendrocytes, while sulfatide-containing domains are restricted to the cell body, suggesting a correspondence between "micro" and "macro" domains in the highly specialized oligodendrocyte membrane. Moreover, the effect of sulfatide on the microdomain association and diffusion rate of PLP is strongly dependent on the presence of different extracellular matrix (ECM) proteins. The interplay of sulfatide with ECM proteins is confirmed by the lateral association of sulfatide with integrin α6 in cultured oligodendrocytes, allowing the interaction with laminin-2 that is essential for oligodendrocyte maturation. Remarkably, laminin-sulfatide interaction was disrupted by the presence of fibronectin. On the other hand, anti-sulfatide antibodies disrupted the lateral organization between sulfatide, integrin α6 and other myelin proteins in oligodendrocytes cultured on laminin-2, resulting in demyelination, while the same antibodies were able to stimulate myelin formation in oligodendrocytes in

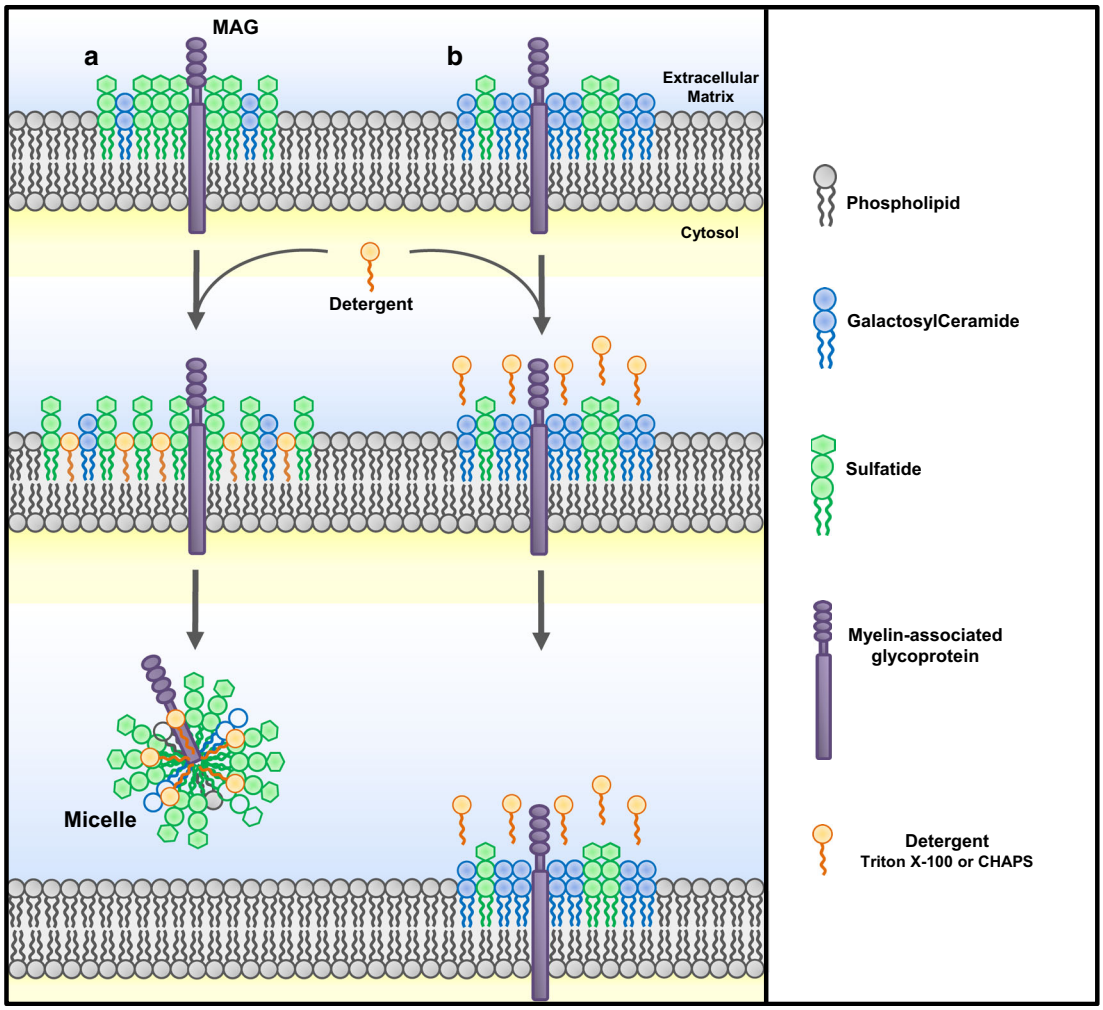

Fig. 3 The behavior of MAG in lipid rafts with different lipid composition. The detergent-resistant association of MAG with the myelin membrane is dependent on sulfatide (**a**) but not on GalCer (**b**). In fact, in CST knock-out mice, the detergent extractability of MAG is increased [98]

