Chapter 5

A role for gangliosides in morphological oligodendrocyte differentiation and myelin membrane integrity

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Abstract

Studies with transgenic animals lacking complex gangliosides show impaired myelin stability, which has been suggested to be due to disturbed axo-glial interactions. Gangliosides expression in axon appears to be essential, however, a role for gangliosides in the integrity of oligodendrocyte (OLG) derived myelin membranes has not been properly defined yet. Here, we addressed this issue in primary OLGs and an OLG-derived cell line, OLN 93. Pharmacological inhibition of glycosphingolipid (GSL) biosynthesis influenced the stability of OLG membranes, resulting in the formation of extracellular galactosylceramide (GalCer) containing deposits, surrounding OLG cell bodies, which had detrimental consequences on morphological OLG differentiation. Moreover, in GalCer-over expressing OLN 93 cells that lack complex gangliosides similar deposits were formed, as visualized by immunocytochemical and electronmicroscopical analyses. The deposits contained components of the outer leaflet (GalCer), transmembrane spanning proteins (NCAM 140) and β-tubulin cytoskeleton, whereas actin cytoskeleton and components associated with the inner leaflet (CNP) were absent. Additional work indicated that GalCer deposition could be prevented by exogenous administration of the ganglioside GT1b, but not ganglioside GM1. Taken together, the results suggest that a proper ratio of GalCer/ ganglioside (GT1b) needs to be maintained for the formation of stable myelin membranes.

Introduction

Gangliosides are a major class of glycosphingolipids (GSLs) that contain one or more sialic acid residues, and are abundantly present in vertebrate brain (Stults et al., 1989). Thus, it is not surprising that defects in their metabolism could have a most severe impact on this organ. In fact, inherited defects in degradation of gangliosides cause two types of disease in human, i.e., the GM1 and GM2 gangliosidoses (Gravel et al., 1979; Itoh et al., 2001). Furthermore, other genetically determined diseases, not directly related to defects in ganglioside metabolism, such as Niemann-Pick and Gaucher disease, may be associated with a significant accumulation of specific gangliosides (Siegel and Walkley, 1994; Schuchman and Miranda, 1997; Gornati et al., 2002). Differences in ganglioside levels have also been observed in neuroinflammatory pathologies, like the demyelinating disease multiple sclerosis (MS). In MS, the levels of ganglioside GM1, the most abundant ganglioside in myelin (Ueno et al., 1978; Cochran et al., 1982a,b), decreases (Yu et al., 1974), whereas elevated levels of ganglioside GD3 have been reported in the cerebrospinal fluid of MS patients (Miyatani et al., 1990). Likely, activated microglia secrete GD3, which triggers OLG apoptosis (Simons et al., 2002), thus raising the issue as to how gangliosides modulate MS pathology, and remyelination in particular.

However, next to a potentially detrimental role in MS pathology, gangliosides are nevertheless important for myelin stability. For example a role of gangliosides in the maintenance of myelin stability *in vivo* has been suggested by several studies. In *Galgt1*-null mice that fail to express complex brain gangliosides, including GD1a and GT1b, a selective and progressive decrease in expression of myelin-associated glycoprotein (MAG) results in demyelination, and is accompanied by motor behavioral deficits (Sun et al., 2004). Furthermore, the spinal cord of mice lacking complex gangliosides are characterized by disordered myelination and degeneration of nerve fibers (Ma et al., 2003), further suggesting involvement of brain gangliosides in myelin stability. Interestingly, complex ganglioside GT1b mediates axo-glia interactions (Vinson et al., 2001), whereas its level decreases during development of chronic relapsing experimental allergic encephalomyelitis (EAE), an animal model for MS (Zaprianova et al., 1998), and thus might impair remyelination events in MS.

In OLGs, the myelin forming cells of the central nervous system (CNS), the expression of gangliosides is developmentally regulated. In particular, gangliosides (GT3, *O*-acetylated-GT3 and GD2) recognized by the A2B5 antibody (Farrer and Quarles, 1999**)** and GD3 are present on precursor cells, whereas their expression is lost upon cell differentiation (Levine et al., 1993; Farrer and Quarless, 1999). Furthermore, gangliosides GM1, GM2, GM3, GD1a and GT1b are expressed in OLGs throughout development (Satoh et al., 1996), GM1 ganglioside being the major ganglioside in

myelin (Ueno et al., 1978; Cochran et al., 1982a,b). The function of developmentally-regulated gangliosides in OLG differentiation is not yet known. However, gangliosides almost exclusively localize to the outer leaflet of the membrane (Hansson et al., 1977), and in other cell types a role in cell adhesion (Blackburn et al., 1986; Probstmeier et al., 1999), process outgrowth (Cannella et al., 1998) and transmembrane signaling by participation in the formation of membrane microdomains (McKerracher, 2002; Sorice et al., 2004) has been reported. These reported functions are important for OLG development as well, and a role for gangliosides in these functions could thus be readily envisioned. Membrane microdomains, for example, have been shown to be important signaling platforms for OLG survival (Baron et al., 2003, Decker and ffrench-Constant, 2004) and myelination (Kramer et al., 1999). Moreover, adhesion of OLG to its target, the axon, is necessary for OLGs to survive *in vivo* (Barres et al., 1993; Barres and Raff, 1999) and an important mediator in this axo-glia adhesion is the axonally-derived extracellular matrix molecule, laminin-2 (Ln2, Colognato et al. 2002). Ln2 also appears to be important in myelin membrane formation (Buttery and ffrench-Constant, 1999; Relvas et al., 2001) and process outgrowth (Šišková et al., submitted (*chapter 4*)). For example, OLG adherence to fibronectin (Fn), which is normally absent in healthy CNS and present under pathological conditions (Sobel and Mitchell, 1989; Šišková et al., *chapter 2*), results in impaired process outgrowth and myelin membrane formation (Buttery and ffrench-Constant, 1999; Maier et al., 2005; Šišková et al., 2006 (*chapter 3*); Šišková et al., submitted (*chapter 4*)).

Taken together, although knowledge about gangliosides structure, metabolism and function gradually emerges, knowledge of how gangliosides control OLG behavior and myelin membrane integrity, is fairly scanty. In the present *in vitro* study, evidence is provided that highlights the importance of ganglioside GT1b in myelin membrane integrity, a prerequisite for myelin compaction. Based on these results, we propose a mechanism, explaining the role of GT1b in myelin stability, which is impaired in complex ganglioside deficient animals.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, penicillin/streptomycin and geneticin (G418) were from GIBCO BRL (Life Technologies, Paisley, Scotland). Fetal calf serum (FCS) was obtained from Bodinco (Alkmaar, Netherlands). Paraformaldehyde (PFA) and Triton-X 100 (TX100) were obtained from Merck (Darmstadt, Germany). FGF-2 and PDGF-AA were purchased from Peprotech (Rocky Hill, NJ). Protease inhibitor cocktail tablets (Complete Mini) were obtained from Roche Diagnostic Corp (Mannheim, Germany). DL-threo-1-phenyl-2-palmitoylamino-3-

pyrrolidino-1-propanol-HCL (PPPP) and N-butyldeoxynojirimycin-HCL (NBDNJ) were obtained from Biomol international, L.P. (Exeter, UK). Gangliosides GT1b and GM1 were from Alexis Corp. (Läufelfingen, Switzerland). Sep-Pak 18 cartridges were purchased from Waters Corp. (Milford, MA). HRP-conjugated cholera toxin subunit B (CTB) was from EMD biosciences Inc. (La Jolla, CA, USA). HPTLC plates, chloroform and methanol were from Merck (Amsterdam, The Netherlands). All other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO).

