Chapter 1

Introduction and scope of the thesis
Exploiting oligodendrocyte biology to target myelin repair: what does it take to (re)myelinate?

In vertebrates, myelination of the axons of neurons in the central nervous system is required for saltatory pulse conductance, whereas demyelination causes severe diseases, such as multiple sclerosis. Myelin constitutes a dynamic multilamellar membrane system, which is produced by oligodendrocytes (OLGs), and insight into regulation of its biosynthesis is therefore crucial to a better understanding of disease development and potential therapeutic treatment. Accordingly, since the discovery that remyelination may occur within the central nervous system (CNS) (Bunge et al., 1961) there has been a great interest in clarifying the molecular and cell biology of OLGs, including factors that influence their proliferation, migration, differentiation and ability to (re)myelinate nerve axons. This chapter provides an overview of key regulators that control developmental myelination, and discusses how alterations at pathological conditions could interfere with remyelination events. Special attention will be given to the role of extracellular matrix proteins in these processes.

1. Organization of the mammalian nervous system

Next to the cardiovascular system, the nervous system is one of the first organ systems that functions during embryonic life in mammals. For generating a human brain, more than 25 billion neurons and up to 5-10 times more glial cells are needed, which requires the production and differentiation of precursor cells throughout the entire period of prenatal life (Sigelman and Rider, 2005). Despite a large variability among the organization of the vertebrate’s nervous system, the formation of two distinct parts, i.e., the central nervous system and the peripheral nervous system (PNS), is a common feature. From a cellular point of view, the CNS and PNS are composed of the same structural elements and consist of neurons, neuroglia [astrocytes, myelin forming cells (oligodendrocytes in CNS, Schwann cells in PNS), and ependymal cells] in the interstitium, and microglia and blood vessels in the connective tissue. Neurons are highly specialized cells that transmit impulses that may change the behavior of a target cell, such as, for example, muscle effectors cells. Morphologically, a neuron is characterized by i) a soma or cell body, which harbors the functions relevant to cell biogenesis and metabolism, ii) dendrites, which are branching extensions that receive signals and conduct impulses to the cell body, and iii) an axon, which is an extension that ends in terminal branching fibers through which chemical signals are being released. Strikingly, although the interacting pathways and networks of cells present in the CNS are far more complex than those operating in the PNS, the neurons
of the CNS have a very similar structure as those operating in the PNS (Peters et al., 1991; Hildebrand et al., 1993).

The extensive communication network between neurons depends on fast nerve impulse conduction, which is facilitated by myelin, an insulating layer of tightly packed membranes wrapped around the axons. Myelin membranes separate axonal surfaces into functionally discrete domains, which are defined as internode, juxtaparanode, paranode and the node of Ranvier (Ishibashi et al., 2003) (figure 1). The node of Ranvier, comprised of sodium channels, is the bare axonal segment that is flanked by paranodal loops that are being formed by terminal expansions of myelinating cells and the juxtaparanode, which, in turn, is located distal to the paranodal domain and the node of Ranvier.

Figure 1: The myelin sheath separates axons into specific regions. The axonal segment which is not covered by myelin is referred to as the node of Ranvier, characterized by the abundant presence of sodium channels. This structure allows fast action potential transmission – saltatory conduction.

The evolutionary invention of insulating myelin (internodes) has the advantage that impulses do not have to be constantly regenerated along every micron of the axonal surface, but can be propagated by a saltatory mechanism. Therefore, the metabolic requirements along myelinated fibers are greatly reduced and the action potential can jump from one node of Ranvier to another along the axon (saltatory conduction), allowing rapid impulse transmission (Huxley, 2002). Thus the evolution of the nervous system of vertebrates, including humans, seems to be tightly connected to the existence of myelin, allowing rapid and efficient impulse transduction as required for controlling such an extensive system as a human body.
Origin of neural cells

All three major classes of neural cells, i.e., neurons, astrocytes and oligodendrocytes, are derived as precursor cells from neuroepithelial cells of the neural tubes (Peters et al., 1990), and their developmental appearance in the brain is in that order (Altman and Bayer, 1984; Liu et al., 2002). Oligodendrocyte precursors (OPCs) arise in highly restricted ventricular domains (reviewed by Miller, 2002) and their initial appearance is controlled by local signals. Two different pools of OPCs have been identified based on expression of either PDGFRalpha (Hall et al., 1996) or PLP/DM20 (Spassky et al., 2001). Several factors have been suggested to play a role in the commitment of OPCs to develop into the OLG lineage, including Olig genes and Notch (Fu et al., 2002; Park and Appel, 2003). For example, the OLG lineage genes, Olig 1 and Olig2, have been proposed to control the appearance of motor neurons and early OLG precursor cells from a common precursor. Lack of Olig2 is lethal to mice (Ligon et al., 2006), whereas Olig1 deficient mice show a small delay in OLG development (Xin et al., 2005), although Olig1 is essential for remyelination (Arnett et al., 2004).

The formation of the new neurons and glial cells continues by mitosis in specific areas of the brain throughout adulthood, as evidenced by the presence of proliferating OPCs in adult CNS (Wolswijk and Noble, 1989). However, in contrast to the restricted distribution in developing CNS, in adult CNS the OPCs are widely distributed (Polito and Reynolds, 2005).

