Myelin glycolipids and their functions

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During myelination, oligodendrocytes in the CNS and Schwann cells in the PNS synthesise myelin-specific proteins and lipids for the assembly of the axon myelin sheath. A dominant class of lipids in the myelin bilayer are the glycolipids, which include galactocerebroside (GalC), galactosulfatide (sGalC) and galactodiglyceride (GalDG). A promising approach for unravelling the roles played by various lipids in the myelin membrane involves knocking out the genes encoding important enzymes in lipid biosynthesis. The recent ablation of the ceramide galactosyltransferase (*cgt*) gene in mice is the first example. The *cgt* gene encodes a key enzyme in glycolipid biosynthesis. Its absence causes glycolipid deficiency in the lipid bilayer, breakdown of axon insulation and loss of saltatory conduction. Additional knock-out studies should provide important insights into the various functions of glycolipids in myelinogenesis and myelin structure.

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Abbreviations

C	cholesterol
CGT	ceramide galactosyltransferase
ER	endoplasmic reticulum
GalC	galactocerebroside
GalDG	galactodiglyceride
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
PL	phospholipids
PLP	proteolipid protein
PNS	peripheral nervous system
sGALC	galactosulfatide

Introduction

Oligodendrocytes in the CNS and Schwann cells in the PNS develop plasma membrane processes that spirally ensheath axons in segments between nodes of Ranvier (internodes). Compared to unmyelinated axons, myelinated axons have a more rapid saltatory conduction, smaller axon diameter, greater energy conservation and a greater capacity for miniaturisation. The structural and physical properties of the oligodendrocyte-specific and Schwann-cell-specific proteins and lipids are crucial for the long-term stability of the tightly packed multilamellar myelin membrane. Unlike membranes of intracellular compartments, the stacked lamellar plasma membrane processes are tightly attached at their cytosolic and external surfaces, resulting in a compact structure, which is seen ultrastructurally as a major dense line and interperiod lines.

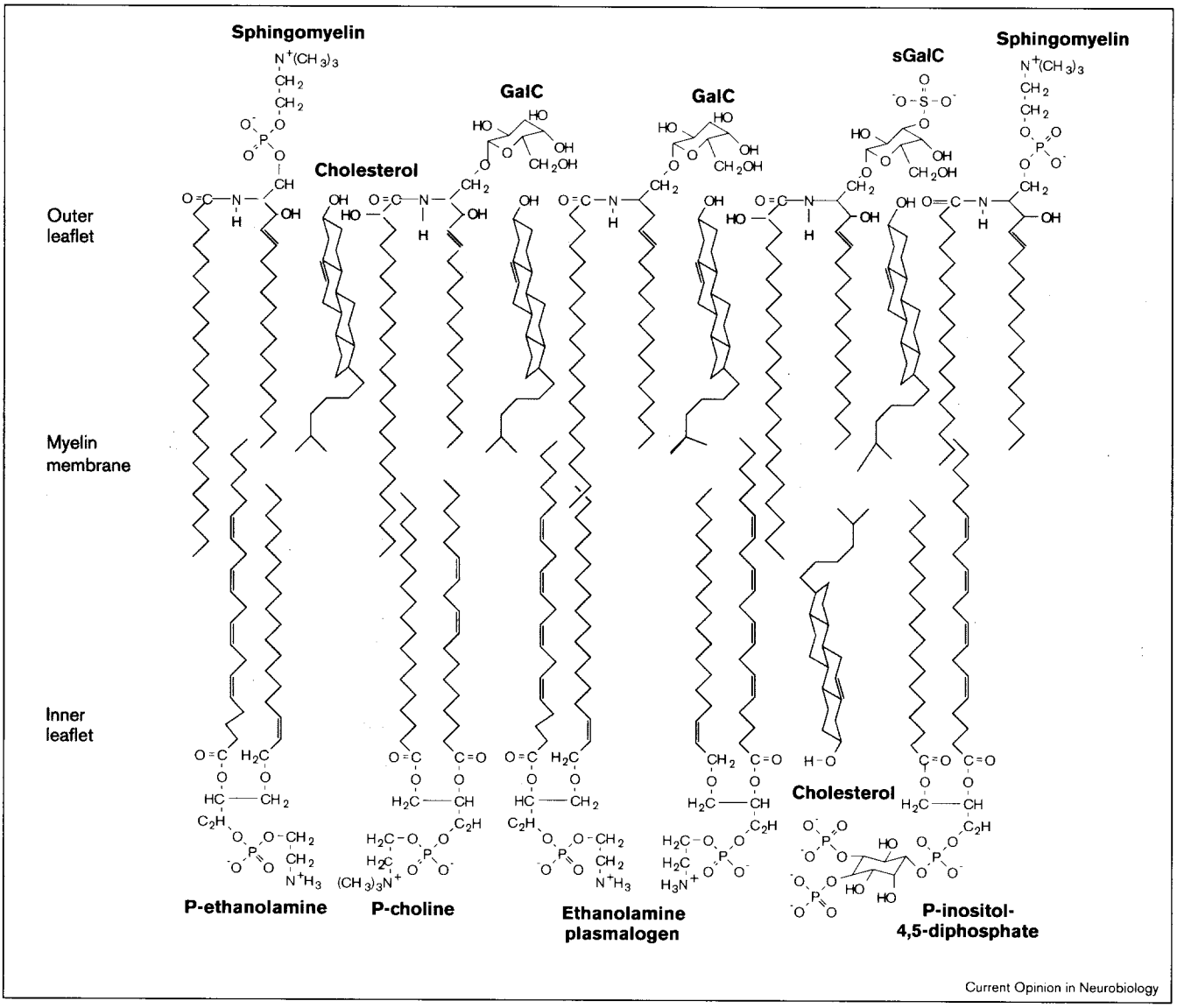
Below, we briefly summarise the structural, biochemical and biophysical properties of myelin glycolipids essential for our understanding and interpretation of the functions of this class of unusual complex lipids.