Antibodies

The R-mAb and O1 hybridoma (Ranscht et al., 1982) were kind gifts of Guus Wolswijk (NIBR, Amsterdam, The Netherlands). Mouse anti-CNPase (IgG1), mouse anti-β-tubulin (IgG1) and mouse NCAM 140/180 (IgG2b) were purchased from Sigma Chemical Co. (St. Louis, MO). Rat anti-MBP was obtained from Serotec (Oxford, UK). FITC and TRITC-conjugated antibodies were purchased from Jackson ImmunoResearch (West Grove, Pa., USA).

Constructs

The cDNA encoding ceramide galactosyltransferase (cgt) was a kind gift of Dr. Brian Popko (Chapel Hill, NC). For cloning, the cgt gene was inserted into the *EcoRI* restriction site of the retroviral vector pLXIN (Clontech Biosciences, Mountain View, CA). Recombinant plasmids were grown in TOP10 cells, and plasmids with the cDNA insert in the correct orientation with respect to transcription were identified by restriction analysis. The orientation and the integrity of the obtained pLXIN constructs were confirmed by DNA sequencing. The production of retroviral particles and the subsequent infection and selection of OLN 93 cells were performed according to Maier et al. (2006). An OLN-MOCK and an OLN-G, overexpressing GalCer, were generated. OLN-MOCK was obtained by retroviral infection with vector only (pLXIN). OLN-G is retrovirally infected with the pXLIN-cgt and selected for at least 14 days with 2 mg/ml G418. Infection efficiency was in all cases nearly 100%.

OLN 93 cultures

The oligodendroglia derived cell line OLN 93, a kind gift of Dr. Christiane Richter-Landsberg (Richter-Landsberg and Heinrich, 1996), were cultured in DMEM supplemented with 10% heat-inactivated FCS, L-glutamine, and the antibiotics penicillin and streptomycin (proliferation medium). Retroviral infection (see above) was performed on passage 28. Cells were trypsinized when they reached near-confluency and experiments were performed with passage 30-38. To test the effect of ECM components, the cells were cultivated on pre-coated poly-L-lysine (PLL, 5 μg/ml), laminin-2 (Ln2, 10 μg/ml) or fibronectin (Fn, 10 μg/ml) tissue culture plastics (Corning

Incorporated Corning, NY). Tissue culture plastics were pre-coated for at least 3 hrs at 37ºC. For TLC analysis OLN 93 cells were plated at a density of 500.000 per T75 flask and cultured till 70% confluency in 10% FCS. For immunocytochemical and electronmicrosopical studies cells were grown overnight on Lab-Tek chamber slides (Nalge Nunc) at a density of 4.000 cells per well and grown for at least 3 days in either 10 or 0.5% FCS. To determine the influence of gangliosides on the membrane integrity, 25 µM GT1b or 25 µM GM1 were added directly after attachment of OLN 93 cells to the substrate and kept in culture medium for the duration of the experiment.

Primary oligodendrocyte culture

Primary OPCs were obtained from 1-3 day old Wistar rats as described previously (Baron et al., 2002, Maier et al., 2005). Briefly, rats were decapitated, forebrains were collected and a single cell suspension was obtained by mechanical and enzymatic (papain) digestion. Cells were cultured in DMEM, supplemented with 10% FCS, L-glutamine, penicillin and streptomycin for 10-14 days on PLL-coated tissue culture flasks (Nalge Nunc, Naperville, IL). OPCs, growing on top of an astrocyte monolayer, were then isolated by a shake off procedure (McCarthy and de Vellis, 1980). After a 1 hr pre-shake to remove contaminating microglia, flasks were shaken overnight at 240 rpm on an orbital shaker. The floating OPCs obtained by this procedure were further purified by differential adhesion on bacterial culture dishes, to remove astrocytes and microglia. Enriched OPCs were plated on pre-coated dishes (see above) or well chamber slides, and synchronized in SATO medium (Maier et al., 2005), containing PDGF-AA (10 ng/ml) and FGF-2 (10 ng/ml) for 2 days. Differentiation was induced by growth factor withdrawal and cells were grown for 7 days (mature myelinating stage) in SATO medium supplemented with 0,5 % FCS with medium changes twice a week. For immunocytochemical studies, primary OPCs were plated on ECM-precoated 8 well chamber slides at a density of 15.000 cells per well, whereas for GM1 quantification, the cells were plated at the density of 250.000 cells per PLL-precoated 35 mm dish (Nalge Nunc). To inhibit the *de novo* synthesis of gangliosides, 200 µM NBDNJ and 0.5 µM P4 (unless otherwise indicated) were added to the culture medium immediately after initial adhesion and have been re-applied every 72 hrs till the duration of the experiment.

Immunohistochemistry

Antibody staining of cell surface components was performed either on live cells at 4°C or on PFA-fixed cells. After blocking aspecific binding with 4% BSA in phosphate buffered saline (PBS) for 10 min, primary OLGs or OLN 93 cells were incubated with R-mAb (1:10; Ranscht et al., 1982) or O1 (1:20) for 30 min, washed three times with PBS and incubated for 25 min with appropriate TRITC or FITC-conjugated secondary antibodies (1:50). After washing with PBS, the nuclei were stained with DAPI. For internal staining, the cells were first gently fixed with 2% PFA in PBS at room temperature (RT) for 20

min, followed by 15 min fixation with 4% PFA. Cells were permeabilized and blocked with 0.1% TX100 and 4% BSA, respectively and subsequently incubated with the appropriate antibody (NCAM 140/180 1:50, CNP 1:100, β-tubulin 1:200) for 60 min in 4% BSA. After washing with PBS, cells were incubated for 30 min at RT with the appropriate fluorescently-conjugated secondary antibodies (FITC- and TRITC-conjugated antibodies 1:50) in combination with DAPI. Actin filament staining on fixed and permeabilized cells was performed with TRITC-labeled phalloidin (1µg/ml, 30 min) as described previously Šišková et al., 2006 (*chapter 3*). For all stainings, mounting medium (1 mg/ml DABCO in 90% glycerol) was added to prevent image fading. The cells were analyzed with a conventional fluorescence microscope (Olympus ProVis AX70) and data were processed using Paint Shop Pro or Adobe Photoshop 7.0 software.