Developmental regulation of oligodendrocytes

The very early generation of OLG precursors in the cerebral cortex is stimulated by increasing concentrations of fibroblast growth factor-2 (FGF2) (Fisher, 1997) and by neuregulin-1 in the spinal cord (Vartanian et al., 1999). A second step involves the migration of OPCs over a considerable distance towards the axons that are to be myelinated. OPC migration is strictly controlled by specific cues. For example, cell surface components like adhesion molecules (Wang et al., 1994) and extracellular matrix receptors (Frost et al., 1996; Garcion et al., 2001) have been proposed to play a role in directing migration. During migration, proliferation occurs, first in the ventricular and subventricular zone and later in developing white matter (Miller et al., 1997). The platelet-derived growth factor (PDGF-AA) is known to be a very powerful proliferating and surviving factor for OPCs (Noble et al., 1988). Its proliferative capacity is modulated by interactions with other molecules such as chemokine CXCL1 (Robinson et al., 1998), neurotrophin-3 (NT3) (Barde et al., 1994), FGF-2 (McKinnon et al., 1990) and vitronectin (Baron et al., 2002). For example, in combination with PDGF, FGF-2 is capable of extending proliferation (Bögler et al., 1990), whereas vitronectin enhances its sensitivity
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Once migrating OPCs have reached their destination, they differentiate into immature OLGs, which is accompanied by dramatic changes in morphology and the concomitant expression of myelin-associated proteins (figure 2). IGF-I, neuregulins (NRGs) and FGF-2 appear to be involved in terminal OLG differentiation, either positively or negatively (Goddard et al., 2001; Palacios et al., 2005; Lemke, 2006). The acquisition of target-dependent survival signals is a prerequisite for the establishment of a mature myelinating phenotype. Important players in this target-dependent survival are both soluble factors (e.g. NRG, PDGF, and IGF) and axonal-contact dependent signals (laminin-2, NRG) (Colognato et al., 2002; reviewed in Bozzali and Wrabetz, 2004). Molecules like L1, MAG, NCAM and N-cadherin, which are expressed on the axonal surface, possibly control the final step of myelination (Payne and Lemmon, 1993).

Another mechanism known to regulate OLG maturation is connected to Notch signaling. Developmental differentiation of OPCs is inhibited by axonal expression of Jagged, which signals via Notch receptors expressed on OPCs (Wang et al., 1998). Depending on potential ligands, Notch signaling can also provide beneficial stimuli for OLG maturation. For example, it has been shown that interaction with F3/contactin triggers a signaling pathway that promotes OLG differentiation (Hu et al., 2003). Moreover, laminin-2, an ECM molecule present in developing white matter tracts (Colognato et al., 2002) promotes myelin membrane formation (Buttery and ffrench-Constant, 1999; Relvas et al., 2001).

The final stage of OLG development involves compaction of the myelin sheath in order to ensheath the axon, thereby exerting its insulating function. A first attempt to explain

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**Figure 2:** Schematic representation of the developmental stages of an oligodendrocyte cell lineage, reflected by morphological and antigenic properties characteristic for each of the developmental stages from oligodendrocyte precursor cell to mature myelinating oligodendrocyte. Integrin receptors present throughout development are listed and their function is indicated. The appearance of major myelin components is also indicated at the various stages. Adapted from Baron et al., 2005.
how compaction is regulated stems from work of the group of Birchmeier (2000), which suggested that products of the Nrg1 gene, the neuregulins, might be involved. Indeed, recently it has been shown that the amount of the axonal Nrg1 regulates the number of wraps (Michailov et al., 2004).

Oligodendrocytes are the myelin-forming cells in the CNS

A crucial event in the formation of myelin is the extension of multiple OLG processes, which results in a dramatic increase in total surface area (figure 2), leading to an extensive morphological alteration in appearance of the OLG. Accordingly, myelin can be seen as a distinct specialised domain of the OLG. OLG development starts with a highly migratory and proliferatory progenitor stage, characterized by a bipolar morphology and by the presence of specific markers, including ganglioside GD3 (Hardy and Reynolds, 1991) and chondroitin sulfate proteoglycan NG2 (Nishiyama et al., 1996). After migration in the CNS, these precursor cells will transform into multiprocessed cells, which are less motile, but still possess the capacity of cell division. These pre-OLGs acquire reactivity towards the O4 antibody (Sommer and Schachner, 1981; Bansal et al., 1989), which recognizes sulfatide (Bansal et al., 1989). After this stage the cells develop into immature OLGs, typified by the disappearance of GD3 and the appearance of GaIC (recognized by an antibody known as O1). At this stage the first myelin-specific protein, CNP, emerges, together with RIP and CAII (Sprinkle, 1989; Friedman et al., 1989; Butt et al., 1995). These immature OLGs have lost their capacity for migration and cell division, but most importantly, for survival in vivo the cells have to contact an axon (Barres et al., 1993; Burne et al., 1996). Upon axonal recognition, mature myelinating OLGs are formed, that are characterized by an arborized morphology in which primary processes are branched into secondary and tertiary ones, thus forming flat myelin sheets (Bunge et al., 1962; Bunge, 1968). At this stage, the major myelin proteins MBP and PLP are expressed, as well as the minor myelin protein MAG (Dubois-Dalcq et al., 1986; Monge et al., 1986). Based upon a characterization of antigenic properties, the evidence suggests that differentiation of precursor cells into mature OLGs in monocultures closely mimics that in vivo (Trapp et al., 1997). However, compared to the morphology in in vitro monocultures, that in the in vivo situation differs substantially, as all secondary and primary OLG processes disappear at the onset of myelination (Hardy and Friedrich, 1996). Upon axonal contact, the primary processes of OLG will wrap around the axon with the concomitant formation of a compacted multilamellar membrane, i.e., myelin, which represents the final stage of OLG differentiation. Both in vivo and in vitro, morphological criteria as described above are often not sufficient for characterization of the OLG lineage. In order to make a reliable distinction between developing and mature OLGs, a characterization of the cells relying on a panel of
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The primary function of the OLG is to create the very specialized myelin sheath as a distinct part of the OLG membrane. However, not all OLGs are necessarily involved in myelin production. Thus, it has been shown that also so called satellite OLGs exist, whose function is probably connected to regulation of the microenvironment of adjacent neurons (Ludwin 1997).