The myelin membrane is composed of specific proteins and lipids

Cholesterol and complex phospholipids and glycosphingolipids, comprising about 80% of the dry weight of both CNS and PNS myelin, form the lipid bilayer. Specifically, the myelin bilayer contains cholesterol, ethanolamine plasmalogens and the glycolipids galactocerebroside (GalC) and galactosulfatide (sGalC) as typical major myelin lipid classes, together with galactodiglyceride (GalDG) and ganglioside GM1 as minor components [1]. Figure 1 depicts representative lipid molecules of the myelin lipid bilayer and indicates the asymmetric arrangement of glycolipids (outer leaflet) and phospholipids (inner leaflet). These components are assembled with myelin-specific membrane proteins, the most abundant of which are the integral membrane proteins proteolipid protein (PLP) in the CNS and P_0 in the PNS and the peripheral myelin basic protein (MBP), with myelin-associated glycoprotein (MAG) as a minor constituent. The lipid bilayer of myelin membranes also has highly specialised properties as a result of its unique lipid composition, which is not only particularly rich in glycolipids, but is also the membrane with the highest cholesterol content. Most of the phosphatidyl ethanolamines are plasmalogens, in which the fatty acids in position 1 are substituted by a long-chain alkenylether.

Cerebrosides were first described more than 100 years ago ([2]; see also [3]). They comprise a large family of compounds that differ in the chemical structure of their ceramide moiety. Like the diacylglycerol backbone of phospholipids (e.g. phosphatidyl ethanolamine in Figure 1), the ceramide backbone of sphingolipids consists of a long-chain fatty acyl residue linked by an amide bond to a long-chain sphingosine base that also presents the functional group for N-acylation (e.g. sphingomyelin, GalC and sGalC in Figure 1). The predominant long-chain base is sphingenine (sphingosine) 2S-amino-3R-hydroxy-octadec-4t-ene-1-ol and less abundantly the saturated parent compound sphinganine (dihydrosphingosine) [4,5]. The primary alcohol group of sphingosine is either part of a phosphodiester bond (as in sphingomyelin) or glycosylated to glycosphingolipids.

