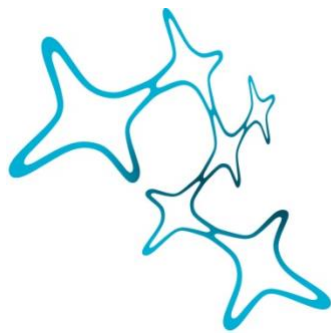


# CNS MYELINATION INVOLVES NONVESICULAR GLYCOLIPID TRANSPORT FROM ENDOPLASMIC RETICULUM TO PLASMA MEMBRANE IN OLIGODENDROCYTES

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## Abstract

In the central nervous system (CNS), myelin is formed as oligodendrocytes wrap their plasma membrane multiple times around axons. Myelination, the process to generate myelin, requires a massive expansion of oligodendroglial plasma membrane. An outstanding question is how oligodendrocytes efficiently deliver lipids to the plasma membrane to facilitate myelination. Utilizing volume electron microscopy and immunohistochemistry, I demonstrated that mouse oligodendrocyte endoplasmic reticulum (ER) extends into developing myelin as tubules and is in close proximity to plasma membrane. Furthermore, tubular ER is highly correlated with myelination. Myelinating oligodendrocytes have elevated levels of tubular ER markers compared to premyelinating oligodendrocytes, suggesting oligodendrocytes increase the amount of tubular ER when they start to myelinate. When developmental myelination is complete, tubular ER is downregulated in white matter.

I hypothesized that lipids can be transferred directly from the ER to the plasma membrane at these contact sites. I focused on one lipid transfer protein abundant in myelin—glycolipid transfer protein (GLTP). Like tubular ER markers, GLTP is abundant in myelinating oligodendrocytes compared to premyelinating oligodendrocytes. I generated *Gltp* flox mouse lines and conditionally knocked out *Gltp* in oligodendrocytes by crossing the flox line with the *Cnp-Cre* mouse line. These conditional knockout (cKO) mice exhibit ER pathology in developing myelin, possibly due to lipid accumulation in the ER. Subsequently, these mice experience hypomyelination and degeneration. Myelin purified from cKO mice has a reduction in glycolipid, and primary oligodendrocytes with *Gltp* knockout and knockdown have a deficiency in glycolipid delivery to the cell surface, indicating that GLTP contributes to glycolipid transport during myelination.

In summary, my research suggests that non-vesicular lipid transport plays an important role in myelin development.

## List of abbreviations

Abbreviation	Explanation
ABC transporters	ATP-binding cassette transporters
ATUM-SEM	automated tape collecting ultramicrotomy- scanning electron microscopy
BCAS1	breast carcinoma-amplified sequence 1
Cnp	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CNS	central nervous system
cKO	conditional knockout
EM	electron microscopy
ER	endoplasmic reticulum
E-syt	extended synaptotagmin
GalCer	galactosylceramide
GFP	green fluorescent protein
GLTP	glycolipid transfer protein
GRAMD	glucosyltransferases, Rab-like GTPase activators and myotubularins domain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HexCer	hexosylceramide
HFA	hydroxy fatty acid
KO	knockout
LTP	lipid transfer protein
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MCS	membrane contact sites
Mospd2	motile sperm domain-containing protein 2
MS	mass spectrometry
MSMS	tandem mass spectrometry
NFA	normal fatty acid
NPC	Niemann-Pick type C
n.s.	not significant
OSBP	oxysterol-binding protein
ORP	oxysterol-binding protein (OSBP)-related protein
P14/28	postnatal day 14/28

## List of abbreviations

PBS	phosphate-buffered saline
PC	phosphatidylcholines
PCA	Principal Component Analysis
PE	phosphatidylethanolamine
PFA	paraformaldehyde
PI	phosphatidylinositol
PITP	phosphatidylinositol transfer protein
PM	plasma membrane
PNS	peripheral nervous system
PS	phosphatidylserine
REEP5	receptor expression-enhancing Protein 5
RFP	red fluorescent protein
rpm	rotations per minute
RTN1	reticulon 1
RTN4	reticulon 4
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SMP	synaptotagmin-like mitochondrial-lipid binding protein
SNARE	soluble NSF attachment protein receptor
StART	steroidogenic acute regulatory transfer
TEM	Transmission electron microscopy
TMEM24	transmembrane protein 24
WT	wildtype

\*For abbreviations of all lipid classes, please refer to “Box 1 Lipid nomenclature” in Section 3.6.

# 1. Introduction

## 1.1 Myelination, a fundamental process in the vertebrate nervous system

First emerging in jawed vertebrates, myelin represents a key innovation in vertebrate evolution (Ghosh et al., 2024). As a multilayered membrane wrapping around the axon (Fig 1.1), myelin acts as insulation comparable to the insulation material surrounding an electric wire, and can increase nerve conduction velocity by 20 to 200 times (Nave & Werner, 2021; Stadelmann et al., 2019). Additionally, myelin and myelin-generating cells offer metabolic support to axons, including the provision of pyruvate and lactate (Fünfschilling et al., 2012; Lee et al., 2012), and antioxidant defense (Mukherjee et al., 2020). Myelin is formed by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) (Nave & Werner, 2014). In this dissertation, I will focus on CNS myelin and myelination unless specified.

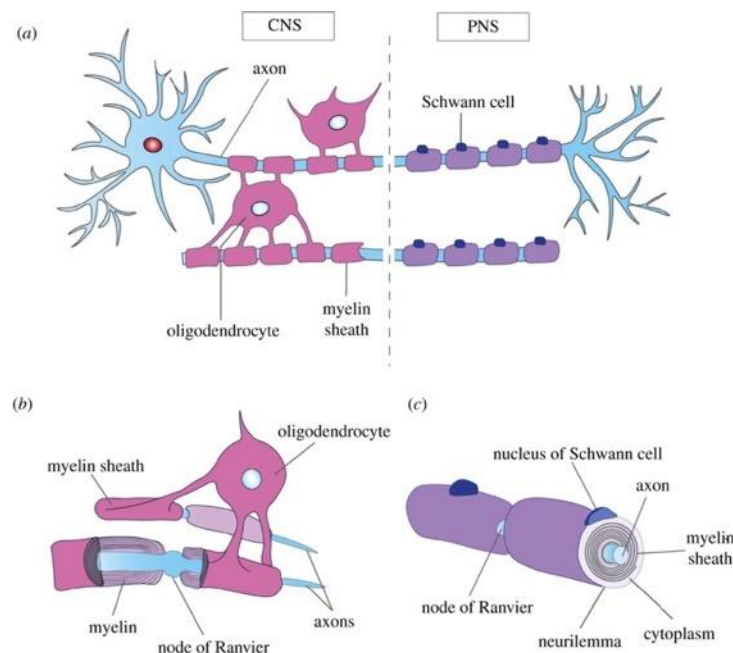


Figure 1.1 Myelination in the CNS and PNS. Oligodendrocytes in the CNS and Schwann cells in the PNS both ensheath axons to provide insulation. However, while oligodendrocytes can ensheath multiple axons, Schwann cells typically myelinate only one axon each, with the cell body lying adjacent to the myelin sheath. Figure taken from open access article (C. Z. Chen et al., 2021) DOI:<https://doi.org/10.1098/rsob.200352>

## 1. Introduction

Myelination is a critical process that occurs not only during developmental stages but also in adulthood (Snaidero & Simons, 2017), where it is closely associated with learning and memory (Bonetto et al., 2021; Gibson et al., 2014; Hughes et al., 2018; McKenzie et al., 2014; Xin & Chan, 2020). In the context of some neurodegenerative diseases such as multiple sclerosis, the failure of remyelination drives disease progression (Fancy et al., 2011; Stadelmann et al., 2019). Therefore, it is essential to understand the molecular and cellular mechanism underlying myelination.

