CHAPTER 4

Identification of oligodendrocyte counterreceptors for galectin-4 that promotes myelin membrane formation together with the remyelination inhibitor fibronectin

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ABSTRACT

Neuronal galectin-4 (Gal-4) is an inhibitor of oligodendrocyte progenitor cell differentiation that is transiently re-expressed upon demyelination. Gal-4 expression persists in demyelinated multiple sclerosis (MS) lesions, likely contributing to remyelination failure. As a step to disclose its mechanism of action, pull-down assays led to mass spectrometric identification of counterreceptors presented on the surface of oligodendrocytes. UDP-galactose: ceramide galactosyltransferase (UGT8, also named CGT), and contactin-1, an adhesive protein involved in the onset of myelin basic protein expression, were identified as the prominent oligodendroglial surface binding partners, along with two Rab proteins. Using a sulfatide-specific antibody, chlorate treatment and engineered cell lines, this glycosphingolipid was found to be a minor ligand for Gal-4 in these cells, in contrast to the situation in axons and enterocytes. UGT8, the enzyme that turns ceramide by β-galactosylation into galactosylceramide (GalCer), was found to be transiently present at the proximal part of primary processes of immature oligodendrocytes. Upon binding of the bivalent Gal-4, GalCer levels in membrane microdomains were reduced on an inert substratum, but not on a fibronectin substratum, a remyelination inhibitor present in MS lesions. Rather unexpectedly, in functional assays designed to direct (re) myelination, and in contrast to their individual activities, simultaneous presence of Gal-4 and fibronectin effectively promoted myelin membrane formation *in vitro*. The presented results guide to analysis of post-binding effects of Gal-4, likely involving cross-linking and signaling, with the aim to find innovative ways to eventually promote remyelination in MS lesions.

INTRODUCTION

Loss of myelin by a chronic autoimmune process and neurodegeneration are the hallmarks of multiple sclerosis (MS)[1]. Remyelination, i.e., the regeneration of myelin membranes, could restore saltatory conduction and prevent (secondary) neurodegeneration [56–58,436], but the efficiency of this process is often compromised in progressive MS [41,59,279]. Oligodendrocyte progenitor cells (OPCs) that are present in approximately 70% of MS lesions [14,15,40], are relatively quiescent, also at the transcriptional level [437,438], and likely fail to differentiate into mature myelinating oligodendrocytes (OLGs) [15,59]. A non-permissive microenvironment, including a dysregulated remodeling of extracellular matrix (ECM) components can account for the failure of OPC differentiation [439-441]. For example, fibronectin is a major ECM glycoprotein that accumulates in MS lesions in aggregates and impairs remyelination [30,442].

Giving this study direction, the observation that neurons secrete the endogenous lectin galectin-4 (Gal-4), a negative regulator in the timing of OPC differentiation [49], suggests that glycan-protein recognition is involved in the dysregulation of OPC differentiation. In principle, endogenous lectins are the 'readers' of glycan-encoded information presented by cellular glycoconjugates [72,350]. Whereas negative neuronal effectors for OPC differentiation, including Gal-4, are transiently re-expressed upon a demyelinating event, they persist in MS lesions [8,35,36,42,147,359,443]. Since enzymatic removal of the polysialic acid chains of NCAM by endoneuraminidase N and presence of anti-LINGO1 antibodies increase remyelination in experimental rodent models [35,359,443], a targeted intervention of neuronal effectors has potential for establishing remyelination, hereby halting disease progression. In this sense, the elucidation of binding partners for Gal-4 and the characterization of its complete functional profile *in vitro* are steps into this direction.

Gal-4 belongs to a family of multifunctional β-sandwich-type proteins that translate glycan(β-galactoside)-encoded signals into diverse context-dependent cellular effects [80,365,444]. Its modular architecture with two different carbohydrate recognition domains (CRDs) predestines the protein to bridge counterreceptors, for example hereby acting as a raft stabilizer when leading to the formation of "superrafts" and as

cargo transporter during routing of distinct glycoproteins in enterocytes (digestive enzymes such as dipeptidyl peptidase IV) and axons [112,152,167,367]. Fittingly, the integrity of the bivalency is essential, also in its role as negative regulator of OPC differentiation during CNS development [49].

With the aim to contribute to find a way to restore the capacity for myelin regeneration, the present study was undertaken to identify the nature of the counterreceptor(s) of Gal-4 at the surface of oligodendrocyte lineage cells. Since the profile of binding partners is known to depend on the cell type, with sulfatide as well as the cell adhesion molecule L1 and contactin-1 on the surface of neurons [152,153], sulfatide and carcinoembryonic antigen on enterocyte-like cells along with the apically transported digestive enzymes [112,167,445] and CD14 on monocytes [446], a simple extrapolation is not valid. Due to its abundance in the nervous system, sulfatide is a prime candidate for being a counterreceptor on the oligodendroglial surface. Favoring this idea, the sulfatide headgroup is known to be present on the surface of developing OLGs [121,447] and acts - as neuronal Gal-4 does [49] - as a negative regulator of OPC differentiation [184,185,448]. Our findings based on immunocytochemistry and blocking access by antibody, on metabolic inhibition of biosynthesis and assays on engineered cells revealed that sulfatide was not a main counterreceptor for Gal-4 in OLGs. Instead, Gal-4 bound to UDP-galactose: ceramide galactosyltransferase (UGT8), the enzyme producing galactosylceramide (GalCer), and contactin-1 at primary processes of immature OLGs. GalCer is a major myelin-stabilizing constituent and the substrate for generating sulfatide by its 3-O-sulfotransferase [449]. When tested as effector, Gal-4 interfered with the lateral membrane distribution of GalCer of OLGs plated on a non-natural substratum, but not when plated on MS-relevant fibronectin. Intriguingly, the presence of both myelination-inhibiting components, i.e., Gal-4 and fibronectin, induced myelin membrane formation *in vitro*. This may open a new therapeutic avenue to overcome remyelination failure in MS lesions.

MATERIALS AND METHODS

Primary oligodendrocyte cultures

Experimental animal protocols for primary cell cultures were approved by the Animal Ethics Committee of the University of Groningen (the Netherlands). All methods were carried out in accordance with the European Directive (2010/63/EU) on the protection of animals used for scientific purposes. Primary glial cell cultures were generated from postnatal day 1-3 Wister rats (Charles-River) as described [375,419]. In brief, a digestion mix (papain at 1.2 U/ml (Sigma), L-cysteine at 0.24 mg/ml (Sigma), DNase I at 40 mg/ml (Roche Diagnostics)) was added to the meninges-free cortices, which were then incubated for 45 minutes at 37°C. Pelleted cells were resuspended and plated in mixed glia culture medium (DMEM supplemented with 10% fetal bovine serum (FBS; Capricorn), 1% L-glutamine and 1% penicillin/streptomycin) on poly-L-lysine (PLL, 5 µg/ml; Sigma)-coated 80 cm^2 flasks (Nunc). Medium was exchanged every 3-4 days until after 11-12 days a confluent layer of astrocytes was formed to which microglia and OPCs adhere. Contaminating microglial were removed by a 1-hour shake-off procedure at 37°C on an orbital shaker (Innova 4000; Newbrunswick Scientific at 150 rpm). OPCs were thereafter detached by a 16-hour shake-off at 240 rpm. Contaminating microglia and astrocytes were removed by differential adhesion on non-tissue cultured dishes (Greiner). Pelleted OPCs were resuspended in SATO medium [375,419] and plated on PLL- or fibronectin (Fn, 10 µg/ml; Sigma)-coated 10-cm dishes (Nunc) at a density of 800,000 cells/dish or on 13-mm PLL-coated glass coverslips (VWR International) at a density of 30,000 cells/coverslip. After 1 hour, the medium was supplemented with FGF-2 (10 ng/ml; Peprotech) and PDGF-AA (10 ng/ml; Peprotech). To induce differentiation, medium was changed to SATO supplemented with 0.5% FBS two days after plating. After initiating differentiation, cells were either left untreated or treated for 4 days with fumonisin B1 (FB1, 15 µM; Sigma) that inhibits sphingolipid biosynthesis by inhibiting the sphingosine-N-acetyltransferase or sodium chlorate (30 µM; Sigma) that inhibits sulfation of galactosylceramide to produce the sulfatide (3-O-sulfated galactose) headgroup. Cells were either cultured for 4 days to obtain immature OLGs or for 6 days to obtain mature OLGs.

Cell lines

The rat-derived oligodendroglia lines OLN-PLP-MOCK (vector only) and OLN-PLP-G (expressing UGT8 and GalCer) were generated and cultured as described [186,187,450]. Cells were cultured in DMEM supplemented with penicillin/streptomycin, L-glutamine and 10% FBS. For biochemical analysis, cells were plated on 10 cm the surface of dishes (Corning Costar) at 400,000-600,000 cells per dish and for immunocytochemical analysis on PLL-coated 13-mm glass coverslips (VWR) in a 24-well plate (Corning Costar) at a cell count of 12,000-24,000 cells per well. Cells were analyzed after 3 days in culture.