presence of fibronectin. It is known that fibronectin accumulates in MS lesions and, this suggests that treatment with anti-sulfatide antibodies might be able to restore the lateral organization of oligodendrocyte membrane, thus eliciting a response from quiescent oligodendrocyte progenitor cells eventually resulting in axon remyelination [109]. MBP and PLP, both described as rafts-associated myelin proteins, deeply differ for their mode of association with the membrane and membrane topology. MBP is a small peripheral membrane protein associated with the internal side of the myelin membrane; PLP is a highly hydrophobic transmembrane protein. MBP associates with lipid rafts relatively early during myelination, at the stage when GalCer and sulfatide synthesis reach significant levels [102, 103], while PLP translocates to lipid rafts only in the final stages of myelination [102]. For both proteins, association with lipid rafts is lost or reduced in CGT-null mice. Previous studies have suggested that PLP targeting to and association with the myelin membrane involves its interaction with GalCer- and cholesterol-enriched membrane domains in the Golgi complex (that is supposedly a critical step in the sorting of components destined to the myelin membrane more in general) [94]. However, it has been suggested that PLP is specifically associated with sulfatide in transport vesicles targeting neosynthesized material to the myelin membrane [110]. On the other hand, a recent paper nicely demonstrated that the newly synthesized PLP is transported from the trans-Golgi to the cell body plasma membrane in oligodendrocytes in Triton-resistant membrane domains, that do not require sulfatide for their assembly. At the cell surface, a sulfatide-assisted change in the conformation or oligomerization of PLP occurs, shifting the protein from sulfatide-independent Triton-insoluble microdomains to sulfatide-dependent, CHAPS-insoluble domains, and eventually resulting in the transport of PLP to myelin membranes via a transcytotic mechanism [111].