2. Structure and properties of myelin

The first reference to myelin was made by Virchow in 1854, when he described a spiral multilamellar structure consisting of extensions of the plasma membrane of the myelinating glial cells. Myelin is characterized by a periodic structure with alternating concentric electron-dense (major dense line) and light layers (intraperiod line, Sjöstrand, 1949; figure 3). A unique feature of myelin is the relatively high lipid-to-protein ratio, i.e., 70% lipids to 30% proteins (Norton, 1984; table 1), which is usually the reverse in other cellular membranes, including the plasma membrane of OLGs. Without this rather specific feature, the rapid nerve conduction velocity would be impossible. Although myelin is enriched in lipids, it does not contain myelin-specific lipids. However, the proportion of cholesterol and glycosphingolipids in this membrane is particularly enhanced, and both galactosylceramide (GalCer) and its sulfated derivate sulfatide represent major fractions of the galactolipid pool, while in mature myelin this pool amounts approx. 30% of its dry weight (Morell et al., 1994, table 1). In developing brain the fraction of GalCer is directly proportional to the amount of myelin present (Norton et al., 1983). In contrast, proteins that are highly specific for myelin can be discerned, emphasizing the special role these proteins play in the assembly and organization of the multilayered membrane structure of the myelin sheath. Myelin basic protein (MBP) and proteolipid protein (PLP) are the major CNS myelin proteins, accounting for 30 and 50%, respectively, of the total myelin content (Macklin et al., 1987). Minor myelin proteins include CNP, MAG, MOG, P2 protein, oligodendrocyte-myelin glycoprotein (OMgp; Habib et al., 1998), myelin/oligodendrocyte specific protein (MOSP; Dyer et al., 1991), RIP antigen, Ni-35/250 proteins and small basic proteins including myelin-associated oligodendrocyte basic protein (MOBP). The latter has been proposed to play a role in myelin compaction (Holz and Schwab, 1997). Members of the tetraspan-protein family, such as myelin and lymphocyte protein (MAL), oligodendrocyte-specific protein (OSP), Cx32 and tetraspan-2 are also present in myelin.
Table 1: Composition of mature human CNS myelin with major protein and lipid components represented as a percentage of its dry weight. Adapted from Norton, 1984.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>% of dry weight</th>
<th>Protein</th>
<th>% of protein weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>27.7 %</td>
<td>Myelin basic protein (MBP)</td>
<td>22.5 %</td>
</tr>
<tr>
<td>Total galactolipid</td>
<td>27.5 %</td>
<td>Proteolipid protein (PLP)</td>
<td>30.0 %</td>
</tr>
<tr>
<td>GalCer</td>
<td>22.7 %</td>
<td>MAG</td>
<td>&lt;1.0 %</td>
</tr>
<tr>
<td>Sulfatide</td>
<td>3.8 %</td>
<td>CNP</td>
<td>4.0 %</td>
</tr>
<tr>
<td>Total phospholipid</td>
<td>43.1 %</td>
<td>Others</td>
<td>42.5 %</td>
</tr>
<tr>
<td>Ethanolamine phosphatides</td>
<td>15.6 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylycholine</td>
<td>11.2 %</td>
<td></td>
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<tr>
<td>Sphingomyelin</td>
<td>7.9 %</td>
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<td></td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td>4.8 %</td>
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<td></td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>0.6 %</td>
<td></td>
<td></td>
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<tr>
<td>Plasmalogens</td>
<td>12.3 %</td>
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Myelin basic protein is encoded by one MBP gene, but exists in different isoforms with different molecular weights. The major isoforms are 18.5 and 17.2 kDa in humans and 18.5 and 14 kDa in mice (Roach et al., 1983). These isoforms are further subjected to posttranslational modifications like phosphorylation, NH₂-terminal acetylation and methylation and indirect evidence suggests that some of these processes could be important for myelin membrane maturation (Campagnoni and Macklin, 1988). 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 (Fig. 3; Privat et al., 1979).

The PLP gene encodes for two proteins, PLP (30 kDa) and DM-20 (25 kDa), derived via alternative splicing, with the predominant appearance of PLP in myelin. These proteins appear to be involved in the correct apposition of the extracellular leaflets of the membrane (major dense line, fig.3), stabilizing the multilayered myelin membrane structure after compaction (Klugmann et al., 1997). Other functions of PLP have been suggested as well, like a role in survival (Yang and Skoff, 1997; Gow et al., 1998) and adhesion in relation to migration (Gudz et al., 2006). Remarkably, in PLP null mice myelination of axons still occurs, albeit that the physical stability of the myelin structure in these animals diminishes as a function of time (Boison et al., 1995), similarly as has been noted in jimpy mice, in which the PLP gene has mutated spontaneously, as well as
in demyelinating diseases in human (Hudson et al., 1989; Fannon and Moscarello, 1990; Yool et al., 2002).

In myelin biogenesis, CNP is one of the first myelin-specific proteins that are synthesized, representing 4% of the total myelin protein pool, and existing in two isoforms (46 and 48 kDa). Isoprenoid modification allows its association with the inner leaflet of the myelin membrane (Braun et al., 1991). In addition, CNP displays binding affinity for the actin-cytoskeleton, which presumably mediates its close interaction with the OLG plasma membrane (Braun et al., 1991; De Angelis and Braun, 1996). Interestingly, overexpression causes aberrant OLG membrane formation and perturbs myelination (Gravel et al., 1996). A role for CNP in process formation, i.e., initial sites where myelin membrane extensions originate, is further corroborated by a recent finding that OLGs from CNP-deficient mice extend smaller and less branched processes (Lee at al., 2005). In addition, a role for CNP in the formation of the paranodes has been suggested (Rasband et al., 2005). MAG belongs to the immunoglobulin superfamily and is another minor constituent of myelin (1% of the total protein fraction). Thus far, two MAG proteins have been identified, i.e., large MAG (L-MAG, 72 kDa) and small MAG (S-MAG, 67 kDa). Both these isoforms share identical extracellular and transmembrane domains but differ in their cytoplasmic domains. MAG is mainly localized in the periaxonal region of the paranodal loops and therefore appears to be involved in axo-glial adhesion. In MAG-deficient mice, the CNS develops with a prominent defect in the formation of the periaxonal cytoplasmic collar that is missing in most of the internodes. These observations suggest a role of MAG in directing OLG processes towards myelinated and unmyelinated axons in the CNS (Li et al., 1994). The gangliosides GM3, GM1, GM1b, GD1a, and GD1b, which are abundantly present on the neuronal surface, have been proposed to function as potential ligands for MAG (Yang et al., 1996), and as such MAG has been shown to be a potent inhibitor of axonal regeneration and axonal growth promoter in young neurons (Domeniconi et al., 2005; Domeniconi et al., 2002).

