Sorting and trafficking of proteins in oligodendrocytes during myelin membrane biogenesis

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Chapter 1

General Introduction

and

Scope of Thesis

General introduction

The nervous system can be divided into two main systems, i.e., the central nervous system (CNS), consisting of the brain and spinal cord, and the peripheral nervous system (PNS), consisting of bundles of neurons (nerves), connecting the CNS to other parts of the body. The CNS is composed of white and gray matter, the latter being the most external layer of the brain, which predominantly contains neuronal cell bodies. Projections from these cell bodies, called axons, extend to other areas of the brain, thereby enabling these cells to actively participate in the storage and processing of information. White matter consists primarily of axons coated with the light-colored myelin, which is produced by oligodendrocytes (OLGs). The myelin sheath, which is wrapped around axons, is interrupted at regular intervals along these axons, called nodes of Ranvier. Importantly, this anatomical construction allows saltatory conduction in which nerve impulses 'jump' between nodes, rather than they pass in a continuous and monotonous fashion.

The elaboration of the myelin sheath by OLGs is one of the most complex and remarkable features of neural development. Not surprisingly therefore, myelin biogenesis is a highly regulated process that requires the coordination of several oligodendrocytic events including lipid and protein synthesis, intracellular membrane trafficking and morphological changes. Moreover, a carefully regulated balance of myelin synthesis and turnover during adult life is crucial for the maintenance of a functional myelin sheath. Imbalance in these processes or disruption of this myelin membrane may lead to irreparable consequences, resulting in diseases like multiple sclerosis (MS) (de Vries and Hoekstra, 2000; Baumann and Pham-Dinh, 2001). In MS, the myelin is destructively removed from around the axon in a process known as demyelination, which slows nerve impulses. Damage to the myelin can be caused by macrophages that, in the presence of infiltrating T cells, are capable of phagocytosis of apparently 'normal' sheaths. This abnormal immune response may be triggered by genetic, environmental, and/or viral factors. In contrast to this generally held opinion, Barnett and Prineas (2004) recently presented data that extensive OLG apoptosis is the major pathological feature of myelin forming lesions. Consequently, rather than a cause, autoimmunity might well be considered a secondary amplifying response to massive OLG apoptosis (Barnett and Prineas, 2004; Matute and Pérez-Cerdá, 2005).

Nevertheless, for a successful search of an effective therapeutic strategy, which is still conspicuously lacking, a detailed understanding of the key events of myelination, and hence potential remyelination, including the characteristics of the sorting and trafficking pathways of (myelin) membrane proteins in myelinating OLGs, is imperative. In doing so, it is relevant to take into account that OLGs can be considered as polarized cells in which the overall myelin membrane composition differs dramatically from that of the plasma membrane of the OLG cell body, in spite of the continuous nature of these two membrane domains. As polarized sorting and trafficking have largely been characterized in Madin-Darby canine kidney cells (MDCK), the general aspects of the sorting and trafficking of membrane proteins will first be described in these cells. Subsequently, by extrapolating these findings the focus will be directed to whether similar mechanisms of sorting and trafficking of membrane proteins play a role in the biogenesis and maintenance of myelin and plasma membrane in OLGs.

Membrane biogenesis in epithelial cells

Introduction

Epithelial cells are characterized by an asymmetric distribution of different sets of proteins and lipids in their plasma membranes. Specifically, the plasma membrane of these cells is separated into two distinct membrane domains, the apical domain, facing the organ lumen, and the basolateral domain facing neighboring cells and the underlying extracellular matrix (ECM) (Schuck and Simons, 2004). The two domains are separated by tight junctions, which form a morphological border and act as an intramembrane diffusion barrier. The distinct membrane domains have striking differences in their protein and lipid composition which correlates with the specific and distinct biological functions of these domains, dictated by environmental differences faced by either surface. The apical domain is enriched in glycospingolipids and cholesterol, whereas compositional-wise the basolateral domain resembles the plasma membrane of nonpolarized cells. In epithelial cells, newly synthesized membrane proteins can reach these surfaces by different cellular pathways, a direct pathway from the *trans*-Golgi network (TGN) to either the apical or basolateral surface, and an indirect or transcytotic pathway in which internalized apical and basolateral proteins are sorted in endosomes and recycled back to a specific plasma membrane domain (fig. 1) (Ait Slimane and Hoekstra, 2002; Schuck and Simons, 2004).

