Review

Cholesterol: A Novel Regulatory Role in Myelin Formation

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Abstract

Myelin consists of tightly compacted membranes that form an insulating sheath around axons. The function of myelin for rapid saltatory nerve conduction is dependent on its unique composition, highly enriched in glycosphingolipids and cholesterol. Cholesterol emerged as the only integral myelin component that is essential and rate limiting for the development of CNS and PNS myelin. Experiments with conditional mouse mutants that lack cholesterol biosynthesis in oligodendrocytes revealed that only minimal changes of the CNS myelin lipid composition are tolerated. In Schwann cells of the PNS, protein trafficking and myelin compaction depend on cholesterol. In this review, the authors summarize the role of cholesterol in myelin biogenesis and myelin disease.

Keywords

myelin, cholesterol, ER exist, oligodendrocyte, Schwann cell

Cholesterol may have a bad reputation in the mass media, but it is a critical lipid for mammalian life. At the cellular level, cholesterol contributes to the semipermeable property of cellular membranes and regulates membrane fluidity by increasing the lateral density of saturated fatty acid chains (Ikonen 2008). Cholesterol is also crucial for the formation of lipid nanodomains, better known as membrane lipid rafts, which may act as platforms for signal transduction (Simons and Ikonen 1997; Brown and London 2000; Hanzal-Bayer and Hancock 2007). Although the existence of membrane lipid rafts in cells has been debated, transient cholesterol-containing complexes of small size (<20 nm in diameter) have recently been demonstrated in living cells using stimulated emission depletion (STED) far-field fluorescence microscopy (Eggeling and others 2009).

In addition to influencing the biophysical properties of membranes, cholesterol can interact directly with certain proteins, possibly modulating their functions. For instance, the structural and functional properties of the nicotinic acetylcholine receptor (AChR) are affected by cholesterol. Low cholesterol reduces the number of functional AChR by reducing its trafficking and cell surface clustering and enhancing its endocytosis. However, the remaining AChR show increased probability of channel opening (Barrantes 2007). The signaling protein sonic hedgehog is covalently bound to cholesterol. The relevance of this modification may be required for restricting local dilution and unregulated spreading of the sonic hedgehog activity gradient in mice (Huang and others 2007; Guerrero and Chiang 2007). Although significant progress has been made to elucidate the function of cholesterol at the subcellular level, its role in the context of organs and tissue development is not well established yet.

Cholesterol Homeostasis in the Nervous System

In newborn mice, the brain cholesterol concentration is about 3.5 mg/g and thus in the same range as in all other organs (Dietschy and Turley 2004). However, three weeks later, the brain cholesterol level has almost tripled (12 mg/g) and reaches about 19 mg/g in the adult (Dietschy and Turley 2004). This major increase reflects postnatal myelin formation by oligodendrocytes. Myelin in the adult brain accounts for around 80% of the brain cholesterol content (Muse and others 2001; Quarles and others 2006; Dietschy 2009). In contrast to the dynamic cellular pool of cholesterol, the turnover of cholesterol in myelin is very low with

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an estimated half-life of about one year as measured by deuterium labeling and mass spectrometry (Ando and others 2003). Therefore, myelin could be seen as a "cholesterol drain" within the developing nervous system.

All nucleated mammalian cells can synthesize cholesterol. The cholesterol biosynthesis starts with acetyl-CoA molecules that build isoprenoid moieties via the isoprenoid biosynthesis pathway (Fig. 1). The conversion of HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) to mevalonate is catalyzed by HMG-CoA reductase (encoded by *Hmgr*) and represents the rate-limiting step in cholesterol biosynthesis. Schwann cells up-regulate the expression of *Hmgr* and increase endogenous cholesterol biosynthesis when grown in lipoprotein-deficient media (Fu and others 1998). Mice treated with low doses of tellurium, an inhibitor of squalene epoxidase, initially show increased expression of *Hmgr* in sciatic nerve, implying a compensatory increase of cholesterol biosynthesis by Schwann cells in vivo (Toews and others 1997). At higher tellurium doses, however, mature Schwann cells undergo autophagic cell death, rendering these cells especially vulnerable to cholesterol biosynthesis inhibition (Berciano and others 1998). With this partial Schwann cell loss, also the initial increase of *Hmgr* mRNA is lost, and demyelination becomes apparent (Toews and others 1997). Isoprenoids, in addition to being intermediates in cholesterol biosynthesis, are involved in other cellular functions. For example, as membrane anchors, isoprenoids facilitate signal transduction by small GTPases and several G-protein-coupled receptors (Burger and others 2000; Meske and others 2003). Furthermore, they are intermediates in the formation of dolichol (carrier in sugar metabolism) and ubiquinone (electron carrier in energy metabolism).

The first biochemical step dedicated to cholesterol biosynthesis is the formation of squalene, which is catalyzed by squalene synthase (SQS; farnesyl-diphosphate farnesyl transferase; encoded by Fdft1). After cyclization of squalene to the first sterol intermediate, lanosterol is converted into cholesterol by a series of enzymatic reactions (Fig. 1) in the endoplasmic reticulum (ER) and the nuclear membrane (Goldstein and Brown 1990; Gaylor 2002). The biosynthesis of cholesterol is energy demanding, requiring 18 ATP and 25 reduction equivalents (mostly NADPH/H+) in addition to 18 acetyl-CoA molecules for one molecule of cholesterol.

In the clinic, the most widely used cholesterol-lowering drugs are statins, a chemically heterogeneous group of competitive HMG-CoA reductase inhibitors. Besides their role in cholesterol biosynthesis inhibition, statins exert immunomodulatory effects: By inhibiting isoprenoid formation (see above), posttranslational protein prenylation is attenuated. This inhibits small GTPases and other prenylated proteins, which modulates many proinflammatory pathways (Greenwood and others 2006; van der Most and others 2009). To date, four small clinical trials in relapsing-remitting multiple sclerosis (MS) with lovastatin (Sena and others 2003), simvastatin (Vollmer and others 2004), or atorvastatin (Paul and others 2008; Lanzillo and others 2010) have been performed, with a total of 92 patients. A reduction in lesion number was consistently demonstrated on MRI, but patients showed no clinical improvement. The studies were too short to monitor any effect on the relapse rate (up to two years of follow-up). The data are inconsistent as to whether the combination therapy with interferon-β is beneficial (Paul and others 2008; Birnbaum and others 2008; Lanzillo and others 2010). In animal models of MS, experimental autoimmune encephalomyelitis (EAE), short-term treatment with statins can reduce initial disease severity (Weber and Zamvil 2008). When administered after the onset of disease, lovastatin or atorvastatin ameliorates the clinical outcome, maybe because inflammatory processes are modulated (Paintlia and others 2005; Stuve and others 2006; Luccarini and others 2008). The combination treatment of statin with a second immunomodulatory drug such as glatiramer, minocycline, or rolipram might be beneficial in EAE. Here, reactive gliosis is reduced and/or the secretion of anti-inflammatory cytokines is promoted (Stuve and others 2006; Luccarini and others 2008; Paintlia and others 2009). However, inconsistent readouts relating to clinical score, axonal degeneration, and myelination reveal the complexity of the disease. Given that statins, when administered after the onset of disease, cannot prevent any immune response, their potential negative impact on remyelination by lowering cholesterol biosynthesis should be considered (see below). Although statin treatment is well tolerated, the use of more specific inhibitors for the subset of isoprenoids that is responsible for the immunomodulatory effect of statins may be a more promising approach for treating MS.