Figure 1



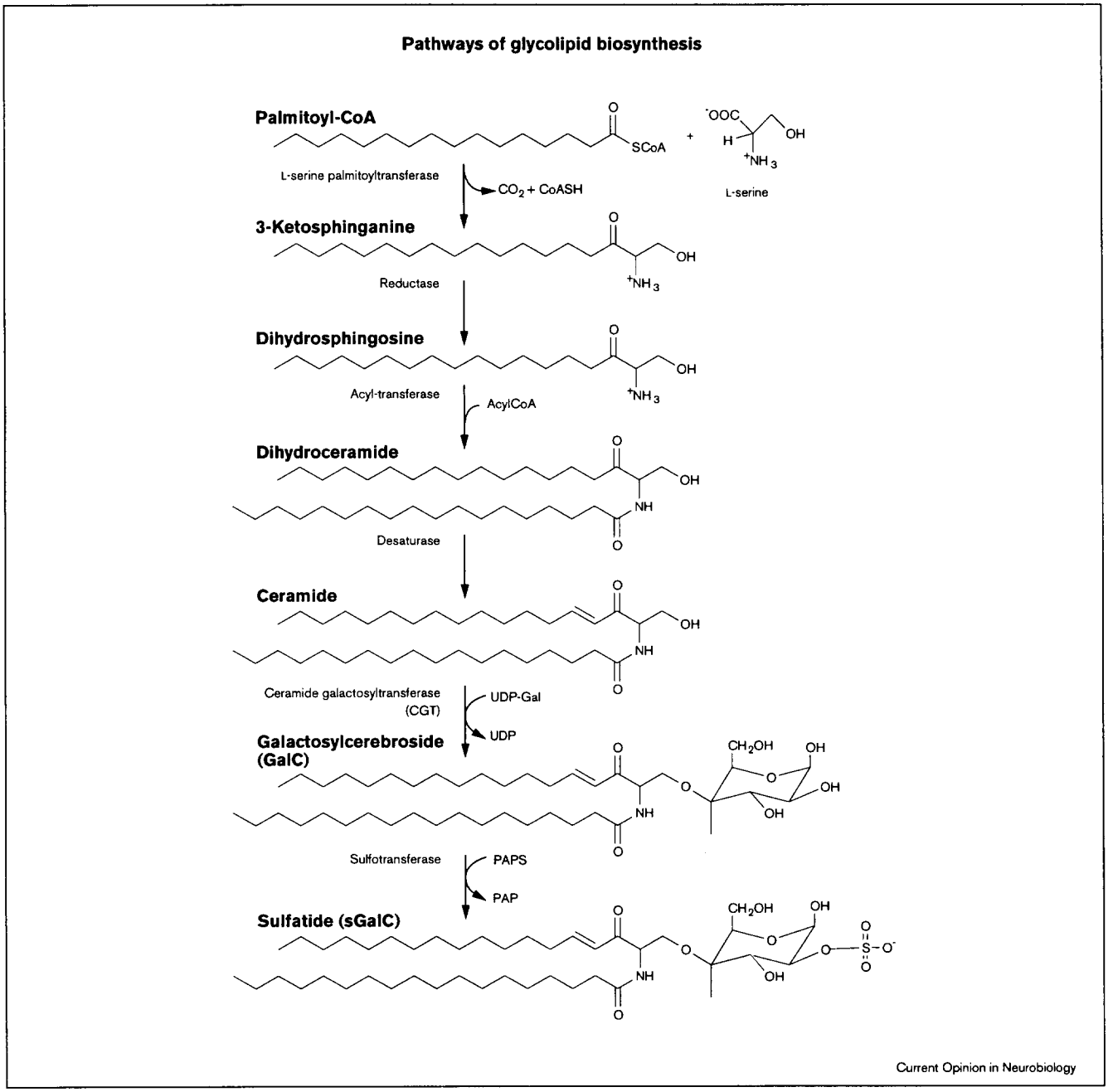
Hypothetical arrangement of complex lipids in the bilayer of the myelin membrane. Notice the asymmetric arrangement of glycolipids in the outer leaflet. P, phosphatidyl.