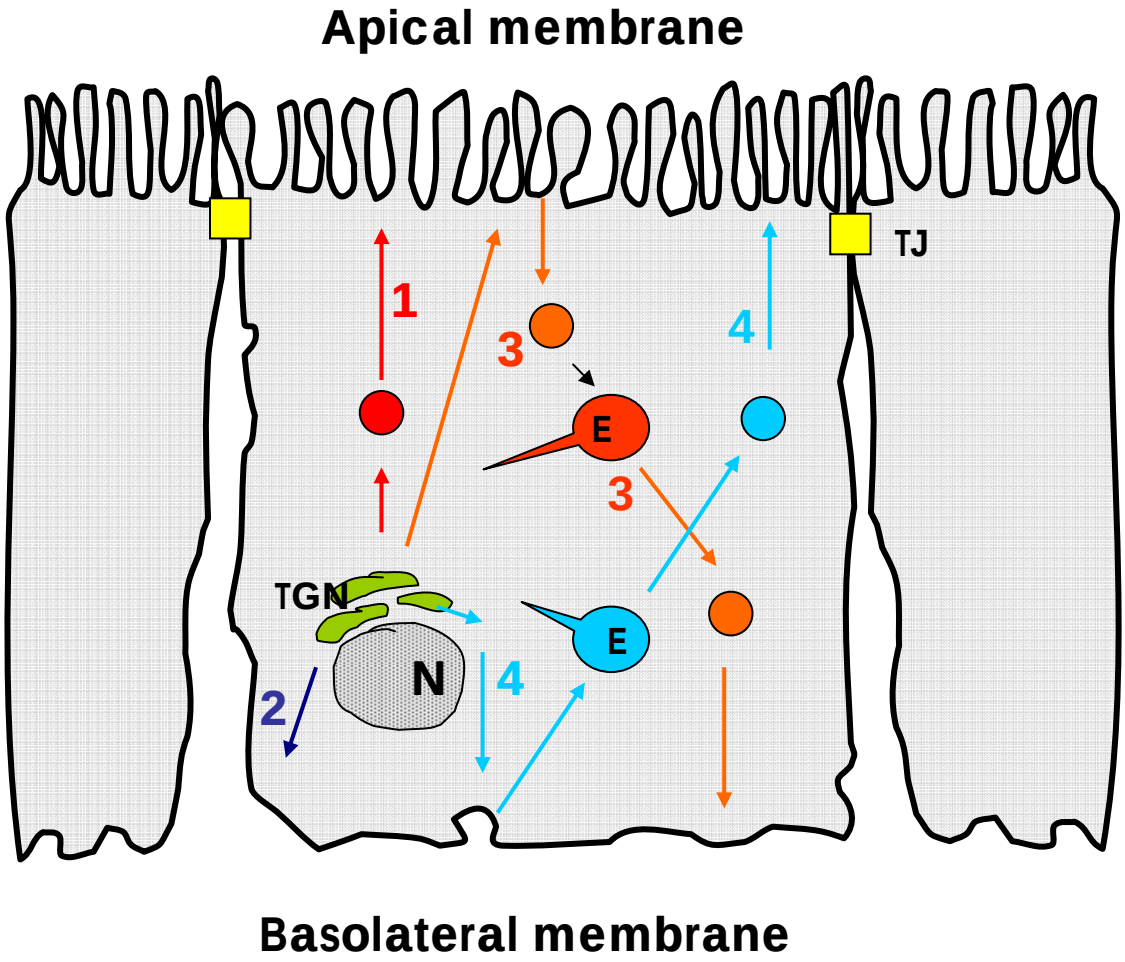

Figure 1: Sorting and trafficking routes of membrane proteins in polarized epithelial cells. In polarized epithelial cells two plasma membrane domains, the apical and basolateral, separated by tight junctions can be distinguished. In these cells targeting of newly synthesized membrane proteins to these membrane domains exist by different cellular pathways: in the 'direct' pathway membrane proteins are sorted in the Golgi apparatus and directed by vesicular transport to the apical (red arrows, no. 1) and basolateral (blue arrow, no. 2) membranes. In the 'indirect' pathway, newly synthesized membrane proteins are first transported from the TGN to either the apical (orange arrows, no. 3) or basolateral (light blue arrows, no. 4) surface and are then endocytosed into endosomes and recycled back to a specific membrane domain. Abbreviations: N – nucleus; TGN – *trans*-Gogi network; TJ – tight junctions; E – endosomes.

For instance, after its biosynthesis, the polymeric immunoglobulin receptor (pIgA-R) is transported from the Golgi complex to the basolateral surface after which this protein reaches its apical destination following endocytosis from the basolateral surface and subsequent transport by transcytosis to the apical surface (Hoppe *et al.*, 1985). Its dynamics is illustrated by the notion that *Hepatitis A virus* (HAV) associated with IgA is translocated from the apical to the basolateral compartment of polarized epithelial cells

(Dotzauer *et al.*, 2005). In the next paragraph sorting and trafficking mechanisms in polarized cells will be discussed in more detail.

Basolateral sorting

The molecular basis of basolateral sorting and targeting is only partially understood. Interestingly, in the absence of specific sorting signals, transmembrane proteins accumulate in the Golgi apparatus, suggesting that none of the routes to the cell surface is an efficient default pathway (Gut *et al.*, 1998; Benting *et al.*, 1999). Basolateral sorting and targeting of transmembrane proteins is mediated by specific short amino acid sequences located in their cytoplasmic tails and facilitated by cytosolic machineries that recognize these signals (Rodriguez-Boulan *et al.*, 2004). These short amino acid sequences contain a crucial tyrosine (Y) residue, within a consensus sequence NPXY or YXXΦ, where Φ is a bulky hydrophobic residue and X is any amino acid. Other basolateral signals include Leu-Leu and di-hydrophobic motifs (Odorizzi and Trowbridge, 1997; Bello *et al.*, 2001). Given the similarity between basolateral tyrosine sorting determinants and endocytosis signals, basolateral cargo and molecules endocytosed from the plasma membrane meet in recycling endosomes and move to the basolateral membrane (Matter and Mellman, 1994; Marks *et al.*, 1997; Traub and Apodaca, 2003; Schuck and Simons, 2004).

Apical sorting

Apical sorting and targeting of transmembrane proteins is mediated by a number of different types of sorting signals, including N-linked or O-linked carbohydrates, specific transmembrane domains or glycosylphosphatidylinositol (GPI) anchors, and cytoplasmic domain determinants (Ikonen and Simons, 1998; Rodriguez-Boulan and Gonzalez, 1999). Whereas the presence of N- or O-glycans in the ectodomain is necessary for mediation of apical transport of some transmembrane proteins (Fiedler and Simons, 1996), glycosylation does not constitute a universal apical sorting signal, as an inhibition of glycosylation does not necessarily lead to missorting while unglycosylated membrane proteins are able to reach the apical surface (Lisanti *et al.*, 1989; Alonso *et al.*, 1997; Meerson *et al.*, 2000; Bravo-Zehder *et al.*, 2000). Apical delivery of transmembrane proteins is mediated through lipid-lipid or lipid-protein interactions (Schuck and Simons, 2004) wherein specific residues in the transmembrane domains of such proteins - or in the case of GPI-anchored proteins, the lipid anchor - are essential for their proper integration into specialized glycolipid and

cholesterol-enriched membrane microdomains or 'rafts' (Simons and Ikonen, 1997; Scheiffele *et al.*, 1997; Brown and London, 1998; Keller and Simons, 1998; Pike, 2006). These cholesterol-sphingolipid membrane rafts can be isolated based on their insolubility in cold detergents like Triton X-100 (TX-100) and 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). Oligomerization of raft components can initiate raft clustering, a mechanism which has also been proposed to be involved in triggering apical delivery (Schuck and Simons, 2004). Although the raft mechanism is still considered a typical apical sorting mechanism, there is evidence that raft association is not sufficient to ensure apical sorting (Ait-Slimane *et al.*, 2003).

Proteins involved in polarized trafficking

Several proteins have been proposed to function in either the basolateral or apical sorting machinery. All identified basolateral sorting signals of integral membrane proteins are located in the cytoplasmic domains of the proteins. The tyrosine-dependent and dileucine-dependent sorting signals that specify endocytosis or lysosomal transport interact with adaptor proteins (APs), AP-1, AP-2, AP-3 and AP-4 (Bonifacino and Dell'Angelica, 1999; Robinson and Bonifacino, 2001; Hirst *et al.*, 1999). Adaptor proteins are heterotetrameric complexes of 'adaptins' that link clathrin to membrane proteins playing an important role in membrane invagination and protein trafficking (Dell'Angelica *et al.*, 1997). Adaptor protein-2 complex AP-2 (adaptins γ, β2, μ2, σ2) is involved in endocytosis at the plasma membrane, while at the TGN and/or endosomes AP-1 (α, β1, μ1, σ1), AP-3 (δ, β3, μ3, σ3) and AP-4 (ε, β4, μ4, σ4) contribute to lysosomal targeting. In general, μ subunits interact with tyrosine-based sorting determinants and β subunits with dileucine signals (Matter, 2000).

Cholesterol-binding caveolin-1 is one of the proteins involved in the apical sorting machinery. Specifically, large caveolin-1 homooligomers are targeted to the apical surface and play a role in apical transport of viral proteins like HA in MDCK cells (Scheiffele, *et al.*, 1998). Another protein implicated in apical transport is the myelin and lymphocyte proteolipid (MAL), also known as MVP17 (myelin vesicular protein 17; Kim *et al.*, 1995), and VIP17 (vesicular integral protein 17; Zacchetti *et al.*, 1995). MAL is a 17 kDa non-glycosylated four transmembrane protein that is localized to membrane rafts (Kim *et al.*, 1995; Puertollano *et al.*, 1999), being a component of the transport machinery for the raft mediated apical pathway (Zacchetti *et al.*, 1995). Downregulation of MAL expression

specifcally inhibited transport to the apical surface (Puertollano *et al.*, 1999; Marin-Belmonte *et al.*, 2000, 2001). Additionally, depletion of MAL resulted in an impairment of overall apical sorting and transport in MDCK cells (Puertollano *et al.*, 1999). Consistently, overexpression of MAL increased apical delivery but also disturbed the morphology of the MDCK cell layers due to an elaboration of the apical membrane (Cheong *et al.*, 1999).