Immunocytochemistry

Staining of surface components was performed on live cells on ice. All antibody dilutions were prepared in PBS containing 4% bovine serum albumin (w/v; BSA). For ligand binding assays, non-specific binding was blocked by incubating cells with this solution for ten minutes. This period was followed by a 30-minute incubation with solution containing biotinylated Gal-4 (bGal-4, 20 µg/ml; prepared under activity-preserving conditions and checked for maintained activity as described [451,452]). Full-length Gal-4, its two types of CRDs and a variant without the sequence of the 42-amino-acid linker were produced and processed as described [176]. After washing, cells were incubated with solution containing streptavidin (SA)-TRITC (30 µg/ml; Jackson-ImmunoResearch Laboratories) for 25 minutes on ice. For assays in competitive setting, the incubation with labelled Gal-4 is either preceded or followed by exposure to either antibody O1 (anti-GalCer, 1:10; kind gift of Guus Wolswijk [453]) or antibody O4 (anti-sulfatide, 1:1; kind gift of Guus Wolswijk [453]) and its detection by FITC- or TRITC-labeled anti-IgM antibodies (Jackson-ImmunoResearch). For double or single stainings of endogenous antigens, cells were fixed with 4% paraformaldehyde (PFA) and nuclei were counterstained with DAPI (1 µg/ml; Sigma). For co-localisation after the fixation step during the ligand-binding assay and for immunocytochemistry on PFA-fixed cells, cells were permeabilized and protein-binding sites were saturated for 30 minutes by using PBS containing 0.1% Triton X-100 (TX-100) and 4% BSA. This was followed by 30 minutes of incubation with solution containing a primary antibody, i.e., anti-UGT8 (1:100; Abcam, AB100964), anti-MBP (1:250; Serotec, MCA409), or anti-contactin-1 (1:25; Santa Cruz, SC20297). Subsequently, cells were washed three times with PBS and then incubated for 30

minutes with solution containing the appropriate FITC- or TRITC--labeled secondary antibody (30 µg/ml; Jackson) and DAPI. Cells were washed three times with PBS, mounted using Dako mounting medium and analyzed with a Leica TCS SP8 confocal laser scanning microscope or a Leica DMI 6000 B conventional fluorescence microscope. Data are processed using Adobe Photoshop software. In order to determine the extent of differentiation, approximately 250 DAPI-stained cells were scored as either myelin basic protein (MBP)-negative or MBP-positive in each independent experiment. In addition, to assess myelin membrane formation, MBP-positive cells were classified as myelin membrane-forming or non-myelin membrane-forming.

Surface biotinylation

Cells were washed twice with ice-cold PBS followed by a 1-hour incubation with solution containing sulfo-NHS-L-C-biotin (100 µg/ml; Pierce) at 4°C. Cells were washed three times for two minutes with cell wash buffer (CWB; 50 mM Tris–HCl containing 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at pH 7.5) and twice with ice-cold PBS. To obtain whole cell lysates, cells were scraped in TNE-lysis buffer (50 nM Tris-HCl, 5mM EDTA, 150 mM NaCl, 1% Triton X-100 (Sigma) and protease inhibitor cocktail (Roche), kept on ice for 30 minutes, and stored at -20°C until further use.

Subcellular fractionation

Cells were scraped in subcellular fractionation buffer (20 nM HEPES containing 250 mM sucrose, 10 mM KCl, 1,5 mM $MgCl_2$, 1mM EDTA, 1 mM EGTA, 1 mM dithiotreitol (Roche) and protease inhibitor cocktail; pH 7.4). The suspension was passed 20 times through a 25G needle and left on ice for 20 minutes. To remove the nuclear fraction, the suspension was centrifugated for five minutes at 700g at 4°C. To separate the membrane fraction from the cytoplasmic fraction, the supernatant was centrifugated at 9,300g for 30 minutes at 4°C. Proteins in the supernatant (cytoplasmic fraction) were precipitated with trichloroacetic acid (TCA) 192. TCA-precipitated cytoplasmic and membrane (pellet) (glyco)proteins were dissolved in TNE-lysis buffer, kept on ice for 30 minutes and stored at -20°C until further use.

Affinity pull-down

Protein concentration of membrane fractions was determined using the Bio-Rad DC Protein Assay (Bio-Rad) and BSA as a standard. To obtain Gal-4-binding glycoconjugates for analysis by biotin-streptavidin complex generation, membrane fractions (60 µg) or Optiprep gradient fractions (250 µl) were incubated with streptavidin-agarose beads (SA-beads; Millipore), to which biotinylated Gal-4 (bGal-4, 20 µg/mL) was associated by gentle head-over-head spinning overnight at 4°C. The supernatant was collected and subjected to TCA precipitation; pellet was washed four times with AP-wash buffer (APW; CWB buffer supplemented with 350 nM NaCl and 1% NP-40) and once with PBS. To precipitate (glyco)proteins present at the cell surface, membrane fractions (60 µg) or Optiprep gradient fractions (250 µl) obtained from surface-biotinylated cells were incubated with SA-beads overnight at 4°C, gently mixing the suspension as above, pellet and supernatant processed as above. For Western blot analysis, samples were re-suspended in reducing sample buffer and heated for 5 minutes at 95 °C.

Proteomics

Gal-4-binding glycoconjugates obtained from membrane fractions were subjected to SDS-PAGE under reducing conditions. Gel lanes were cut in small pieces followed by reduction with dithiotreitol, alkylation with iodoacetamide and in-gel trypsin digestion. Peptides were extracted using 66% acetonitrile/5% formic acid, dried in a speedvac, and reconstituted in 0.1% formic acid. Tryptic digests were analyzed by liquid chromatography coupled to a high-resolution Q-Exactive plus (Thermo Fisher Sicentific) using nanoelectrospray as an ion source. Peptide identification was performed with PEAKS software (release 8.5). Of the positive hits (FDR 1%), only proteins that have at least one unique peptide identified, are consistently present in both biological replicates, are known or predicted to be associated with membranes and/or are known to be glycosylated according to UniProt were taken into account.

Optiprep density gradient centrifugation

Discontinuous gradients were prepared from Optiprep™ (Axis Shield) as described [450]. Briefly, equal amounts of protein (250 µg) in 250 µl were added to 500 µl 60% OptiprepTM. This 40% OptiPrep solution was overlaid with a layer of 30% (2.3 ml) and 10% (2.1 ml) Optiprep™. Gradients were centrifugated overnight at 152,000g (Sorvall Discovery™ 90SE) in a SW55 rotor at 4˚C. Seven fractions were collected

from top (fraction 1, 500 µl) to bottom (fractions 2-7, 750 µl), and equal volumes were subjected to affinity precipitation, dot blot and Western blot analyses.

Dot blot analysis

Equal amount of proteins (5 µg in 5 µl) or volumes (10 µl for gradient fractions) were spotted onto nitrocellulose membrane (Bio-Rad). Membranes were exposed to Odyssey blocking buffer for one hour (1:1 with PBS; Li-Cor) to preclude non-specific binding of cellular (glyco)proteins, followed by overnight incubation with solution containing primary antibodies at 4˚C. Used primary antibodies are mouse IgM monoclonal antibody O1 (1:1000), and mouse IgM monoclonal antibody O4 (1:500). To detect ganglioside GM1, biotinylated cholera toxin (subunit B pentamer) (1:1500; Sigma) was used. After washing three times with PBS supplemented with Tween-20 (PBS-T), membranes were incubated with solution of the appropriate IRDye-labeled secondary antibodies or SA (1:5000, Li-Cor Biosciences) for 1 hour at room temperature. Membranes were washed three times with PBS-T, after which signals were detected using an Odyssey Infrared Imaging system (Li-Cor Biosciences). Quantification was performed with Scion Image software.

Western blot analysis

Equal volume (gradient fractions, 60 µl) or proteins amounts (cell lysates, 20 µg) were mixed with reducing sample buffer and heated for five minutes at 95˚C. Samples were loaded onto 10% or 12.5% SDS-gels and (glyco)proteins were transferred onto PVDF membrane (Immobilon®; Millipore, Bedford, MA) by electroblotting. The membrane was exposed for one hour to Odyssey blocking buffer followed by incubation overnight with solution containing primary antibodies at 4°C with the following primary antibodies: rabbit polyclonal anti-UGT8 (1:750; Abcam), goat polyclonal anti-contactin-1 (1:100; Santa Cruz), mouse monoclonal antibody anti-actin (1:3000; Sigma, A5441), mouse monoclonal anti-NCAM (1:250; Sigma, C9672), rabbit polyclonal anti-caveolin (1:1000; BD Biosciences, 610060), mouse monoclonal anti-RhoGDI (1:1000; BD Biosciences, 610255), and rabbit polyclonal anti-actin (1:1000; Abcam, AB8227). Membranes were incubated in solution containing the appropriate IRDye-labeled secondary antibody for 1 hour at room temperature. Membranes were thoroughly washed three times with PBS-T, then intensity of signals was measured using an Odyssey Infrared Imaging system (Li-Cor Biosciences). Quantification was

performed with Scion Image software.

Statistics

Data are expressed as mean + standard deviation (SD) of at least three independent experiments. When relative values are compared to control, statistical analysis was performed with a one-sample t-test by setting the control values at 1 in each independent experiment. When values between two groups are compared, statistical analysis was performed using an unpaired two-tailed two-sided t-test. All analyses were performed using GraphPad Prism 6.0 software. A p-value of $p<0.05$ was considered significant (* or # $p<0.05$, ** or ## $p<0.01$, *** or ### $p<0.001$).

RESULTS

Gal-4-binding sites are transiently present at proximal processes of non-myelin membrane-forming oligodendrocytes

To characterize the mechanism underlying Gal-4 involvement in the timing of myelination, we first extended the evidence for transient binding of Gal-4 to the cell surface of maturing OLGs. In a live binding-assay monitoring the level of MBP expression, a marker for differentiated OLGs in parallel, Gal-4 was found to almost exclusively associate to the cell surface at the proximal part of primary processes of OLGs that are MBP-positive, but myelin membrane-negative (Fig. 1A, immature OLGs, arrow). MBP-positive cells with myelin membranes did not bind Gal-4 (Fig. 1A, mature OLGs, arrowhead). Primary processes, but not the cell body of MBP-negative cells were stained with Gal-4 (Fig. 1A, mature OLGs, arrow), confirming previous findings that Gal-4-binding sites are exposed before MBP is expressed [49]. Binding, albeit at a reduced level, was also observed with the separate CRDs and a linkerless Gal-4 variant (Fig. 1B). Specific contacts are thus transiently made at the proximal part of primary processes of immature not yet myelin membrane-forming OLGs. To reveal the nature of Gal-4 counterreceptors and considering the well-documented pairing of Gal-4 with sulfatide, the possibility of this interplay was examined first.