MOG is another CNS specific minor myelin constituent which is highly conserved and present only in mammalian species (Birling et al., 1993). MOG is mainly localized in the abaxonal loop thereby facing the extracellular environment. Accordingly, MOG is the only CNS component that can induce an antibody-mediated response and T-cell mediated immune reaction in an animal model for multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE) (Linnington et al., 1988). This explains the exclusive CNS pathology in MS, as MOG is absent in PNS myelin (Brunner et al., 1989; Lolli et al., 2005; Lalive et al., 2006).
Figure 3: **Structure and composition of myelin.** Compaction of the multilayered myelin structure arises upon apposition of the external face of the membrane, wrapping around the axon. Upon examination by electron microscopy, so-called major dense lines and intraperiod lines can be distinguished. The major dense line is formed by apposition of the internal faces, involving the extrusion of the cytoplasm.

**Myelin biogenesis and intracellular mechanisms involved in myelin membrane formation**

From a quantitative point of view, myelin biogenesis, as observed in rats, is most impressive in that the myelin membrane area expands at a rate of 5-50 × 10³ µm² /cell/day, compared with a cell body surface area of only approx. 300 µm² (Pfeiffer et al., 1993). In order to generate such an efficient membrane producing system, OLGs must tightly control the synthesis of proteins and lipids, and related trafficking of these compounds to their sites of destination. Also afterwards, in mature myelin, it is crucial to maintain proper synthesis and turnover in order to maintain a proper state of myelination (Niemann et al., 1999). Formation of the myelin membrane involves the distinct participation of a variety of mechanisms, including synthetic and transport machineries. In case of the major myelin protein MBP, its mRNA rather than the protein *per se* is transported to the tips of the OLG processes, where it is synthesized on demand (Colman et al., 1982). In contrast, PLP is processed in the Golgi and transported via a vesicular trafficking mechanism to the myelin sheet (Kalwy and Smith, 1994).

As the composition of myelin and plasma membrane of the OLG cell body differ, mature OLGs can be considered as polarized cells. Indeed, it has been shown that distinct apical and basolateral pathways are operating in OLGs (de Vries et al., 1998).
Surprisingly, the myelin sheet, being enriched in glycosphingolipids and cholesterol and therefore resembles a typical apical domain, appears to be served by a ‘basolateral-like’ pathway. Similarly, trafficking to the cell body plasma membrane shows ‘apical-like’ features. Obviously, trafficking to either membrane domain should be tightly regulated and controlled. In polarized epithelial cells, proteins of the SNARE machinery are known to play an important role in the targeting of transport vesicles to distinct polarized target membranes (Weimbs et al., 1997; Lafont et al., 1999). In these events, specific SNARE molecules, present on vesicles (v) and target (t) membranes combine, thus resulting in the formation of v/ t-SNARE complexes, instrumental in specific docking and fusion (Sollner et al., 1993). A similar mechanism likely operates in OLGs as well, as inferred from the notion that mRNA expression of specific SNARE proteins is highly upregulated upon OLG differentiation. Thus, it has been suggested that the v-SNARE VAMP-2, the small GTP-binding protein Rab3a, and the t-SNARE syntaxin-4 play an important role at discrete stages of OLG development (Madison et al., 1999). Intriguingly, the t-SNARES syntaxin-3 and -4, which are specifically enriched in polarized epithelial cells at the apical and basolateral surface, respectively, localize in OLG at the plasma membrane and myelin sheet, respectively, consistent with the polarity features of these membrane domains in these cells (de Vries and Hoekstra, 2000; Klunder et al., submitted). Moreover, in a functional sense, PLP transport to the myelin sheath is dependent on syntaxin-3, but not syntaxin-4 (Klunder et al., submitted), indicating that transcytosis, another important pathway operating in polarized epithelial cells, might exist in OLG, mediating PLP transport from plasma membrane to myelin sheath. In fact, recently it has been proposed that all membrane components in OLGs follow a similar secretory pathway to the plasma membrane of the cell body, after which local myelin extensions are produced by subsequent endocytic uptake, and sorting and recycling to glial processes and growing myelin sheath, which may include prior storage of endocytosed compounds in the endosomal/lysosomal track (Trajkovic et al., 2006). Moreover, it has been shown that neuronal signaling is involved in controlled release of PLP from such late endosomal/lysosomal storage depots. The latter suggests the existence of regulated exocytosis, i.e specific control by axonal (-derived) factors of the formation of local membrane extensions on the OLG surface, leading to myelin outgrowth, and eventually, axonal wrapping.

3. Role of extracellular matrix in (re)myelination

Extracellular matrix (ECM) molecules are important environmental factors in directing cell behavior, including OLGs, and importantly, are known to be altered in MS lesions. The ECM is a complex structural entity that is found within almost any cell type in multicellular organisms. The three most common forms of ECM are bone, cartilage and
basement membrane. Collagen, that serves as a structural scaffold, and proteoglycans are the major components of the ECM. In addition, adhesion proteins like tenascin, fibronectin and laminin are part of the ECM. Recently, a role of ECM in cell-cell signaling, cell adhesion and tissue repair has become apparent (Karram et al., 2005; Fuja et al., 2006; Laurens et al., 2006; Rangaswami et al., 2006).

CNS development very much depends on such ECM molecules like laminins, fibronectin, tenascins, collagens and proteoglycans. (Garcion et al., 2004; De Winter et al., 2002; Probstmeier et al., 2000; Pires-Neto et al., 1999). Among these, laminin-2 is the only one from several tested ECM proteins (vitronectin and fibronectin) that is capable of enhancing myelin membrane formation by OLGs (Buttery and ffrench-Constant, 1999; Relvas et al., 2001). In addition, laminin-2 promotes OLG survival (Frost et al., 1999, Corley et al., 2001, Colognato et al., 2002). Laminins are key molecules of the ECM and are secreted as trimers generated from the five α, four β and three γ laminin subunits. Each laminin molecule has binding sites for several receptors, mainly integrins but also for receptors, such as α-dystroglycan and sulfatide (Li et al., 2005). Prominent laminin expression suggests multiple roles for these molecules during developing and adult CNS, and not surprisingly there are many abnormalities connected with laminin deficiency. For example, CNS changes are connected to laminin-2 (merosin) deficiency found in humans (Farina et al., 1998), and laminin-α2-deficient dystrophic mice show a change in CNS myelination in that the number of myelinated axons are reduced and myelin shows a thinner appearance (Chun et al., 2003; reviewed in Colognato et al., 2005). Based upon a transgenic approach, it has been shown that another ECM molecule, tenascin-C, regulates both OLG migration and proliferation during development (Garcion et al., 2001). However, no abnormalities in myelination per se were observed in these animals (Kiernan et al., 1999). Recently, a novel role for osteopontin as a modulator of myelination and remyelination in the CNS has been proposed (Selvaraju et al., 2004). This secreted glycoprotein that contains an RGD binding sequence, has anti-apoptotic, chemotactic and cytokine-like properties, and importantly, this protein has the potential to enhance myelin formation in vitro (Selvaraju et al., 2004). As such, in the cuprizone-induced demyelination mice model, osteopontin is expressed by microglia and astrocytes in the demyelinating brain areas.