Oligodendrocytes synthesise cerebrosides and sulfatides that characteristically contain in their ceramide part very long-chain normal fatty acids with 18 to 24 carbon atoms (stearic to lignoceric acid) or monounsaturated fatty acids with a *cis* double bond in position 15, such as nervonic acid ($24:1^{15}$). These fatty acids are present as α-hydroxy fatty acid groups and linked to the long chain sphingosin base. Cerebrosides are esterified to sulfoesters in position 3 of galactose, which renders the polar head group strongly negative. Myelin glycolipids shift from glucoceramides to galactoceramides during species development in the ascending phylogenic tree [6]. Similarly, the ratio of hydroxy to nonhydroxy cerebrosides increases with the complexity of the CNS [6,7]. The nature of the hydrophobic ceramide core, as well as the polar head groups of GalC and sGalC, is important for the fluidity of the lipid bilayer and the polar properties of the membrane surface.

Glycolipids have unusual physical properties

Several physical methods have been applied to the study of the properties of glycolipids isolated from myelin, including X-ray diffraction, spectroscopy (Raman, UV, NMR), the Langmuir technique and calorimetry. A remarkable property of cerebrosides and sulfatides is that their fluid/solid phase transition temperature ('melting point') is far above physiological body temperature, so that glycolipids have a para-crystalline structure at body temperature. Therefore, it is assumed that glycolipids form patches in the lipid bilayer either associated with membrane proteins (rafts) or as islands. The biophysical state of glycolipids depends on the composition of the

Figure 2



Biosynthetic pathway of long-chain sphingosine bases and glycosphingolipids. The pathway outlines the biochemical reactions that lead to ceramide and the long chain sphingosine bases. Ceramide is the branching point in the biosynthesis of sphingomyelin, neutral glycosphingolipids and gangliosides. Sphingosine and ceramide have been recognized as important potential lipid second-messenger molecules in different cell types. PAPS, 3′-phosphoadenosyl-5′-phosphosulfate.

apolar ceramide moiety: chain length, α-hydroxylation, and the number and position of *cis* double bonds in the hydrocarbon chain of the amide-linked acyl group. For example, saturated GalCs have a phase transition temperature of up to 85°C; the introduction of a double bond into a saturated fatty acid, such as lignoceric (24:0) yields nervonic acid (24:1[15]). This reduces the transition temperature by 10°C, which is a small effect compared to, for instance, the introduction of a double bond in stearic acid (C18:0) of the phosphatidylcholine 16:0, 18:0 PC (phase transition at 49°C) to form 16:0, 18:1[9] PC (phase transition at –3°C) [8–13].

The monolayer technique has been used to study the physical properties of galactolipids, such as the average molecular area, the compressibility of the monomolecular

lipid film, and the lateral film pressure at monolayer collapse point. Alkyl chains with an all-*trans* conformation and α-hydroxy fatty acids stabilise the glycolipid monolayer, contrary to *cis* unsaturated fatty acids (A Bosio, E Binczek, W Stoffel, unpublished data; see Note added in proof). X-ray analysis of lamellar cerebroside structures has shown that alkyl chains of cerebrosides are tightly aligned and that their polar head groups are oriented almost parallel to the plain of the bilayer [14]. Each cerebroside molecule may form up to eight inter- or intramolecular hydrogen bonds by lateral interaction between the polar hydrogens of the sugar and the hydroxy and amide groups of the sphingosine base of the ceramide moiety. This dense network of hydrogen bonds is believed to contribute to the high transition temperature and the compact alignment of the galactolipids. Similar interactions can be envisioned between cerebrosides and the polar groups of phospholipids and cholesterol in the bilayer of the myelin membrane.

In principle, the behaviour of sulfatides is similar to that of GalCs. The negatively charged polar head groups form a polyanionic surface on the outer leaflet of the bilayer, which then forms additional interactions with positively charged complementary molecules [15,16].