Transmission electron microscopy

Cells were fixed with 2% glutaraldeyde in 0.1 M phosphate (pH 7.4) for 20 min at RT. Cells were washed once with 0.1 M phosphate pH 7.4 containing 6.8% sucrose, after which the cells were osmificated with 1% OsO_4 in 0.1 M phosphate for 2 hrs at RT. Cells were dehydrated in a graded series of ethanols (30, 50, 70 and 100% respectively), and embedded in a 1:1 Epon / propylene oxide mixture overnight, followed by embedding in pure Epon. Ultra thin sections were counterstained with uranyl acetate and lead citrate and samples were examined using a Philips CM 100 (FEI electron optics, Eindhoven, The Netherlands).

Analysis of cellular glycosphingolipids

Cells were washed three times with PBS and harvested by scraping in 1 ml PBS. Cells were centrifuged at 10.000 rpm at RT, followed by lipid extraction from the cell pellet according to Bligh and Dyer (1959). Briefly, the cell pellet was resuspended in water and sonicated. Total lipid extraction was performed with 3 volumes $CHCl_3/CH_3OH$ (2:1, v/v). The chloroform layer was washed once with 1 volume of CH_3OH/water (1:1, v/v and dried with a nitrogen flow. Phospholipid content was determined according to Bőttcher et al. (1961), using phosphate (Pi) as a standard. From equal amounts of phospholipid, acylglycerolipids were hydrolyzed in $CHCl_3/CH_3OH$ (1:1, v/v) containing 0.1 M NaOH (0.1M) for 1 hr at 37 ºC The remaining lipids were re-extracted as described above and applied onto high performance thin layer chromatography (HPTLC) plates. Lipids were separated in $C_3H_6O_2/CH_3CH(OH)CH_3/CHCl_3/CH_3OH$/25% KCl (25:25:25:10:9, v/v/v/v/v), after which the plates were dried, and sprayed with 10% H_2SO_4 and 5% CH_3OH and heated to 120 ºC to visualize lipids. Glycosphingolipids were quantified using Scion Image software (Scion Corp., Frederick, MD).

Gangliosides extraction

Cells were washed three times with PBS and harvested by scraping in 1 ml PBS. Cells were centrifuged at 10.000 rpm at RT, and gangliosides were isolated from cell pellets, as described (Senn et al., 1998). Briefly, cell pellets were extracted in $CHCl_3/CH_3OH$ (1:1, v/v) and $CHCl_3/CH_3OH$ (2:1, v/v). The supernatants were pooled and dried with a nitrogen flow. Lipids were redissolved and sonicated in $CHCl_3/CH_3OH$ (1:1, v/v). After centrifugation and overnight storage at –20°C, the supernatants were collected and dried, and their phospholipid content was determined as described above. Equal amounts of phospholipid were redissolved in diisopropylether-1-butanol (3:2, v/v) by sonication after which 17 mM NaCl was added. The aqueous phase was re-extracted with diisopropylether-1-butanol and subsequently dried. Samples were dissolved in CH_3OH/water (1:1, v/v) and loaded onto prewashed Sep-Pak C18 cartridges. After washing with water, gangliosides were eluted with CH_3OH followed by $CHCl_3/CH_3OH$ (1:1, v/v). The eluate was concentrated by drying and loaded onto HPTLC plates. Gangliosides were separated in $CHCl_3/CH_3OH$/0.2% (w/v) $CaCl_2$ (11:9:2, v/v/v). Dried plates were sprayed with Ehrlich reagent, covered with a glass plate and heated to 180°C. Gangliosides were quantified using Scion Image software (Scion Corp., Frederick, MD).

GM1 quantification (dot blot)

The cells were washed three times with PBS and harvested by scraping into 1 ml PBS. Cells were centrifuged at 10.000 rpm at RT and pellets were lysed in TNE-lysis buffer (50mM Tris-HCL, 5mM EDTA, 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail). Protein determination was performed by a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA), using BSA as a standard. Equal amounts of protein in equal volumes (max. 10 µl) were applied onto nitrocellulose membrane. When dried, membranes were incubated for 1 hr at RT in blocking solution (5% nonfat dry milk in PBS). After washing, the membranes were incubated incubated for 2 hrs with HRP-conjugated in 1% nonfat dry milk in PBS containing 0.1% Tween 20. The membranes were washed three times with PBS containing 0.1% Tween 20. Signals were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech), scanned and processed using Paint Shop Pro and quantified with Scion Image software (Scion Corp., Frederick, MD).

Results

Glycosphingolipid characterization of primary rat oligodendrocytes and OLN 93 cells

In previous studies we and others have shown that ECM molecules play an important role in myelin membrane formation (Buttery and ffrench-Constant, 1999; Maier et al., 2005; Šišková et al., 2006 (*chapter 3*)). Whereas Ln2 is beneficial, Fn severely retards morphological OLG differentiation, and therefore myelin formation (Maier et al., 2005; Šišková et al., submitted (*chapter 4*)). As laminin is able to upregulate ganglioside synthesis in primary Schwann cells (Farrer and Quarles, 1996), the myelinating cells of the PNS, a role of gangliosides in ECM-dependent myelination could be anticipated.