Sulfatide is not a main Gal-4 counterreceptor in immature oligodendrocytes

Toward assessing the status of sulfatide as a putative Gal-4 counterreceptor, assays with sequential live binding of biotinylated Gal-4 and the anti-sulfatide antibody O4 were performed. In parallel, to determine the percentage of Gal-4- and antibody O4-binding cells, immature OLGs were also labeled separately with the lectin and antibody O4. The competition assays revealed that binding of Gal-4 was hardly, if at all, blocked when cells had first been incubated with a solution containing the antibody O4 (Fig. 2B,G, white bars), as the antibody O4 did also not reduce Gal-4 binding when interacting with cells after Gal-4 (Fig. 2A,G, white bars). Also, the percentage of antibody O4-stained cells was not reduced upon prior exposure of cells to labeled Gal-4 (Fig. 2A,E, bGal-4 vs O4). However, binding of antibody O4 was slightly, but reproducibly, reduced (by about 15%) when following a treatment step with Gal-4 (Fig. 2B,E, O4 vs bGal-4, $p=0.065$).

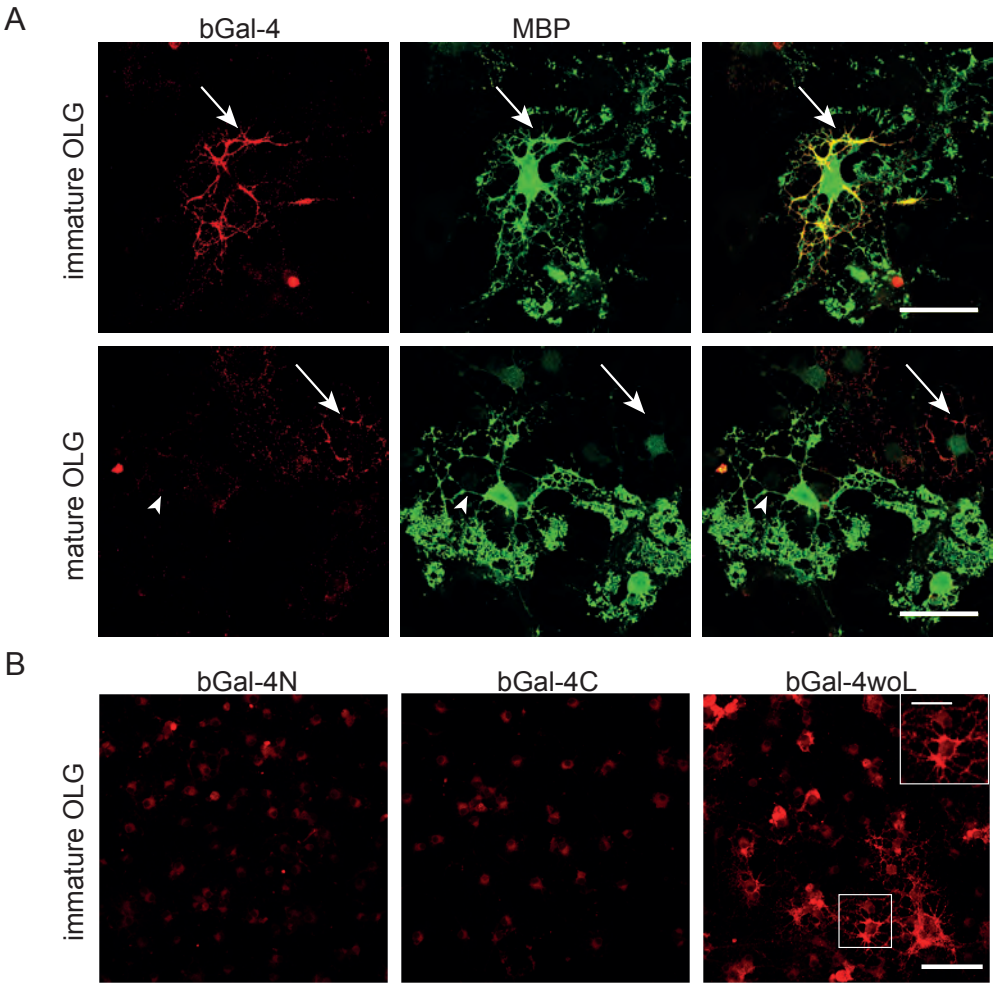

Figure 1. Binding sites for labeled Gal-4 are transiently exposed on non-myelin membrane-forming immature oligodendrocytes (OLGs). (**A**) Live binding assays of biotinylated galectin-4 (bGal-4, red) on immature (4 days after initiating differentiation, upper panel) and mature OLGs (6 days after initiating differentiation, lower panel) combined with immunocytochemistry for the OLG differentiation marker MBP (green). Representative images of three independent experiments are shown. Note that bGal-4 binds at the surface of proximal processes of MBP-negative (lower panel, arrow) and non-myelin membrane-forming MBP-positive cells (upper panel, arrow), but not to myelin membrane-bearing MBP-positive cells (lower panel, arrowhead). Scale bar is 50 µm. (**B**) Live binding assays with the biotinylated N-terminal CRD of Gal-4 (bGal-4N), C-terminal CRD (bGal-4C) and a labeled linkerless Gal-4 variant in which the two CRDs are connected without including the physiological linker peptide (bGal-4woL) on immature OLGs. Representative images of three independent experiments are shown. Scale bars are 50 µm and 5 µm (inset), respectively.

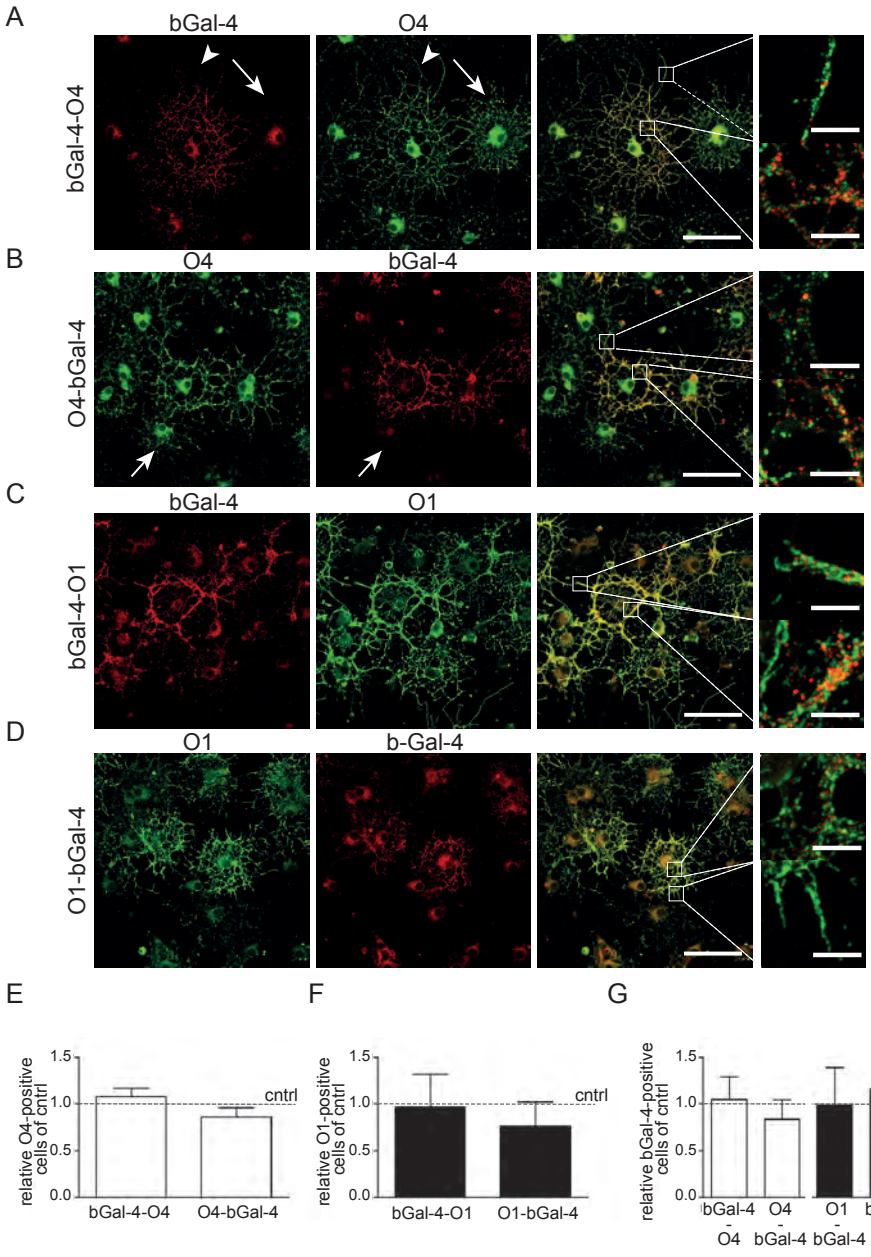
A
bGal-4
O4
bGal-4-O4
B
O4
bGal-4
O4-bGal-4
C
bGal-4
O1
bGal-4-O1
D
O1
b-Gal-4
O1-bGal-4
E
F
G
relative O4-positive cells of cntrl
1.5
1.0
0.5
0.0
cntrl
bGal-4-O4
O4-bGal-4
relative O1-positive cells of cntrl
bGal-4-O1
O1-bGal-4
relative bGal-4-positive cells of cntrl
bGal-4 → O4
O4 → bGal-4
O1 → bGal-4
bGal-4 → O1

Figure 2. Galectin-4 and anti-sulfatide (O4)/anti-GalCer (O1) antibodies do not compete for binding sites at the cell surface of immature OLGs. (**A–D**) Sequential live binding assays of biotinylated galectin-4 (bGal-4, red) and live staining with anti-sulfatide antibody O4 (**A,B**; green) or anti-galactosylceramide (anti-GalCer) antibody O1 (**C,D**; green) on immature oligodendrocytes (4 days after initiating differentiation). Cells were first incubated with bGal-4 in a (bGal-4-O4) and c (bGal-4-O1), with antibody O4 in b (O4-bGal-4) and with antibody O1 in d (O1-bGal-4). Representative images are shown. Scale bars are 50 µm and 5 µm (inset). **(E,F)** Quantification of the data shown in panels a–d. Counting at least 180-360 cells was performed per condition. Bars represent relative means to single O4 labeling **(E)**, single O1 labeling **(F)** or single bGal-4 labeling **(G)**, which were set to 1 in each independent experiment (cntrl, horizontal line, n=3). Error bars represent standard deviation. Statistical analyses are performed with a one sample t-test to test for differences with single labelings (cntrl, not significant). Absolute values for the percentage positive cells of single labelled cells are for O4 55.6% ± 2.4 (e), for O1 42.7%±13.1 (e) and for BG4 30.2%±4.0 **(G)**. Note that bGal-4 binding does not interfere with binding of antibodies O4 or O1; similarly, prior incubation with antibody O1 and O4 did also not affect bGal-4 binding.