Cells meet their cholesterol demand by de novo synthesis (see above) or by uptake from the extracellular milieu, mainly by endocytosis of cholesterol-rich lipoprotein particles. The blood-brain and blood-nerve barriers shield the nervous system from the cholesterol metabolism of the body and nutritional cholesterol sources (Björkhem and Meaney 2004). Feeding mice a high-cholesterol diet does not alter the synthesis rate or the content of brain cholesterol (Quan and others 2003). Hence, virtually all cholesterol of the nervous system is synthesized in situ.

Within the nervous system, the intercellular transport of cholesterol and other lipids is mediated by lipoprotein particles (Herz and Chen 2006; Bu 2009). The lipoprotein particle receptors belong to the low-density lipoprotein receptor (LDLR) family with LDLR and LRP1 (LDLR-related protein 1) as the main receptors involved in cholesterol homeostasis. Proteins of the LDLR family are

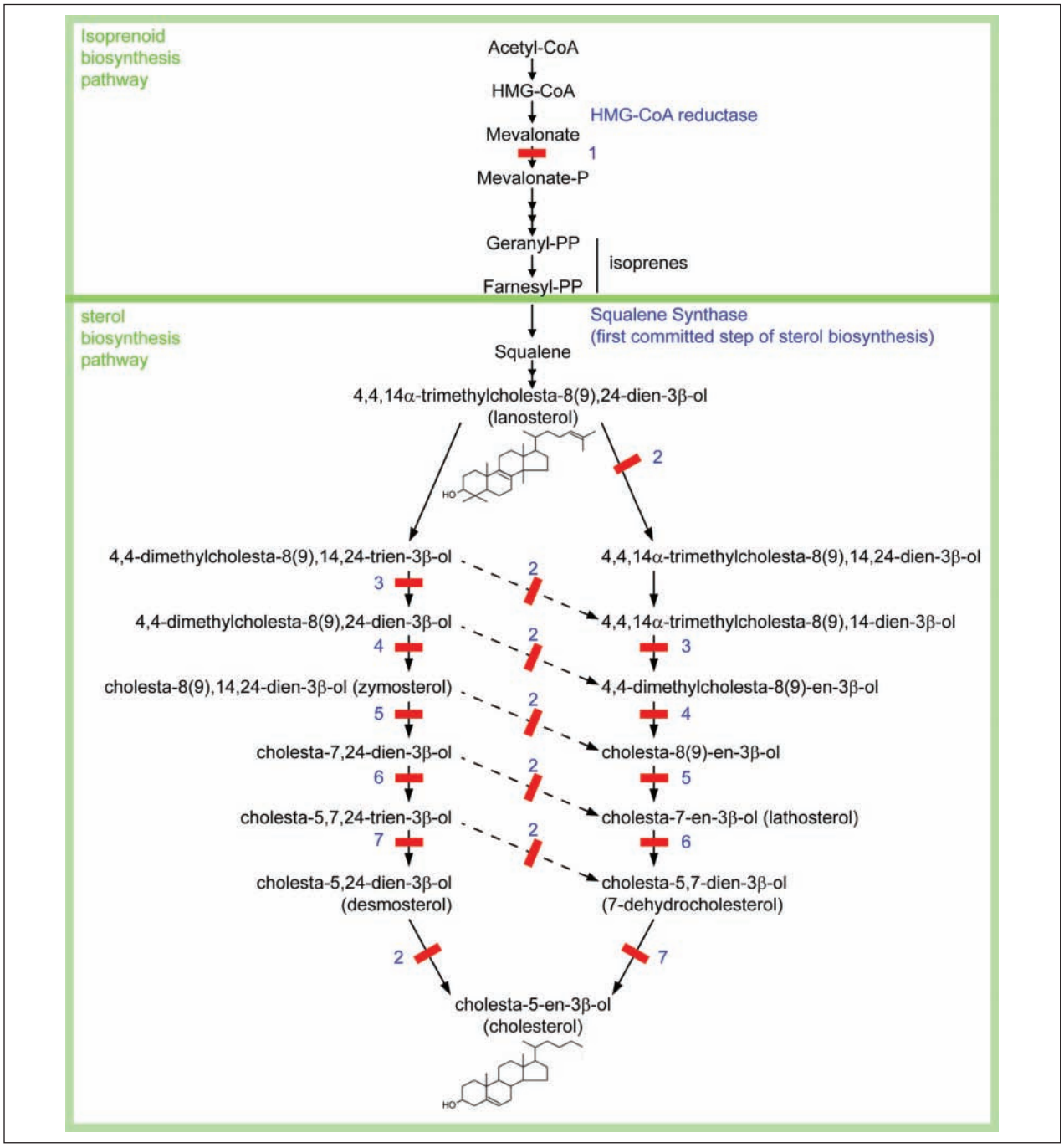

Figure 1. The cholesterol biosynthesis pathway. The isoprenoid biosynthetic pathway (*top*) produces numerous biologically active compounds (i.e., isoprenoids) involved in a variety of important cellular processes. In addition to sterol biosynthesis (*bottom*), isoprenoids give rise to farnesyl and geranylgeranyl moieties (protein modification), dolichol (protein glycosylation), ubiquinone, and heme A (respiratory chain). Enzyme activity of mevalonate kinase (1) is lost in patients with mevalonic aciduria and strongly reduced in patients with hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) (Houten and others 1999; Drenth and others 1999) (red bar). After cyclization of squalene (composed of six isoprene units), lanosterol is synthesized and converted into cholesterol in a series of enzyme reactions. Indicated are the two major routes of cholesterol synthesis. In this segment of the pathway, six inherited disorders have been linked to specific enzyme deficiencies (indicated by red bars). Numbering of the enzymes: 2 = 24-dehydrocholesterol reductase (desmosterolosis; Waterham and others 2001); 3 = sterol C14 reductase (hydrops-ectopic calcification-moth-eaten [HEM] skeletal dysplasia; Waterham and others 2003); 4 = 3β-hydroxysteroid dehydrogenase (congenital hemidysplasia with ichthyosiform erythroderma and limb defects, CHILD syndrome; Konig and others 2000); 5 = delta-8, delta-7 sterol isomerase (Conradi-Hünemann-Happle syndrome, CDPX2; Derry and others 1999); 6 = sterol C5 desaturase *SC5DL* (lathosterolosis); 7 = 7-dehydrocholesterol reductase (Smith-Lemli-Opitz syndrome, SLO; Wassif and others 1998; Fitzky and others 1998). Reproduced from *FEBS Letters* with modifications (Waterham 2006), with permission from Elsevier.