ECM molecules exert their signaling capacity mainly via the integrin receptor family. Integrins, the major receptor class involved in cell-ECM and cell-cell interactions, are a large family of heterodimeric transmembrane glycoproteins. They consist of a large α-subunit (120-170 kDa) and of a smaller β-subunit (90-100 kDa) and contain binding sites for divalent cations (Mg²⁺, Mn²⁺ and Ca²⁺), which are involved in expressing their adhesive functions (reviewed in Etzioni, 2000). Integrins are capable of bidirectional signaling, i.e. from inside to the extracellular environment and also in the reverse direction (Calderwood et al., 2000; Liang et al., 2004). Intracellularly, integrins bind to a
number of cytoskeletal proteins, such as talin (Calderwood et al., 2002) and plectin (Geerts et al., 1999), thus providing a connection with actin (reviewed in Wiesner et al., 2005). Moreover, integrins also bind to molecules that establish their interaction with important signaling transduction pathways (Karna et al., 2006; Legate et al., 2006; Brancaccio et al., 2006). In this way, integrins are very potent regulators of intracellular events based on ECM properties. Of the 20 known integrin species (Caswell and Norman, 2006), OLGs express a limited repertoire, i.e. $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 8$ and $\alpha 6\beta 1$ (Milner and ffrench-Constant, 1994; Shaw et al., 1996; Milner et al., 1997; Cousin et al., 1997). Whereas $\alpha v\beta 8$ and $\alpha 6\beta 1$ are expressed throughout OLG development, the expression of the other $\alpha v$ integrins is very tightly regulated during OLG differentiation, i.e., in the order $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$ (Milner et al., 1994; Blaschuk et al., 2000). Interestingly, in contrast to growth factor regulation of OLG behavior, the expression of an integrin receptor is specifically associated with one aspect of OLG behavior. For example, $\alpha v\beta 1$ integrin is implicated in the migratory phenotype of an OPC (Milner et al., 1996), whereas integrin $\alpha v\beta 3$ is involved in OLG proliferation (Blaschuk et al., 2000; Baron et al., 2002) and integrin $\alpha v\beta 5$ in differentiation (Blaschuk et al., 2000). During the final stage of OLG differentiation and myelin membrane formation, an essential role for $\alpha 6\beta 1$ integrin has been proposed as an effective receptor for myelin membrane propagation (Buttery and ffrench-Constant, 1999; Relvas et al., 2001) and survival (Frost et al., 1999; Corley et al., 2001; Colgnato et al., 2002). Recently, it has been reported that beta 1 integrin is responsible for survival of premyelinating OLGs but CNS myelination and also remyelination is not affected in animals lacking $\beta 1$ integrin mediated signals (Benninger et al., 2006). Interestingly, integrins are important regulators of growth factor-mediated OLG behavioral signaling. Thus, association of PDGF$\alpha$R with integrin $\alpha v\beta 3$ results in proliferation, whereas its association with integrin $\alpha 6\beta 1$ is necessary for PDGF-mediated survival (Baron et al., 2002; 2003; Colognato et al., 2004). An important role in these associations is the nature of the ECM encountered and the subsequent lateral membrane compartmentalization. Laminin-2, for example, induces clustering of integrin $\alpha 6\beta 1$ and PDGF$\alpha$R into a specific membrane microdomain, whereas vitronectin sequesters PDGF$\alpha$R into another membrane microdomain (reviewed by Baron et al., 2005). Similarly, laminin-2 is able to trigger activation of the NRG intracellular signaling pathway, favoring survival and differentiation signaling pathways (Colognato et al., 2002; Baron et al., 2005). Fibronectin, present at pathological conditions in adult CNS (Mitchel and Sobel, 1989), has been proposed to interfere with formation of membrane microdomains (Baron et al., 2003, Maier et al., 2005). For example, the association of the oligodendrocyte-specific isoform of neurofascin, NF155 (155 kDa isoform) with these microdomains is perturbed. Consistently, raft association of NF155 is reduced in the spinal cord of EAE rats. Thus,
fibronectin may alter differentiation of OLGs by hampering axo-glia interactions that are required for myelin integrity (Maier et al., 2005).

In MS and other CNS pathologies, rupture of the vasculature, represented by breakdown of the blood-brain barrier (BBB), allows the entry of blood-derived cells and proteins into the brain. Fibrin is an example of a blood-derived protein that is not normally expressed by cells of the nervous system, but accumulates only after disease-associated vasculature rupture (Adams et al., 2004). In MS pathology, fibrin deposits correlate with the border of demyelination (Kwon and Prineas, 1994) and coincide with macrophage infiltration, astrocyte swelling (Kirk et al., 2003) and axonal damage (Gveric et al., 2001). In 1989, Mitchel and Sobel reported fibronectin accumulation in MS lesions, the expression of which correlated with the degree of inflammation. Fibronectin, which is normally absent from healthy adult CNS, has been proposed to be secreted by perivascular infiltrates and also accumulated by the leakage from the blood serum due to BBB disruption. Deposition of ECM molecules (several isoforms of laminin, fibronectin, collagen IV and heparan sulfate proteoglycans) has been observed also in inflammatory cuffs in fiber-like structures (van Horssen et al., 2005). These ECM molecules may help facilitating the transport of myelin-containing phagocytes towards peripheral lymph nodes out of the CNS, and thus contribute to an enhanced inflammatory response in MS. Interestingly, the molecular composition of the vascular and astroglial basement membrane in chronic active and active MS lesions differs in expression of specific laminin chains (van Horssen et al., 2005). Upon injury, OLGs themselves are capable of production of the chondroitin sulfate proteoglycans, versican, phosphocan and neurocan, which are other major constituents of the ECM (Asher et al., 2000; 2002). These proteins are able to interfere with axonal regeneration contributing to the limited healing capacity. Furthermore, versican and dermatan sulfate proteoglycan are upregulated at the edges of an active MS lesion (Sobel, 2001). Recently, it has been reported that another ECM molecule, the glycosaminoglycan, hyaluronan, accumulates in demyelinated lesions (Back et al., 2005). In mice with EAE, this protein reversibly inhibits maturation of OPCs and hence could contribute to remyelination failure in MS.