### 1.2 Myelination requires vast membrane expansion.

To generate myelin, an oligodendrocyte can wrap its plasma membrane around up to 50 axons (Chong et al., 2012; Hughes et al., 2018). It has been estimated that myelination requires oligodendrocytes to expand the plasma membrane up to many thousand folds (Pfeiffer et al., 1993). Remarkably, the majority of this vast expansion is achieved during development within just a few hours in zebrafish (Czopka et al., 2013) and two weeks in mouse (Djannatian et al., 2023; Foran & Peterson, 1992; Omoto et al., 2010; Snaidero & Simons, 2014). As a result, myelination requires oligodendrocytes to operate with exceptional efficiency in generating membranes, particularly lipids, since lipids account for 70-80% of the myelin membrane's dry weight (Aggarwal et al., 2011). This makes oligodendrocyte one of the most powerful lipid-producing cells in the human body.

Vesicular trafficking plays a crucial role in the expansion of the plasma membrane (Pfenninger, 2009). Recent studies have highlighted its significance in myelination, revealing that vesicular transport mediated by SNARE proteins VAMP2, VAMP3, and VAMP7 are essential for oligodendrocyte development and myelin formation (Fekete et al., 2023; Feldmann et al., 2011; Lam et al., 2022; Pan et al., 2023).

However, based on the 3D structure of myelin, I envisioned an additional layer to the story.

### 1.3 A 3D perspective on myelination

Myelination involves intricate cellular behavior of oligodendrocytes (Edgar et al., 2021; Meschkat et al., 2022; Nawaz et al., 2015; Snaidero & Simons, 2014; Snaidero et al., 2014, 2017; Zuchero et al., 2015). The process is illustrated in Fig 1.2, which includes three views: the 3D view (middle), the cross-sectional view (bottom left) and

## 1. Introduction

the unwrapped view (upper right). Firstly, an oligodendrocyte extends its process to grow around an axon (Fig 1.2 A). At the end of one wrap, the growing front enters underneath the existing wrap and keeps growing while the outer wraps start to compact (Fig 1.2 B-D), by zippering the cytoplasmic leaflets of plasma membrane (Fig 1.3). However, this compaction is partial, leaving certain areas uncompact to form the cytoplasmic channels, which serve as a highway for transportation of essential materials from oligodendroglial cell body to myelin's growing front. This growing front, when viewed in the cross-sections of developing myelin (Fig 1.2 B-D bottom left), is the uncompact compartment known as inner tongue, situated between the myelin and the axon.

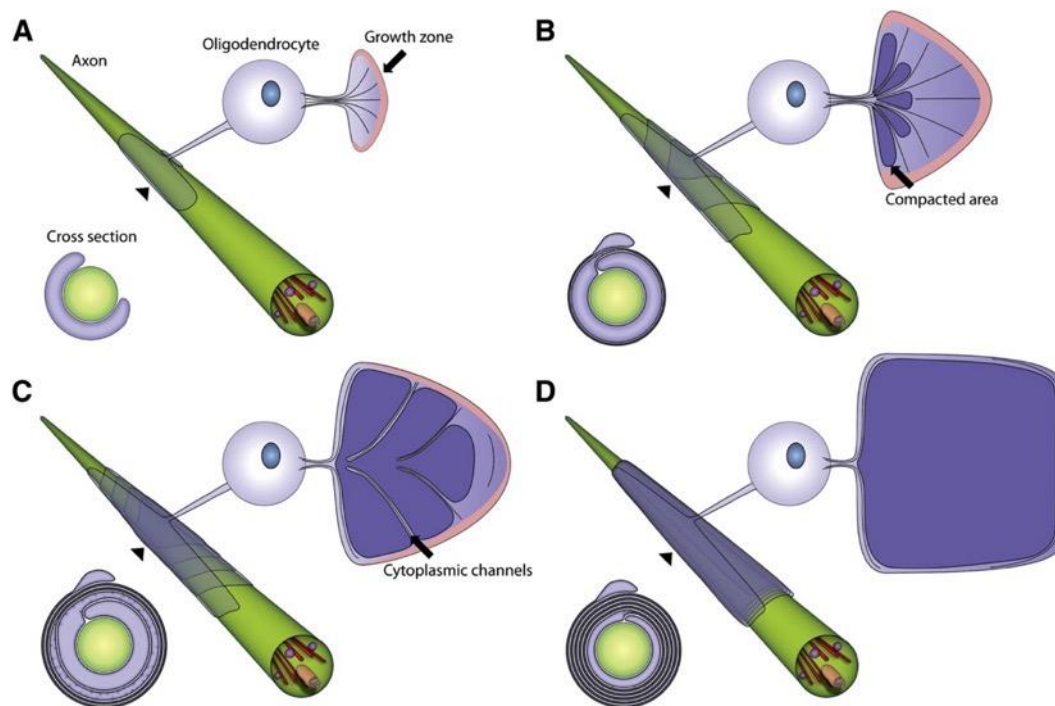


Figure 1.2 Model for myelin ensheathment with 3D (middle), unwrapped (upper right), and cross-sectional views (bottom left). The unwrapped view illustrates the sheath's geometry and development, including cytoplasmic channels (arrow in C) linking the cell body to the growth zone (pink area). Dark violet indicates the compacted myelin. The 3D view depicts ensheathment around the axon, and cross-sections display varying compaction levels throughout growth. Figure taken from (Snaidero et al., 2014), with permission from the publisher Elsevier, license number 5743700289301.

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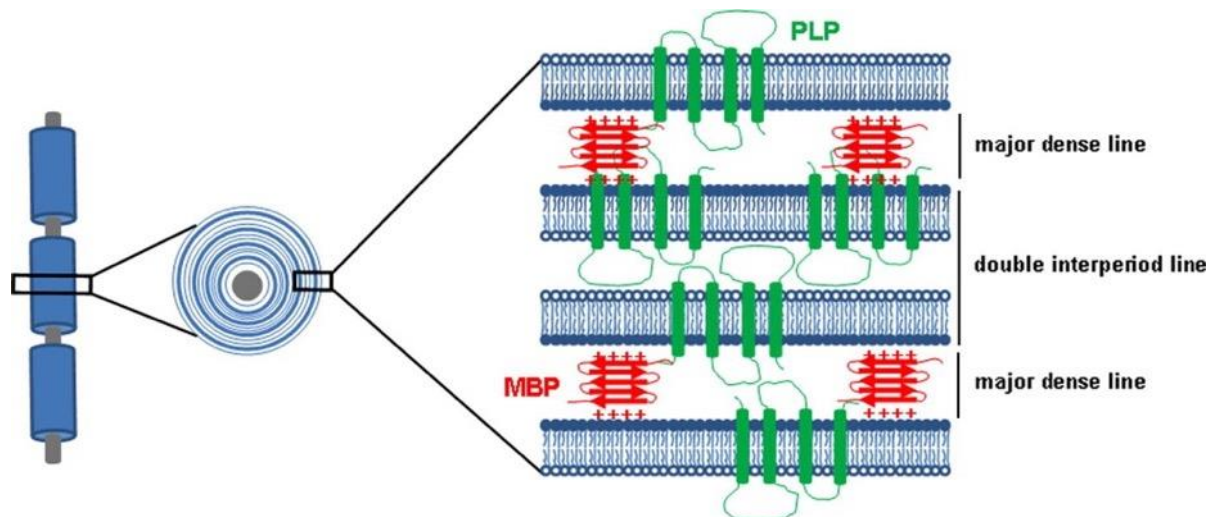


Figure 1.3 Structure of compact myelin. Compact myelin is formed when oligodendroglial cytosolic protein MBP mediates the alignment of intracellular leaflets of plasma membrane and the expulsion of cytoplasm, resulting in the formation of the major dense line, while plasma membrane protein PLP mediates juxtaposition of the bilayer membrane's extracellular leaflets, creating the double intraperiod line. Figure taken from (Baron & Hoekstra, 2010), with permission from the publisher ELSEVIER BV, license number 1462821-1.