single-span transmembrane proteins with a large extracellular portion that harbor characteristic domains like the ligand-binding repeats, epidermal growth factor (EGF) domains, and YWTD-containing β propeller domains (Bu 2009). Lipoprotein particles are composed of a lipid core of esterified cholesterol and other lipids that is surrounded by a shell of phospholipids, unesterified cholesterol, and apolipoproteins. At a diameter of 14 to 22 nm, the size range of CNS lipoproteins is between those of plasma high-density lipoprotein (HDL) and LDL. Apolipoprotein E (ApoE) and ApoA-I are the major lipoproteins of the nervous system, but also ApoA-IV, ApoD, ApoH, and ApoJ have been found in human cerebrospinal fluid (Koch and others 2001). Mainly astrocytes next to microglia/macrophages have been attributed to be net producers of cholesterol, providing ApoE-containing lipoprotein particles to other cell types, including mature neurons in the CNS (Pitas and others 1987; Uchihara and others 1995). Oligodendrocytes and Schwann cells are able to produce all the cholesterol needed for myelin synthesis cell autonomously (see below) (Morell and Jurevics 1996; Saher and others 2005). However, they can also profit from cholesterol synthesized by other cell types (Saher and others 2005; Saher and others 2009; Verheijen and others 2009). Accordingly, oligodendrocytes express LDLR and very low-density lipoprotein receptor (VLDLR; Zhao and others 2007). However, direct evidence for a role of LDLR and ApoE in the lipid metabolism of the CNS is lacking (Gee and Keller 2005; Rogers and Weeber 2008), probably because of redundancy of apolipoproteins and their receptors (Herz and Chen 2006; Bu 2009). In the PNS, the *Ldlr* gene follows the same expression pattern as myelin protein genes (i.e., increasing early postnatally and decreasing in adulthood; Verheijen and others 2003). Peripheral myelin formation appears normal in the absence of LDLR or ApoE (Goodrum and others 1995; Goodrum and others 2000; Genden and others 2002). However, Schwann cells strongly up-regulate LDLR during remyelination after nerve injury (Boyles and others 1989). The apparent lack of defects in nerve remyelination in the absence of LDLR or ApoE may relate to redundancy of apolipoproteins and their receptors (Goodrum and others 1995; Goodrum and others 2000). ApoE and ApoB are also required for normal myelin debris clearance (Genden and others 2002).

Human Neurodegenerative Diseases Associated with Cholesterol

Several inherited disorders of cholesterol biosynthesis and intracellular transport of cholesterol affect the nervous system. Out of seven genetic disorders of the cholesterol biosynthesis pathway known to date, six enzymatic defects affect postsqualene sterol synthesis (Fig. 1). Whereas sterol precursors may serve some cholesterol functions, they cannot fully substitute for cholesterol itself. Patients generally suffer from lower cholesterol levels and accumulation of potentially teratogenic intermediate sterol precursors. Several mouse mutants have been generated that model inherited diseases affecting cholesterol biosynthesis. In mice, the developmental malformations are less severe, probably because of more efficient utilization of maternal cholesterol by the mouse fetus (Wechsler and others 2003; Marcos and others 2007). Inactivation of the cholesterol biosynthesis pathway at early stages (i.e., disrupting Hmgr or Fdft1) is lethal during embryonic development (Tozawa and others 1999; Ohashi and others 2003).

The most common defect of cholesterol biosynthesis is the autosomal recessive Smith-Lemli-Opitz syndrome (SLOS) with an incidence of 1:20,000 to 1:50,000 in northern European and American populations (Lowry and Yong 1980; Ryan and others 1998). Similarly, defects of the gene *DHCR7* (encoding 7-dehydrocholesterol reductase) induce multiple malformations, including delayed and incomplete myelination, microcephaly, mental retardation, and behavioral problems (Nwokoro and others 2001). Despite these severe developmental defects, 7-dehydrosterol and desmosterol can be incorporated into myelin (Banik and Davison 1971; Wechsler and others 2003; Marcos and others 2007). Mild forms of sterol metabolism defects have been associated with autism spectrum disorders (Bukelis and others 2007). Niemann-Pick type C disease (NPC) is caused by mutations in the *Npc1* or *Npc2* gene (Karten and others 2009). The prevalence of NPC has been estimated to be 1:120,000 to 1:150,000 in Western Europe (Vanier and Millat 2003). The hallmark of this lethal neurodegenerative disorder is an intracellular trafficking defect of lipids, mainly cholesterol and glycosphingolipids. The pathology of NPC patients is characterized by generalized brain atrophy and hypoplasia of white matter (Palmeri and others 1994). *Npc1* null mice show severe dysmyelination and progressive loss of glial cells in the corpus callosum (Weintraub and others 1987; German and others 2002).

Cholesterol in Myelin

Myelin is formed as an extension of the plasma membrane of glial cells, with multiple layers of membranes forming a compacted sheath around the axon (Fig. 2). Myelin exhibits a periodic ultrastructure with electron dense and light layers in electron microscopic images. Electron dense layers represent the closely apposed hydrophilic ends of extracellular membranes (intraperiod lines) and the tight