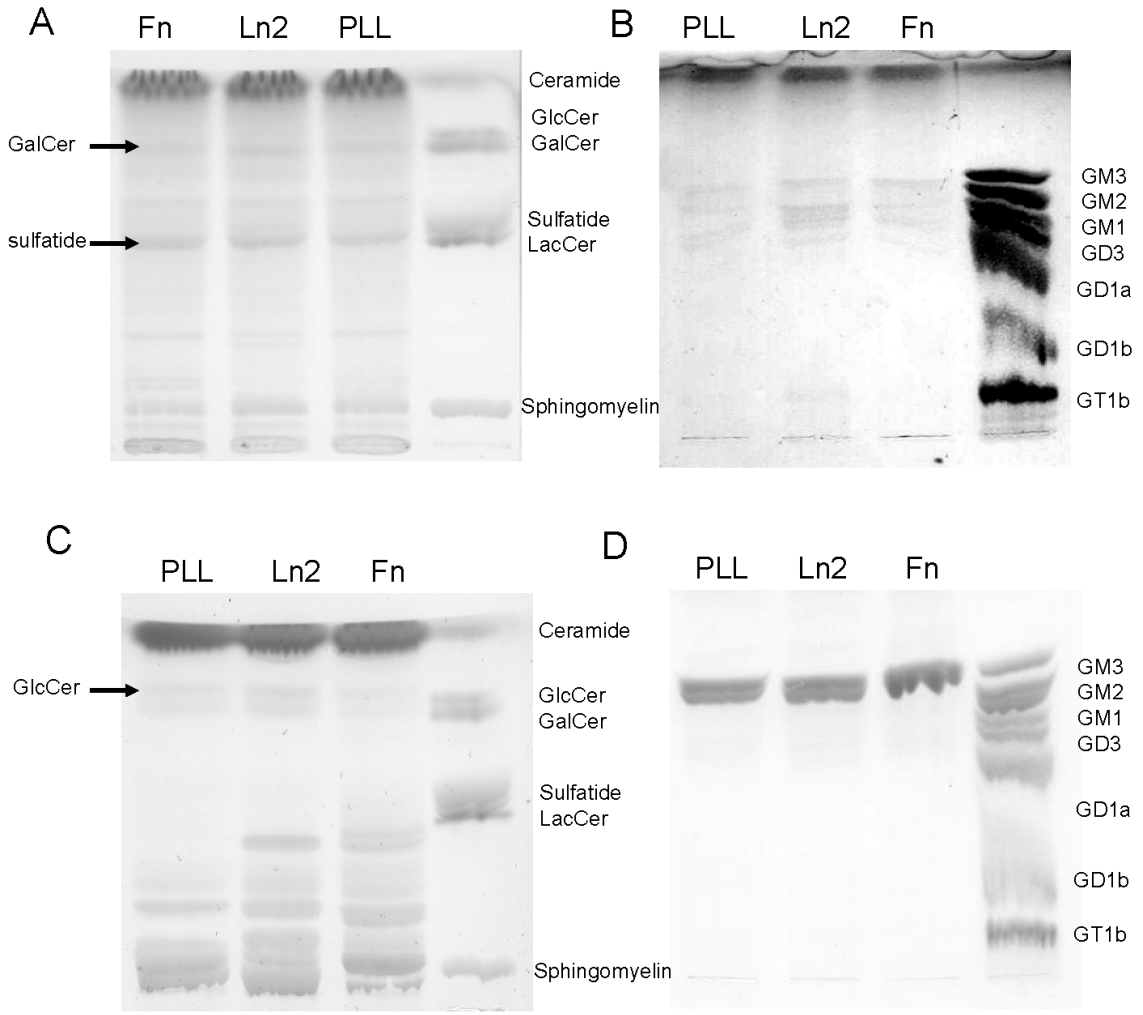

Figure 1. ***Glycosphingolipid expression in primary OLGs and OLN 93 cells on different extracellular matrix molecules.*** *Primary OLGs (A, B) and OLN 93 (C, D, 10% FCS) cells were cultured on laminin-2, fibronectin or poly-L-lysine for 7 days and 3 days respectively, after which neutral glycosphingolipids (A, C) and ganglioside (acidic glycosphingolipids, B, D) expression was analyzed as described in Materials and Methods. Representative TLCs of at least 3 independent experiments are shown. Note, the slight increase in the general expression of gangliosides, and GM3, GM1, GD3 and GT1b in particular, on laminin-2 as compared to poly-L-lysine and fibronectin in primary OLGs. In OLN 93 cells only ganglioside GM3 is expressed in detectable levels.*

Therefore, we examined ganglioside expression in mature myelinating OLGs, cultured on different ECM substrates, i.e. inert poly-L-lysine (PLL), Fn and Ln2. In parallel, the expression of the galactosphingolipids, galactosyl ceramide (GalCer) and its sulfated derivate, sulfatide, were analyzed, both of which are abundant in primary OLGs (Menon et al., 2003) and important for OLG maturation (Pfeiffer et al., 1993; Bansal et al., 1999; Hirahara et al., 2004). As shown in figure 1A, in primary OLGs major ECM-mediated differences in glycosphingolipid (GSL) expression were not observed. In contrast, ganglioside expression (GM3, GM1, GD3 and GT1b) on Ln2 as compared to PLL and Fn was slightly increased. (fig.1B). Next, we determined the GSL profiles in OLN 93 cells, previously shown to express similar properties in terms of trafficking of myelin proteins and cell differentiation as primary OLGs, when grown on different ECM substrates (Šišková et al., 2006 (*chapter 3*); Šišková et al., submitted (*chapter 4*)). As shown in figure 1C, proliferating OLN 93 express on all substrates similar levels of glucosylceramide (GlcCer), ceramide and the phospholipid sphingomyelin (SM), and hardly, it at all, the myelin typical lipids GalCer or sulfatide. Interestingly, on Ln2 and Fn additional yet unidentified lipids appeared. Intriguingly, however, ganglioside GM3 is the only detectable ganglioside in OLN 93, implying that these cells are completely lacking structurally more complex gangliosides (fig. 1D). Again, no quantitative effect on the extent of GM3 was seen when the cells were grown on different substrates. Hence, these data suggested that ECM molecules hardly, if at all, modulate glycosphingolipid expression in OLGs. To assess if gangliosides are involved in myelin membrane formation per se, we next examined, if OLG development is ganglioside-dependent.

Inhibition of glycosphingolipid biosynthesis impedes primary process extension in OLGs and gives rise to GalCer positive extracellular deposits

In order to reveal a role for gangliosides in OLG maturation *in vitro*, *de novo* biosynthesis of GSL was inhibited using pharmacological inhibitors. GlcCer synthase catalyzes the transfer of glucose of UDP-glucose to ceramide to form GlcCer, which is the common precursor of high-order glucosylceramide-based, GSL, including gangliosides. N-butyldeoxynojirijmycin (NBDNJ) inhibits both ceramide-specific glucosyltransferase and α-glucosidases and is a potent and non-cytotoxic inhibitor of GlcCer synthesis *in vitro* (Platt et al., 1998). DL-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol-HCL (PPPP) is another specific inhibitor of GlcCer synthase, thereby also inhibiting ganglioside synthesis, without significantly affecting intracellular levels of ceramide (Lee et al., 1999), GalCer and sulfatide (our unpublished observations). These inhibitors were added to OPCs, i.e. before the onset of myelin sheet formation. Furthermore, their effect was analyzed on all substrates, but only the results on the inert PLL substrate are shown, as virtually identical results were obtained on all ECMs examined. Given the limited amount of cells available, and hence the need

for microanalysis of the lipid pools we improved the sensitivity of lipid detection, a dot blot assay was applied.