Trafficking of membrane proteins is mediated by vesicular transport and their integration within the membrane of their final destination, for example the plasma membrane, is finalized by a membrane fusion process. During this process, membrane vesicles target and fuse with their correct target membranes. Three central players in vesicle targeting and fusion can be distinguished (Hay and Scheller, 1997; Jahn and Sudhoff, 1999; Ludger and Galli, 1998; Pfeffer, 1996): (1) the Sec proteins, (2) small-molecular-weight GTP-binding proteins, the rabs, and (3) the SNAREs, a group of cytoplasmically oriented integral membrane proteins that are present on vesicles (v-SNAREs) or target membranes (t-SNAREs) (also reffered to as Q- and R-SNARE; see Weimbs *et al.*, 1997; Fasshauer *et al.*, 1998). In the following sections the three protein families involved in vesicle targeting and fusion will be briefly discussed.

Exocyst

The exocyst is an evolutionarily conserved multiprotein complex implicated in tethering secretory vesicles at specific sites of the plasma membrane preceding SNARE assembly and membrane fusion (Novick and Guo, 2002; Lipschutz and Mostov, 2002; Hsu *et al.*, 2004). The exocyst is an octameric complex, consisting of the Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Ex084p components. All are hydrophilic proteins that exist in the cytosol and associate with plasma membrane (Terbush and Novick, 1995; Terbush *et al.*, 1996; Guo *et al.*, 1999). The exocyst is also important during earlier steps in the transport pathway, specifically in post-translational regulation of protein synthesis (Lipschutz et al., 2000; 2003). Exocysts mediate vesicle delivery to restricted regions of the cell surface discriminating whether and/or where a vesicle docks (Terbush and Novick, 1995). Mammalian homologs to the exocyst subunits are ubiquitously expressed and also form a multimeric complex that is mainly peripherally associated with the plasma membrane (Ting *et al.*, 1995; Hsu *et al.*, 1996; Kee *et al.*, 1997). For instance, the mammalian counterparts of the yeast Sec6, Sec8 and Sec10 exocyst have been localized to tight junctions in MDCK cells. In epithelial cells, exocyst components stimulate the

synthesis of basolateral but not apical proteins, and subsequently favor membrane addition to the epithelial basolateral compartment during tubulogenesis (Lipschutz et al., 2000; 2003).

Rab proteins

Rab proteins comprise the largest subgroup of small GTPases and belong to the Ras superfamily. Rab proteins act as molecular switches regulating intracellular membrane traffic by binding to specific organelle membranes (Miaczynska and Zerial., 2002; Pfeffer, 2003), thereby recruiting effector proteins that control fusion, and perhaps sorting and budding of transport vesicles. Rab proteins lack efficient guanine nucleotide exchange and hydrolysis activity, and their interactions with effectors are regulated by guanine nucleotide exchange factores (GEFs) and GTPase-activating proteins (GAPs) that promote the critical assembly of Rab containing protein complexes (Pfeffer, 2001; Bernards, 2003; Spang, 2004). Effector complexes formed in response to Rab activation can perform a variety of functions. They couple membranes to the cytoskeleton through the recruitment of kinesin- and myosin -based motors (Hammer and WU, 2002; Karcher *et al.*, 2002), direct the recruitment of tethering factors to initiate transport container docking (Moyer *et al.*, 2001; Allan *et al.*, 2000), potentially facilitate the function of proteins that alter membrane lipid composition (Gruenberg, 2003), and may organize the activity of SNARE components that mediate membrane fusion (Pfeffer, 2001; Gerst, 2003; Spang, 2004). All together, Rab proteins regulate distinct transport steps along the biosynthetic/secretory and endocytotic pathway and are involved in vesicle formation, vesicle movement, and cytoskeletal organization.

SNARE proteins

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein) (Nuoffer and Balch, 1994; Rothman and Wieland, 1996; Jahn and Sudhof, 1999; Larocca and Rodriguez-Gabin, 2002) proteins are integral membrane proteins that form stable complexes which are present on both vesicle (v-SNARE) and the target mambranes (t-SNARE) (Chen and Scheller, 2001). The vesicle and target membranes are pulled together upon assembly of the v-t- SNARE complex, together with a variety of accessory proteins, thus bringing apposed bilayers in close approach, eventually leading to the fusion of vesicle and target membrane (Chen and Scheller, 2001). SNARE proteins are found in all tissues and cell types

and there are more than 9 VAMP isoforms, 19 syntaxin isoforms, and 3 SNAP25 isoforms across the animal and even the plant kingdoms (Jahn and Sudhoff, 1999). Syntaxins are the prototype family of SNARE proteins, constituting an important subunit of the t-SNAREs. Syntaxins usually consist of three main regions: a very short extracellularly/luminally directed COOH terminus, a central SNARE transmembrane domain which is characteristic of and conserved in all syntaxins, and a long cytoplasmic NH_2-terminal region encompassing two coil-coil domains (Foster *et al.*, 2000). Most importantly, the apical and basolateral plasma membrane domains of polarized epithelial cells may contain specific syntaxins, e.g. syntaxin 3 is located exclusively at the apical membrane, as well intracellularly in endosomes and lysomes (Gaisano *et al.*, 1996; Low *et al.*, 1996; Fujita *et al.*, 1998) wheras syntaxin 4 is located entirely at the basolateral membrane surface. By expression of chimeric variants of syntaxin 3 and 4 in MDCK cells, it was demonstrated that these syntaxins play a role in determining the specificity of membrane targeting by regulating protein delivery to only certain target membranes (ter Beest *et al.*, 2005). In syntaxin 3 overexpressing cells or cells treated with anti-syntaxin 3 antibodies only the apical delivery was inhibited (Lafont *et al.*, 1999; Low *et al.*, 1998).

Vesicle targeting and fusion at the basolateral membrane was inhibited by addition of anti-NSF antibodies, NEM, mutant NSF, rab-GDI, or tetanus and botulinum F neurotoxins, whereas the transport from the TGN to the apical domain was not changed (Ikonen *et al.*, 1995; Apodaca *et al.*, 1996), suggesting the involvement of a distinct protein machinery in finalizing transport to either surface. Specifically, membrane traffic to the apical plasma membrane is regulated by Syntaxin 3 and T1-VAMP, which are associated with rafts (Lafont *et al.*, 1999), whereas syntaxin 4 has been implicated in TGN to basolateral traffic, requiring NSF, rab proteins and VAMP-2 (Low *et al.*, 1998; Lafont *et al.*, 1999). How Rab GTPases, SNARE proteins, and their associated effectors and regulators confer membrane identity and coordinate the dynamics of cargo flux through sequential compartments to define the highly distinct subcellular organizations found in different mammalian cell and organ systems, remains largely unknown.