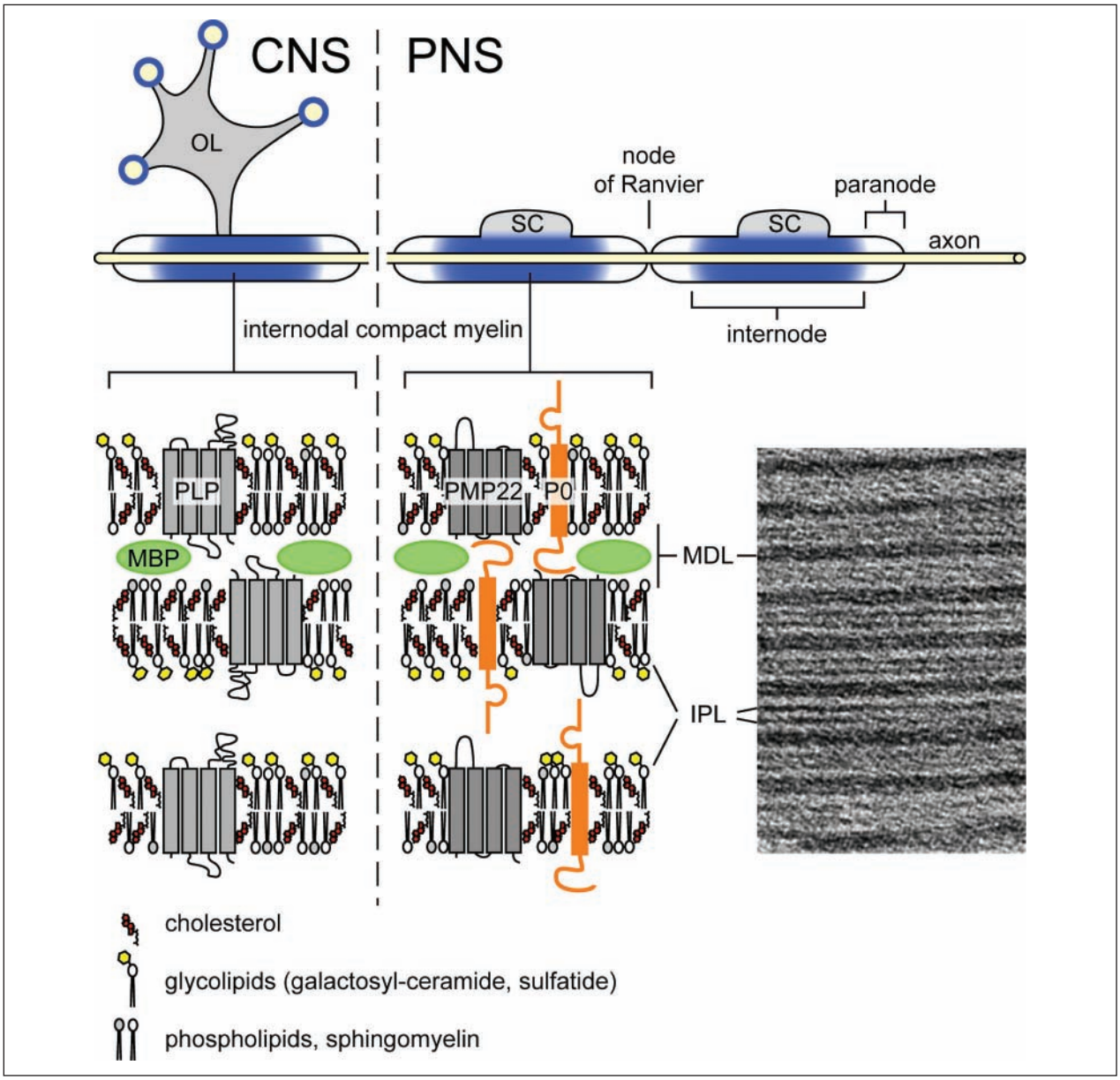

Figure 2. Schematic depiction of myelin. Oligodendrocytes (OL) in the CNS myelinate multiple axons, whereas Schwann cells (SC) associate with one axon and form only one myelin sheath. The morphology of internodal myelin being a compacted stack of glial membranes is reflected by its unique molecular composition. IPL = intraperiod lines; MBP = myelin basic protein; MDL = major dense line; P0 = myelin protein zero; PLP = proteolipid protein; PMP22 = peripheral myelin protein 22.

apposition of cytoplasmic membranes (major dense line; Fig. 2). The distance between the midpoints of two successive major dense lines represents one myelin period. By X-ray diffraction of unfixed tissue of young adult mice, PNS myelin has a period of 17.45 nm, whereas the period of CNS myelin is about 10% smaller (15.65 nm; Agrawal and others 2009). Measuring aldehyde-fixed and dehydrated tissue by electron microscopy typically reveals shorter lengths of the myelin period, because of tissue shrinkage. However, the myelin period remains dynamic in aging animals, increasing in the sciatic nerve (PNS) and decreasing in the optic nerve (CNS; Agrawal and others 2009). The unique structure of compact myelin reduces the capacitance of the axon surface and increases the transmembrane resistance. Myelinated segments, termed *internodes*, are interrupted by small gaps, the nodes of Ranvier (Salzer and others 2008). At these specialized sites, sodium channels are clustered and action potentials

are generated for saltatory impulse conduction. Flanking each internode, paranodal loops seal the internodal periaxonal space from the outside milieu and represent uncompacted myelin. In addition to paranodal loops, noncompact myelin comprises the adaxonal layer of internodal myelin, as well as the abaxonal myelin layer and Schmidt-Lanterman incisures of PNS myelin. Noncompacted myelin contains some cytoplasm and may facilitate the transport of metabolites, proteins, and ions (Nave 2010).

Myelin Lipids

The unique structure of myelin is reflected in its special molecular composition. Some myelin proteins are present in high abundance, although the complexity of the myelin protein composition may not be below that of other membranes (see below). Another prominent feature of myelin is the high content of lipids that constitute about 70% to 80% of its dry weight (Norton and Cammer 1984), in contrast to most plasma membranes that show a lipid to protein ratio of around 1:1. In addition, CNS myelin contains the exceptional molar ratio of cholesterol/phospholipids/galactolipids/plasmalogen of 3:3:2:1 to 4:3:2:1 with cholesterol constituting about 26% to 28% of dry weight of myelin (Norton and Cammer 1984). The content of 20% to 30% galactolipids comprises galactosylceramides (GalC) and their sulfated derivatives, sulfatides. Galactolipids are almost exclusively located at the extracellular leaflet of the membrane (i.e., facing the intraperiod lines), constituting up to two-thirds of the outer surface of the myelin sheath (Eckhardt 2008). Whereas cholesterol is rate limiting for myelin membrane growth (see below), galactolipids have a more distinctive function as revealed by several mutant mouse lines. The role of sulfatides has been studied in mice with a mutation of the *Ga13st1* (galactosylceramide 3'-sulfotransferase) gene. Mutants lack sulfatides but form almost normal myelin, with regional instabilities of compact myelin (Honke and others 2002; Marcus and others 2006). Although paranodal loops are morphologically disrupted (with the absence of transverse bands from the septate-like junctions), impulse conduction is preserved (Honke and others 2002). In contrast, mutant mice that lack the enzyme UDP-galactose/ceramide galactosyltransferase (*Ugt3a1*), and thus sulfatides as well as GalC, show severe reduction of nerve conduction velocity (Coetzee and others 1996; Bosio and others 1996; Dupree and others 1998; Dupree and others 1999). The phenotype of *Ugt3a1* null animals is similar to *Ga13st1* null mice but more severe. It was hypothesized that the absence of galactosylceramide is partially compensated by glucosylceramide. However, the phenotype of mice with ablated UDP-glucose ceramide glucosyltransferase (*Ugcg*) in myelinating glia on top of the *Ugt3a1* null allele is identical to *Ugt3a1* null animals (Saadat and others 2010). This reveals that myelin formation is possible in the complete absence of glycolipids.