In quantitative terms, 42.0±21.6% of antibody O4-binding cells were not Gal-4 positive (Fig. 2A,B, arrows), and the antibody O4 only occasionally co-localized with Gal-4 in primary processes (Fig. 2A,B, insets). In fact, whereas Gal-4 bound mainly to the proximal part of primary processes, binding of antibody O4 was also present at the cell surface of the distal part of primary processes (Fig. 2A, arrowheads). Similar findings were obtained when performing competition assays between the labeled Gal-4 and antibody O1, which is directed against GalCer, the metabolic precursor of sulfatide (Fig. 2C,D,F,G). Thus, no differences were observed in the percentage of Gal-4-binding cells upon single-reagent staining (cntrl) or with the application of antibody O1 (Fig. 2G, black bars). Similarly, the percentage of antibody O1 antigen-positive cells was not reproducibly altered upon pre-labeling with Gal-4 (Fig. 2F, bGal-4 vs O1) or following Gal-4 labeling (Fig. 2F, O1 vs bGal-4). Some level of colocalization of labeled Gal-4 and the antibody O1 was observed, in particular when the lectin was applied first (Fig. 2C,D, insets).

To further investigate whether Gal-4 binding to the cell surface depends on the presence of GalCer and/or sulfatide, we performed live binding assays on immature OLGs that had been treated at the onset of OPC differentiation with inhibitors of GalCer sulfation (sodium chlorate) or sphingolipid biosynthesis (fumonisin B1 (FB1)). Treatment with FB1 did not prevent binding of Gal-4, an about 15% decrease in the

percentage of Gal-4-binding cells was observed upon sodium chlorate treatment (Fig. 3A,B). These findings indicate that GalCer is not and sulfatide is likely not a main surface counterreceptor for Gal-4 in immature OLGs, directing interest to glycoproteins for the pairing.

Gal-4 binds to UGT8 and contactin-1 in membrane fractions of immature oligodendrocytes

To identify Gal-4-binding (glyco)proteins on the surface of immature OLGs, we next performed affinity pull-down followed by mass-spectrometrical identification of the precipitated proteins. As starting material, membrane fractions were prepared from immature OLGs by subcellular fractionation. Proteomic analysis identified 13 (glyco) protein species in two independent biological replicates. Using criteria of predicted localization in or association with membranes and/or known glycosylation reduced the number of this list of six proteins (Table 1), namely UGT8 (also called CGT), contactin-1 (also referred to as F3), Rab5c, vimentin, ITIh3 (inter-α-trypsin inhibitor heavy chain H3) and Rab10. Intriguingly, expression of UGT8, the galactosyltransferase specific for GalCer synthesis, is known to be upregulated at early stages of OPC differentiation [454]. Likewise, contactin-1 has been implicated in regulation of early OPC differentiation [172,174,455,456] and has also inferred as a contact side of Gal-4 in neurons [153]. Affinity precipitation from membrane fractions of immature OLGs with Gal-4 followed by Western blot analysis to detect presence of UGT8 or contactin-1 confirmed their contact with Gal-4 (Fig. 4A). Part of UGT8 also interacted with each of the two CRDs tested separately (Fig. 4B), indicating the remarkable potential of this glycoprotein for being cross-linked by Gal-4 and thus then forming a lattice. While contactin-1 is anchored to membranes via its GPI-anchor and known to be present at the cell surface [172,465], UGT8 localizes as type I transmembrane protein in ultrathin cryosections of CHO cells in the endoplasmic reticulum and nuclear envelope [469,470]. Therefore, to assess whether UGT8 and contactin-1 are counterreceptors for Gal-4 at the proximal part of primary processes of immature OLGs, we next examined their localization and presence at the cell surface.

Table 1. (Glyco)proteins that interact with recombinant Gal-4 in membrane fractions of immature OLGs*

protein	mass	location	glycosylation	% cov	(potential) function in oligodendrocytes	ref
UGT8	61.1	membrane	N-linked	1.5	expression increases upon OPC differentiation, catalyzes the transfer of galactose to ceramide, galactolipids modulate the rate of OPC differentiation, the number of OLGs, and trafficking of myelin protein	454,457–461
Contactin-1	~113.5	plasma membrane	N-linked	2.3	regulates the initiation of MBP expression	172-174,177,455
Rab5c	23.4	plasma membrane, endosome	–	7.4	small GTPase, involved in early endosome fusion, in other cells involved in endocytic traffic of Notch	462–464
Vimentin	53.7		mucin-type O-linked	6.9	intermediate filament that is downregulated upon OPC specification	465,466
ITIh3	~99,8		N-linked	1.0	acts as binding protein between hyaluronan and matrix glycoproteins to regulate localization, synthesis and degradation of hyaluronan, no data available on function in OPC differentiation	467
Rab10	~22.8	ER, Golgi, endosome	–	11.5	small GTPase involved in exocytosis, TGN/RE to plasma membrane, essential for OPC maturation	464,468

**binding of galectins to distinct peptide motifs is known at the F- and S-faces of the CRD Asn, asparagine;* ER, endoplasmic reticulum; kDa, kiloDalton; ITIh3, inter-α-trypsin-inhibitor-heavy chain 3; MBP, myelin basic protein; OLG, oligodendrocyte; OPC, oligodendrocyte progenitor cell; Rab, Ras-related protein; RE, recycling endosomes; TGN; trans-Golgi network; UGT8, UDP-galactose: ceramide galactosyltransferase

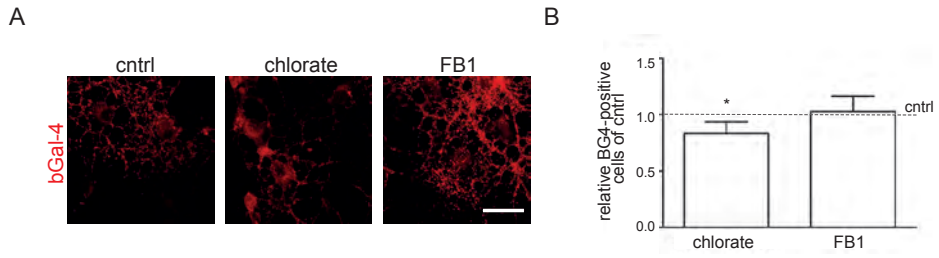

Figure 3. Galectin-4 binding is slightly altered upon inhibition of sulfatide synthesis in differentiating OLGs. (A) Live binding assays with biotinylated galectin-4 (bGal-4, red) on immature OLGs (4 days after initiating differentiation) that were either left untreated (ctrnl) or exposed to medium containing sodium chlorate (chlorate; 30 μM) or fumonisin B1 (FB1; 15 μM) to impair sulfatide or (galactosyl)ceramide biosynthesis, respectively. Representative images are shown. Scale bar is 25 μm. **(B)** Quantification of the data presented in panel a. At least 180 cells per condition were counted. Bars represent relative means to untreated control, this value was set to 1 in each independent experiment (cntrl, horizontal line, n=4). Error bars represent standard deviation. Statistical analyses are performed with a one sample t-test to test for differences with untreated control (cntrl, *p<0.05). Absolute value for the percentage of bGal-4-positive cells in untreated control is 32.0%±8.8. Note that sodium chlorate treatment slightly but significantly decreased extent of bGal-4 binding to the cell surface of immature OLGs.

UGT8 and contactin-1 are present at the cell surface of immature OLGs and co-localize with bound Gal-4

Subcellular fractionation demonstrated that UGT8 and contactin-1 were enriched in membrane fractions of immature OLGs (Fig. 4C,D). Cell surface biotinylation followed by precipitation of biotinylated surface proteins from membrane fractions by streptavidin-exposing beads revealed that about 36% of membrane-localized UGT8 and 48% of contactin-1 were present at the cell surface of immature OLGs (Fig. 4E,F). In addition, both UGT8 and contactin-1 were localized at the cell surface of primary processes of immature OLGs and colocalized with bound Gal-4 (Fig. 5A,B, arrows, insets). Of note, the anti-UGT8 and anti-contactin-1 antibodies recognize intercellular epitopes. Remarkably, while all cells expressed UGT8, mainly perinuclear as is expectable, in myelin membrane-forming OLGs, it was absent in processes in myelin-membrane forming OLGs (Fig. 5C, arrowhead). Fittingly, this lack of UGT8 presence at primary processes in myelin membrane-forming OLGs correlated with loss of Gal-4 binding (Fig. 1).