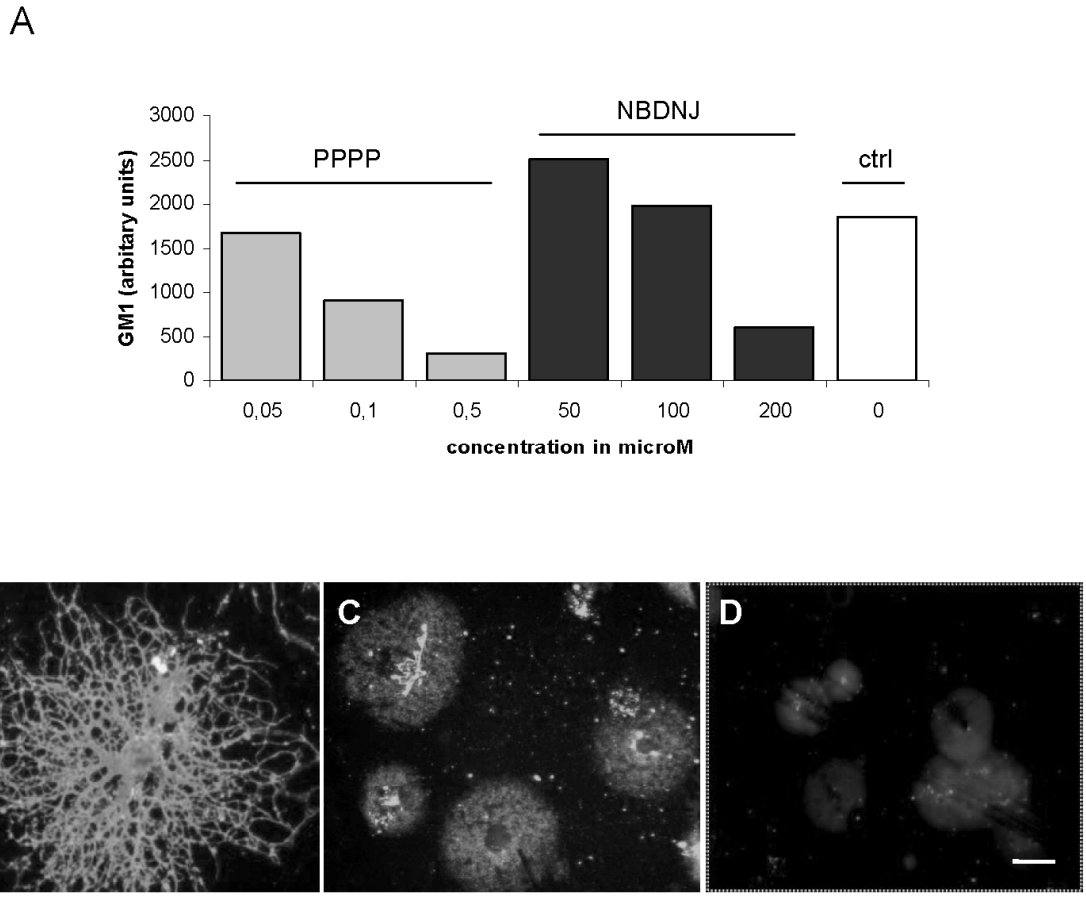

Figure 2. ***Localization of GalCer in primary OLGs upon inhibition glucosylceramide synthesis****. Primary OPCs were plated on poly-L-lysine, allowed to adhere, after which, NBDNJ and P4 at the indicated concentrations were added. These glucosylceramide synthesis inhibitors were continuously present in the culture medium. The cells were grown for 7 days, after which GM1 expression was determined (A) and GalCer distribution visualized (B-D) with immunofluorescence as described in Materials and Methods. A) cellular GM1 expression as quantified with a dotblot. Note, the dose-dependent decrease in GM1 expression by both inhibitors. A representative experiment out of two is shown. B) GalCer distribution in control cells, C) GalCer distribution in PPPP (0.5 µM)-treated cells, D) GalCer distribution in NBDNJ (200 µM)-treated cells. Representative pictures of at least 3 independent experiments are shown. Scale bar is 2 µm. Note that inhibition of glycosylceramide synthesis by both inhibitors (C, D) prevented morphological differentiation of OLG, and induced extracellular deposition of GalCer.*

Figure 2A shows a dose-dependent inhibition of GM1 expression with both inhibitors, and for further experiments, 0.5 µM PPPP (80-85 % inhibition) and 200 µM NBDNJ (65-70 % inhibition) were used. Whereas in control cells a highly branched GalCer positive network of processes extending from the cell body was formed (fig. 2B), in cells in which GlcCer biosynthesis was inhibited, process formation was impeded and extracellular deposits containing GalCer appeared, closely surrounding cell bodies. Inhibition of morphological differentiation and GalCer positive extracellular deposits were observed with both inhibitors (PPPP, fig. 2C, NBDNJ, fig. 2D). Hence, these data suggest that GlcCer synthesis is essential for morphological OLG differentiation. To assess if the depositing of GalCer-containing membrane structures was due to the absence of *de*

novo GlcCer biosynthesis *per se*, or due to the absence of a GlcCer-based GSL, such as gangliosides, we next examined whether OLN 93 cells, which lack complex gangliosides and only express ganglioside GM3 (fig. 1D), could also form similar deposits.

GalCer-expressing OLN-G cells, but not OLN-MOCK cells, form extracellular deposits

In order to investigate the relation between a particular GlcCer-based GSL and deposition of GalCer positive extracellular membrane structures, we examined GalCer deposition in OLN 93 cells that only express the relatively simple gangliosde GM3 (fig.1D). As they also express rather low GalCer levels (fig.1C), we first retrovirally overexpressed galactosyl ceramide transferase (cgt) to improve GalCer biosynthesis in OLN 93 cells, hereafter referred to as OLN-G. As compared to control mock transfected cells (MOCK; fig. 3A, line 1), infected OLN-G cells significantly overexpress GalCer (fig. 3A). A double band is visible, representing non-hydroxylated (upper band) and hydroxylated (lower band) GalCer. In addition, low but detectable levels of sulfatide were seen in OLN-G cells, however these levels were too low for immunofluorescent detection.