In cells, glycosphingolipids, especially when esterified with saturated (or monounsaturated) long and very long fatty acid chains, together with cholesterol promote the formation of membrane subdomains that are resistant to cold detergent extraction, termed *membrane lipid rafts* (Ikonen 2008). This specific composition of lipids is assumed to favor an increased lateral compaction of the fatty acid chains that is likely to contribute to the insulator function of myelin. Moreover, it has been speculated that membrane lipid rafts might be one type of transport unit that delivers myelin proteins and lipids to the growing myelin sheath (de Vries and Hoekstra 2000; Kramer and others 2001; Lee 2001). Given their high abundance in myelin and their association with several myelin proteins, it is still unsolved whether membrane lipid rafts are important for myelin because they act as transport units during myelin biogenesis (see below) or because the raft-like composition of compact myelin is essential for myelin function.

Lipid Interacting Myelin Proteins

In contrast to the myelin lipids, the most abundant myelin proteins are unique to myelin. Several of the myelin proteins associate with membrane lipid rafts, as they are enriched in detergent-resistant extracts of myelin. In the CNS, proteolipid protein (PLP) and its minor splice variant DM20 constitute about 17% of the protein content as calculated in a recent proteomics approach using liquid chromatography (Jahn and others 2009). This small (24-kDa/20-kDa) four-helix bundle protein can be isolated from detergent-resistant membranes and has been shown to associate with cholesterol (Brophy and others 1984; Simons and others 2000). In agreement with this, PLP/DM20 contains a four–amino acid motif that could be involved in direct cholesterol binding (Epand 2006). Surprisingly, *Plp1* null mice develop normally with respect to myelin formation and nerve conduction (Klugmann and others 1997). They show only minor defects in myelin ultrastructure (Rosenbluth and others 2006) and exhibit a late-onset axonal degeneration phenotype (Griffiths and others 1998), rendering the function of PLP/DM20 still elusive. It had been speculated that PLP/DM20 is involved in the extracellular compaction of myelin, but the lack of a corresponding phenotype in *Plp1* null mice argues for a functional redundancy of PLP/DM20 with other myelin proteins. In Pelizaeus-Merzbacher disease (PMD), the most frequent cause of dysmyelination is overexpression of the *PLP1* gene. It has been hypothesized that the binding of PLP/DM20 to cholesterol results in abnormal accumulation

of cholesterol in the endosomal/lysosomal compartment (Simons and others 2000; Simons and others 2002), leading essentially to an oligodendrocyte-specific "lysosomal storage disease." Myelin basic protein (MBP) constitutes the second most abundant protein of compact CNS and PNS myelin (Jahn and others 2009). MBP represents a heterogeneous group of cytoplasmic but membrane-associated proteins. Although encoded by a single gene, several splice isoforms (four in humans) and multiple posttranslational modifications have been described (Boggs 2006). Generally, it is assumed that the high density of basic amino acids in MBP is responsible for its association with the negatively charged polar head groups of membrane lipids. This strong interaction with the myelin membrane may also mediate its partial cyclodextrin-sensitive association with the detergent-resistant membrane fraction of myelin (Musse and others 2009). Especially the highly charged phosphatidyl-inositol-(4,5)-bisphosphate appears responsible for the membrane association of MBP (Musse and others 2009; Nawaz and others 2009). In addition to binding specific lipids by MBP in the lateral dimension (Fitzner and others 2006), MBP strengthens the adhesion of the cytosolic surfaces of myelin membranes, thus promoting the cytoplasmic compaction of myelin (Martini and others 1995; Boggs 2006). To date, MBP is the only myelin protein that has been shown to be essential for myelin formation and is apparently rate limiting, as demonstrated by the mouse mutant *shiverer* (Roach and others 1985; Readhead and others 1987; Popko and others 1987).

Myelin protein zero (P0) is the major myelin protein of the PNS. Mutations in the *MPZ* gene are associated with different forms of hereditary neuropathies, for example, Charcot-Marie-Tooth disease (CMT) type 1B (CMT1B), Dejerine-Sottas syndrome (DSS), congenital hypomyelination (CH), and CMT2 (Shy and others 2004). P0 is a single-span transmembrane protein containing an extracellular domain with one immunoglobulin (Ig)–like domain and a cytoplasmic domain with many basic amino acids. The adhesive properties of its extracellular and cytosolic domains are involved in the extracellular and cytosolic compaction of PNS myelin (Giese and others 1992). In addition, P0 associates with photo-cholesterol (Saher and others 2009) and localizes to detergent-resistant membranes (Erne and others 2002; Bosse and others 2003; Saher and others 2009). Its transmembrane domain contains a cholesterol recognition amino acid consensus (CRAC) motif (Epand 2006) that mediates cholesterol association (Saher and others 2009).

PMP22 (peripheral myelin protein 22) is found predominantly in compact myelin of the PNS. This tetraspan membrane glycoprotein associates with detergent-resistant membranes (Bosse and others 2003) and possibly with cholesterol itself. The overexpression of PMP22 is associated with the development of hereditary neuropathies (Charcot Marie Tooth disease type 1A, CMT1A). Reduced expression of PMP22 is associated with dysmyelination, demyelination, and local hypermyelination with the formation of sausage-like outfoldings (termed *tomaculae*), as well as axonal loss (Adlkofer and others 1995). Lateral association of PMP22 with integrins and secondarily with the extracellular matrix might contribute to the etiology of the neuropathies, but the pathomechanisms are not known (Amici and others 2006).

Not all transmembrane myelin proteins are enriched in detergent-resistant membranes. One extensively studied example is myelin-associated glycoprotein (MAG). Although MAG also contains a CRAC motif (Saher and Simons 2010), it most likely does not associate with cholesterol. MAG is a member of the noncompact fraction of PNS and CNS myelin. In accordance with its localization, MAG is not found in detergent-resistant membranes (Erne and others 2002; Saher and others 2009). MAG is a single-span transmembrane glycoprotein with five extracellular Ig-like domains that are involved in adhesion of glial cells to axons. MAG supports long-term axon-myelin stability, despite its low abundance (Schnaar and Lopez 2009). Axonal degeneration of MAG null mice is preceded by alterations of the axonal cytoskeleton (Schnaar and Lopez 2009). Especially at the node of Ranvier, the correct distribution of axonal proteins (i.e., adhesion molecules and ion channels) is influenced by MAG. To date, sialoglycans and Nogo receptors have been described as functional receptors for MAG (Kelm and others 1994; McGee and Strittmatter 2003).

Cholesterol Is Rate Limiting for Myelin Formation in the CNS

The role of cholesterol in myelination has been studied in conditional mouse mutants that lack cholesterol biosynthesis specifically in myelinating glia, oligodendrocytes in the CNS, and Schwann cells in the PNS. These mutants have been obtained by inactivating the first committed enzyme of the cholesterol biosynthesis pathway, squalene synthase (SQS; *Fdft1* gene) (Saher and others 2005).