In conclusion, ECM components are important regulators of OLG behavior during normal development. In MS lesions, the ECM environment may be severely perturbed as consequence of BBB disruption and deposition by activated microglia, astrocytes and inflammation-associated cells. Evidently, such a perturbed ECM environment upon injury may have detrimental consequences for the physiological development of OLGs, and hence (re)myelination. Among others, remyelination-based therapies should therefore be focused on the beneficial support of the ECM, with a particular aim of precluding (further) deposition of detrimental ECM or, alternatively, specifically stimulating its degradation.
4. Involvement of glycosphingolipids in (re)myelination

*Galactosphingolipids – essential components of myelin membrane*

As described above, myelin is abundant in galactosphingolipids, GalCer and its sulfated derivate, sulfatide. These lipids comprise almost one third of all myelin lipids (*table 1*). Previous studies showed that both GalCer forms (hydroxylated and non-hydroxylated) are encoded by a single gene (Bosio et al., 1996). GalCer is synthesized upon attachment of a galactose to ceramide by UDP-galactose: ceramide galactosyltransferase (CGT) (Morell and Radin, 1969). Synthesis occurs in several compartments, including endoplasmatic reticulum (ER), Golgi compartment as well as in myelin (reviewed in Coetzee et al., 1998). Recent studies reveal that GalCer synthesized at the ER is transported to the outer leaflet of the myelin membrane and part of GalCer is assembled into lipid microdomains in the Golgi apparatus. Formation of GalCer/cholesterol-containing lipid domains on the ER is influenced by Sigma-1 receptors that play a role in regulating transport of ER-formed lipids and in this manner, important signaling platforms might be assembled possibly serving in OLG differentiation (Hayashi and Su, 2004).

Sulfatide is synthesized by the transfer of a sulfate molecule to the third carbon of the GalC sugar ring by galactosylceramide 3′-sulfotransferase (CST), which is thought to occur on the lumen of the Golgi apparatus (Vos et al., 1994). There is mounting evidence that galactolipids excerpt several important functions during OLG development. For example, GalC and sulfatide are important stage-specific differentiation markers (see figure 3; Pfeiffer et al., 1993). Moreover, a functional role for sulfatide has been proposed in OLG maturation, as experiments with anti-sulfatide antibodies show a reversible inhibition of OLG maturation (Bansal et al., 1999). The underlying mechanism possibly relies on modulation of the cytoskeleton and Ca\(^{2+}\) influx related events (Bansal and Pfeiffer, 1989; Dyer, 1993). Furthermore, the trafficking of PLP in OLGs appears to depend on galactolipids (Folch and Lees, 1951; Gow et al., 1998; Simons et al., 2000), although PLP trafficking to the cell surface as such has been shown to be galactolipid-independent (van der Haar et al., 1998). In addition, GalCer and sulfatide are important components of membrane microdomains, which have been implicated in the transport of various proteins (Simons et al., 2000). Such domains also serve as signaling platforms for OLG survival (Baron et al., 2003; Decker and ffrench-Constant, 2004) and myelination (Kramer et al., 1999). In addition, a role in stabilization of the myelin membrane has been proposed, involving a role in myelin compaction and axon ensheathment (Koynova and Caffrey, 1995). In fact, sulfatide knock-out mice form abnormal structures of myelin, showing the appearance of vacuoles in CNS white matter and resulting in generalized tremors followed by hind-limb paralysis (Honke et al., 2004).
In GalCer knock-out mice, myelin displays a seemingly normal ultrastructure. Nevertheless, these animals manifested behavior which suggests a disrupted nerve function, presumably due to failure of myelination (Bosio et al., 1996). GalCer and sulfatide appear also essential for proper formation of the node of Ranvier (Dupree et al., 1998), and nerve impulse transmission is interrupted in these animals (Coetzee et al., 1996). In addition, sulfatide null mice show enhanced numbers of mature OLGs, suggesting a role for sulfatide as a negative regulator of OLGs differentiation (Hirahara et al., 2004), which has been corroborated in in vitro OLG cultures (Bansal and Pfeiffer, 1989).

**Gangliosides**

Gangliosides are a family of sialoglycosphingolipids that are present in the outer surface of the plasma membrane within all vertebrate cells and tissues. In brain tissue they account for 6% of the total lipid weight. In OLGs, ganglioside expression is developmentally regulated, as reflected by the transient appearance of certain gangliosides (GT3, O-acetylated-GT3 and GD2), recognized by the A2B5 antibody, and GD3, which are present in precursor cells, but disappear upon differentiation (Levine et al., 1993; Farrer and Quarless, 1999). The expression of GM1, GM2, GM3 and GD1a has been confirmed within OLGs through development (Satoh et al., 1996), with GM1 ganglioside as the major ganglioside in myelin (Cochran et al., 1982).