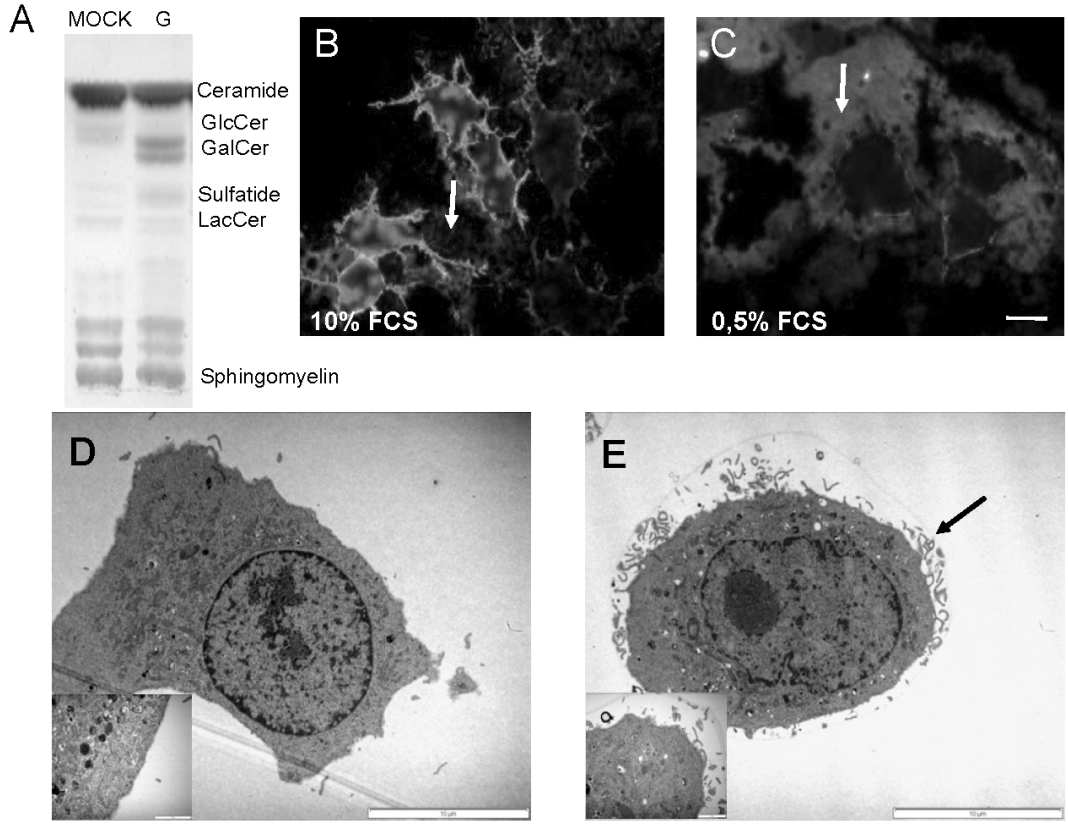

Figure 3. ***GalCer localization in GalCer-overexpressing OLN 93 cells.*** *OLN-MOCK (D) and GalCer-overexpressing OLN-G cells (B, C, E) were generated as described in Materials and Methods. Cells were cultured on poly-L-lysine for 3 days (B, C) or 24 hrs (D, E) in either 10% (B) or 0.5% FCS (C-E) and GalCer distribution (B, C) and membrane integrity using transmission electronmicroscopy (D, E) were analyzed as described in Materials and Methods. A) Neutral glycosphingolipid expression in OLN-MOCK and OLN-G cells. Note that GalCer is significantly overexpressed in OLN-G as compared to OLN-MOCK. B) GalCer distribution in OLN-G cells, 10% FCS, C) GalCer distribution in OLN-G cells, 0.5% FCS. Representative pictures out of at*

least 3 independent pictures are shown. Scale bar is 2 μm. Note, that as in primary cells, GalCer-positive deposits are formed (arrows). D) OLN-MOCK, 0.5% FCS, E) OLN-G, 0.5% FCS. Representative images out of at least 3 independent experiments are shown. Note that the membrane integrity is severely impeded in OLN-G cells as compared to OLN-MOCK cells, indicating that GalCer expression is essential for extracellular deposit formation. Scale bar is 10 μm.

Remarkably, the OLN-G cells were in general more spread and fewer processes were formed (data not shown). Interestingly, however, the OLN-G cells spontaneously formed GalCer-positive extracellular deposits surrounding the cell body (fig. 3B), similarly as observed for primary OLGs, except that in these cells the effect was only seen upon inhibition of GlcCer biosynthesis. Furthermore, GalCer deposition was even more prominent upon serum withdrawal, i.e. upon triggering differentiation of the cells (fig. 3C). Importantly, as in OLN 93 cells, only ganglioside GM3 is expressed in OLN-MOCK and OLN-G cells (data not shown). Intriguingly, as examined with transmission electron microscopy (TEM), in OLN-G cells substantial degeneration of the plasma membrane was observed, as evidenced by secreted membranous structures (fig. 3E). These secreted membrane-like structures were not detectable in OLN-MOCK cells (fig. 3D) that express relatively low levels of GalCer (fig. 3A), thus suggesting that a relative excess of GalCer, artificially induced in control cells in which overall GSL biosynthesis is impaired, is instrumental in the formation of these deposits.

Extracellular deposits in OLN-G cells contain membrane proteins and cytoskeletal components

To further analyze the composition of the extracellular deposits formed in OLN-G cells, the distribution of several plasma membrane proteins at the cell surface was compared in control cells and OLN-G cells, using immunofluorescence. Whereas in OLN-MOCK, the distribution of the transmembrane spanning protein NCAM 140 randomly localized to the plasma membrane (fig. 4A), in OLN-G cells, NCAM 140 is not associated with the cell boundaries (fig. 4B) but co-localized with the GalCer positive deposits (fig. 4D). In contrast, CNP, known to be associated with the inner leaflet of the plasma membrane only (Braun et al., 1991) is similarly distributed in OLN-G (fig. 4F) as in OLN-MOCK (fig. 4E) cells, i.e. very little if any colocalisation (fig. 4H) with the GalCer positive deposits (fig. 4G). As process formation was severely impeded both upon inhibition of GlcCer synthesis in primary OLGs and in OLN-G cells, we next analyzed the localization of the cytoskeletal proteins actin and β-tubulin. The distribution of the actin cytoskeleton, as visualized with phalloidin-TRITC, is similar in OLN-MOCK (fig. 4I) and OLN-G (fig. 4J), and no colocalisation of actin with extracellular deposits was detected (fig. 4J-L). In contrast, β-tubulin, known to be primarily present in primary processes of OLGs (Wilson an Brophy, 1989), colocalised with the extracellular deposits in OLN-G (fig. 4N-P), whereas in OLN-MOCK the β-tubulin distribution is confined to the cells (fig. 4M).

Therefore, these data suggest that the plasma membrane of OLN-G cells is severely disorganized, with a concomitant membrane destabilization, which apparently resulted in the extracellular deposition of outer leaflet membrane components (GalCer), transmembrane spanning components (NCAM 140) and β-tubulin, whereas the actin cytoskeleton, and inner leaflet associated proteins (CNP) remained cell-associated. Intriguingly, in spite of this apparent perturbation, OLN-G cells are still capable to grow and proliferate. As similar deposits were observed in primary OLGs in which overall GlcCer biosynthesis had been inhibited, and spontaneously in OLN-G cells, that overexpress GalCer and only express GM3 as the sole ganglioside, we next examined whether a complex ganglioside might be involved in affecting OLG membrane integrity.