Sorting and transport of myelin components: concepts of membrane traffic

Composition and formation of myelin

To discriminate between the various mechanisms involved in the sorting and transport of myelin components, it is relevant to first provide insight into the molecular

composition of the myelin sheet. During myelin biogenesis, OLGs mature in a process in which distinct developmental stages can be identified. The cells differentiate from a bipolar progenitor cell (O2A stage) to a cell with branched primary processes (GalC stage), when the earliest myelin specific protein 2', 3' –cyclic nucleotide 3'-phosphodiesterase (CNP, Pfeiffer *et al.*, 1993) is expressed. Final maturation into myelin-forming OLGs and the assembly of the sheath as such is characterized by the sequential expression of the proteins that ultimately make up the myelin, including myelin-associated glycoprotein (MAG), myelin basic protein (MBP), proteolipid protein (PLP), and myelin-oligodendrocyte glycoprotein (MOG) (Pfeiffer *et al.*, 1993; Baron *et al.*, 2003) (fig. 2).

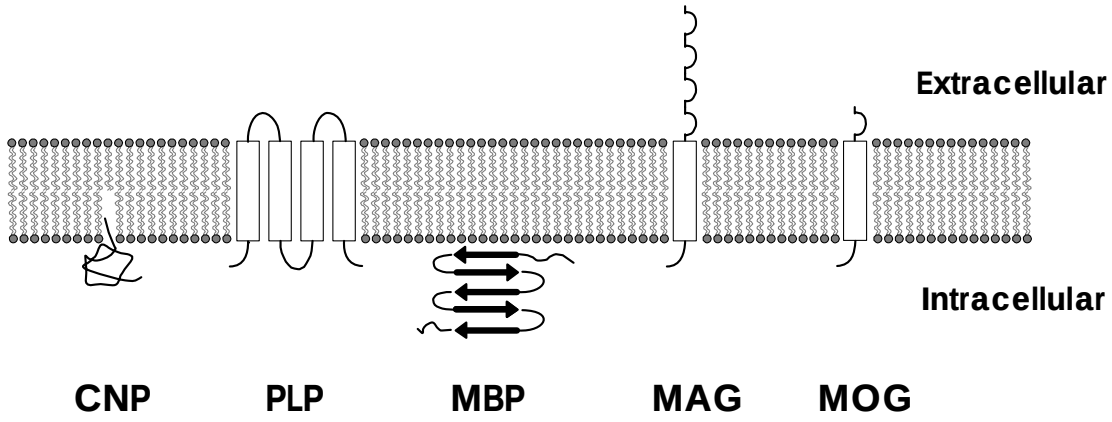

Figure 2: Schematic representation of the membrane interactions of the different myelin proteins in CNS myelin. CNP is associated with the cytoplasmic plasma membrane by isoprenylation. PLP is a transmembrane protein with a large extracellular domain. MBP, rich in positively charged lysine and arginine, interacts with the cytoplasmic faces at the phospholipid bilayer. MAG contains both a membrane-spanning domain and an extracellular region that contains five immunoglobulin domains. MOG, contains only one transmembrane domain and one immunoglobulin segment. Abbreviations: CNP - 2',3'-cyclic nucleotide 3'-phosphodiesterase; PLP - proteolipid protein; MBP – myelin basic protein; MAG – myelin-associated glycoprotein; MOG – myelin oligodendrocyte glycoprotein.

Apart from a distinct biochemical composition when compared to the plasma membrane of the glial cell soma, the myelin membrane is segregated into distinct subdomains with an asymmetric protein distribution. Indeed, myelin can be considered to be subdivided in a compact region, responsible for its physical insulation properties (Arroyo and Scherer, 2000) and a non-compact region. The compact myelin is formed by the apposition of the external faces of the membrane of the myelinating cell, forming the double intraperiodic line; the apposition of the internal faces followed by the extrusion of the cytoplasm, gives rise to formation of the major dense line. A tight junctional array is

located between compact and non-compact myelin, which likely serves as a diffusion barrier between these myelin subdomains (Gow *et al.*, 1999; Morita *et al.*, 1999; Bronstein *et al.*, 2000). The compact region, enriched in the glycosphingolipids galactocerebroside (GalCer) and sulfatide, represents the bulk of the sheath. In this region the major myelin proteins PLP/DM20 and MBP are located as well as MAL (see above), as a minor non-specific myelin protein (Lees and Brostoff, 1984; Griffiths *et al.*, 1998; Norton and Cammer, 1984; Frank, 2000). The non-compact myelin contains the minor myelin-specific proteins CNP, MAG and MOG.

Myelin specific proteins

The proteolipid protein PLP is the major integral membrane protein of CNS myelin. PLP spans the membrane four times and is highly hydrophobic with 50 % hydrophobic amino acids (fig. 2). Alternative splicing of the *plp* gene generates two proteins, PLP and DM-20, which lacks amino acids 116-150 of the PLP sequence (Macklin et al., 1987; Nave et al., 1987). As a structural protein, PLP plays a major role in assembly and stabilization of the myelin sheath in that the protein brings about the correct apposition of the extracellular leaflets of the membrane, thereby stabilizing the multilayered myelin membrane structure after compaction (Gudz *et al.*, 1996; Klugmann *et al.*, 1997, Rosenbluth *et al.*, 2006). Indeed, in PLP-null CNS axons often lack myelin entirely or are surrounded by abnormally thin myelin sheaths. Moreover, mutations in the plp gene, such as jimpy and jimpy^{msd} results in premature arrest of oligodendrocyte differentiation and early death, while overexpression of the normal *plp* gene in transgenic mice (Kagawa *et al.*, 1994; Readhead *et al.*, 1994) leads to severe dysmyelination (Griffiths et al., 1995; Dimou *et al.*, 1999). In humans, a variety of mutations, including missense mutations, deletions, and duplications, of the PLP gene are known to cause the dysmyelinating disorders like Pelizaeus-Merzbacher disease (PMD) and spastic paraplegia (Garbern *et al.*, 1999). In fact, gene duplications of the human PLP locus are responsible for the majority of cases of PMD, leading to enhanced expression of the PLP protein and patients die in their early teens (Hodes and Dlouhy, 1996; Griffiths *et al.*, 1998; Garbern *et al.*, 1999; Yool *et al.*, 2000). Although it is thus evident that PLP plays a vital role in myelin assembly and maintenance, on a functional and molecular level, its precise role remains undefined.

MBP is a highly basic protein, located at the cytoplasmic surfaces of compact myelin membranes (fig. 2)(Korngurth and Anderson, 1965). It is a strongly positively charged

protein, which binds primarily via electrostatic interactions with charged lipid headgroups (Smith, 1992; Ter Beest and Hoekstra, 1993). In aqueous solution MBP is relatively unfolded, while in the presence of lipids, it seems to have a tendency to fold (Stuart, 1996; Polverini *et al.*, 1999). MBP is derived by splicing from a common mRNA (Campagnoni, 1988) and various alternatively spliced isoforms of 14, 17, 18.5, 21.5 kDa (in rodents) are known. These isoforms are targeted, dependent on the presence or absence of the exon 2 encoded peptides, to distinct subcellular locations (Staugaitis *et al.*, 1990; Allinquant *et al.*, 1991). The major isoforms of MBP in compact myelin, i.e., the 14 kD and 18.5 kD isoforms (exon 2-minus), display a strong affinity for cellular membranes. In contrast, the less abundant 17 kD and 21.5 kD isoforms (exon 2-plus) (Carson *et al.*, 1983) localize to the nucleus and are diffusely distributed throughout the cytoplasm of transfected cells (Pedraza *et al.*, 1997). As for the mRNA, exon 2-minus mRNAs are preferentially transported into the cell's processes, while exon 2-plus mRNAs are confined to the cell body (de Vries *et al.*, 1997). MBP plays a major role in myelin compaction as revealed by studies of the shiverer mutant mouse, where a large deletion of the MBP gene results in severe perturbation of myelin compaction (Privat *et al.*, 1979). In fact, MBP is thought to be required for facilitating the approach of apposed inner leaflets of the plasma membrane, structurally characterized by the intraperiod line.