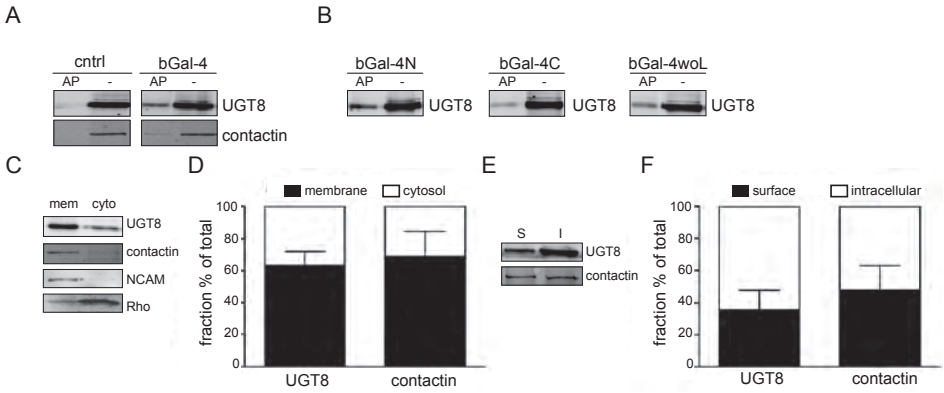

Figure 4. Galectin-4 binds to cell surface-present UGT8 and contactin-1 in membrane fractions of immature OLGs. (A,B) Affinity precipitation (AP) from membrane fractions of immature OLGs (4 days after initiating differentiation) with biotinylated galectin-4 (bGal-4, a), its N-terminal CRD (bGal-4N, b), C-terminal CRD (bGal-4C, b) or the linkerless variant (bGal-4woL, b) followed by Western Blot analysis on precipitated (AP) and non-precipitated (-) fractions of UGT8 **(A,B)** or contactin-1 **(A)**. Control (cntrl) represents control precipitation without addition of biotinylated Gal-4. Note that UGT8 and contactin-1 co-precipitate with bGal-4. **(C,D)** Western blot analysis of UGT8 and contactin-1 on membrane and cytosol fractions of immature OLGs. NCAM and RhoGDI serve as control of membrane and cytosol fractionation, respectively. Representative blots are shown in **(C)** quantification of data in **(D)** (n=5). Note that UGT8 and contactin-1 are enriched in membrane fractions. **(E,F)** Cell surface biotinylation followed by streptavidin-based precipitation of biotinylated surface (glyco)proteins from membrane fractions followed by Western blot analysis of UGT8 and contactin-1 on precipitated (S, surface) and non-precipitated (I, intracellular) fractions. Representative blots are shown in **(E)** and quantification of data in **(F)** (n=6). Note that both UGT8 and contactin-1 are present in membrane fractions. Bars represent means of the fraction percentage of membrane **(D)** and cell surface localization **(F)**. This percentage was calculated by dividing protein intensity in the indicated fraction by protein intensity of both fractions. Error bars represent standard deviation.

To obtain further information on binding of Gal-4 to UGT8, the rat-derived oligodendroglial progenitor cell line OLN-93 that can not produce GalCer and thus no sulfatide [187,450,471] was used. By compensating the defiency for UGT8, the OLN-93 cell line that presents GalCer (OLN-G) on the cell surface had been obtained ([186,187,450], Fig S1A). Of particular note in this context, these two cell lines do not express contactin-1. Western blot analysis revealed two additional UGT8-immunoreactive bands in extracts of vector only-transduced OLN cells (OLN-MOCK) and of OLN-G cells with a slightly higher molecular weight (Fig S1B, arrowheads). These bands may represent isoforms of UGT8 either generated by alternative splicing or harboring

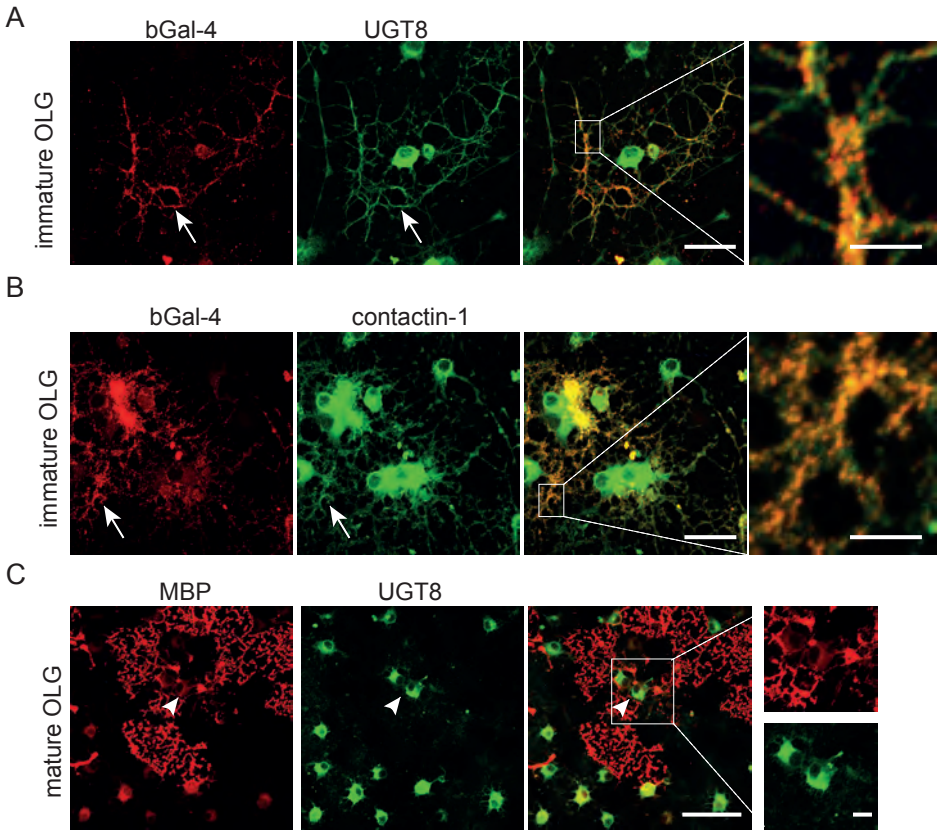

Figure 5. Galectin-4 co-localizes with UGT8 and contactin-1 at the cell surface of immature OLGs. (A,B) Live binding assays of biotinylated galectin-4 (bGal-4, red) on immature (a,b, 4 days after initiating differentiation) OLGs, followed by immunocytochemistry for detection of UGT8 (a, green) or contactin-1 (b, green). Note that UGT8 and contactin-1 co-localize with bGal-4 (arrow) at the cell surface. **(C)** Co-staining of UGT8 (green) and the OPC differentiation marker MBP (red) on mature OLGs (6 days after initiating differentiation). Note that UGT8 is mainly present in the cell body in myelin membrane-bearing mature OLGs (arrowhead). Representative images of three independent experiments are shown. Scale bars are 25 µm **(B)** and 50 µm **(C)** and 5 µm (insets).

different posttranslational modifications. As both thin layer chromatography analysis and staining with antibody O1 had previously documented that OLN-MOCK cells do not produce GalCer ([186,187,450], see also Fig. S1A), these potential isoforms of UGT8 are likely non-functional. More detailed analysis on OLN-G cells showed that UGT8 was present in membrane fractions (Fig. S1C, arrow), not detected at the cell surface

but mainly intracellularly localized (Fig. S1D, arrow).Live binding assays demonstrated that OLN-G cells exposed no Gal-4 counterreceptors at the cell surface (Fig. S1E). In addition, affinity pull-down assays disclosed no evidence for a Gal-4 contact (Fig. S1F, arrow), indicating that intracellular UGT8 did not bind to Gal-4 in OLN-G cells. Considering binding capacity of both types of CRDs, bridging of counterreceptors and thus post-binding effects may occur, which was examined next.

GalCer levels are reduced in detergent-resistant membrane microdomains upon Gal-4 treatment

Membrane microdomains play an important role in OPC differentiation, among others by amplification of growth factor signaling, process extension, onset of MBP expression and trafficking to myelin membranes [172,186,187,375,450,455,461,472–475]. Therefore, interactions of Gal-4 with membrane microdomains may underlie its role in the timing of OPC differentiation. As the presence of UGT8 in membrane microdomains has not been reported, we first characterized the lateral membrane distribution of UGT8 using detergent extraction followed by Optiprep density gradient centrifugation and Western blot analysis. Of the obtained fractions, fractions 3, 4 and 5 are considered as membrane microdomain or raft fractions, i.e., to contain TX-100-resistant (glyco) proteins and (glyco)sphingolipids. TX-100-soluble proteins and lipids appear in fractions 6 and 7. UGT8 was equally present both in raft and in non-raft fractions, and no alteration in UGT8 distribution was apparent upon Gal-4 exposure of immature OLGs for three days (Fig. 6A,B).

Consistent with previous results [172,476], contactin-1 integrated into TX-100-resistant membrane microdomains, and its lateral distribution was not altered upon treatment with Gal-4 (Fig. 6A,C). Similarly, the lateral distributions of the membrane microdomain marker caveolin, and the TX-100-soluble protein RhoGDI were similar in untreated and Gal-4-treated OLGs (Fig. 6A). To determine whether cell surface-localized UGT8 is present in membrane microdomains, raft and non-raft fractions of surface-biotinylated OLGs were subjected to streptavidin-based affinity precipitation and resulting material was analyzed by Western blot. While 55-60% of UGT8 present in membrane microdomains was present at the cell surface, this applied to only 25-30% of the UGT8 in the non-raft fraction (Fig. 6D,F). The lateral membrane