Enzymes involved in glycolipid biosynthesis

The structural determinant of all glycosphingolipids are the long-chain bases sphinganine (dihydrosphingosine), sphingenine (sphingosine) and phytosphingosine. Their enzymic synthesis is initiated by the rate-limiting condensation of serine and palmitoyl-CoA to 3-ketosphinganine, which is catalysed by L-serine palmitoyltransferase (SPT), a pyridoxalphosphate-dependent enzyme that resides in the endoplasmic reticulum (ER) membrane (see Figure 2). The 3-oxo-intermediate is stereospecifically reduced by a NADPH-dependent reductase to the long-chain base sphinganine (dihydrosphingosine, 2S-amino-3R-hydroxyoctadec-4t-ene-1-ol). Neither the 3-oxo-intermediate nor the long-chain base occur free in the cell. An acyltransferase immediately forms sphinganine-containing ceramides, followed by the desaturation of their sphinganine moiety to the sphingenine-containing ceramides.

Oligodendrocytes and Schwann cells synthesise very long-chain acylated ceramides, which are the acceptors in galactosphingolipid synthesis for the transfer of galactosyl residues from UDP-Gal, which is catalysed by the enzyme UDP-galactose-ceramide galactosyltransferase (EC 2.4.1.45). This reaction takes place on the luminal side of the ER. A special UDP-galactose transporter shuttles the substrate into the lumen of the ER. A sulfotransferase esterifies the 3-position of galactose of GalC, with 3′-phosphoadenosyl-5′-phosphosulfate (PAPS) as the sulfate donor. This reaction occurs in the Golgi stacks through which GalC passes during sorting to the plasma membrane of oligodendrocytes and Schwann cells. Despite the fact that a sulfotransferase that uses GalC, diacylglyceryl-1-alkyl-2-acylglycerylgalactoside and lactoside as acceptor molecules has been purified [17] and cloned [18•] from a human renal cancer cell line, this sulfotransferase has not been found to be expressed in brain tissue. Its size differs considerably from that of another less well characterised sulfotransferase (31 kDa) from mouse brain [19]. Therefore, the nature of sulfotransferase activity in oligodendrocytes awaits a definitive answer.

UDP-galactose-ceramide galactosyltransferase, the key enzyme in glycolipid synthesis in oligodendrocytes and Schwann cells

A major recent breakthrough in myelin membrane research was initiated by the isolation of UDP-galactose-ceramide galactosyltransferase (CGT), the key enzyme of galactolipid biosynthesis, and was followed by the molecular characterisation of the human and murine *cgt* gene, which led to the generation of a *cgt* knock-out mouse by gene targeting through homologous recombination. The CNS and PNS myelin membranes of these mice are completely deficient in GalC and sGalC structures and have provided insights into the pivotal function of these glycolipids in the normal myelin sheath.

This development started with the protein isolation, purification and characterisation of CGT from myelinating rat brain [20]. The complete sequence of the 541 amino acid residue long polypeptide was derived from a CGT-specific cDNA. Protein analysis revealed a 20-residue-long signal sequence that is cleaved during co-translational translocation into the ER membrane. Post-translational N-glycosylation at sites Asn78, Asn333 and Asn442 yields a 64 kDa mannose-rich glycoprotein. CGT contains a carboxy-terminal hydrophobic α-helical transmembrane domain and a KKVK sequence that is regarded as an ER retention signal (Figure 3) [21].