In the non-compact region, the minor myelin specific proteins are located. CNP, present in two isoforms (46 and 48 kDa), is one of the earliest myelin-related proteins to be expressed in differentiating OLGs. It is located periaxonally and in the outer loops, associated with the inner leaflet of the myelin membrane and the actin/cytoskeleton (De Angelis and Braun, 1996) (fig. 2). A proposed role for CNP in process formation, i.e., initial sites where myelin membrane extensions originate, comes from the observations that overexpression of CNP causes aberrant OLG membrane formation and perturbs myelination (Gravel *et al.*, 1996). It has also been reported that CNP-deficient mice extend smaller and less branched processes (Lee *et al.*, 2005). In addition, a role for CNP in the formation of the paranodes has been suggested (Rasband *et al.*, 2005).

MAG, a cell adhesion molecule belonging to the immunoglobulin superfamily, is a transmembrane glycoprotein with a molecular weight of 100 kDa (Quarles *et al.*, 1972; 1973), of which approximately 30 % is carbohydrate (Frail and Braun, 1984) (fig. 2). As a result of alternative splicing of the primary MAG transcript, MAG exists as two isoforms, designated small-MAG (S-MAG, 67 kDa) and large-MAG (L-MAG, 72 kDa) (Lai *et al.*, 1987;

Tropak *et al.*, 1988). The polypeptide chains of the two isoforms differ only by the carboxy terminus of their respective cytoplasmic domains, which most probably determine the isoform-specific functions (Fujita *et al.*, 1998). L-MAG, containing a larger cytoplasmic intracellular domain, is expressed early in development and has been implicated in signalling, whereas S-MAG, representing the major isoform in mature myelin is upregulated later during development. The cytoplasmic domain of L-MAG contains two putative tyrosine internalisation signals, which may account for selective endocytosis of L-MAG (Bo *et al.*, 1995). Since MAG is mainly localized in the periaxonal region of the paranodal loops while in MAG/deficient mice the CNS develops with a prominent defect in the formation of the periaxonal cytoplasmic collar, these observations suggest a role of MAG in directing OLG processes towards myelinated and nonmyelinated axons thereby being intimately involved in axo/glial interaction (Li *et al.*, 1994).

MOG is another CNS specific minor myelin constituent, which is highly conserved and present only in mammalian species (Birling *et al.*, 1993). MOG is a glycosylated protein with a molecular mass of 26-28 kDA that can form dimers of 52-54 kDa (Amiguet *et al.*, 1992; Birling *et al.*, 1993), and which is mainly localized in the abaxonal loop thereby facing the extracellular environment. Besides MOG 's role in defining the structural integrity of the myelin sheath, it has also been suggested that it might interact with proteins of the immune system. MOG, located at the cell surface, is the only CNS component that can induce an antibody-mediated response and a T-cell mediated immune reaction in an animal model for MS, experimental autoimmune encephalomyelitis (EAE) (Linington *et al.*, 1988).

Lipids

Lipids comprise 70–80 % of the dry weight of the myelin, containing a major fraction of cholesterol and the glycosphingolipids (GSL) galactosylceramide (GalCer) and its sulfated derivative, sulfatide. The outer leaflet of the myelin membrane is enriched in GSL and cholesterol, whereas the inner leaflet is enriched in phospholipids (Stoffel and Bosio, 1997). GalCer is synthesized in the endoplasmatic reticulum (ER) upon attachment of a galactose to ceramide by UDP-galactose ceramide galactosyltransferase (CGT). Part of the GalCer is converted to sulfatide by the transfer of a sulfate molecule to the third carbon of the GalC sugar ring by galactosyl ceramide 3'-sulfotransferase (CST) in the lumen of the Golgi apparatus. The remaining part is present in the periphery of the myelin sheets in GalCer-enriched ER globules. These globules are separated from the Golgi

apparatus and used as loci for compartmentalization and transport of GalCer and cholesterol. Thus, an ER-plasma membrane transport pathway for GalCer inside OLGs exists that bypasses the Golgi apparatus just like that known for transport of cholesterol. Sigma-1 receptors colocalize with cholesterol and neutral lipids and form detergent-insoluble lipid microdomains on the ER subcompartments. Upregulation of sigma-1 receptors affects the levels of plasma membrane lipid rafts by changing the lipid components therein, suggesting that sigma-1 receptor in OLGs might be involved in myelination by direct interference with cholesterol biosynthesis. Galactosphingolipids are possibly involved in affecting the orientation and/or lateral movement of signaling proteins in the outer leaflet of the plasma membrane. Possibly, for effective signaling, molecules like growth factor receptors and cell adhesion molecules require oligomerization, which could be driven by the clustering capacity of glycosphingolipids (Iwabuchi *et al.*, 1998). These observations underscore the vital role that glycosphingolipids play during development and differentiation, and that they are involved in crucial processes that trigger these events. In the next section the sorting and trafficking of the myelin proteins, and their dependence on the presence of glycosphingolipids, will be discussed.

Sorting and trafficking in oligodendrocytes

To maintain the myelin specific composition and its spatial organzation, OLGs not only have to synthesize large amounts of proteins and lipids, the cells also must specifically target these compounds to locations where cell processes emerge and eventually wrap around axons. Thus, specific lipid and protein sorting mechanisms are required during myelinogenesis and myelin maintenance.

PLP trafficking

PLP and its alternatively spliced isoform DM20, are synthesized in the endoplasmic reticulum and subsequently transported via vesicles to the Golgi, followed by transport to the compacted internodal region of the myelin sheath. As indicated above, the myelin sheath is enriched in glycosphingolipids and cholesterol, thereby resembling the apical plasma membrane of polarized cells. De Vries *et al.* (1998) demonstrated that the biogenesis of the myelin sheet in vitro involved basolateral-like sorting features rather than parmameters typically directing apical transport. Thus, the apical domain marker influenza hemagglutinin (HA) was transported to the cell body plasma membrane, localizing to

detergent resistant microdomains, whereas the basolateral membrane marker vesicular stomatitis virus glycoprotein (VSV G) accumulated in the myelin sheet, being fully solubilized upon detergent treatment of the cells (fig. 3).