Elevated brain ganglioside expression is a hallmark of various pathologies with neuroinflammatory background, including MS (Simon et al., 2002). For example, elevated GD3 levels have been found in cerebrospinal fluid of MS patients (Miyatani et al., 1990) and expression of GD3 is increased in MS lesions, in contrast to GM1 which is decreased. In vitro experiments identified activated microglia by inflammatory stimuli, as the source of secreted GD3 which triggered caspase-independent OLG apoptosis by disrupting plasma membrane integrity. Another member of the ganglioside family, GM3 enhanced differentiation of OLG progenitors towards myelin production (Yim et al., 1994). This effect was specific for this molecule and was not produced by adding GM1, GM2, GD3 or GD1a to the cultures. Interestingly, an important role for the neuronal gangliosides has been proposed with regard towards regulation of myelin stability via interactions with their ligand, myelin protein MAG (Vyas and Schnaar, 2001). In this way brain gangliosides appear to be an important factor in maintaining myelin membrane stability by facilitating axo-glial interactions. Taken together, these observations introduce gangliosides as promising therapeutic target for promoting survival and axo-glial “communication” that appear to be derailed in demyelinating diseases like MS (Matthews et al., 1998).
5. On the role of matrix metalloproteinases in (re)myelination

Matrix metalloproteinases (MMPs) play an important role in tissue remodeling, associated with various physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, and metastasis. They are also known as important mediators of CNS development, pathology and regeneration (reviewed in Yong, 2005). For example, MMP activity is necessary for morphological differentiation of OLGs, and an altered activity of MMP in CSF of MS patients has been observed. Initially, MMPs were described by Gross and Lepiere in 1962 in vertebrates, but have been since detected in invertebrates and plants as well. MMPs are zinc-dependent endopeptidases that belong to the larger family of proteases, known as the metzinc superfamily. Thus far, 24 mammalian MMP members have been identified, each of them being a product of a unique gene. They share a common domain composition, generally consisting of a pro-peptide, the catalytic domain and the haemopexin-like C-terminal domain, which is linked to the catalytic domain by a flexible hinge region (Sternlicht & Werb, 2001). Most MMPs are secreted into the extracellular environment, where they are capable of degrading extracellular matrix proteins. Furthermore, MMPs can also process a number of bioactive molecules, i.e. they are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands and modulation of chemokine activity (Vos et al., 2000; van den Steen et al., 2000; Zhang et al., 2003). In fact, in OLGs it has been shown, that MMP(s) is (are) involved in the shedding of the paranodal protein, NF155 (Maier et al., 2006).

Several MMPs are localized within adult mammalian CNS (Karkkainen et al., 2000; Yong, 2005). Regarding OLG biology, three MMPs have been shown to be of major interest, i.e. MMP-2, 9, and 12. It has been shown that MMP-12 plays an important role in the morphological differentiation of OLG, rationalizing that their maturation is limited in MMP-12 null mice (Larsen and Yong, 2004). An important event like process extension by OLG, representing an initial step in myelination, is very likely controlled by regulated release of proteases (Uhm et al., 1998). In vitro, (protein kinase C) PKC activators increase MMP-9 activity, which has been linked to an enhanced process extension by OLGs (Uhm et al., 1998). In vivo, an elevated MMP-9 activity parallels myelination in the corpus callosum, rich in myelin and OLGs (Larsen et al., 2006). In pathology, MMP-9 is capable of facilitating remyelination by degrading a myelinating inhibitory molecule, NG2 proteoglycan (Larsen et al., 2003). In addition, mice lacking MMP-9 fail to remyelinate lysolecithin-induced demyelinated lesions (Larsen et al., 2003). Moreover, by using a transgenic approach it has been shown that MMP-2 and -12 null mice display more severe EAE, accompanied with an earlier onset (Esparza et al., 2004; Weaver et al., 2005). In contrast, MMP-9 null mice show a less severe course of EAE disease development (Dubois et al., 1999). Hence, MMP activity appears
essential at the onset of myelination, in particular in creating a path for OLG process outgrowth towards the axon.

Upon CNS injury, including MS, detrimental effects have been reported for MMPs, such as BBB dysfunction, demyelination, neurotoxicity, and neuroinflammation. Interestingly, elevation of their expression occurs in all CNS diseases and in addition, expression of additional MMPs accompanies CNS pathology (Yong et al., 2001). On the other hand, effects of MMPs in CNS may depend on the stage of CNS injury. Specifically, MMPs released early after insult are considered to be rather detrimental (Noble et al., 2002), whereas at later stages after injury they could be beneficial (Godin et al., 2000; reviewed in Lo et al., 2002). Several lines of evidence implicate MMPs as pathogenic factors in MS and its animal model, EAE (Yong et al., 2001). In MS pathology, MMPs may act in three steps, (i) in cellular invasion and BBB disruption, (ii) in myelin breakdown and (iii) in the first steps of OLGs process extension. Thus, MMPs (particularly MMP-7, -9 and -12) are elevated in brain of MS individuals and animals with EAE. MMP-9 levels are significantly increased in serum of MS patients as compared to healthy individuals. In MS patients, a higher MMP-9 content is observed during clinical relapse, relative to periods of stability (Lee et al., 1999). In addition to their role as effector molecules in BBB disruption and cell infiltration, MMPs are also known to degrade MBP in vitro (Chandler et al., 1995), and increased MMP-9 activity is essential for remyelination (Larsen et al., 2003), probably required for process extension in OLGs (Uhm et al., 1998). In conclusion, tight control of the function of MMPs is a relevant factor, as MMPs contributes to the function of OLGs in the CNS, both during development and upon CNS injury and therefore can be considered as an interesting therapeutic target in MS.

6. Demyelinating diseases and multiple sclerosis

Demyelinating diseases with myelin as the (primary) target, fall into two main categories, i.e., hereditary neurodegenerative disorders (e.g., the leukodystrophies) and acquired diseases, such as multiple sclerosis (MS). Although the outcome of CNS demyelination is very similar, etiology and cause of these disorders can be very different. Persistent demyelination will delay or completely block nerve pulse conduction and lead to neurological symptoms. Clinical signs and symptoms include paralysis, sensory, visual and additional neurological impairments (McFarlin and McFarland, 1982a).

MS is the most well-known acquired demyelinating disease, and is one of the most disabilitating diseases in young adults. Its etiology is very likely multifactorial with genetic and environmental factors contributing to disease susceptibility. Geographically, the risk of developing MS is highest in northern Europe, US and Canada, southern Australia and New Zealand (Kurtzke, 1980). Ethnic background also plays a significant role in the prevalence of MS, since it occurs very frequently in Caucasians and only
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rarely in Asians and Africans (Compston, 1997). MS can be distinguished into four well characterized forms. First, a relapsing-remitting (RRMS) form that is characterized by relapses during which new symptoms of disease can appear while old ones can re-appear and worsen. These periods are followed by remission periods, during which full recovery may occur. The vast majority of MS patients (70-85%) are first diagnosed with this form of MS (Lubin and Reingold, 1996). Second, secondary progressive MS (SPMS) develops a number of years after the onset of RRMS. This form is characterized by a gradual worsening of the disease between relapses, and some remission but not a genuine recovery may take place (Runmarker and Andersen, 1993). Third, the primary progressive form of MS is characterized by a serious progression of the disease from the onset, without any remissions. This form differs from others in that the disease emerges at an age in the late thirties or early forties, and is also sex-related, males being relatively less susceptible, and the initial disease activity is localized in the spinal cord (Lubin and Reingold, 1996). Fourth, a very mild form of MS is occasionally seen in a very small group of patients, typified by little progression in severity of the disease over time (reviewed by Ramsaransing and de Keyser, 2006).