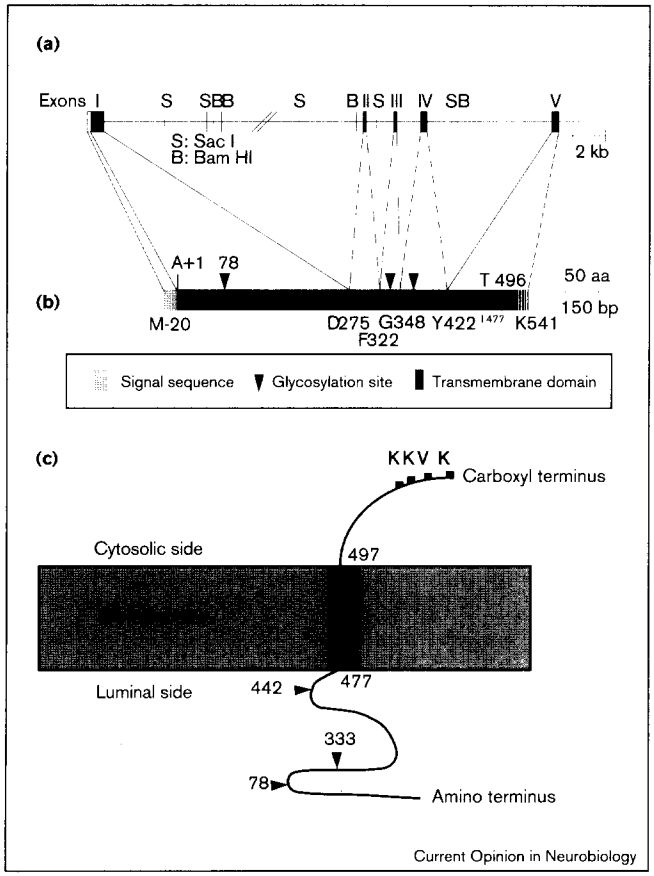
Sequence analysis has revealed that CGT is remarkably homologous to the members of the UGT family (UDP-glucuronosyltransferase). These isozymes are located in the hepatic ER and detoxify a wide range of endogenous and xenobiotic toxic compounds by glucuronyl transfer to form a water-soluble product for excretion.

The rat, mouse and human CGT-specific cDNA sequences, as well as the organisation of the gene (see Figure 3), are strongly conserved. The *cgt* locus spans approximately 70 kilobases and has been mapped to the distal region of human chromosome 4 band q26 and to mouse chromosome 3 bands E3–F1 [22–24].

Divergent functions of glycolipids are suggested by immunochemistry of oligodendrocytes in culture

The cell- and development-specific patterns of glycolipids reflect their function in differentiation, signal transduction and cell adhesion. This is particularly well illustrated by the differentiation of oligodendrocytes [25,26]. Antibodies

Figure 3



The schematic presentation of the CGT gene, protein and membrane integration. **(a)** Organisation of the *cgt* gene. The human *cgt* locus is on chromosome 4 band q26. **(b)** Schematic presentation of the protein, depicting the signal sequence, glycosylation site, transmembrane domain and ER retention signal. **(c)** Proposed integration of CGT in the endoplasmic reticulum membrane.

that recognize GalC and sGalC have been used to study the functions of glycolipids in the development of cell lineage. GalC has been used to identify the emergence of differentiated oligodendrocytes, indicating that ceramide galactosyltransferase is expressed at an early stage of myelinogenesis. GalC synthesis occurs in parallel to the expression of MBP and PLP [20].

Interestingly, the interaction of antibodies to GalC but not to sGalC alters the organisation of the CNS and PNS myelin membrane sheets [27]. Anti-GalC antibodies reversibly inhibit the differentiation of O2A precursor cells [28] and the formation of peripheral myelin [29]; however, the monoclonal antibody O4 [30], which is specific for sGalC, enhances the rate and extent of oligodendrocyte differentiation and aggregation [31,32]. Antibodies to GalC and sGalC cause a Ca^{2+} influx followed by degradation of microtubules in cultured oligodendrocytes. GalC and sGalC can mediate this Ca^{2+} response independently [33,34], suggesting that GalC and sGalC play different roles in the regulation and maintenance of myelination *in vivo* (and that antibody binding disrupts this differentiation process). It has been suggested that GalC acts as a receptor for intracellular signals that regulate sheet formation of the oligodendroglia by interacting with a G protein, leading to Ca^{2+} channel activation via a second messenger [34]. However, no component of this speculative signal transduction system has been identified so far.