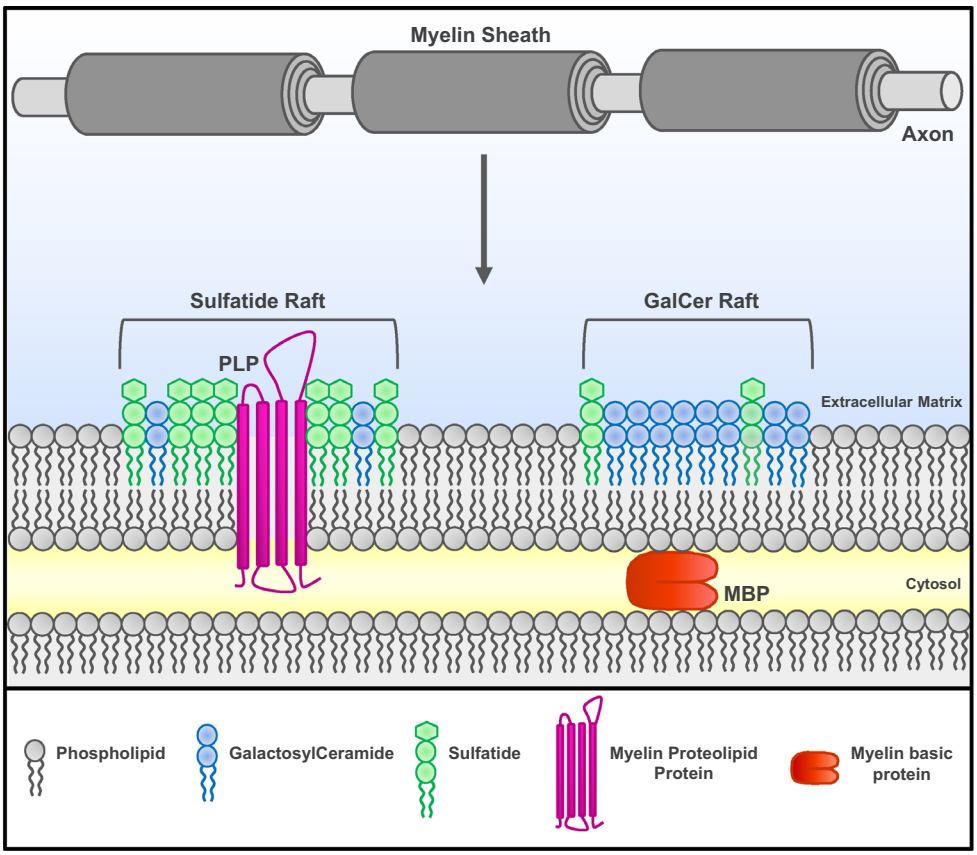

Fig. 4 Association with specific sulfatide-rich domains regulates the function of PLP. The membrane association and dynamics of PLP are sulfatide-dependent while those of MBP are GalCer-dependent [99]

Future Directions

The pieces of evidence discussed in this overview article convincingly support the following conclusions: (1) GalCer and sulfatide in the membrane of myelin-forming cells are able to laterally organize specific membrane microdomains; (2) the synthesis of galactolipids and the consequent formation of laterally organized membrane domains in the membrane of myelinating cells does not simply reflect the need of building components instrumental to the extension of the myelin sheath, but rather is linked to the regulation of specific biological processes; (3) the role of galactolipids and especially of sulfatide is very different in the nervous system at the early stages of myelination or in mature myelin. GalCer and sulfatide control the proliferation/differentiation of oligodendrocyte precursors, sulfatide in particular regulates the transition from the pro-oligodendroblast/late progenitor stage to the immature oligodendrocyte stage, and the terminal differentiation of oligodendrocytes; on the other hand, sulfatide is involved in the organization of specific substructures in mature myelin, with different roles in the different subdomains of myelin and in particular of the node of Ranvier. These specific functions require the interaction with specific myelin proteins in a very dynamic fashion; (4) sulfatide-driven lateral organization of specific subdomains is altered in pathologies.

In our opinion, sulfatide might represent a pivotal target for future therapeutic approaches. Particularly intriguing is the interplay between sulfatide-rich membrane domains in myelin and the niche environment, with two aspects being particularly relevant. One is the influence of external molecules on sulfatide-mediated membrane organization and regulated biological events. In particular, a number of recent papers point out the role of ECM proteins in this sense, with opposite effects on myelin (i.e., demyelination *versus* promotion of myelin formation) [99, 109]. The other is the multifaceted interaction between myelin sulfatide and the immune system [61, 65]. Myelin sulfatide is increased in biological fluids from patients with different pathologies, the most notable being AD and MS. This is frequently accompanied by the appearance of anti-sulfatide antibodies, which likely reflects only one aspect of sulfatide interplay with ad activation of a plethora of immune cells subpopulations. This aspect is particularly relevant in sight of future perspectives, and the role of sulfatide in this sense still has to be fully elucidated. As mentioned above, anti-sulfatide antibodies can interact with the surface of cultured oligodendrocytes

and affect the lateral organization of sulfatide with myelin proteins with opposite consequences (demyelination *versus* stimulation of myelin formation), depending on the type of ECM protein prevalent in the culture environment [99, 109]. Thus, anti-sulfatide antibodies might participate to the onset of the disease, but on the other hand, they might represent an important immunological tool for the treatment of neurological diseases involving myelin lesions or altered myelin-axon interactions [112, 113]. Sulfatide has been described as an activator of inflammatory response in brain-resident immune cells, suggesting that sulfatide released by damaged myelin regions might participate to the worsening of pathological conditions in the brain [62]. On the other hand, sulfatide seems to be involved in an immune-mediated regulatory pathway leading to the modulation of the functions of different NKT subsets, dendritic cells and CNS-resident microglia. Remarkably, sulfatide administration in mice with experimental autoimmune encephalomyelitis, a well-studied animal disease model for multiple sclerosis, resulted in a reduced susceptibility to disease and in the reversal of ongoing chronic and relapsing disease [63, 64], suggesting that the sulfatide-mediated pathway represent a realistic therapeutic target in CNS autoimmune diseases.

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