Considering this 3D structure of developing myelin, vesicular trafficking poses a significant challenge: it would require transporting lipids from the ER to the Golgi apparatus located within the cell body and proximal processes, followed by formation of vesicles at the Golgi and their transport through the cytoplasmic channels to myelin, and the eventual fusion of these vesicles with target membranes (Alberts et al., 2017; Bonifacino & Glick, 2004; Feldmann et al., 2011; Jahn & Scheller, 2006; Trajkovic et al., 2006). Furthermore, given that cytoplasmic channel is highly curved and narrow, vesicular trafficking in myelin faces even greater challenges compared to axonal vesicular transport, which is already known to be complex.

In summary, vesicular trafficking is a multi-step, relatively slow and challenging process. It raises the question whether it could be complemented by a more straightforward pathway, specifically, a direct transfer of lipids from the ER to the plasma membrane during myelin development. Theoretically, this route is feasible since the majority of myelin lipids are synthesized in the ER and bypass the need for Golgi processing.

### 1.4 Nonvesicular Lipid transport

It has been shown that lipids can be transported in a nonvesicular manner between two membranes. The potential routes are summarized in Fig 1.4 (Prinz, 2010).

Among these, the most documented route is the transport by lipid transfer proteins at membrane contact sites (Fig 1.4C). Different organelles can come together in close proximity to a distance of less than 30nm without fusing, often tethered by molecules. Such a short distance facilitates a class of proteins, known as lipid transfer proteins, to transfer lipids between membranes. A variation of this transport mechanism is the bridge-like transfer (Fig 1.4D), exemplified by VPS13, which forms a hydrophobic tunnel and facilitates bulk transport for a wide spectrum of lipids at membrane contact sites, making it a potentially crucial mechanism for membrane expansion (Melia & Reinisch, 2022). Transient hemifusion could also be an effective method for bulk lipid transport; however, the feasibility of this route is highly controversial. For the remaining mechanisms, additional evidence is required to confirm their physiological relevance.

Nonvesicular lipid transport can facilitate bulk transport of lipid, thereby contributing to membrane expansion, such as neurite growth (Gallo et al., 2020; Petkovic et al., 2014), and the membrane biogenesis of autophagosome (Maeda et al., 2019; Osawa et al., 2019; Valverde et al., 2019), mitochondria and chloroplast (Reinisch & Prinz, 2021). Yet, whether oligodendrocytes employ nonvesicular lipid transport for membrane expansion remains unclear.



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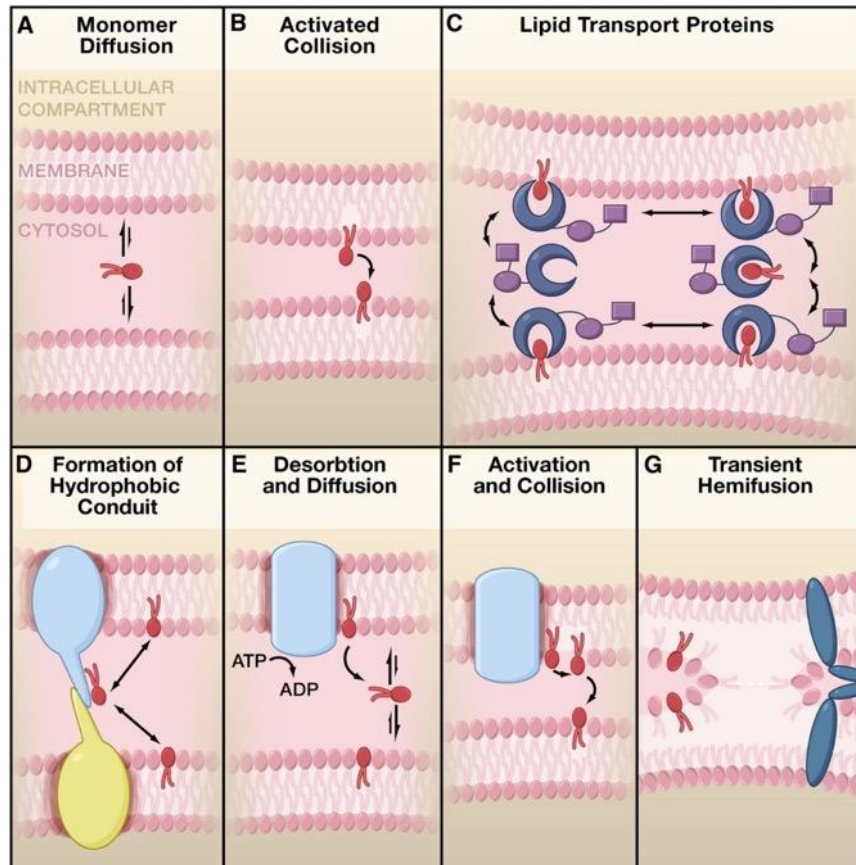


Figure 1.4 Possible routes of nonvesicular lipid transport.

Lipids can move between membranes without proteins' aid in two ways: (A) by diffusing as monomers through the aqueous phase or (B) when two membranes collide.

(C-G) illustrate mechanisms dependent on proteins. (C) Lipid transfer proteins (LTPs) can transfer lipids between membranes of different organelles. LTPs have a hydrophobic pocket (blue) and, often targeting domains (purple) that may direct lipid transfer to particular membranes by binding to certain lipids or proteins. Lipids may be transferred or exchanged at membrane contact sites where two membranes are apposed to each other. In addition, proteins or protein complexes can facilitate nonvesicular lipid transport by (D) creating a tunnel for lipid diffusion between membranes, (E) enhancing lipid detachment from a membrane, (F) activating lipids before membranes collide, or (G) inducing temporary membrane hemifusion.

Figure taken from (Prinz, 2010) with permission from the publisher Elsevier, license number 5747690781232

Nonvesicular lipid transport also occurs between ER-plasma membrane (Saheki & De Camilli, 2017). In fact, the first evidences supporting nonvesicular transport came from studies on ER-to-plasma membrane transport (Prinz, 2010): A wide range of lipids synthesized on the ER, including glycerophospholipids, sterols and monoglycosylceramides, have been shown to reach the plasma membrane when vesicular transport is blocked (Table 1) (Baumann et al., 2005; Halter et al., 2007; Kaplan & Simoni, 1985a; Urbani & Simoni, 1990; Vance et al., 1991). Furthermore,

## 1. Introduction

ER-to-plasma membrane nonvesicular transport exhibit significant capacity, as the transfer rate of various lipids remains unaffected even when vesicular trafficking is inhibited (Kaplan & Simoni, 1985a, 1985b; Sleight & Pagano, 1983; Warnock et al., 1994). In recent years, with the advancements and increased accessibility of electron and light microscopy techniques, ER-plasma membrane contacts have been documented across various cell types. This has strengthened the evidence supporting the feasibility and importance of ER-to-plasma membrane nonvesicular transport.

Table 1: Lipid classes that can reach plasma membrane in the absence of vesicular trafficking.

Lipid classes	References
Sterols	(Heino et al., 2000; Kaplan & Simoni, 1985b; Urbani & Simoni, 1990)
Phosphatidylcholine (PC)	(Kaplan & Simoni, 1985a)
Phosphatidylethanolamine (PE)	(Sleight & Pagano, 1983; Vance et al., 1991)
Glucosylceramide	(Warnock et al., 1994)
Galactosylceramide (GalCer)	(Halter et al., 2007)

### 1.5 Potential nonvesicular lipid transport for myelination

For cultured oligodendrocytes, blocking exocytic SNAREs leads to smaller cells that are nonetheless capable of forming myelin-like sheets (Fig 1.5) (Lam et al., 2022). Although inhibiting protein transport significantly impedes these cells' development—evidenced by a reduction in mature oligodendrocyte markers like myelin basic protein (MBP) (Lam et al., 2022)—the analysis of Galactosylceramide (GalCer), a major myelin lipid, on the plasma membrane indicates that GalCer still reaches the plasma membrane and tends to accumulate over time. This points to the potential involvement of a nonvesicular pathway, at least for GalCer.