In the brain and spinal cord, myelin formation of these conditional SQS mutants is severely delayed, taking months rather than weeks (Fig. 3). Oligodendrocyte loss is apparently not the cause of this hypomyelination because oligodendrocyte numbers are unchanged, and the cells appear morphologically healthy. Surprisingly, myelin derived from mutant brains contains normal amounts of cholesterol when compared to other lipids. Thus, despite the low intracellular level of cholesterol, mutant oligodendrocytes enrich cholesterol, presumably as a prerequisite for CNS myelin membrane growth. However, the cholesterol content of

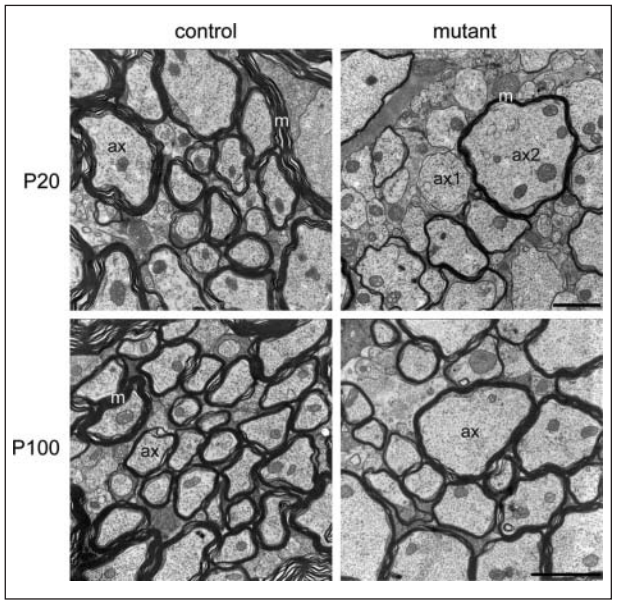

Figure 3. Delayed CNS myelination by oligodendrocytes lacking cholesterol biosynthesis. Ultrastructural analysis of white matter of the ventral spinal cord at P20 shows that virtually all axons (ax) are myelinated in control animals (*left*), whereas fibers in mutants (*right*) possess a thin myelin sheath or lack myelin. At P100 (*lower panel*), as in control animals, all axons in mutant mice were myelinated. Nevertheless, myelination did not reach control levels (ax1 = unmyelinated axon; ax2 = axon with thinner myelin; m = myelin; scale bars, 1 μm). Reproduced from *Nature Neuroscience* with modifications (Saher and others 2005), with permission.

purified myelin is decreased by about 30% when normalized to protein. This implies that mutant oligodendrocytes do not synthesize a "low-cholesterol" myelin but rather a "reduced-lipid" myelin. PLP is present in the same detergent-resistant membrane fractions as in control littermates, implying that the reduced lipid content of myelin suffices to associate with the myelin proteins (Saher and others 2005). By this quality control, cholesterol apparently coordinates the assembly of lipids and proteins to preserve the unique composition of CNS myelin membranes. Interestingly, the cholesterol content of PLP null myelin is reduced, emphasizing the intimate association of the two components (G. Saher, H. Werner, and K. A. Nave, unpublished observation).

The reduced availability of cholesterol limits the expression of myelin proteins, possibly at the transcriptional level, because mRNA levels of myelin protein genes are strongly reduced in conditional SQS mutants (Saher and others 2005). A cholesterol-based feedback mechanism might prevent the toxic accumulation of myelin proteins. The mechanism of cholesterol-regulated gene transcription remains to be elucidated.

Cholesterol synthesis–deficient oligodendrocytes have to take up cholesterol for survival and myelin formation. Astrocytes are a reported source for cholesterol-containing lipoprotein particles in the CNS (Pfrieger 2003). Astrogliosis in the spinal cord of SQS mutant mice, in the absence of neurodegeneration but with astrocytes expressing SQS and HMG-CoA reductase, may facilitate horizontal cholesterol transfer (Saher and others 2005). Increased levels of ApoE and LRP1 point to a lipoprotein-mediated cholesterol transfer.

The importance of cholesterol biosynthesis in myelin maintenance and oligodendrocyte survival has been shown by treating adult rodents with statins (see also above). Although treatment with lovastatin or simvastatin for two weeks does not affect myelin stability (Paintlia and others 2005; Miron and others 2009), treatment with simvastatin for five weeks induces demyelination (Miron and others 2009). Simvastatin treatment regimens decrease the number of mature oligodendrocytes and enhance the number of oligodendrocyte precursors. In addition to inducing oligodendrocyte death, statins may also oppose oligodendrocyte precursor differentiation. In a remyelination paradigm using cuprizone to induce transient CNS demyelination by localized oligodendrocyte death, simvastatin significantly reduces remyelination (Klopfleisch and others 2008; Miron and others 2009), maybe because oligodendrocyte precursors cannot contribute to the oligodendrocyte pool.

In summary, these results demonstrate that the availability of cholesterol is an essential, rate-limiting factor for myelin membrane growth in the CNS. It is not possible for oligodendrocytes to synthesize "cholesterol-free" (or "raft-free") myelin, and only a minor decrease of the cholesterol-to-protein ratio is tolerated.

Cholesterol and Myelination in the Peripheral Nervous System

In the PNS, axonal signals that induce Schwann cells to myelinate (i.e., Neuregulin 1, NRG1) induce also the transcription of cholesterol-biosynthesizing genes (Pertusa and others 2007). This could provide a critical developmental step in the transition of Schwann cell precursors to myelinating Schwann cells in which genes encoding myelin proteins and enzymes involved in the formation of lipids are strongly up-regulated.

To investigate the function of cholesterol in Schwann cells and PNS myelination, two phenotypically related mouse mutants have been studied. The above-mentioned SQS mutant mice lack cholesterol biosynthesis also in the Schwann cell lineage (Saher and others 2009). In the second mouse model, an important player in the regulation of lipid homeostasis, the *Scap* gene, has been inactivated