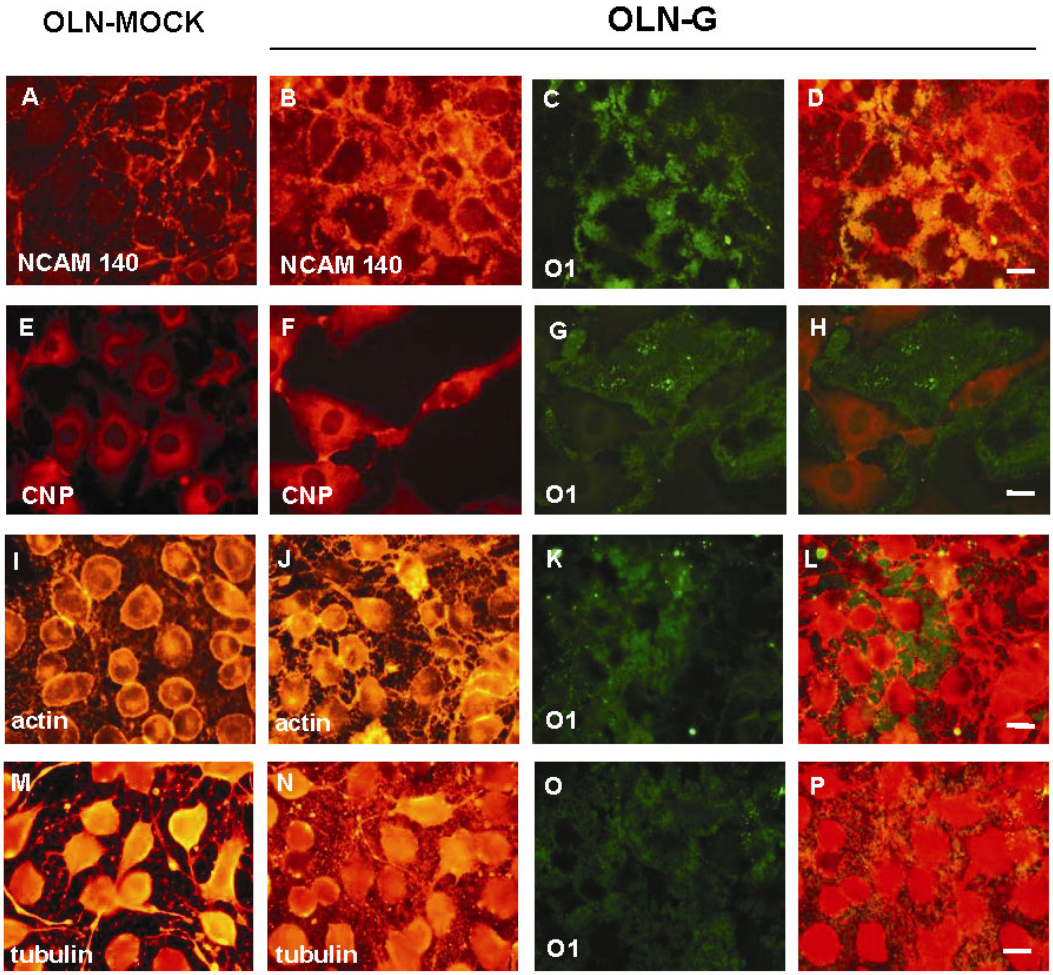

Figure 4. ***Immunofluorescent characterisation of extracellular deposit content.*** *OLN-MOCK (A, E, I, M) and OLN-G cells (B-D, F-H, J-L, N-P) were cultured for 3 days on poly-L-lysine in 0.5% cells were immunolabeled against NCAM 140 (A, B), CNP (E, F) and β-tubulin (M, N). Actin filaments (I, J) were visualized with TRITC-labelled phalloidin. Representative pictures of at least 3 independent experiments are shown. Scale bar is 2 µm. In OLN-G cells deposits were visualized with O1, an anti-GalCer antibody (C, G, K, O), and co-localisation with the indicated components is visualized in the overlay images (D, H, L, P). Note, that NCAM 140 and β-tubulin were part of the extracellular deposits, whereas CNP and actin remained cell-associated.*

Exogenous addition of GT1b counteracts the formation of extracellular deposits

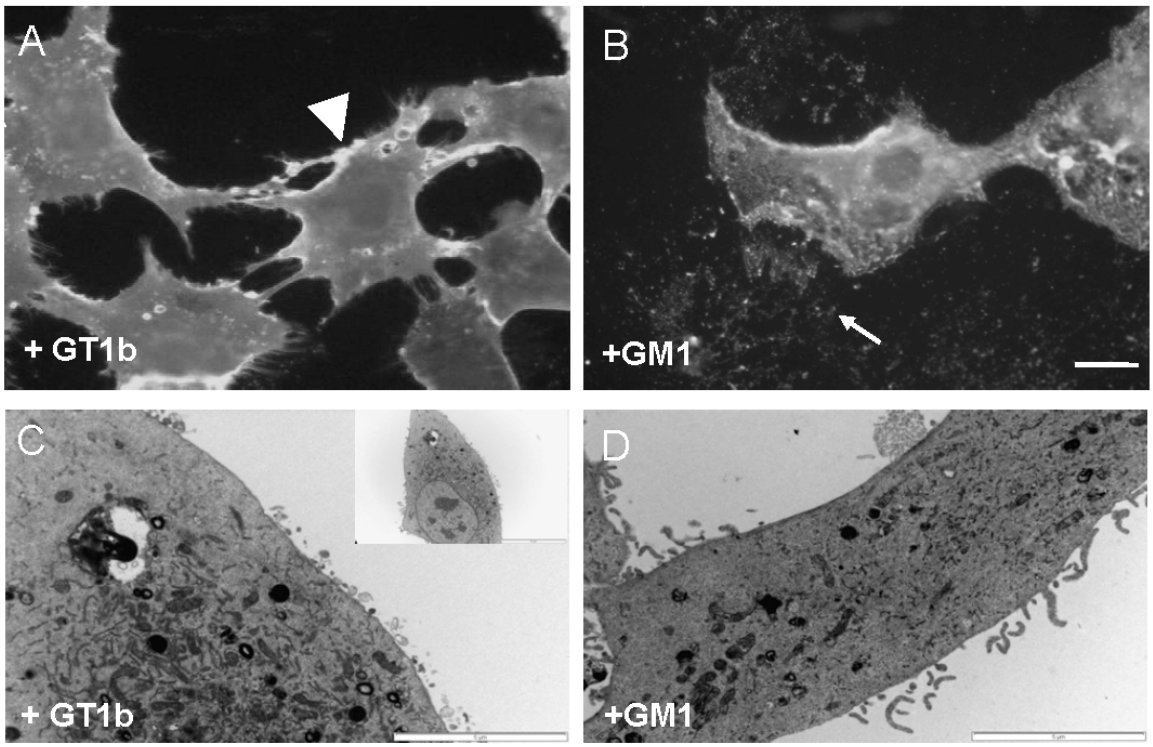

Figure 5. ***Effect of exogenous ganglioside addition on extracellular deposit formation in OLN-G cells.*** *OLN-G cells were plated on poly-L-lysine, allowed to adhere, after which, 25 µM GT1b (A, C) or 25 µM GM1 (B, D) were added, after which the cells were cultured for 3 days in 0.5% FCS and GalCer distribution (A, B) and membrane integrity (C, D) analyzed as described in Materials and Methods. A) GalCer distribution upon GT1b addition, B) GalCer distribution upon GM1 addition. Representative pictures out of at least 3 independent experiments are shown. Scale bar is 2 µm. Note that, in contrast to GM1 addition (B, arrow), GT1b addition (A) prevented extracellular deposit formation, and allowed GalCer incorporation in the plasma membrane (arrowhead). C) representative TEM picture upon GT1b addition, D) representative TEM picture upon GM1 addition. Note that GT1b but not GM1 addition increased membrane integrity in OLN-G cells. Scale bar is 5µm.*

To examine whether complex gangliosides might be involved in modulating myelin membrane integrity, we next determined if exogenous administration of gangliosides could prevent GalCer deposit formation. Indeed, exogenously added gangliosides have been shown to readily insert into the plasma membrane, participating in ganglioside metabolism and cellular signalling (Keenan et al., 1974; Fishman et al., 1977; Masco et al., 1989; Leskawa et al., 1989). Figure 5 shows that addition of structurally complex ganglioside GT1b to OLN-G cells resulted in GalCer positive plasma membranes, i.e., marking a clear membrane boundary without extensive deposit formation or membrane ruffling (fig 5A). Hence, these data suggested that membrane inserted GT1b stabilized the membrane structure. The effect of GT1b appears to be specific, as upon addition of ganglioside GM1, deposit formation in OLN-G cells was not inhibited (fig. 5B), showing a membrane ruffling as in OLN-G control cells (fig. 2). These immunofluorescence data were confirmed with TEM. Thus, addition of GT1b (fig. 5C) to OLN-G cells resulted in a significant diminishment of membrane disruption, in contrast to addition of GM1 (fig. 5D), which was essentially without effect. Administration of GT1b or GM1 had no effect on

the morphology and membrane integrity of OLN-MOCK cells (data not shown). Taken together these data suggest an important role for complex ganglioside GT1b, in membrane integrity, possibly by harbouring excess amounts of GalCer synthesized upon OLG differentiation.