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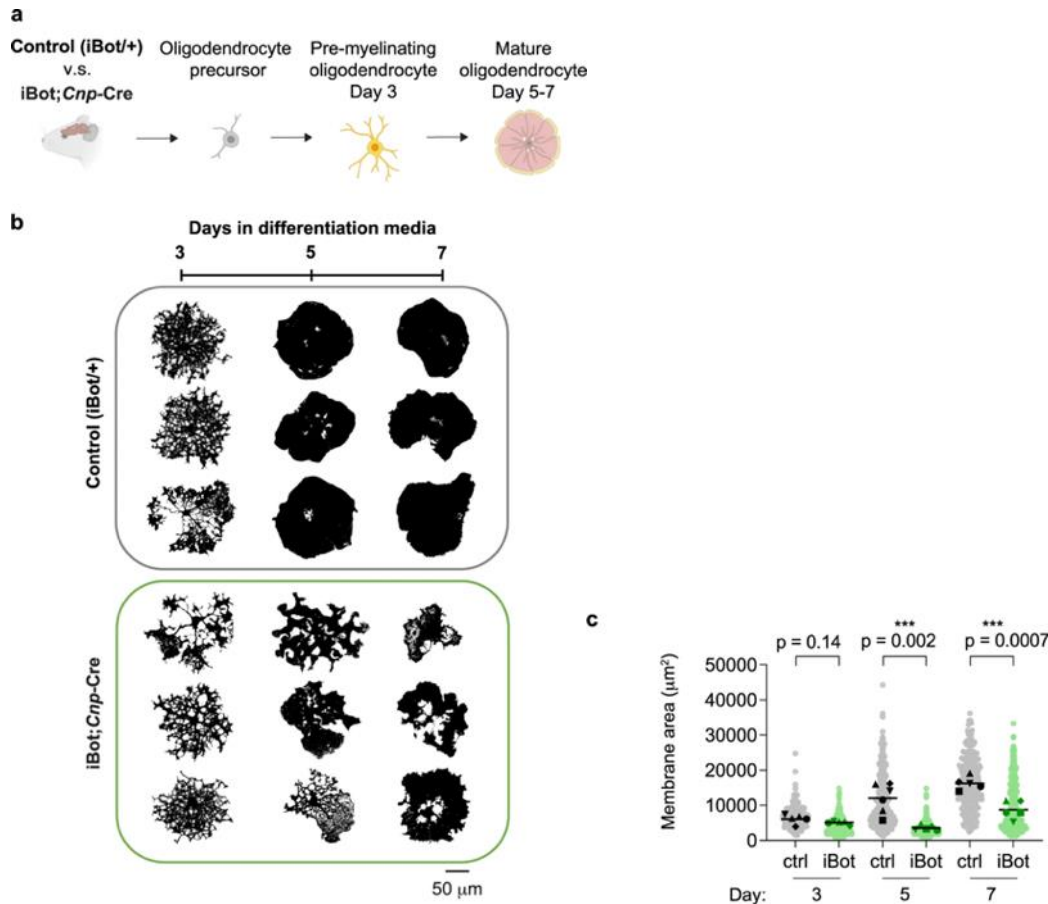


Figure 1.5 Oligodendroglial plasma membrane GalCer under exocytosis inhibition (iBot; *Cnp-Cre*). Figure taken from an open access article (Lam et al., 2022) DOI <https://doi.org/10.1038/s41467-022-33200-4>

Oligodendrocytes also express a number of lipid transfer proteins (Sharma et al., 2015; Y. Zhang et al., 2014), such as cholesterol transporter NPC1 and NPC2 (Prinz, 2010), glycolipid transfer proteins (GLTP) (Mishra et al., 2020) and bridge-like lipid transfer proteins (Braschi et al., 2022).

### 1.6 Glycolipid transfer protein (GLTP)

A particularly intriguing candidate for further study is GLTP, which is significantly more abundant in oligodendrocytes than in other cell types within the CNS. GLTP is known to transfer glycolipids, including GalCer, between liposomes *in vitro* (Mishra et al., 2020). Myelin contains large amount of glycolipids, primarily GalCer, which is synthesized in the ER, and a small quantity of its derivative, sulfatide, which is synthesized in the Golgi apparatus (Norton & Poduslo, 1973). Interestingly, in cell line studies, GalCer transport persists in the absence of vesicular trafficking and *Glt* overexpression stimulates GalCer transport (Halter et al., 2007). Furthermore, GLTP

## 1. Introduction

is upregulated during the progression of myelin development in wildtype mice (C. Zhang et al., 2023), while in contrast, is downregulated in hypomyelinated mutants, along with UGT8, the enzyme responsible for GalCer synthesis (Yang et al., 2019). Taken together, these findings suggest that GLTP plays an important role in myelin development.

### 1.7 Hypothesis and aims of the study.

With the above-mentioned background knowledge, I formulated my hypothesis as follows: Oligodendroglial lipids are transferred directly from the ER to plasma membrane in a nonvesicular manner to contribute to myelin membrane biogenesis. If this hypothesis holds true, two key phenomena should be observable: firstly, the ER would extend into the developing myelin, making contacts with the expanding oligodendroglial plasma membrane. Secondly, lipid transfer proteins would operate at these contact sites, facilitating the transport of lipids. Based on this hypothesis, I have structured the aims of my study as follows:

**Aim 1:** To determine the presence of the ER in developing myelin. Surprisingly, despite the growth zone of myelin (i.e., the inner tongue) being a critical site for essential reactions of myelination and crucial for understanding the process, its detailed contents, particularly the organelles present, remain unexplored. I intend to examine the developing myelin in mice using volume electron microscopy, the benchmark technique for organelle characterization, supplemented by immunohistochemistry.

**Aim 2:** To analyze the distance between the ER and plasma membrane in developing myelin. The application of electron microscopy techniques will facilitate the measurement of such distance.

**Aim 3** (Provided that findings from Aims 1 and 2 support my hypothesis): To investigate the involvement of lipid transfer proteins, specifically GLTP, in myelination. First, I plan to confirm GLTP's abundance and examine its subcellular localization. Subsequently, I aim to assess GLTP's impact on myelin development by generating a mouse mutant lacking GLTP and exploring whether GLTP operates at ER-plasma membrane contact sites.

## 2. Material and Methods

### 2.1 Generation of *Gltf* knockout (KO) and *Gltf* flox mice

To create *Gltf* KO and *Gltf* flox mice, CRISPR/Cas9-mediated genome editing was performed in mouse zygotes, as described previously (Wefers et al., 2023). Briefly, zygotes at the pronuclear stage were collected from matings between C57BL/6J males and superovulated C57BL/6J females (Charles River). These embryos were treated by pronuclear microinjection with a CRISPR/Cas9 ribonucleoprotein (RNP) solution targeting the *Gltf* gene, consisting of SpCas9 protein (IDT), crRNA (IDT), tracrRNA (IDT), and mutagenic long single-stranded DNA (LssDNA). The LssDNA included 200 nucleotides of homology arms and two loxP sites flanking exon 2 (ENSMUSE00000190151) of the *Gltf* gene (264 bp upstream and 487 bp downstream, respectively). After microinjection, the zygotes were implanted into pseudopregnant CD-1 foster mice. Founder mice exhibiting the desired genetic modifications—either an exon 2 deletion or both loxP sites incorporation in cis—were bred with C57BL/6J mice to establish pure lines. The fidelity of genetic alterations in F1 progeny was confirmed through a combination of restriction fragment length polymorphism (RFLP) analysis, Sanger sequencing, and quantitative PCR for gene copy numbers. To exclude off-targets, CRISPOR online tool (Concordet & Haeussler, 2018) was utilized to predict potential off-target sites, which were PCR-amplified and verified by Sanger sequencing. Only mice free of unintended genetic modifications were chosen for further breeding.

### 2.2 Mouse experiments

All mice were handled according to the institutional guidelines sanctioned by the Animal Welfare and Use Committee of the Government of Upper Bavaria. Mice were group-housed in individually ventilated cages (IVC) in a specific pathogen-free and temperature-controlled ( $21 \pm 2^\circ\text{C}$ ) facility on a 12-hr light/dark cycle, with free access to food and water. Experiments involved both male and female mice, with cohorts being sex-matched for comparisons. The study utilized C57BL/6j mice as the wildtype group. The *Cnp-Cre* mouse strain, generously provided by Department Nave of the Max Planck Institute for Multidisciplinary Sciences, was kept heterozygous through backcrossing with C57BL/6j mice. In crossing of *Cnp-Cre* X *Gltf* flox, the *Cre* was

## 2. Material and Methods

always from maternal source. All *Cnp-Cre* samples examined in this study are heterozygous for the *Cnp-Cre* gene.