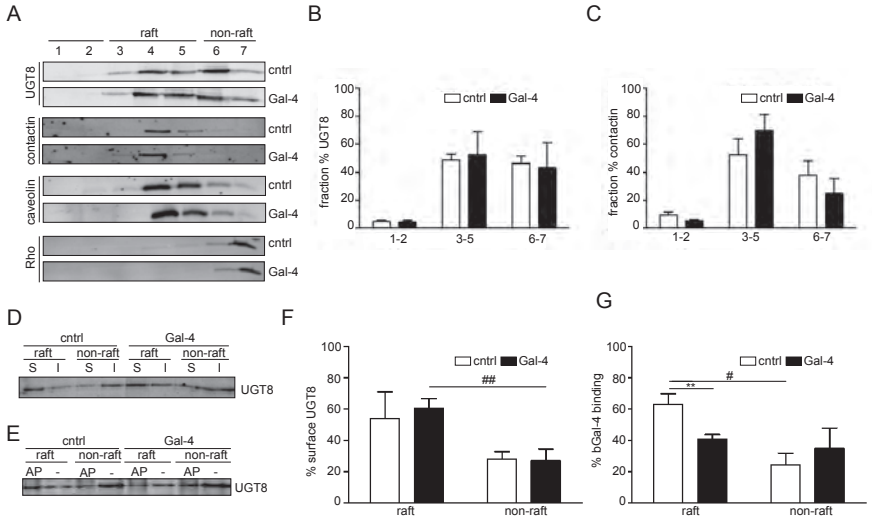

Figure 6. Galectin-4 does not alter the presence of surface-localized UTG8 in Triton X-100-resistant microdomains.(**A-C**). Western blot analysis of the distribution of UGT8 (**A,B**) and contactin-1 (**A,C**) in membrane microdomains ('raft') and non-membrane microdomains ('non-raft') isolated from untreated (cntrl) or Gal-4 (G4)-treated immature OLGs by detergent after OptiPrep density gradient centrifugation. Caveolin and RhoGDI serve as controls for membrane and non-membrane microdomains, respectively. Representative blots are shown in (**A**), quantification of data in (**B**) (n=6) and (**C**) (n=5). Total protein presence was calculated by adding the intensity of all fractions. Protein percentage of each fraction was then calculated by dividing the protein intensity of all fractions. Bars represent the mean of the pooled fraction percentage of fractions 1-2, 3-5 and 6-7. Error bars represent standard deviation. (**D,F**) Streptavidin-based precipitation of biotinylated cell surface proteins from raft and non- raft fractions followed by Western blot analysis of UGT8 in precipitated (S, surface) and non-precipitated (I, intracellular) fractions. Representative blots are shown in (**D**) and quantification of data in (**F**) (n=3). Note that 55-60% of the UGT8 found in membrane microdomains (raft in untreated control cells (cntrl)) is present at the cell surface (untreated, cntrl) and remained at this level upon Gal-4 treatment. (**E,G**) Affinity precipitation (AP) on raft and non-raft fractions obtained from untreated (cntrl) or galectin-4 (Gal-4)-treated immature oligodendrocytes with biotinylated Gal-4 (bGal-4) followed by Western Blot analysis on precipitated (AP) and non-precipitated (-) fractions of UGT8. Representative blots are shown in (**E**) and quantification of data in (**G**) (n=4). Note that Gal-4 treatment significantly reduced bGal-4 binding to UGT8 that is present in membrane microdomains (raft), but not UGT8 present in non-membrane microdomains. Bars represent the mean of percentage of cell surface-localized (**F**) or bGal-4-binding (**G**) UGT8. The protein percentage was calculated by. dividing the protein intensity in the indicated fraction by protein intensity of both fractions. Error bars represent standard deviation. Statistical analyses are performed with a two-sided t-test to test for differences between untreated control (cntrl, b,c,f,g, *p<0.05, **P<0.01) or fraction (f,g, #p<0.05, ##p<0.01).

distribution of cell surface UGT8 remained similar upon Gal-4 treatment (Fig. 6D,F). Affinity precipitation demonstrated that of the UGT8 pool in membrane microdomains about 60% interacted with Gal-4, while only 20-25% of the UGT8 in the non-raft fractions was doing so (Fig. 6E,G). Notably, the fraction of the UGT8 pool that is at the cell surface in either membrane microdomains or non-raft fractions correlated with the respective fractions of UGT8 that binds Gal-4. Treatment with Gal-4 significantly reduced binding to UGT8 that is present in membrane microdomains (Fig. 6E,G). Gal-4 binding to UGT8 in non-raft fractions was not affected (Fig. 6E,G).

Having identified that Gal-4 primarily associated to UGT8 in membrane microdomains, we next examined the effect of Gal-4 on the levels of GalCer, the product of UGT8 action, and sulfatide. Analyses of the lateral membrane distribution of GalCer disclosed that the relative percentage of GalCer in TX-100-resistant membrane microdomains significantly decreased, GalCer presence in non-raft fractions significantly increased (Fig. 7A,B). The lateral membrane distributions of sulfatide and ganglioside GM1 were apparently unaffected upon Gal-4 addition (Fig. 7A,C,D). Remarkably, exposure to Gal-4 only slightly decreased the total levels of GalCer, those of sulfatide were not affected (Fig. 7E,F). The levels of ganglioside GM1, the major ganglioside in mature OLGs, slightly increased in Gal-4-treated OLGs (Fig. 7E,F). The reduced levels of GalCer in membrane microdomains may contribute to the role of Gal-4 in the timing of OPC differentiation.

The lateral membrane distribution of GalCer is not affected upon Gal-4 treatment of oligodendrocytes cultured on a fibronectin matrix

Proceeding from PLL to the MS-relevant fibronectin to coat the surface, we next answered the question whether Gal-4 interferes with the lateral membrane distribution of GalCer when OPCs are differentiated on a fibronectin substratum. Primary processes of immature OLGs that were cultured on a surface coated with fibronectin bound Gal-4 (Fig. 8A), in a similar manner to what had been detected on PLL (Fig. 1A). Also, UGT8 was found to be present in membrane microdomains in OLGs, and its lateral membrane distribution was not affected by Gal-4 treatment (Fig. 8B,C). In contrast to what had been observed when OLGs were grown on PLL, features of lateral membrane distribution of GalCer were maintained in the presence of Gal-4 (Fig. 8B,D).

Affinity pull-down assays on raft and non-raft fractions demonstrated that the lectin interacted to a similar extent with UGT8 in both fractions (Fig. 8E,F). Intriguingly, its binding to UGT8 in non-raft fractions was significantly reduced in Gal-4-treated OLGs that were cultured on a fibronectin substratum, the binding to UGT8 in membrane microdomains not being affected by Gal-4 (Fig. 8E,F). Of note, GalCer, sulfatide and ganglioside GM1 levels were similar in untreated and Gal-4-treated OLGs grown on a fibronectin substratum (Fig. 8G,H). Therefore, in contrast to OLGs grown on PLL (Fig. 6), Gal-4 and UGT8 equally well form a complex in membrane microdomains and non-membrane microdomains, while extent of binding was only diminished in non-membrane microdomains after Gal-4 treatment. Since it had previously been described that Gal-4 treatment reduces the percentage of MBP-positive cells perturbing OPC differentiation when cultured on PLL [49], whereas a fibronectin substratum impairs myelin membrane formation [30,355,375,477,478], we next assessed whether Gal-4 will also negatively affect OPC differentiation on a fibronectin substratum.

Gal-4 overcomes the inhibitory effect of fibronectin on myelin membrane formation

To measure what Gal-4 will do on OPC maturation in the presence of fibronectin, OPCs were plated on a fibronectin substratum and either left untreated or treated for three days with the lectin from the immature OLG stage onwards. As expected from evidence of previous reports [30,355,375,477,478], OLGs plated on a fibronectin substratum were MBP-positive, but did form less myelin membranes than OLGs that were grown on PLL (Fig. 9A-C). Rather surprisingly, treatment of immature OLGs grown on fibronectin with Gal-4 essentially reversed the inhibitory effect of fibronectin on myelin membrane formation (Fig. 9A-C). More specifically, a 1.5-fold increase in the percentage of OLGs that have MBP-positive myelin membranes was observed compared to untreated cells cultured on the fibronectin matrix (Fig. 9C). Thus, under these conditions, and in contrast to what was previously observed in case of OLGs grown on PLL [49], Gal-4 promoted myelin membrane generation rather than preventing OPC differentiation. This mode of process was further reflected by similar percentages of MBP-positive cells in the presence or absence of Gal-4 (Fig. 9B). Hence, fibronectin appears to counteract the inhibitory effect of Gal-4 on differentiation. Gal-4, in contrast, might overcome the inhibitory effect of fibronectin on myelin membrane formation, pointing to a crosstalk between these two biomodulators in a network.

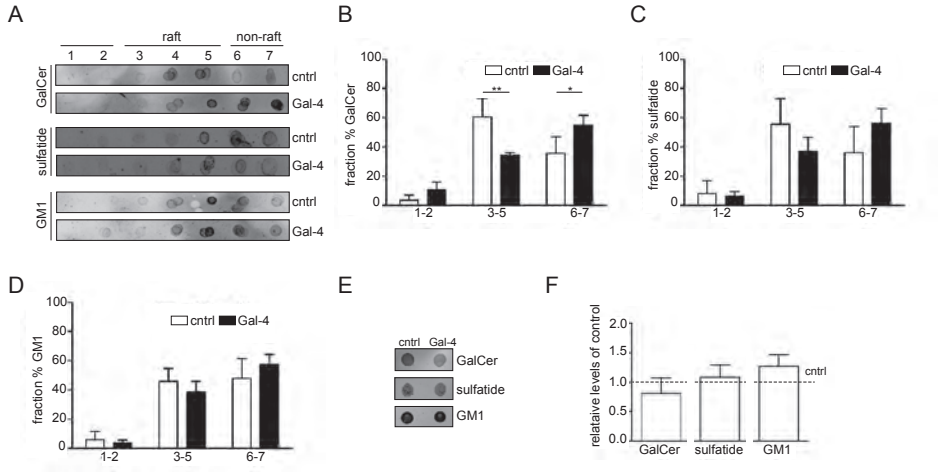

Figure 7. Galectin-4 reduces galactosylceramide levels in Triton X-100-resistant membrane microdomains. **(A-D)** Dot blot analysis of the distribution of galactosylceramide (GalCer, a,b), sulfatide **(A,C)** and ganglioside GM1 **(A,D)** in membrane ('raft') and non-membrane microdomains ('non-raft') isolated from untreated (cntrl) or galectin-4 (Gal-4)-treated immature oligodendrocytes by exposure to detergent and OptiPrep density gradient centrifugation. Representative blots are shown in **(A)**, quantification of data in **(B-D)** (b n=4, c n=4, d n=3). The total lipid expression was calculated by adding the intensity of all fractions. The lipid percentage of each fraction was then calculated by dividing the lipid intensity of all fractions. Note that Gal-4 treatment significantly reduced GalCer levels in membrane domains along with an increase in GalCer levels in non-membrane microdomains. Bars represent the mean of the pooled fraction percentage of fractions 1-2, 3-5 and 6-7. Error bars represent standard deviation. Statistical analyses are performed with a two-sided t-test to test for differences between untreated control (cntrl, *p<0.05, **P<0.01). **(E,F)** Dotblot analysis of GalCer, sulfatide and ganglioside GM1 levels in untreated (cntrl) and Gal-4-treated immature oligodendrocytes. Representative blots are shown in **(E)** and quantification of data in **(F)** (GalCer and sulfatide n=5, ganglioside GM1 n=3). Bars represent the relative mean to untreated control, which was set to 1 in each independent experiment (cntrl, horizontal line). Error bars represent standard deviation. Statistical analyses are performed with a one sample t-test (not significant) to test for differences with untreated control (cntrl).