Discussion

In the present study we show that modulation of the level of the GSL GalCer, relative to that of other GSLs, either by its overexpression per se (in OLN cells) or upon inhibition of GlcCer biosynthesis, which also blocks complex ganglioside biosynthesis but not that of GalCer, destabilizes the membrane structure of OLG, the myelinating cells in brain. The severe degeneration of the membrane is evidenced by extracellular deposition of GalCer-positive membranous structures surrounding the cell body. Since exogenous addition of the ganglioside GT1b counteracts this disintegration the data might support the notion that a perturbation of GSL homeostasis is detrimental for OLG membrane stability and hence myelin assembly and stability. The data might thus suggest a role for complex gangliosides in adjusting the lateral membrane environment to harbour high GalCer levels. Indeed, excess GalCer, as induced in overexpressing OLN-G cells is adequately sequestered within the membrane without extensive disruption upon exogenous addition of GT1b, but not GM1. Hence, these data suggest potential specificity of a role for particular gangliosides in controlling myelin membrane integrity, allowing morphological OLG differentiation and process extension in particular. Consistent with this notion, myelination defects are observed in transgenic mice lacking complex gangliosides (Sheikh et al., 1999; Kawai et al., 2001; Ma et al., 2003, Yasmashita et al., 2005). Furthermore, ganglioside synthesis and expression in axons is essential for proper axon to glia communication (Vinson et al., 2001; Vyas and Schnaar, 2001). Myelin-associated glycoprotein (MAG) localised in the myelin membrane adjacent to the axon, is a ligand for neuronal GD1a and GT1b (Yang et al., 1996, Vinson et al., 2001; Vyas and Schnaar, 2001; Vyas et al., 2002), and this interaction stabilizes axon-myelin interactions (Sun et al., 2004). However, the data in the present study indicate that for stabilization of the myelin membrane, ganglioside expression, and possibly GT1b in particular, in OLGs is essential as well. Thus our findings that membrane integrity appears to be highly dependent on a proper ratio of GalCer and complex gangliosides, could provide an indication for possible cellular mechanisms underlying the disordered myelination observed in all transgenic animals, lacking complex gangliosides, and hence expressing a relative excess of GalCer. The observation that the phenotypes of GalCer-deficient or complex ganglioside-deficient mice are very similar,i.e., in both cases reflecting a perturbation in GalCer/Ganglioside

ratio, is in support of this (Coetzee et al., 1996; Honke et al., 2002; Yamashita et al., 2005).

The present study showed also an impaired morphological differentiation of primary OLG progenitors upon inhibition of GlcCer synthesis. Strikingly, process outgrowth was severely impeded, and primary process were hardly, if at all, observed, suggesting that gangliosides may play a functional role in this process. Indeed, exogenous addition of ganglioside GM3 stimulates process outgrowth by cultured bovine OLGs, resulting in process elongation and extensive process branching (Yim et al., 1991, 1994). Similarly, exogenously administered gangliosides to primary neurons in vitro stimulates neurite sprouting and extension (Roisen et al., 1981; Ledeen, 1984; Skaper et al., 1985), and accumulation of gangliosides within neurons in ganglioside storage diseases results in extensive neurite outgrowth (Purpura, 1987). Extensive cytoskeletal remodelling is needed for process sprouting and extension, and in OLGs microtubules are specifically present in primary processes (Wilson and Brophy, 1989). In this respect, our finding that β-tubulin, but not actin, is present in the extracellular deposits, corroborates the notion that in the absence of complex gangliosides and high GalCer levels, primary process formation is deregulated. Whether ganglioside GT1b is involved in primary process formation remains to be determined, however, since GT1b in OLGs is specifically localized to the cell body and in a patchy appearance at branching points (Schnaar et al., 1996), such a role could be readily foreseen.

Gangliosides reside in the outer leaflet of the plasma membrane and there is ample evidence (Ledeen, 1985; Thomas and Brewer, 1990; Yim et al., 1991; Probstmeier et al., 1999) that next to a structural function, ganglioside have a role as active signaling partners, especially in regulating cellular interactions. For example, gangliosides may participate in the organization of the membrane by directing adhesion receptors to discrete areas. Indeed, the function of integrins, the main receptors of ECM molecules, are affected by endogenous metabolism and exogenous addition of gangliosides (Ono et al., 2001; Satoh et al., 2001). In OLGs, ganglioside-mediated signaling regulates integrin-mediated adhesion of OLGs progenitors to the ECM molecule tenascin-R (Probstmeier et al., 1999). Intriguingly, previous studies (Matyas et al., 1986) and our preliminary experiments suggest that fibronectin binds to ganglioside GT1b, which could explain the hampered process formation and myelination on fibronectin (Buttery and ffrench-Constant, 1999, Maier et al., 2005, Šišková et al., 2006 (*chapter 3*); Šišková et al., submitted (*chapter 4*), i.e. fibronectin-mediated adhesion to ganglioside GT1b could preclude its role in morphological OLG differentiation. Therefore, ganglioside GT1b-ECM interactions may contribute to OLG myelination.

Interestingly, in an MS animal model, chronic relapsing experimental allergic encephalomyelitis, GM1 levels increase and GT1b levels decrease, suggesting a role for gangliosides in autoimmune demyelination (Zaprianova et al., 1998). Indeed, numerous

studies have focused on the involvement of gangliosides in autoimmune demyelinating diseases (Rapport and Huang, 1985; Schwerer et al., 1986; Stevens et al., 1992). In fact, an MS-like disease develops in rabbits upon immunization with brain gangliosides (Cohen et al., 1981). Moreover, antisera against GM1, GD1a, GD1b and GT1b induce demyelination in spinal cord and spinal roots (Schwerer et al., 1986). Intriguingly, the cellular mechanisms underlying gangliosides-induced demyelination are unknown. However, with the proposed role of complex gangliosides in OLG membrane integrity, it is very well possible that modulation of ganglioside levels disturbs the organization of myelin membranes, resulting in extracellular GalCer deposition, and leading to demyelination. These considerations justify further detailed work on the role of glycosphingolipids in determining myelin membrane stability, in particular in the context of a search for novel targets in treatment of demyelinating diseases.

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