### 2.3 Cell line and plasmid

U2OS cells (ATCC HTB-96), a kind gift from Christian Behrends laboratory at SyNergy Munich, were cultured in DMEM medium with 10% FBS and 1X penicillin-streptomycin at 37°C with an atmosphere of 5% CO<sub>2</sub>. Transfections were performed using Lipo3000 (Invitrogen). The plasmid “pCMV-UGT8-FusionRed” is constructed by Gibson assembly (NEB), assembling three fragments from 5' to 3': (1) pCMV from Addgene plasmid #36412; (2) mouse Ugt8 cDNA (synthesized as gblock fragment by IDT); (3) FusionRed from Addgene plasmid #54778. Because UGT8 is a Type I ER membrane protein, it is designed to fuse with a non-cytotoxic fluorescence protein, FusionRed, at its C-terminal.

### 2.4 Primary antibodies

Antibodies	Source	Application
Rat anti-MBP	Abcam ab7349	1:500 for IHC/ICC
chicken anti-MBP	Thermo PA1-10008	1:500 for IHC/ICC
anti-Rtn4	Abcam ab47085	1:500 for IHC/ICC
anti-Reep5	Proteintech 14643-1-AP	1:500 for IHC/ICC
anti-BCAS1	Santa Cruz sc136342	1:400 for IHC
anti-MAG-Alexa647	Santa Cruz sc-166849 AF647	1:100 for IHC 1h at room temperature
anti-Rtn1	Sigma HPA044249	1:250 for ICC
anti-Sec61b	Sigma HPA049407	1:400 for ICC
anti-KDEL	Enzo ADI-SPA-827-D	1:250 ICC
anti-GLTP	Sigma ATA- HPA056461-100	1:300 for IHC/ICC with antigen retrieval, 1:500 for Western Blot
anti-GalCer	Merck MAB342	1:1500 for ICC
anti-GalCer- Alexa488	Merck MAB342A4	1:100 for ICC
anti-UGT8	Proteintech 17982-1-AP	1:500 for ICC
anti-CD140a	Biolegend 135902	1:300 for primary culture isolation



### 2.5 siRNA for oligodendrocyte culture

Cell permeable siRNAs were purchased from Horizon/Dharmacon, as the “SMARTpool” format (a mixture of four siRNA): nontargeting (D-001910-10-20), mouse *Rtn4* (E-059578-00-0010), mouse *Glt<sub>1</sub>* (E-058085-00-0010). 100x stock solution was prepared according to manufacturer’s instruction. The siRNA was added to the cells when switching to differentiation medium, with a final concentration of 1  $\mu$ M, and incubated for 3 days.

### 2.6 Transmission and scanning electron microscopy

Optic nerve and spinal cord tissue were dissected and immersion fixed (P14) or perfusion fixed (P28) in 4% PFA (EM-grade, Science Services), 2.5% glutaraldehyde (EM-grade, Science Services), 2 mM calcium chloride in 1xPBS, pH 7.4 (Science Services) for 24h. Short (2-3 mm) pieces of the optic nerve and the 1 mm thick spinal cord slices were incubated in the same fixative for another 24h and stored in 0.1 M cacodylate buffer at 4°C. We applied a rOTO en bloc staining protocol including postfixation in 2% osmium tetroxide (EMS), 1.5% potassium ferricyanide (Sigma) in 0.1 M sodium cacodylate (Science Services) buffer (pH 7.4) (Kislinger et al., 2020). The contrast was enhanced by incubation in 2% thiocarbohydrazide (Sigma) for 45 min at 40°C. The tissue was washed in water and incubated in 1% aqueous osmium tetroxide, washed and further contrasted by overnight incubation in 1% aqueous uranyl acetate at 4°C and 2h at 50°C. Samples were dehydrated in an ascending ethanol series and infiltrated with the epoxy-araldite resin LX112 (Ladd Research). Blocks were cured for 48h at 60°C, trimmed (EM TRIM, Leica) and sectioned at 100 nm thickness using a 35° ultra-diamond knife (Diatome) on an ultramicrotome (UC7, Leica). Sections were collected onto 1x0.5 cm carbon nanotube tape strips (Science Services) for scanning EM (SEM) analysis or onto formvar-coated copper grids (Plano) for transmission EM (TEM). For SEM imaging the samples on tape were attached to adhesive carbon tape (Science Services) on 4-inch silicon wafers (Siegert Wafer) and grounded by adhesive carbon tape strips (Science Services). EM micrographs were acquired on a Crossbeam Gemini 340 SEM (Zeiss) with a four-quadrant backscatter detector at 8 kV using ATLAS5 Array Tomography (Fibics). Medium lateral resolution images (100-200 nm) allowed the identification of regions of interest that were in turn reimaged at 4-10 nm lateral resolution. TEM micrographs for higher resolution representations were acquired on a JEM 1400plus (JEOL) equipped

with a XF416 camera (TVIPS) and the EM-Menu software (TVIPS). Image analysis was performed in Fiji (Schindelin et al., 2012)

### 2.7 Volume electron microscopy

For volume EM analysis of myelinated axons in the optic nerve we applied Automated Tape Collecting Ultramicrotomy (ATUM) (Kislinger, Niemann, et al., 2023). The blocks were trimmed at a 45° angle on four sides using a trimming machine (EM TRIM, Leica). Serial sections were taken with a 35° ultra-maxi diamond knife (Diatome) at a nominal cutting thickness of 50 nm on the ATUMtome (Powertome, RMC). Sections were collected onto freshly plasma-treated carbon coated Kapton tape (kindly provided by Jeff Lichtman and Richard Schalek). Plastic tape stripes were assembled onto adhesive carbon tape (Science Services) attached to 4-inch silicon wafers (Siegert Wafer) and grounded by adhesive carbon tape strips (Science Services). ATUM-SEM acquisition was performed on an Apreo S2 SEM (Thermo Fisher Scientific) with the T1 detector using the Maps2 (Thermo Fisher Scientific) software. The tissue was hierarchically imaged as previously described (Kislinger et al., 2020). Target regions were identified and acquired at high resolution (4x4x50 nm). Serial section data were aligned by a sequence of automatic and manual processing steps in Fiji TrakEM2 (Kislinger, Fabig, et al., 2023; Schindelin et al., 2012).

### 2.8 EM analysis

The VAST software was used for manual segmentation (Berger et al., 2018) and rendered in Blender (Brito, 2018) for three-dimensional visualization.

Organelle identification followed the detailed protocol (Park, 2023) with inputs from Martina Schifferer (SyNergy EM hub, Munich, Germany) and Shujun Cai (Southern University of Science and Technology, Shenzhen, China). Organelles were categorized into three groups: ER, mitochondria and others. The ER is characterized as a continuous structure, while mitochondria are distinguished by their inner and outer membranes (cristae), which were absent in developing myelin. The 'others' category encompasses endosomes, vesicles, and organelles of uncertain identity. Endosomes are typically ball-shaped with a light lumen; among these, multivesicular bodies (MVBs) are a special type that contain small vesicles. The Golgi apparatus is identified by its ribbon-like appearance, featuring interconnected stacks, and surrounding small vesicles. In this dataset, some membrane structures of unclear identity might



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represent the Golgi, which were potentially missed due to the ATUM-SEM's technical limitation with a z-resolution of 50nm. Nonetheless, if these structures are indeed Golgi, they are relatively rare.