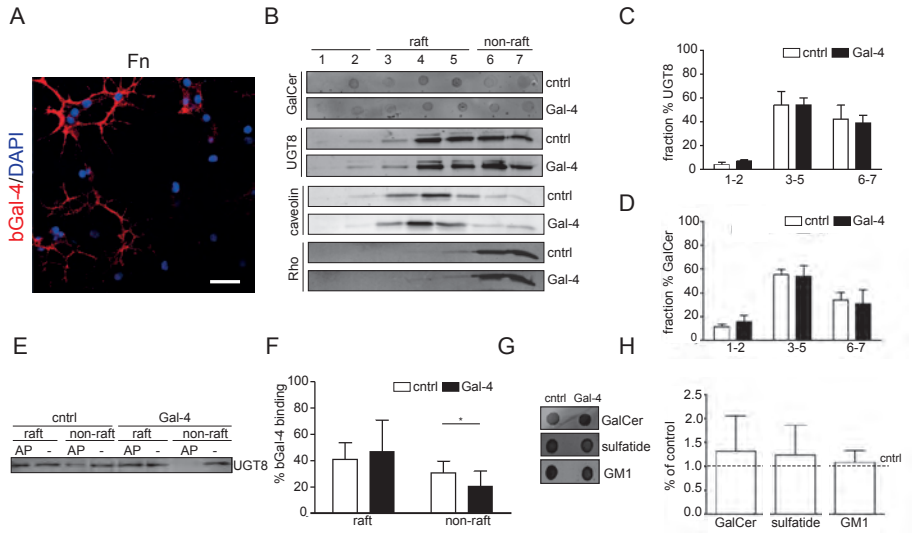

Figure 8. Galectin-4 does not alter the lateral membrane distribution of galactosylceramide and of UGT8 of OLGs cultured on fibronectin. (A) Live binding assays of biotinylated galectin-4 (bGal-4, red)) on immature (4 days after initiating differentiation) OLGs cultured on a fibronectin substratum. **(B-D)** Dot blot or Western blot analysis of the distribution of galactosylceramide (GalCer, b,c) and UGT8 **(B,D)** in membrane ('raft') and non-membrane microdomains ('non-raft') isolated from untreated (cntrl) or Gal-4 (G4)-treated immature oligodendrocytes cultured on a fibronectin substratum after exposure to Triton X-100 and OptiPrep density gradient centrifugation. Caveolin and RhoGDI serve as controls for membrane and non-membrane microdomains, respectively. Representative blots are shown in **(B)**, quantification of data in **(C,D)** (c n=4, d n=4). The total lipid/protein expression was calculated by adding the intensity of all fractions. The lipid/protein percentage of each fraction was then calculated by dividing the lipid/protein intensity of all fractions. Note that Gal-4 treatment did not alter the lateral membrane distribution of GalCer and UGT8. Bars represent the mean of the pooled fraction percentage of fractions 1-2, 3-5 and 6-7. Error bars represent standard deviation. **(E,F)** Affinity precipitation (AP) on raft and non-raft fractions obtained from untreated (cntrl) or Gal-4-treated immature oligodendrocytes cultured on a fibronectin substratum with bGal-4 followed by Western Blot analysis on precipitated (AP) and non-precipitated (-) fractions of UGT8. Representative blots are shown in **(E)** and quantification of data in **(F)** (n=5). Note that Gal-4 treatment significantly reduced bGal-4 binding to UGT8 that is present in non-membrane microdomains (non-raft). Bars represent the mean of the protein percentage of bGal-4-binding UGT8. The protein percentage was calculated by dividing the protein intensity in the indicated fraction by protein intensity of both fractions. Error bars represent standard deviation. **(G,H)** Dotblot analysis of GalCer, sulfatide and. ganglioside GM1 levels in untreated (cntrl) and Gal-4-treated immature OLGs cultured on a fibronectin substratum. Representative blots are shown in **(G)** and quantification in (h) (n=4). Bars represent the relative mean to untreated control, which was set to 1 in each

Figure 8 continued independent experiment (cntrl, horizontal line). Error bars represent standard deviation. Statistical analyses are performed with a two-side t-test (c,d,f, *p<0.05) or a one sample t-test (h, not significant) to test for differences with untreated control (cntrl) and a two-side t-test to test for differences between fractions (f, not significant). Scale bar is 25 µm.

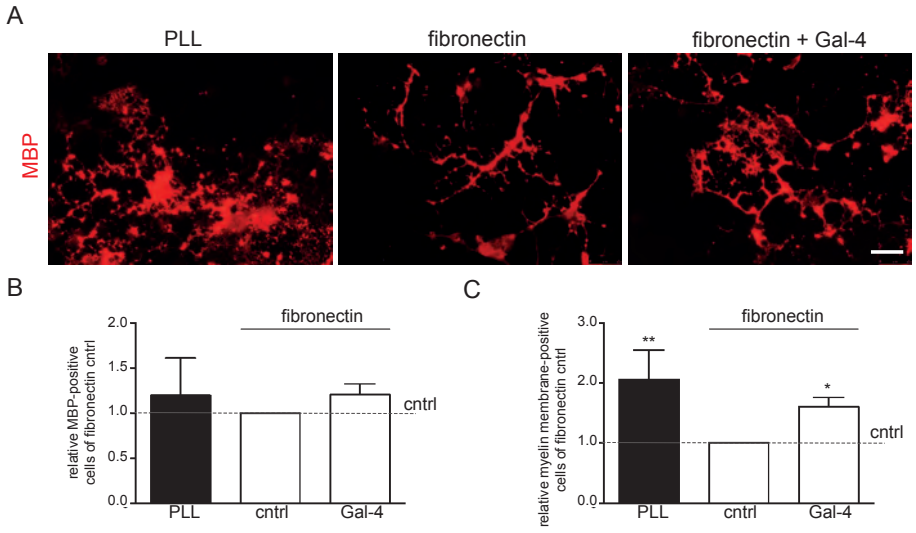

Figure 9. Galectin-4 overcomes the inhibitory effect of fibronectin on myelin membrane formation. (**a-c**) Immunocytochemistry of the OLG maturation marker MBP (red) on OLGs that were plated fibronectin substratum and left untreated (cntrl) or treated with galectin-4 (Gal-4) at the immature OLG stage for three days. Oligodendrocytes that were plated on PLL serve as a control. Representative images are shown in (**a**), quantification of the percentage of MBP-positive cells (differentiation) in (**b**) (n=6), and quantification of the percentage of MBP-positive that bear MBP-positive myelin membranes in (**c**) (n=6). Note that Gal-4 promotes myelin membrane formation on a fibronectin substratum. Bar represent the relative mean to untreated cells cultured on a fibronectin substratum, which was set to 1 in each independent experiment (cntrl, horizontal line). Error bars represent standard deviation. Statistical analyses are performed with a one sample t-test to test for differences with control (cntrl, *p<0.05, **p<0.01). Absolute value for the percentage of MBP-positive cells of control is 22.2% ± 1.9 and for the percentage of myelin membrane-bearing MBP-positive cells 29.8% ± 5.3. Scale bar is 25 µm.

DISCUSSION

Gal-4 is a negative regulator of OPC differentiation that is persistently present in MS lesions so that its expression is assumed to be associated with remyelination failure in MS [49,147]. This connection between the tissue lectin and a clinically relevant dysregulation, deserved to be explored further by identifying the oligodendroglial counterreceptor(s) of this galectin. Using pull-down assays, we identified a small set of proteins, including UGT8 and contactin-1, as binding partners. Interestingly, co-localization studies and testing engineered cell lines excluded sulfatide from being a main contact site in OLGs. Functionally, Gal-4 treatment reduced GalCer levels in membrane microdomains when OLGs were cultured on a PLL, but not on a fibronectin substratum. Rather than negatively regulating OPC differentiation, the lectin counteracted fibronectin-mediated inhibition of myelin membrane formation *in vitro*. These findings inspire the idea that modulating counterreceptor presence or post-binding effects of Gal-4 at the level of the oligodendroglial cell surface has potential to overcome remyelination failure in MS lesions.