Pathology of MS

Histologically, MS is characterized by areas in the brain that show severe demyelination, i.e. plaques, perivascular infiltrates, decreased numbers of OLGs and axons, and gliotic scaring. Plaques have been demonstrated to occur anywhere within the white matter of the CNS. Recently it has been reported that lesions can occur also in grey matter, although with a different pathology (Bö et al., 2006). The histopathology of MS lesions suggests that MS involves repetitive rounds of inflammation in the CNS ultimately leading to damage of the myelin sheaths, the mature OLGs, and axons. MRI investigation revealed that BBB disruption accompanies the onset of the disease but it is not known whether demyelination precedes or is secondary to the inflammation. Recent insights suggest that apoptosis of OLGs may constitute a primary event, followed by local microglia activation which triggers the inflammation (Barnett and Prineas, 2004; Lucchinetti et al., 2000; figure 4). These observations raise questions concerning the inflammatory etiology of the disease. Alternatively, primary axonal damage followed by demyelination has also been suggested as a cause for the onset of lesion formation (Wang et al., 2005). In active MS lesions, for example, an average of more than 11,000 transected axons/mm$^3$ were observed in comparison to control brain tissue that had less than 1 transected axon/ mm$^3$. These data indicate that axonal injury could also be a significant hallmark in the early onset of the disease (Bruck, 2005; Petzold et al., 2005). This, whether OLG or axon pathology is the primary event in the onset of MS clearly
requires further work, but likely emphasizes the potential involvement of multiple factors in causing the disease.

![Diagram of relevant events in the development of multiple sclerosis]

**Figure 4:** Relevant events in the development of multiple sclerosis. An autoimmune attack, directed against myelin, is generally considered to represent a major mechanism in disease development. Two possible pathways of disease etiology are shown, the CNS etiology, represented by the black line, starts with demyelination (2) that leads to microglial activation (3) and subsequent T cell activation (4) that develops into T-cell mediated demyelination (5). Another possible mechanism, shown in grey, may be initiated in the peripheral vascular system, where an unknown factor activates T cells (1) that are crossing blood-brain barrier (2), which is followed by an attack of oligodendrocytes and axons, accompanied by microglia activation (3).

**Therapy for MS**

Currently, there is no medication available for curing MS, not in the least due to the fact that the exact etiology of the disease is unknown. Therapeutic approaches focus therefore on immunomodulatory therapies that are specifically directed to suppress the response of the immune system. Also, much effort is devoted to repair-promoting therapies that include replacement and/or enlargement of the endogenous pool of OPCs by transplantation of stem cells and/or stimulation of remyelination by the endogenous OPC pool. Finally, therapies are aimed at neuroprotective treatments.

OPCs have been observed in MS lesions, even in chronic plaques (Scolding et al., 1998; Wolswijk et al., 1998; Chang et al., 2002). These observations are of particular interest since the potential ability for remyelination may thus exist in quite an advanced
stage of disease, as these cells may constitute an appropriate target for therapeutic approaches. However, endogenous remyelination fails as evidenced by limited remyelination that occurs in the first stage of the disease, which further decreases when the disease progresses. This suggest that other factors, like an altered extracellular microenvironment in MS lesions [e.g. ECM, MMPs or growth factors], might play a significant role in the perturbation of remyelination (Mitchell and Sobel, 1989; Sobel, 2001; Charles et al., 2002; Frohman et al., 2006; Kanesaka et al., 2006; Gerlach and Tanus-Santos, 2006). These observations should thus be taken into the account when designing remyelination strategies relying on either stimulation of endogenous remyelination or therapies involving cell replacement. For example, stem cell mediated remyelination in the adult CNS is a realistic therapeutic solution (Liu et al., 2000; Wu et al., 2002) since in principle, newly supplied myelinogenic cell populations can be generated. However, these cells will encounter the same environmental limitations as the endogenous pool of OPCs that fail to remyelinate denuded axons. Hence, insights into the extracellular microenvironment of MS lesions and the behavior of OPCs in this microenvironment will add to designing therapies in overcoming this environmental restriction.

Scope of this thesis

The aim of this thesis was to obtain insight into the role and the underlying mechanisms of ECM molecules in myelination from the perspective of OLG biology. To this end, the role of specific ECM molecules was investigated and how they contribute to the failure of remyelination in MS, and, in particular, how to overcome this impairment. In chapter 2, we show that fibronectin, an ECM molecule absent in adult healthy human brain, is accumulated within demyelinated lesions in individuals with MS. In lysolecithin-induced lesions, its presence precludes remyelination, suggesting a correlation between the presence of this ECM molecule and a failure of remyelination. We have observed fibronectin deposits in close proximity to activated microglia and astrocytes within injured tissue. In chapter 3 and chapter 4 the underlying mechanism(s) of myelination failure in the presence of fibronectin was investigated. Fibronectin appears to perturb intracellular vesicular transport of myelin-sheet directed proteins by PKC and subsequently interfering with actin cytoskeleton assembly, required for functioning of the vesicular trafficking route to the myelin sheet (chapter 3). In chapter 4 we demonstrate that fibronectin prevents process outgrowth by OLGs by causing a mislocalization of MMP-9 activity. In cells, cultured on fibronectin, the activity of this enzyme is not present along extending processes, as observed for cells cultured on laminin-2, another ECM component, which promotes myelination. The data emphasize that MMP-9 plays a crucial role in the onset of the myelination process, Chapter 5 aims at elaborating the
role of gangliosides during OLG development and its possible function in maintaining myelin integrity. Chapter 6 summarizes major achievements of the work described in this thesis and provides suggestions for future research.

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