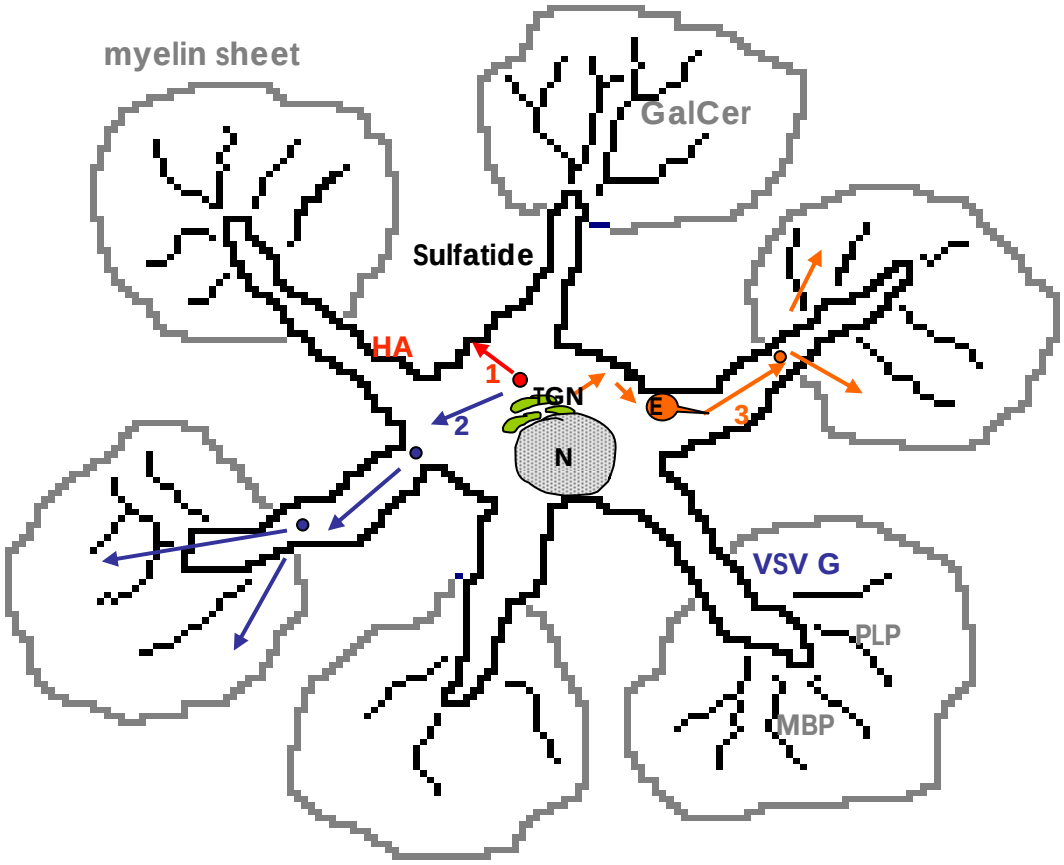

Figure 3: Sorting and trafficking pathways of membrane (associated) proteins in cultured oligodendrocytes. In OLGs the biochemical composition of the plasma membrane of OLG cell body is different from the membrane of the myelin sheet. Targeting of newly synthesized proteins to these distinct surfaces exist by different cellular pathways: in the 'direct' pathway membrane proteins are sorted in the Golgi apparatus and served by an apical-like transport route to OLG cell body plasma membrane (red arrow, no. 1) and a basolateral-like transport route to the myelin sheet (blue arrows, no. 2). In the 'indirect' pathway, proteins are first transported from the TGN to either cell body plasma membrane surface and are then endocytosed into endosomes and recycled back to the myelin sheet (orange arrows, no. 3). Abbreviations: N – nucleus; TGN – *trans* Golgi network; E – endosomes

In OLGs it is still unknown how PLP/DM20 is transported to its final destination, i.e., the compact domain of the myelin sheath. Thus, evidence as to a role of glycosphingolipids and cholesterol in sorting and trafficking of PLP/DM20 is controversial. Bansal and Pfeiffer

(1994) demonstrated that in primary OLGs the sorting and trafficking of PLP/DM20 to the processes and the myelin sheet is not affected by inhibition of sulfation, indicating that PLP/DM20 was not raft-associated (van der Haar *et al.* 1998). In the latter study (van der Haar et al., 1998) it was shown that the delivery of overexpressed PLP to the plasma membrane in GalC- and sulfatide-deficient Chines hamster ovary (CHO) cells was not affected and was very similar to that observed in cells, expressing these glycosphingolipids. Moreover, also in vivo, mice which were unable to synthesize GalC and sulfatide could still transport PLP to myelin (Coetzee *et al.*, 1996). In contrast, other data demonstrated that PLP/DM20 is sorted by a raft-dependent mechanism, indicating that the association of PLP with cholesterol and galactosylceramide-enriched membrane domains during biosynthetic transport in primary cultures of OLGs, is critical. In isolated brain slice the translocation of PLP into myelin was 50 % reduced by inhibition of GSL synthesis through the effect of L-cycloserine, whereas the incorporation of MBP and overall protein synthesis was unaffected (Pasquini *et al.*, 1989). Furthermore, in OLG infected with influenza virus, PLP colocalized with HA, which is transported via a raft-mediated mechanism to the apical domain in polarized epithelial cells (Simons *et al.*, 2000). In other studies (Brown *et al.*, 1993) claims has been made suggesting PLP co-transport with sulfatide, although it should be noted that this GSL species largely resides in the plasma membrane of the OLG cell body rather than in the myelin membrane, which is strongly enriched in GalCer (Baron et al., manuscript in preparation).

Endocytic sorting and transcytosis may be an alternative pathway of trafficking of PLP/DM20 to the myelin sheath. Trajkovic *et al.* (2006) demonstrated that myelin membrane sorting and trafficking is regulated by neurons. They showed that in the absence of neurons, PLP is internalized and stored in late endosomes/lysosomes (LEs/Ls) by a cholesterol-dependent and clathrin-independent endocytosic pathway. Upon maturation, the rate of endocytosis reduced, and a cAMP-dependent neuronal signal triggered the transport of PLP from LEs/Ls to the plasma membrane. These findings revealed a fundamental and novel role of LEs/Ls in OLGs. Krämer *et al.* (2001) demonstrated in vitro by immunofluorescence labelling that in differentiating OLGs PLP/DM20 colocalized with the late endosome marker LAMP-1, also indicating that PLP/DM20 was sorted to the endocytic pathway. In summary, it is still unclear how PLP is transported to its final destination, but an indirect route, involving an intermediate endocytic step, where PLP is (partly) associated with rafts, is optional.

MBP trafficking

MBP is synthesized close to the site of myelin assembly where compaction takes place (Colman *et al.*, 1983; Trapp *et al.*, 1987; Barbarese *et al.*, 1995; Ainger *et al.*, 1997). Therefore this protein is not detectable in rough ER, lysosomes or any other cytoplasmic organelles. The mRNA of MBP has been observed to be bound to free polysomes near the myelin membrane (Colman *et al.*, 1982). After exiting the nucleus, MBP mRNA assembles into ribonucleoprotein complexes termed granules, preventing adhesion via electrostatic interaction to inappropriate intracellular components, which move along microtubules for reaching their final destination, the myelin compartment (Ainger *et al.*, 1993, 1997; Carson *et al.*, 1997). In cultured OLGs exon 2-minus MBP mRNAs are transported into the processes, whereas exon 2-plus transcripts are not, indicating that in OLGs the exon 2 sequence is crucial in determining the destination of an MBP mRNA. Due to the secundary structure in exon 2, an OLG (co)factor responsible for MBP mRNA trafficking cannot bind to exon 2-plus mRNA. This OLG (co)cofactor could be involved in the linkage of exon 2-minus mBP mRNA transport particles to the cytoskeleton. This proces is dependent on the selective recognition of A2RE, a *cis*-acting element in MBP mRNA, heterogenous nuclear ribonucleoprotein (hnRNP) A2 (Hoek *et al.*, 1998; Munro *et al.*, 1999; Maggipinto *et al.*, 2004).