New molecular membranology tackles glycolipid functions *in vivo*

The biophysical properties of glycolipids of the myelin membrane have been derived largely from *in vitro* studies on isolated lipid species, but not in the context of the natural environment of the myelin lipid bilayer or in association with the peripheral and integral membrane proteins. The interaction of anti-GalC and anti-sGalC antibodies with their antigens in the oligodendrocyte and Schwann cell plasma membrane has been interpreted as 'removal' of these glycolipids from the surface of these cells. However, antibody binding perturbs not only the order of the polar head groups in the outer surface but this perturbation may also be transmitted to the apolar ceramide part in the bilayer core. It is conceivable that the cellular response may therefore express the conformational perturbation of a special lipid class in its environment within the bilayer. Cellular responses to the antibody binding to its membrane-bound antigens GalC or sGalC discussed above make it highly desirable to devise an experimental system that will give insights into the functions of GalC and sGalc in an unperturbed system *in vivo*.

The powerful method of gene targeting by homologous recombination in the mouse has now provided a tool for studying glycolipid functions *in vivo* by making it possible to ablate (knock out) a single gene, leading to the loss of its protein product and function. This approach has been used previously to analyse the functions of myelin proteins [35–38] and has now been used to study the function of glycolipids in the myelin membrane. Knocking out *cgt* leads to myelin membranes deficient in the glycolipids GalC, sGalC and GalDG. The altered membrane properties have provided insights into these glycolipids' functions.

After isolating the mouse *cgt* gene, we [39••] and Coetzee *et al.* [40••] generated a null mutant mouse ($cgt^{-/-}$) in which CNS and PNS myelin was completely depleted of GalC, sGalC and GalDG. The complete lack of glycolipids in the brain and peripheral nerves in the $cgt^{-/-}$ mutant mouse proved that only one *cgt* gene copy is responsible for galactosphingolipid synthesis. The expression of oligodendrocyte- and Schwann-cell-specific genes relevant for the myelination of axons — namely, the genes for MBP, PLP, MAG and P_0 — was found to be normal in these mice.

A minor part of the ceramides containing the long-chain fatty acids (normal, α-hydroxylated and mono-unsaturated) is used as an acceptor for a glucosyl transfer by UDP-glucose-ceramide glucosyltransferase and phosphocholine transfer by CDP-choline:ceramide phosphocholine transferase to glucoceramides and sphingomyelin, respectively. Schwann cells transfer a sulfo-group to the newly formed glucocerebroside but oligodendrocytes do not. In *cgt*$^{-/-}$ mice, these glucocerebrosides and their sulfate esters are unable to compensate for the loss of galactosphingolipids. The hydrophobic core of the bilayer and particularly the carbohydrate polar head group and charge pattern of the myelin membrane surfaces of the *cgt*$^{-/-}$ mouse are seriously altered despite the utilisation of long-chain n- and hydroxy-fatty acyl ceramides for glucocerebroside and sphingomyelin synthesis [39••].

Oligodendrocytes and Schwann cells are unable to replace the lost structure of these galactosphingolipids (A Bosio, E Binczek, W Stoffel, unpublished data; see Note added in proof). Their abolished function was manifested in the dramatically reduced velocity of nerve conduction from saltatory conduction of myelinated to the continuous conduction of unmyelinated axons in homozygous *cgt*$^{-/-}$ mice [39••]. The insulation of the internodal myelin membranes is lost as a result of the severely altered ion permeability of the lipid bilayer. A most striking phenotype develops after postnatal day 10 (p10): a dysmyelinosis, with whole-body shivering, seizures, peripheral pareses, increasing loss of locomotor activity, reduced growth and death from p20 onwards. Therefore, GalC and sGalC are not only essential for the unperturbed lipid bilayer of the myelin membrane of CNS and PNS, but are also of vital importance.

Although the phenotype of the *cgt*$^{-/-}$ mutant is strikingly similar to dysmyelinosis due to severe hypomyelination (as seen in *shiverer* and *jimpy* mice and in *myelin-deficient* rats), the myelin sheath of both wild-type and *cgt*$^{-/-}$ mice appears (ultrastructurally) to display regular periodicity of the main and intermediate dense lines in the PNS sciatic nerve. However, in the CNS of these mice, the inner mesaxon and lateral loops of myelin appear to have lost contact with the axolemma: the periaxonal space is wide and irregular; the myelin membranes are no longer concentrically wrapped around the axon; and redundant myelin foldings often cover an already myelinated axon. In addition, the CNS myelin in the paranodal and nodal areas appears disorganised. Interestingly, the Na^+ channels remain concentrated at the nodes of Ranvier [41].