G-ratio and inner tongue diameter were measured semi-automatedly using MyelTracer(Kaiser et al., 2021). Outfolding and paranodes are excluded for quantification. Axon, inner and outer edge of myelin are recorded. Diameter of each compartment is calculated as  $2X\sqrt{area/\pi}$ . G-Ratio is calculated as  $\sqrt{\frac{inner\ myelin\ area}{outer\ myelin\ area}}$ ,

i.e. the ratio of inner to outer myelin diameter. Notably this is an updated definition of g-ratio, as opposed to the ratio of axon diameter to outer myelin diameter, so that I can take into account of inner tongues present in developing myelin. Inner tongue diameter is calculated as inner myelin diameter - axon diameter.

### 2.9 Immunohistochemistry

The mice were anesthetized with isoflurane, followed by transcardial perfusion with PBS and then 4% paraformaldehyde (PFA). Subsequently, brains and spinal cords underwent postfixation in 4% PFA for 4h and overnight, respectively. The tissues were immersed in 30% sucrose in PBS until they sank, then embedded in Tissue-Tek O.C.T., frozen on dry ice, and stored at -80°C. The frozen tissues were cut to 14-μm coronal sections using a Leica CM 1900 cryostat. Sections were rinsed with PBS containing and permeabilized in 0.3% Triton X-100 for 15min (for brain) or 1h (for spinal cord). For GLTP staining, antigen retrieval was performed for 20min sodium citrate buffer (pH 6) in 80°C using water bath. Sections were incubated in blocking solution (1% FBS, 1% fish gelatin, and 1% bovine calf serum in PBS) for 1h at room temperature and if mouse primary antibodies are used, Fab fragments are added for another 1h. Primary antibodies, diluted in 10% blocking solution, were added, and incubated overnight at 4°C. On the following day, after washing with PBS, sections were incubated with secondary antibodies for 1h at room temperature, followed by washing, staining with 2 μg/ml Hoechst 33342, and mounting with ProLong gold antifade mounting medium (Thermo P36934).

### 2.10 Primary oligodendrocyte culture

Oligodendrocyte progenitor cells were prepared from postnatal day 7 to day 9 mouse cortices by immunopanning(Emery & Dugas, 2013). Briefly, cortices were dissociated

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to single-cell suspension, which was passed through two negative-selection plates coated with BSL1 to remove microglia. The remaining cell suspension was then incubated in a positive-selection plate coated with anti-CD140a antibodies. The attached cells were collected by accutase and cultured on poly-L-lysine-coated coverslips in proliferation medium containing Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, catalog no. 41965), Sato Supplement, B-27 Supplement, GlutaMAX, Trace Elements B, penicillin–streptomycin, sodium pyruvate, insulin, N-acetyl-L-cysteine, D-biotin, forskolin, ciliary neurotrophic factor (CNTF), platelet-derived growth factor (PDGF) and neurotrophin-3 (NT-3). The culture is differentiated by replacing the above-mentioned proliferation medium's PDGF and NT-3 with T3.

### 2.11 Immunocytochemistry

Cells were fixed by 4% PFA in room temperature for 10min, washed with PBS and stored in PBS containing azide at 4°C. For staining, cells were permeabilized with PBS-0.1% Triton X-100 for 30 sec. For GLTP staining, antigen retrieval was performed for 10min sodium citrate buffer (pH 6) at 70°C using water bath. Blocking solution (1% FBS, 1% fish gelatin, and 1% bovine calf serum in PBS) is applied for 30 min before incubating with primary antibody in PBS-10% blocking solution at 4°C overnight. The following day, the cells are stained with secondary antibodies for 1h at room temperature, followed by Hoechst 33342 for 10min and mounted with ProLong glass antifade mounting medium (Thermo P36980). For GalCer measurement, cells are first incubated with blocking solution, stained with mouse anti-GalCer antibody with 10% blocking solution for 1h at room temperature, then anti-mouse Alexa 555 for 1h at room temperature, permeabilized with 0.1% Triton X-100 for 30 sec, blocked again, and stained overnight with chicken anti-MBP (or other primary antibody) in PBS-10% blocking solution at 4°C overnight. The following day, the cells were stained with anti-chicken-Alexa 647 and anti-GalCer conjugated with Alexa 488 for 1h in room temperature, and lastly stained with Hoechst 33342(Sigma B2261) and mounted with mounting medium.

### 2.12 Fluorescence imaging and analysis

All fluorescence images were acquired by the point scanning confocal microscope Zeiss LSM 900 equipped with Airyscan 2 module, and Plan-Apochromat 63x/1.2 oil

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immersion objective, C-Apochromat 40x/1.1 water immersion objective, Plan-Apochromat 20x/0.8 objective, Plan-Neofluar 10x/0.3 objective. Images were analyzed using Fiji(Schindelin et al., 2012).

### 2.13 Western Blot Analysis

Individual P14 mouse brains were homogenized using a sonicator. The resulting whole-brain lysates were loaded at 12 µg per lane onto a 12% TGX™ precast gel (BioRad #4561046). Following separation by SDS-PAGE, proteins were transferred onto a nitrocellulose membrane. The membrane was then blocked in 3% BSA in PBS-Triton for 30min at room temperature, followed by overnight incubation with primary antibodies in PBS at 4°C. After washing, the membrane was incubated with HRP-conjugated secondary antibodies in PBST for 1h at room temperature. Subsequently, targeted proteins were detected using Pierce ECL substrate (Thermo Fisher #32106), and visualized using an Odyssey Fc imager from LI-COR.

### 2.14 Myelin isolation

Myelin was isolated from mouse brain and spinal cord (P14) or mouse brain only (P28), using one animal for each sample. The protocol with two rounds of sucrose density centrifugation and osmotic shocks is as previously described(Gouna et al., 2021), with some modifications. The ultracentrifugation was done using an SW41 Ti rotor. The tissues were homogenized with a sonicator in a solution containing 10 mM Hepes pH 7.4 and 0.32 M sucrose. The homogenized tissue was layered on 0.32/0.85M sucrose gradient and centrifuged at 25,000 rpm for 35 min with low deceleration and acceleration. The crude myelin fraction was recovered from the interface, resuspended in ice-cold distilled water, and centrifuged at 25,000 rpm for 18 min. The hypo-osmotic shock was applied to the pellet two more times and pellets were collected at 10,000 rpm for 18 min. The pellet from the last step was dissolved in Hepes buffer containing 0.32 M sucrose, then all the centrifugation steps and hypo-osmotic shocks were repeated one more round. Eventually, the purified myelin pellet was resuspended in 1 ml PBS and stored at -20°C.

### 2.15 Lipidomics

Each sample is myelin from one animal. Mass spectrometry-based lipid analysis was performed by Lipotype GmbH (Dresden, Germany) as described(Michał A Surma et al., 2021). Lipids were extracted using a chloroform/methanol procedure(Ejsing et al.,

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2009). Samples were spiked with internal lipid standard mixture containing: cardiolipin 14:0/14:0/14:0/14:0 (CL), ceramide 18:1;2/17:0 (Cer), diacylglycerol 17:0/17:0 (DAG), hexosylceramide 18:1;2/12:0 (HexCer), lyso-phosphatidate 17:0 (LPA), lyso-phosphatidylcholine 12:0 (LPC), lyso-phosphatidylethanolamine 17:1 (LPE), lyso-phosphatidylglycerol 17:1 (LPG), lyso-phosphatidylinositol 17:1 (LPI), lyso-phosphatidylserine 17:1 (LPS), phosphatidate 17:0/17:0 (PA), phosphatidylcholine 15:0/18:1 D7 (PC), phosphatidylethanolamine 17:0/17:0 (PE), phosphatidylglycerol 17:0/17:0 (PG), phosphatidyl-inositol 16:0/16:0 (PI), phosphatidylserine 17:0/17:0 (PS), cholesterol ester 16:0 D7 (CE), sphingomyelin 18:1;2/12:0;0 (SM), triacylglycerol 17:0/17:0/17:0 (TAG) and cholesterol D6 (Chol). After extraction, the organic phase was transferred to an infusion plate and dried in a speed vacuum concentrator. The dry extract was re-suspended in 7.5 mM ammonium formate in chloroform/methanol/propanol (1:2:4; V:V:V). All liquid handling steps were performed using Hamilton Robotics STARlet robotic platform with the Anti Droplet Control feature for organic solvents pipetting. Samples were analyzed by direct infusion on a QExactive mass spectrometer (Thermo Scientific) equipped with a TriVersa NanoMate ion source (Advion Biosciences). Samples were analyzed in both positive and negative ion modes with a resolution of  $R_{m/z=200}=280000$  for MS and  $R_{m/z=200}=17500$  for MSMS experiments, in a single acquisition. MSMS was triggered by an inclusion list encompassing corresponding MS mass ranges scanned in 1 Da increments (Michal A Surma et al., 2015). Both MS and MSMS data were combined to monitor CE, Chol, DAG and TAG ions as ammonium adducts; LPC, LPC O<sup>-</sup>, PC and PC O<sup>-</sup>, as formate adducts; and CL, LPS, PA, PE, PE O<sup>-</sup>, PG, PI and PS as deprotonated anions. MS only was used to monitor LPA, LPE, LPE O<sup>-</sup>, LPG and LPI as deprotonated anions, and Cer, HexCer, and SM as formate adducts. Data were analyzed with in-house developed lipid identification software based on LipidXplorer (Herzog et al., 2011, 2012). Data post-processing and normalization were performed using an in-house developed data management system. Only lipid identifications with a signal-to-noise ratio >5, and a signal intensity 5-fold higher than in corresponding blank samples were considered for further data analysis.