Proceeding from defining Gal-4 as pathogenic factor to characterizing its counterreceptors has led to the discovery of transient expression of UGT8 at the cell surface, and this in the proximal, but not distal part of primary processes of immature OLGs. These obtained results are in line with previous immunocytochemical and biochemical observations that UGT8 is present in OLG processes and myelin of developing and adult rat brain [479] and in oligodendroglial plasma membrane fractions [480,481]. Of relevance in this context, *in vitro* and *in vivo* analysis with UGT8 (CGT)-deficient mice has demonstrated that galactosphingolipids modulate the rate of OPC differentiation (reviewed in [458]), the number of OLGs [459] and myelin composition [460]. Differences in OPC differentiation have been attributed to the absence of 3-O-sulfated GalCer (sulfatide). In fact, sulfatide is a negative regulator of OPC differentiation [184], enriched at the surface of OLGs and interacts with Gal-4 in enterocyte-like HT-29 cells and neurons [112,152,176,382,482]. In rodent brain and in maturing OLGs, both length and hydroxylation of the acyl chains of sulfatide are developmentally regulated and reflect myelin maturity. Prior to the onset of maturation, sulfatide (18:0) is predominant, whereas long-chain-fatty-acid sulfatide (24:1), the preferred ligand of Gal-4

[112], is upregulated during myelination and most abundant in the adult brain [483–485]. This temporal regulation of the type of lipid anchor for headgroup presentation may explain why sulfatide with its comparatively short chain fatty acid, mainly present in immature OLGs, and Gal-4 fail to interact prominently. Indeed, a switch in this parameter from incorporating a C18 to a C24 acyl chain appears to have potential to mean much more than a biosynthetic peculiarity of a sphingolipid.

Drawing an analogy to other cases of glycosyltransferases presented on the cell surface such as the β1,4-galactosyltransferase with assumed roles in adhesion or even signal induction [486,487], the localization of UGT8 may serve so far not realized aspects of functionality, and a Gal-4-mediated cross-linking may enhance their impact. In fact, local GalCer levels are linked to membrane association and distribution of MBP [187,488,489], a peripheral membrane protein imperative for myelin membrane formation [490]. Given the exclusive detection of plasma membrane-localized UGT8 at primary processes, it is tempting to suggest that the Gal-4-mediated reduction in GalCer levels in membrane microdomains may preclude premature positioning of MBP at the plasma membrane.

Our affinity capture mass spectrometry analysis identified contactin-1 as another Gal-4 surface counterreceptor present at the proximal part of primary processes. Oligodendroglial contactin-1 orchestrates the initial interaction between immature OLGs and axons that is essential for the onset of MBP expression *in vivo* [171–174,491]. This finding invites the attractive speculation that the restrictive localization of both UGT8 and contactin-1 at primary processes may be an elegant way to prevent ectopic MBP expression at secondary and tertiary processes as well as to confine initial MBP production and associations to the proximal part of primary processes. Neuronal Gal-4 may then act as an antagonist that prevents both premature binding of contactin-1 to the axonal glycoprotein L1, a binding partner for Gal-4 at that site [152], and GalCer accumulation, thereby affecting the timing of the onset of MBP expression in OLGs. Considering this protein's expression, an endogenous role of oligodendroglial Gal-4 is in the p27-mediated activation of the MBP gene and its capacity to interact with this promoter [151].

In addition to UGT8 and contactin-1, Rab5c and Rab10 were found to associate to Gal-4. Rab proteins are a subgroup of small GTP-binding proteins that regulate vesicular trafficking (reviewed in Hutagalung & Novick, 2011). Both proteins are apparently not glycosylated (Table 1). However, in addition to glycans, the galectin CRD is apparently capable to specifically interact with distinct peptide motifs at its β-galactoside-binding F-face, and this in a lactose-inhibitable manner, and alternatively at its S-face. Hereby, a galectin binds biorelevant counterreceptors such as anti-apoptotic Bcl-2, the chemokine CXCL12 or eye lens crystallins [492–494]. Rab5c is expressed in all stages of OLG differentiation [462,463], mainly regulating the protein transport from the plasma membrane to early endosomes [464]. Gal-4 binding to Rab5c at the plasma membrane may well induce its internalization. Rab10 is involved in protein routing from the Golgi apparatus and in recycling endosomes to the plasma membrane[464]. Of note, a disruption of Rab10 can cause delayed OPC maturation *in vitro* and myelination *in vivo* [468]. By binding to these two Rab proteins, Gal-4, which is produced on free ribosomes and is thus present intracellularly [495], may have an impact on intracellular trafficking beyond its role by pairing with glycoproteins, another route for modulating OPC differentiation. The targeted delivery of glycoprotein L1 in axons underscores the capacity for vectorial cargo transport at distinct sites of Gal-4, in this case involving sulfatide [152]. Pull-down assays show that these two Rab proteins can also interact with Gal-3 [496,497], a positive regulator of OPC differentiation, and these observations give research direction to explore the synergistic potential within the galectin network.

Concerning molecular cooperation in situ, the presence of two myelinating-inhibiting compounds, i.e., Gal-4 and fibronectin, induced OPC differentiation and maturation into mature myelin membrane producing OLGs *in vitro*. It is thus possible for Gal-4 to alter fibronectin-mediated signaling from being restrictive to constructive to myelin membrane generation. Our binding assays demonstrated that Gal-4-binding sites were exclusively present at primary processes of OLGs cultured on a fibronectin substratum. However, lateral membrane distribution of GalCer was not affected, which may relate to the effect of fibronectin to alter membrane microdomain formation [187,375,450], including the lateral membrane distribution of sulfatide. Interestingly, Gal-4 associated preferentially to UGT8 in membrane microdomains of OLGs

cultured on a PLL. When using fibronectin instead, it bound equally well to UGT8 in membrane microdomains and non-membrane microdomains.

Taken together, we have identified cell surface-localized UGT8 and contactin-1 as major counterreceptors for Gal-4 on immature OLGs. In addition, in contrast to their individual activities, the simultaneous presence of Gal-4 and fibronectin effectively promotes formation of myelin membrane. Since both compounds are constituents of (chronic) MS lesions [30,147,498], further analysis of the presence of these counterreceptors in MS lesions as well as the nature of their interactions and induction of (any) downstream signaling will now be desirable, considering that neuronal contactin-1 is a binding partner for Gal-4, too [153]. Contactin-1, when expressed by neurons is important for the assembly of the paranodal complex [499,500], for axonal growth [499] and for the initiation of OPC differentiation [501]. Gal-4 takes care of the transport of this axon growth-promoting glycoprotein [152] and of recruitment of neuronal contactin-1 to Gal-4-containing domains that prevent myelination [153]. Due to its bivalency facilitating bridging and clustering in microdomains as platform for signaling, contact formation of Gal-4 with (secreted) oligodendroglial [502] or axonal contactin-1 may inspire to devise remyelination strategies for immature OLGs involving Gal-4. Also, the emerging concept of galectins engaging in teamwork in (re)myelination (reviewed by de Jong, Gabius, & Baron, 2020) and as described in the pathogenesis of osteoarthritis [82,504], their synergistic and antagonistic activities to be exploited by custom-made galectin variants [343,505], directs interest to extend the study to other members of this family and to *in vitro* testing of galectin variants of altered modular architecture with therapeutic intention.

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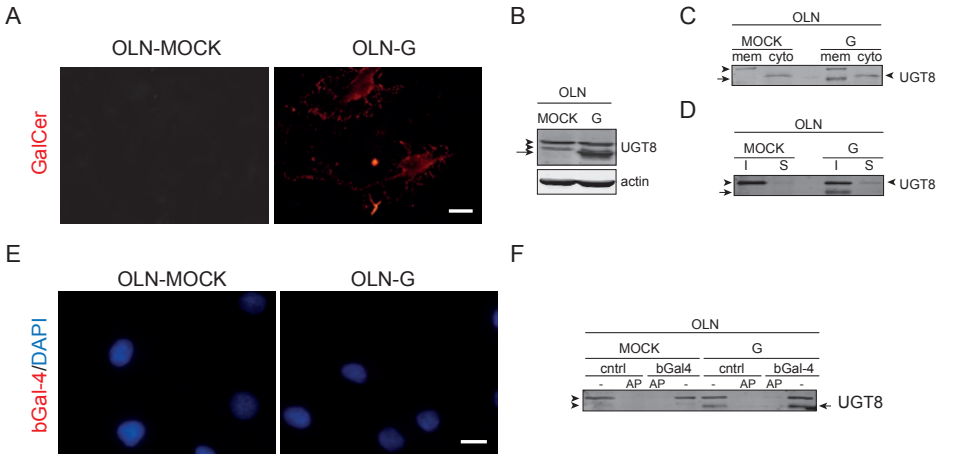
CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CGHMdJ and WB. Performed and analyzed the experiments: CGHMdJ, JCdJ, LW and WB. Contributed reagents/materials/content: HJG. First draft of the manuscript: CGHMdJ . Critical revision of the manuscript: HJG and WB. Study supervision: WB. All contributing authors have seen and approved the manuscript.



Supplementary figure 1. Galectin-4 does not bind to intracellular UGT8 in OLN-G cells. (a) Surface staining by anti-galactosylceramide (GalCer) antibody O1 on OLN-MOCK (control vector only) and OLN-G (transduced with vector coding for UGT8) cells. **(b)** Western blot analysis of UGT8. Actin serves as control. Arrow indicate UGT8, arrowheads indicate two additional UGT8-immunoreactive bands present in OLN-MOCK and OLN-G cells with a slightly higher molecular weight. **(c)** Western blot analysis of UGT8 and on membrane and cytosol fractions. Note that overexpressed UGT8 (arrow) is present in membrane fractions. **(d)** Cell surface biotinylation followed by streptavidin-based precipitation of biotinylated surface proteins from membrane fractions followed by Western blot analysis of UGT8 on precipitated (S, surface) and non-precipitated (I, intracellular) fractions. Note that overexpressed UGT8 (arrow) is localized intracellularly. **(e)** Live binding assays of biotinylated galectin-4 (bGal-4, red)). Note that OLN-MOCK and OLN-G cells do not harbor Gal-4 counterreceptors at the cell surface. **(f)** Affinity precipitation (AP) on membrane fractions with bGal-4 followed by Western Blot analysis on precipitated (AP) and non-precipitated (-) fractions of UGT8. Note that Gal-4 does not bind to overexpressed UGT8 (arrow). Representative image and blots of at least three independent experiments are shown. Scale bars are 10 (e) and 20 (a) µm.