As deduced from their absence in the *cgt*$^{-/-}$ mouse, the dominant function of glycolipids appears to be their contribution to the formation of an ion-impermeable bilayer that is indispensable for saltatory conduction. The ratio of the three lipid classes — cholesterol (C): galactolipids (GalC): phospholipids (PL) — is randomly distributed in the two bilayer leaflets, with C:GalC:PL = 2:1:2. An asymmetric distribution of glycolipids and a cholesterol/polar lipid ratio of 1 in the outer leaflet [25,42] would lead to a stoichiometry of C:GalC:PL = 5:4:1 in the outer leaflet and of C:GalC:PL = 3:0:7 in the inner leaflet. The area/lipid molecule in monolayer films of randomly distributed myelin lipid of the *cgt*$^{-/-}$ mouse is twice that of wild-type myelin. The depletion of galactosphingolipids in the bilayer leads to the loss of the rigid very long-chain fatty acids as stabilising factors within the hydrophobic core of the bilayer, to the loss of polar sugar moieties from the outer leaflet (which weakens the hydrogen bonding network at the membrane surface), and to the depletion of sGalC (which deprives the outer surface of a polyanionic barrier). The lack of GalC and sGalC in the outer leaflet must be replaced by the remaining phospholipids and cholesterol, but no enhanced phospholipid and cholesterol synthesis has been observed.

The phenotype of the *cgt*$^{-/-}$ mouse seems to be nonpleiotropic by all criteria studied

Ablation of the *cgt* gene has no impact on the number and the development of oligodendrocyte and Schwann cell precursors or on the expression of other genes essential for myelination. In addition, it does not appear to affect either plasma membrane sheeting of oligodendrocyte processes and Schwann cells or the periodicities of the main and intermediate dense lines of the internodes.

Conclusions

Integral and peripheral membrane proteins and lipids of the myelin bilayer regulate cooperatively the properties of biological membranes. The over all view of the lipid bilayer of membranes as a 'lipid sea' with membrane proteins floating around is outdated by the accumulating data on the complexity of lipid constituents in different membranes with divergent functions that hint at specialised functions of individual lipid species within the lipid bilayer. However, only scarce information that would allow a molecular view of their individual functions is available.

The powerful methods of molecular biology, including the gene knock-out technique by gene targeting, are now being applied in the field of membranology and should help bridge the gap in understanding the genetic control of the protein–lipid assembly and its function in biological membranes. The selective ablation of single genes and their gene products is now being applied to key enzymes in the biosynthetic pathways of complex lipids. Thus, it is now possible to deprive a lipid bilayer of single lipid classes. The resulting phenotype will allow us to define the function, *in vivo*, of the missing lipid class by the given altered functional properties of the bilayer. The loss-of-structure/loss-of-function of *cgt* encoding the key enzyme of glycolipid biosynthesis is the first study by which the structure/function relationship of a lipid class in the membrane — here, specifically of glycolipids in the membrane of myelin and their function in myelinogenesis — has been addressed. The results

suggest that this is a promising approach to deciphering the various functions of glycolipids.

In the near future, other enzymes involved in the modification of the polar head groups or the hydrophobic core of complex membrane lipids will be identified. Their genes may then be ablated in the mouse. Step-by-step single and multiple gene knock-outs will provide insight into the molecular basis of interactions of membrane enzymes, receptors and transporters with specific lipids and their regulation. Conditional knock-out techniques will give even more sophisticated answers by introducing cell-specific and time-regulated expression during the development and maintenance of biological membranes.

Note added in proof

The work referred to in the text as 'A Bosio, E Binczek, W Stoffel, unpublished data' is now in press [43].

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