MAG Trafficking

MAG is synthesized in the cell body and subsequently transported via a vesicular trafficking route to the myelin membrane (Krämer *et al.*, 2001). Expression of the two isoforms of MAG in MDCK cells demonstrated that L-MAG was primarily targeted to the basolateral membrane, whereas S-MAG was first sorted to the apical plasma membrane and then targeted to the basolateral plasma membrane (Minuk and Braun, 1996). L-MAG contains the consensus motif YXXΦ, YAEI in its carboxyl terminal region, important for transport from the *trans*-Golgi to endocytic compartments (Bonifacino and Dell-Angelica, 1999). Endocytic compartments containing MAG are observed in OLG processes including areas close to the axon where active formation of myelin occurs (Trapp *et al.*, 1989; Bo *et al.*, 1995). Taylor *et al.* (2002) showed that MAG is soluble both in TX-100 and CHAPS, whereas PLP is associated with CHAPS-insoluble microdomains. This differential distribution of MAG and PLP, as reflected by differences in detergent-solubility, is likely accomplished

by the segregation of the two proteins into different vesicle population, thus rationalizing the specific delivery to distinct target membranes. Hence, the different domains discerned in myelin may well be served by transport vesicles operating in distinct pathways. Indeed, electron microscopic immunocytochemical studies indicated that PLP and MAG are present on separate vesicular structures in the cell, presumably originating from a sorting event in the TGN. Accordingly, this segregation into different vesicular structures could be the mechanism by which MAG and PLP are targeted to different membrane domains.

CNP

CNP is a prenylated protein which is synthesized on free ribosomes in the oligodendrocyte perinuclear cytoplasm (Trapp *et al.*, 1988; Gillespie *et al.*, 1990). During development, the expression of CNP is highly upregulated in premyelinating OLGs and is maintained throughout life, suggesting an important role in the myelination process as well as for the lifelong maintenance of the myelin sheath (Scherer *et al.*, 1994). Entry into the myelin/ plasma membrane fraction is similar to that of MBP (Gillespie *et al.*, 1990). CNP is bound to the actin-based cytoskeleton and associated with rafts (Kim and Pfeiffer, 1999). The protein induces microtubule and F-actin reorganization, necessary for process outgrowth and aborization (Lee *et al.*, 2005).

In summary, in OLGs multiple sorting and trafficking mechanism occur. There appears to be no common pathway, which is used for the sorting and trafficking of all myelin (specific) proteins. Rather, prevailing evidence suggest specificity in the pathways, which therefore raises questions as to their communication and regulation of each of these transport steps, given the relevance of each of the myelin components to a proper biogenesis and maintenance of the sheath. In the next section some extracellular factors are described which are involved in biogenesis of myelin.

Involvement of extracellular matrix

Myelination ensues after recognition of the axon by the progenitor cells, followed by the ensheathment of the axon by oligodendrocyte processes and synthesis of the compacted myelin. This cell-cell interaction is highly specific, causing only large-diameter axons rather than dendrites to be myelinated (Lubetzki *et al.*, 1993). The axonal surface plays a pivotal instructive role in determining where and when myelination occurs. In this respect the extracellular matrix (ECM), a complex structural entity that is found around

cells of almost any cell type in multicellular organisms, is of major importance for the development of the CNS (Buttery *et al.*, 1999; Pires-Neto *et al.*, 1999; Probstmeier *et al.*, 2000; De Winter *et al.*, 2002; Garcion *et al.*, 2004). Changes in the extracellular environment may regulate morphological changes by alterating vesicular transport of myelin sheet directed proteins. In the next sections the role of the ECM molecules laminin-2 and fibronectin is discussed on the intracellular vesicular traffic pathways of sheet-directed proteins.

Laminin-2

Laminin-2 (Ln2) is a glycoprotein expressed on axons. It interacts with oligodendrocyte laminin-binding integrins and dystroglycan (Colognato *et al.*, 2007), thereby stimulating OLGs to elaborate the extensive myelin membrane which wraps the axon, thereby forming the myelin sheath (Buttery and ffrench-Constant, 1999). Integrins are a large family of heterodimeric glycoproteins capable of bidirectional signaling, i.e. from inside to the extracellular environment and also in the reverse direction (Calderwood *et al.*, 2000; Liang *et a*l., 2004). Ln2 induces a redistribution of integrins in the membrane, thereby promoting OLG survival (Frost *et al.*, 1999; Corley *et al.*, 2001; Colognato *et al.*, 2002) and myelin formation (Buttery and ffrench-Constant, 1999; Relvas *et al.*, 2001). By observing that Ln2 induces clustering of the laminin binding integrin $\alpha 6\beta 1$ with the PDGFαR-containing lipid raft domains, Baron *et al.* (2003) demonstrated that the interaction between lipid rafts and ECM molecules may play an important role in the development of the myelin sheath.

Fibronectin

Fibronectin (Fn) exists in two main forms: (1) as an insoluble glycoprotein dimer, formerly called cold-insoluble globulin, that serves as a linker in the ECM and (2) as a soluble disulphide linked dimer composed of 250 kDa subunits found in the plasma. Its proposed role in cell adhesion, migration and invasion comes from the observation that it may serve as a general cell adhesion molecule by anchoring cells to collagen or proteoglycan substrates. Fn, absent in adult brain at normal conditions, enters the tissue upon blood-brain-barrier disruption at pathological conditions (Inoue *et al.*, 1997), which occurs during MS. Fn has been proposed to interfere with formation of membrane microdomains (Baron *et al.*, 2003; Maier *et al.*, 2005), thereby interfering with proper myelin sheet formation (Buttery and ffrench-Constant, 1999; Maier *et al.*, 2005, Siskova *et al.*, 2006).

In fact, the data strongly indicate that Fn impedes biosynthetic protein trafficking to the myelin sheet, relying on a mechanism that is most likely related to β1 integrin mediated activation of PKC. Mysristoylated alanine-rich C-kinase substrate (MARCKS), a major PKC substrate, will be translocated into the cytosol, which may lead to an inhibition of dynamic actin cytoskeleton remodeling, highly relevant for myelin sheet-directed protein transport. Permanent displacement of MARCKS, may result in a "frozen"actin cytoskeleton that interferes with vesicle trafficking towards the myelin sheet (Šiškowá *et al.*, 2006).

Expression and function of protein trafficking in oligodendrocytes

As indicate above, multiple pathways are involved in the assembly of mature myelin membranes. To establish polarity and specific transport to either plasma membrane of the cell body or myelin membranes, OLGs likely use similar trafficking and fusion proteins, as has been described for polarized epithelial cell (see above), which will be discussed in the next sections.