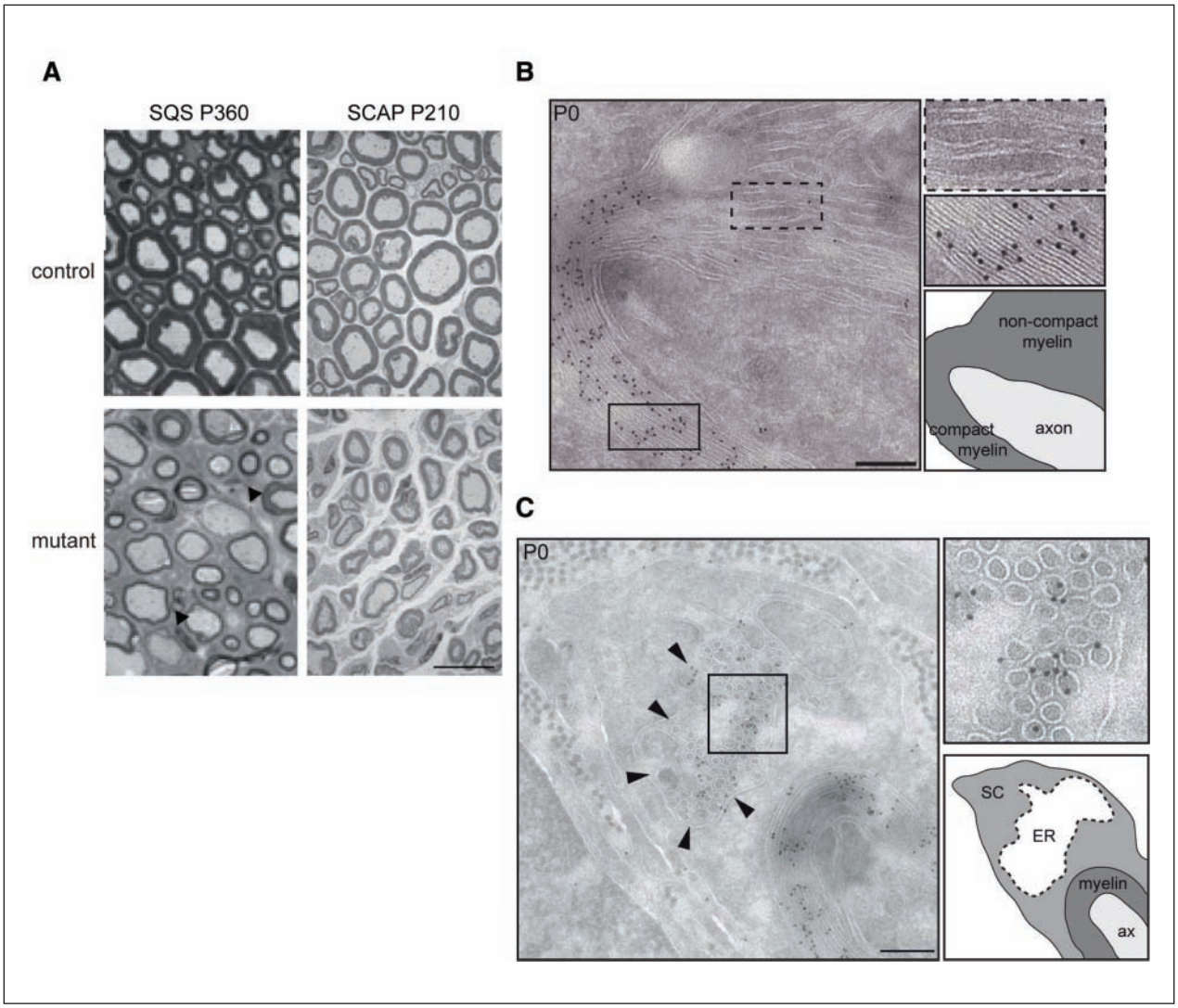

Figure 4. PNS phenotype of squalene synthase (SQS) and SCAP conditional mouse mutants. (*A*) Severely delayed myelination in sciatic nerves from conditional SQS mutants at the age P360 or conditional SCAP mutants at the age P210. (*B–C*) P0 detection by immunoelectron microscopy on P14 sciatic nerve of SQS mutants. P0 is present in compact myelin and absent from noncompact myelin areas. In addition to compact myelin, P0 is found in vesicular/tubular profiles within the Schwann cell ER (arrows and details of boxed area in *top right*). The diagram clarifies structures of the picture. Scale bars in (*A*), 10 μm; in (*B–C*), 250 nm. SC = Schwann cells; ax = axon; ER = endoplasmic reticulum. Reproduced from the *Journal of Neuroscience* and *PNAS* with modifications (Saher and others 2009; Verheijen and others 2009), with permission.

conditionally in Schwann cells (Verheijen and others 2009). SCAP, a sterol responsive element binding protein (SREBP) cleavage activation protein, is needed for the posttranslational activation of SREBP transcription factors. Activated SREBPs translocate to the nucleus, where they induce genes containing sterol responsive elements (Matsuda and others 2001). Hence, inactivating *Scap* affects cholesterol and lipid homeostasis by interfering at the transcriptional level with their biosynthesis and uptake.

Both mouse mutants display a severe congenital hypomyelination of sciatic nerve, which leads to a peripheral neuropathy and hindlimb weakness. Although this PNS phenotype is persistent in SQS mutant mice (and in some cases progressing to a hindlimb paralysis), SCAP mutants recover when reaching adulthood (Fig. 4A; Verheijen and others 2009; Saher and others 2009). In both mutants, affected Schwann cells take up at least some cholesterol/lipids from other (nonrecombined) cells of the nerve, such as fibroblasts and macrophages. Consequently, the expression of genes involved in cholesterol uptake, such as *Apoe* and *Apod*, is increased in peripheral nerves of SQS mutant mice (Saher and others 2009). In SCAP mutants, the altered

fatty acid composition of myelin is compatible with a contribution from external sources (Verheijen and others 2009). As expected, in SCAP mutants, the expression of enzymes in the lipid biosynthesis pathways is dramatically down-regulated. Interestingly, this was also the case in SQS mutants, which implies that Schwann cell cholesterol biosynthesis may regulate intracellular lipid levels.

The importance of lipid uptake by Schwann cells is underscored by co-culture experiments with dorsal root ganglia (DRG) neurons and endogenous Schwann cells cultured in lipoprotein deficient medium. Although SQS mutant Schwann cells show low but significant myelin formation (an amount that is doubled by exogenously added cholesterol), myelination by SCAP mutant cells is negligible and completely depended on extracellular lipids. Even in wild-type co-cultures, exogenously added cholesterol strongly enhances in vitro myelination (Saher and others 2009; Verheijen and others 2009). Thus, even in wild-type Schwann cells, cholesterol is rate limiting in the first days of in vitro myelination. Because this effect is transient, the observation supports the model that cholesterol synthesis is one of the first steps of Schwann cell differentiation.

In both conditional mutants, cholesterol is enriched by Schwann cells as shown by the cholesterol levels in purified myelin membranes. However, cholesterol content is still below control levels with a reduction to 60% in SQS mutant mice and to 80% in SCAP mutants (in SCAP mutant Schwann cells, a residual ability to synthesize cholesterol and lipids might explain this difference). The mechanisms of horizontal cholesterol transfer to mutant Schwann cells remain to be resolved. In both mouse models, the ultrastructure of myelin is affected. In SCAP mutant mice, minor structural changes of the myelin period are associated with an altered lipid composition (i.e., a shift to more polyunsaturated fatty acids). In contrast, in SQS mutants, the altered protein-to-lipid stoichiometry correlates with an increased proportion of uncompacted myelin (Fig. 4B). Accordingly, expression and steady-state level of markers for noncompact myelin, such as MAG, are elevated, whereas proteins of compacted myelin are reduced. The down-regulation of myelin protein genes is also a feature of the SCAP mutant mice. This adjustment of myelin protein synthesis to the availability of cholesterol presumably saves Schwann cells from toxic effects of membrane protein overexpression. The concerted down-regulation of myelin genes can be transcriptionally controlled, as illustrated in the phenotype of mice lacking the relevant transcription factors such as Krox-20 (Topilko and others 1994; Jaegle and others 1996; Le and others 2005).