### 2.16 Statistical analysis

Statistical Analysis were performed using Microsoft Excel 2016 and GraphPad Prism 10. Volcano Plot (Fig. 6a) additionally used Lipid Map statistical tool (Fahy et al., 2007;

## 2. Material and Methods

Ni et al., 2023). Missing values of lipidomics are filled as 0. For mouse analysis, mean value per mouse was calculated and presented as a single data point.

### 2.17 Use of artificial intelligence (AI) tools

The text of this dissertation is revised with assistance from ChatGPT 4, where I wrote initial drafts and utilized the AI for suggestions on phrasing and clarity. I selectively incorporated suggestions and ensured the accuracy and integrity of the dissertation's contents.

## 3. Results

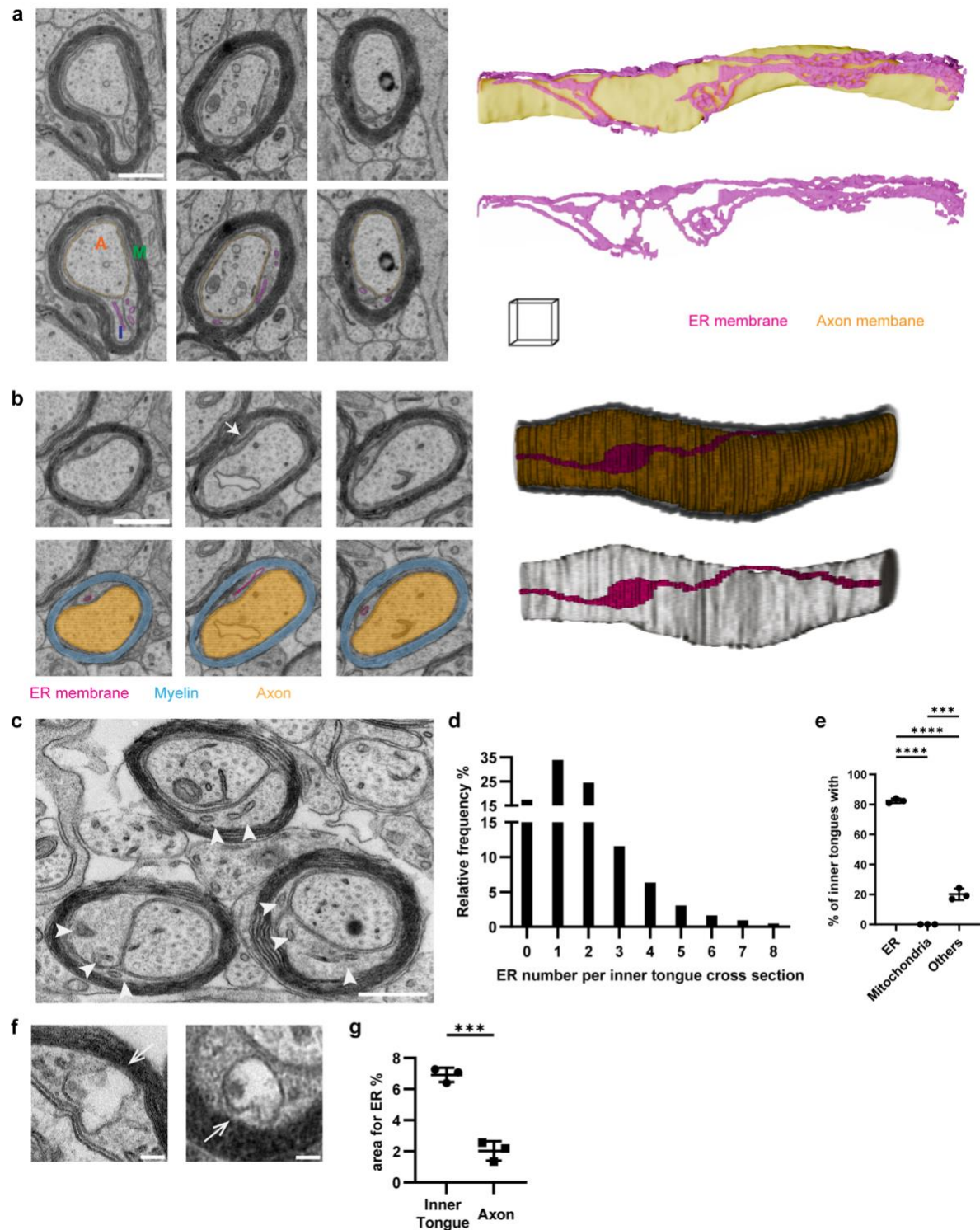
### 3.1 Tubular ER is enriched in developing myelin and contacts the plasma membrane.

The investigation began by examining the presence of the ER in developing myelin, focusing on mice at the postnatal day 14 (P14), a stage when active myelination occurs. A volume electron microscopy dataset from P14 mouse optic nerve was acquired using ATUM-SEM (Automated Tape-collecting Ultramicrotome-Scanning Electron Microscopy) (Kasthuri et al., 2015). Based on the continuity, shape, lumen, and membrane appearance (Heinrich et al., 2021; Park, 2023; Terasaki, 2018), the ER was identified and segmented within the inner tongue, the growing front of myelin (Fig. 3.1a, Movie 1, Fig. 3.1b). The 3D reconstruction revealed an ER network composed primarily ER tubes within the inner tongue. Occasionally, the ER appeared “lumenless” (arrow Fig. 3.1b), reminiscent of the thin ER reported in neuron (Hoffmann et al., 2021; Wu et al., 2017). Quantitative analysis of membrane-bound organelles in three mice at P14 highlighted the ER as the predominant organelle within the inner tongue, with at least one ER present in 82% of examined inner tongues (Fig 3.1c, d, e). Conversely, mitochondria, which are typically prevalent, were absent. The occurrence of ER surpassed that of all other types of membrane-bound organelles, which collectively accounted for 20%, including multivesicular bodies (Fig. 3.1e, f). Despite the spatial constraints of the inner tongue, a significant portion of this area was allocated to the ER by oligodendrocytes, with the ER within the inner tongue occupying more space than that within axons (Fig. 3.1g). The presence of the ER in developing myelin was verified by immunohistochemistry performed on spinal cord cross-sections from P14 mice, which showcased the myelin marker MBP forming distinct rings indicative of myelin structures. Markers for tubular ER, specifically receptor expression-enhancing protein 5 (REEP5) and reticulon 4 (RTN4) (Gia K Voeltz et al., 2006; H. Zhang & Hu, 2016), were detected as puncta on MBP+ rings (Fig. 3.2a). Analysis from three mice indicated that 82% of MBP-positive rings exhibited overlapping REEP5 puncta, and 73% displayed RTN4 puncta (Fig. 3.2b). Moreover, the oligodendrocyte ER was identified in close proximity to the plasma membrane in developing myelin (Fig. 3.3a), with 83% of ER-plasma membrane distances measuring less than 20nm, a