Exocyst complex proteins

It has been recently demonstrated that OLGs express several key components necessary for tethering transport vesicles to areas of rapid membrane growth, including the exocyst components Sec8 and Sec6 (Anitei et al., 2006). The exocyst complex appears to be central in myelin biogenesis, as it has been recently demonstrated that Sec8 expression is important for the recruitment of transport vesicles to the plasma membrane (Anitei *et al.*, 2006). Next to a role in tethering vesicles, Sec8 plays a role during early steps in the secretory pathway, as it coordinates early events in protein synthesis of MBP and MAG (Anitei *et al.*, 2006). Sec6, another component of the exocyst complex, coimmunoprecipitates with Sec8, as are the multidomain scaffolding proteins CASK and Mint1. Thus exocyst complex proteins seem to be important regulators of OLG differentiation and myelin membrane formation.

Rab-GTPases

Thus far, twenty-three different Rab proteins have been detected in OLGs (Burcelin et al., 1997; Bouverat et al., 2000). Among these are two OLG-specific Rab proteins, Rab22b and Rab40c (Rodriguez-Gabin et al., 2001; 2004), which expression are upregulated during OLG differentiation. Three other Rab proteins, Rab 3a, -5a, and -8a,

are also upregulated during OLG maturation (Madison et al., 1999; Bouverat et al., 2000). In addition, the endosomal Rab proteins 4, 5, 11, and 18 are prominently expressed in OLGs (Bouverat et al., 2000), suggesting that endocytic membrane recycling might be an important process in myelin biogenesis (Krämer et al., 2001). The expression of a large number of Rab proteins indicates the complexity of membrane trafficking in OLGs. Yet, thus far no Rab proteins have been identified as being involved in the targeting of myelin proteins.

SNARE complex proteins

Several laboratories have identified SNARE proteins in OLGs. In cultured OLGs, transcripts of the v-SNARE VAMP-2, its t-SNARE partners syntaxin 4 and syntaxin 2 have been detected (Madison et al., 1999), as well as protein expression of another cognate SNARE pair, VAMP-7/syntaxin 3 (Krämer et al., 2000). In addition, two other t-SNAREs, SNAP-25 and SNAP-23 (Hepp et al., 1999; Madison et al., 1999; Krämer et al., 2001) that are also required for the formation of functional SNARE complexes, are localised in myelin. Thus, the components for functional SNARE complexes are present in OLGs, however, knowledge about the extent of SNARE involvement in myelin biogenesis is very scanty. Interestingly, in polarized epithelial cells, syntaxin 3 and syntaxin 4 are involved in apical and basolateral membrane trafficking, respectively (Galli *et al.*, 1998; Low *et al.*, 1998; Lafont *et al.*, 1999), and therefore a similar role in polarised transport in OLGs could be readily envisioned.

MAL protein

Both myelinating cells, i.e., OLGs (CNS) and Schwann cells (PNS), express the MAL protein (Schaeren-Wiemers *et al.*, 1999). In immature, non-myelinating Schwann cells, the MAL proteolipid, having a function in differentiation, is present prior to myelin formation (Frank *et al.*, 1999), whereas in OLGs MAL is upregulated during active myelination (Kim *et al.*, 1995; Schaeren-Wiemers *et al.*, 1995). The MAL proteolipid, is predominantly localized in the compact myelin and is tightly associated with the glycosphingolipids, galactosylceramide and sulfatide. Functional studies, carried out in vitro, suggest that MAL is required for apical protein sorting in epithelia (Cheong *et al.*, 1999; Martin-Belmonte *et al.*, 1999; Puertollano *et al.*, 1999). Together with the observation that in transgenic mice with increased MAL gene dosage both axon-glia interaction and apical membrane

formation in kidney and stomach were altered, these data suggest that MAL is involved in the assembly and targeting of apical transport vesicles, while it also plays a role in the stabilization and maintenance of glycosphingolipid-rich membrane domains in myelinating cells. By MAL gene disruption, Schaeren-Wiemers *et al.* (2004) demonstrated a critical role for MAL in the maintenance of central nervous system paranodes. Major alterations of the structural level seen in MAL-deficient mice include aberrant inclusions of cytoplasm within compact CNS myelin. Furthermore, paranodal loops at the node of Ranvier are detached from the axon and face away from it. Because MAL is expressed in the brain exclusively by OLGs, it is likely that lack of MAL is causing altered axon-glia interactions, and malfunctioning of NF155 would be an excellent candidate in causing impaired axon-glia interactions. This cell adhesion molecule is located at the glial side of the paranodal junction (Tait *et al.*, 2000), is associated with the Caspr-contactin complex (Charles *et al.*, 2002; Collan *et al.*, 2003), and is remarkledly reduced in the absence of paranodal septa (Bhat *et al.*, 2001; Boyle *et al.*, 2001; Poliak *et al.*, 2001). Indeed, NF155 is almost absent in paranodal loops and is recruited in rafts, thus supporting the notion of being a potential candidate for MAL-mediated sorting and/ or trafficking.

Scope of the thesis

During myelin formation OLGs may utilize basic mechanisms of epithelial membrane trafficking, as described and summarized in the introductory chapter (Chapter 1). However, whether specific transport pathways, unique to myelin biogenesis are involved and how such pathways might be regulated in biogenesis and maintenance of the myelin sheath, is largely unexplored. Such insight is of major relevance for devising strategies for exogenous manipulation to stimulate and/or promote de novo biogenesis of the myelin sheath and its (re)assembly at pathological demyelinating conditions, as in the case of multiple sclerosis (MS). In addition, given the special nature of OLGs, consisting of a cell body, bounded by a plasma membrane, and myelin 'protruding' from that cell body, yet maintaining a highly specific and quite distinct membrane composition when compared to the plasma membrane, this kind of work will also contribute to solving fundamental questions of great interest to current cell biology. Accordingly, the main purpose of the work described in this thesis was to acquire insight into the sorting and trafficking of major myelin components such as PLP and MBP, and to reveal regulatory mechanisms,

instrumental in promoting myelin sheath biogenesis. It is felt that this insight is a prerequisite for a prosperous advancement of therapeutic approaches in a disease as complex as MS.

The trafficking and sorting of myelin constituents was primarily studied in vitro, using isolated OLG precursor cells from newborn rats. This approach also allowed us to study myelin biogenesis as a function of cellular development. In chapter 2, molecular parameters that govern the targeting and incorporation of myelin proteins and model proteins into myelin membranes are investigated to further clarify the polarized nature of oligodendrocytes. The purpose of these studies was to reveal the nature of sorting signals in myelin-directed trafficking and whether this pathway was regulated by protein kinase activity. Chapter 3 describes the distribution and functional role of syntaxins 3 and 4 in OLGs, since both proteins usually display a specific, polarized distribution in epithelial cells. We investigated how these syntaxins regulate the trafficking of myelin specific proteins, including PLP and MBP mRNA. Apart from intracellular factors, the role of the ECM was also examined, as described in experiments presented in chapter 4. OLGs were grown on different ECM substrates and their effect on the transport of the PLP and myelin assembly was determined. In chapter 5 the role of MAL in the regulation of sorting and transport of PLP was investigated, using a GFP-MAL construct that was (over)expressed in proliferating oligodendrocyte progenitor cells. The intracellular distribution of MAL was determined and its effect on myelin protein transport was studied as a function of OLG development. Finally, in chapter 6 major observations reported in this thesis are summarized and perspectives for future studies are discussed.