In SQS mutant Schwann cells, P0 is also present in membranes of the ER, suggesting a special role of cholesterol in regulated P0 export from the ER into the growing myelin sheath (Fig. 4C). Indeed, the cholesterol-dependent intracellular transport of P0 could be reconstituted in a heterologous cell culture system (SQS mutant fibroblasts). Viable SQS-deficient cells only shuttle P0 to the cell surface when grown in medium containing sufficient cholesterol. In contrast, MAG transport to the cell surface is cholesterol independent. The cholesterol-dependent transport of P0 uses a so-called CRAC motif (Epand 2006), located at the cytoplasmic face of the transmembrane domain (Saher and others 2009). Site-directed mutagenesis of the CRAC motif renders the transport of CRAC-mutant P0 independent of cholesterol. To date, two mutations (frameshift and nonsense) within the CRAC domain have been found that are associated with DSS or CMT1B, respectively (Shy and others 2004; see also www.molgen.ua.ac.be/CMTMutations/Mutations/Default.cfm).

Summary and Outlook

The analysis of conditional mouse mutants with defects of lipid biosynthesis has revealed five important roles for cholesterol (Fig. 5): 1) Cholesterol is rate limiting for central and peripheral myelination as the biogenesis of myelin requires cholesterol enrichment. 2) Both oligodendrocytes and Schwann cells are able to take up cholesterol from their surroundings and enrich it in myelin membranes. 3) Only a minimal decrease of cholesterol in CNS myelin is tolerated, but it is possible for Schwann cells to synthesize myelin with lowered cholesterol content. 4) A shift in lipid-to-protein stoichiometry leads to structural changes of peripheral myelin membranes because the transport of P0 from the ER to the growing myelin membranes is adapted to cholesterol availability. 5) In the CNS and PNS, also the transcription of myelin proteins is coupled to intracellular cholesterol levels.

Plenty of open questions remain: What is the mechanism by which cholesterol regulates transcription of myelin protein genes? What is the mechanism by which myelinating glia can take up cholesterol from other cells? So far, it has not been possible to generate mice with myelin that contains lipid levels as low as those of plasma membranes. Is the high lipid content of myelin crucial for its function?

The benefit for MS patients treated with statins is ambivalent. Could drugs that preserve the immunomodulatory effect of statins without interfering with cholesterol biosynthesis and therefore remyelination be more suitable for treating MS?

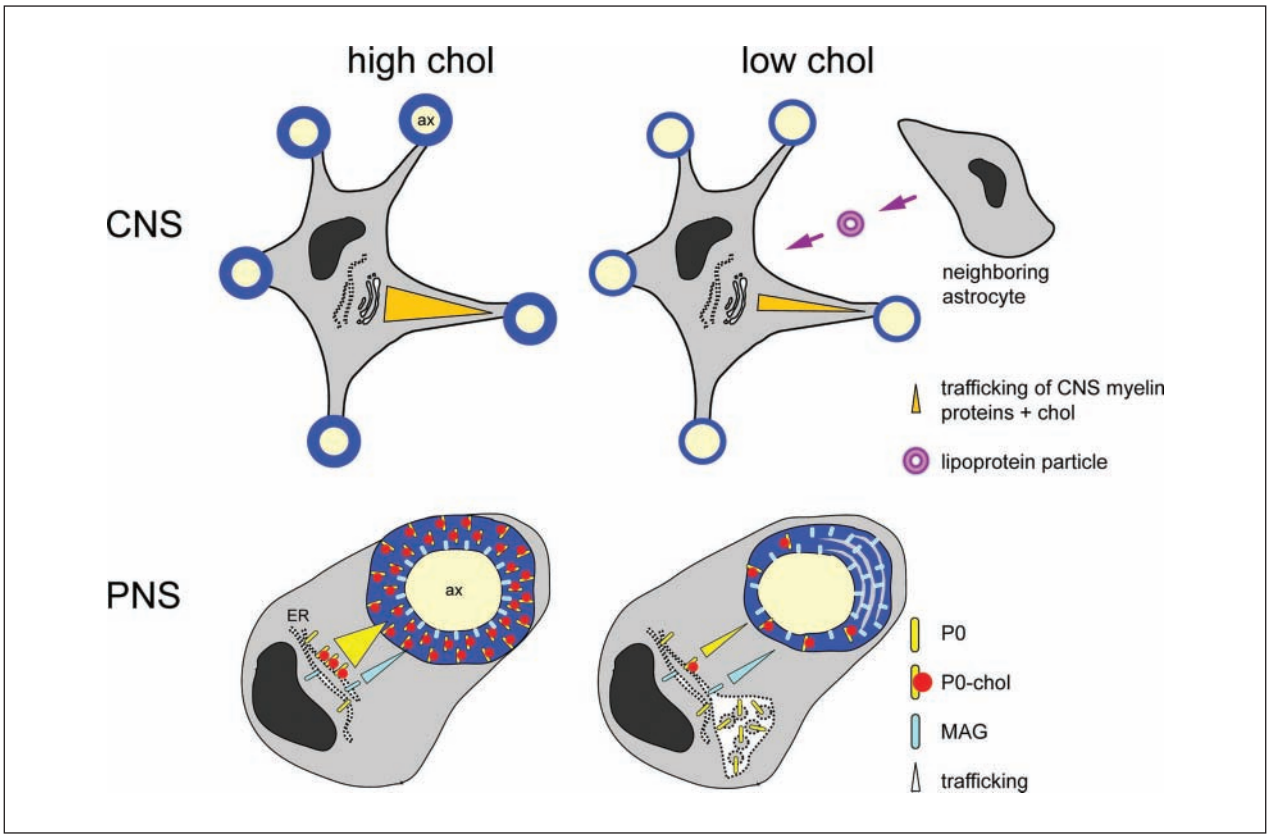

Figure 5. Model of the role of cholesterol in myelin formation. Cholesterol is required for myelin membrane growth. In the CNS, neighboring astrocytes provide cholesterol-rich lipoprotein particles to oligodendrocytes for compensation. In the endoplasmic reticulum (ER) of Schwann cells in the PNS, P0 normally associates with cholesterol, enabling its transport to the myelin sheath (high chol). When cholesterol is limiting (low chol)—that is, during early myelination—P0 transport is adjusted to cholesterol availability, and less P0 is shuttled from the ER to the myelin sheath. In case of severe cholesterol depletion, P0 is stored within endoplasmic vesicular/tubular structures. Note that myelin-associated glycoprotein (MAG) trafficking is independent of cholesterol. Consequently, the ratio of compact myelin (with P0-chol) to noncompact myelin (with MAG) is reduced. ax = axon. Reproduced from the *Journal of Neuroscience* with modifications (Saher and others 2009), with permission.

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Declaration of Conflicting Interests

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