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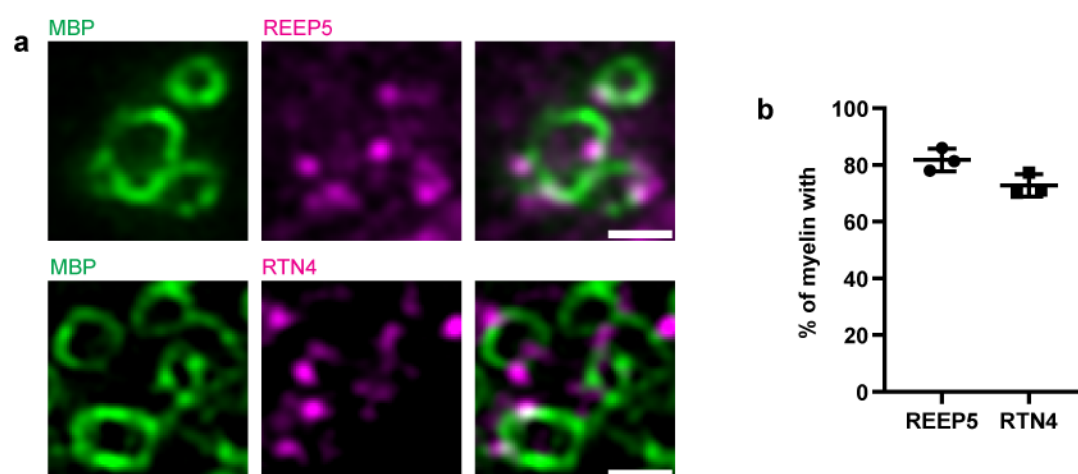
distance feasible for nonvesicular lipid transport (Scorrano et al., 2019; G K Voeltz et al., 2024). Because some inner tongues contain multiple ER, the minimum distance in each inner tongue is more striking, with 94% of ER-plasma membrane distances less than 20 nm.



**Fig. 3.1 | Tubular ER in developing myelin revealed by electron microscopy**

### 3. Results

**a** Volume EM (ATUM-SEM) analysis of developing myelin from postnatal day 14 (P14) mouse optic nerve. Different compartments are marked on the bottom left image: axon (A), myelin (M) and inner tongue (I). ER membrane (magenta) and axon membrane (yellow) are segmented. 3D reconstruction of ER network is shown together with (upper) or without (bottom) axonal membrane, from an 11.25  $\mu\text{m}$ -thick stack (226 slices with 50 nm interval, scale cube 1x1x1  $\mu\text{m}$ ). **b** Another 3D reconstruction of ER in the inner tongue. From a 4.5 $\mu\text{m}$ -thick stack (91 slices with 50 nm interval). Arrow in 2<sup>nd</sup> EM example: “lumenless” ER. Magenta: ER membrane; Blue: Myelin; Yellow: Axon. **c** Representative image of the TEM dataset for quantification of membrane-bound organelles in the inner tongue. Arrow heads: ER. **d** The distribution of ER number per inner tongue cross section, quantified from 425 inner tongue cross sections of three mice. **e** Ratio of inner tongues containing specified organelles. Quantification of (c) showing mean  $\pm$  SD, ER:  $82.36 \pm 1.51$ , mitochondria: 0, others:  $20.13 \pm 3.80$  (n=3 wildtype P14 mice, One-way ANOVA followed by Tukey’s post-hoc test). **f** Examples of other membrane-bound organelles (arrows), many being multivesicular body. **g** ER occupies  $6.91 \pm 0.45\%$  area of inner tongue and  $2.02 \pm 0.62\%$  area of axon (mean  $\pm$  SD). (n=3 wildtype P14 mice, Two-tailed unpaired t-test). Scale bars: 0.5  $\mu\text{m}$  (a, b, c), 0.1  $\mu\text{m}$  (f). \*:  $P \leq 0.05$ , \*\*:  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$ , \*\*\*\*:  $P \leq 0.0001$

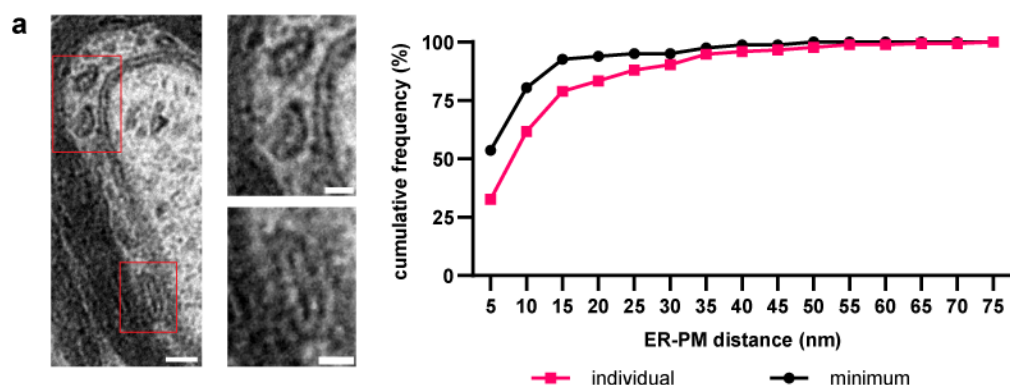


**Fig. 3.2 Tubular ER in developing myelin revealed by immunohistochemistry**

**a** Immunohistochemistry of developing myelin from P14 mouse spinal cord shows tubular ER markers (REEP5 and RTN4) appear as puncta and overlap with myelin (MBP). **b** Ratio of myelin (MBP<sup>+</sup> ring) overlapping with specified tubular ER. Quantification of (a) showing mean  $\pm$  SD, REEP5:  $81.80 \pm 3.97$ , RTN4:  $72.79 \pm 3.91$  (n=3 wildtype P14 mice). Scale bars: 1  $\mu\text{m}$  (a).



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**Fig. 3.3 Tubular ER contacts plasma membrane in developing myelin**

**a** An example with zoom-in views shows short distance between the oligodendroglial ER and plasma membrane in developing myelin, and distribution of ER's distance to plasma membrane. Distribution of individual ER's distance to plasma membrane is shown by magenta squares:  $62\% \leq 10$  nm,  $83\% \leq 20$  nm,  $90\% \leq 30$  nm ( $n=175$  ER-plasma membrane distance from three wildtype P14 mice) and distribution of minimum distance in a given inner tongue is shown by black dots:  $80\% \leq 10$  nm,  $94\% \leq 20$  nm,  $95\% \leq 30$  nm ( $n=82$  inner tongues from three wildtype P14 mice). Scale bars: 100 nm (a left), 50 nm (a zoom-in)

### 3.2 Tubular ER is associated with active myelination.

To investigate if the expression patterns of tubular ER markers are aligned with active myelin development, immunohistochemistry was performed, utilizing antibodies against BCAS1 and MAG to mark premyelinating and actively myelinating oligodendrocytes (Fard et al., 2017). P14 mouse cortex was analyzed. Within the cortex (Fig. 3.4a, b), myelinating oligodendrocytes (BCAS1+MAG+, marked by arrows) exhibited increased levels of the tubular ER markers REEP5 and RTN4 compared to their premyelinating counterparts (BCAS1+MAG-, marked by arrowheads). Data from three P14 mice indicated that a significant majority, 93% for REEP5 and 82% for RTN4, of myelinating oligodendrocytes displayed elevated expression of these markers (Fig. 3.4c). Conversely, premyelinating oligodendrocytes have lower levels of these tubular ER proteins, implying an upregulation of tubular ER proteins concurrent with the onset of myelination. Similar patterns were observed in the cerebellum (Fig. 3.4d, e, f).

In 6-month-old mice, where myelin is mature, a decline in tubular ER within white matter regions was observed in comparison to P14 mice (Fig. 3.5a, b). Analysis of three mice from each time points highlighted a significant decrease of the tubular ER

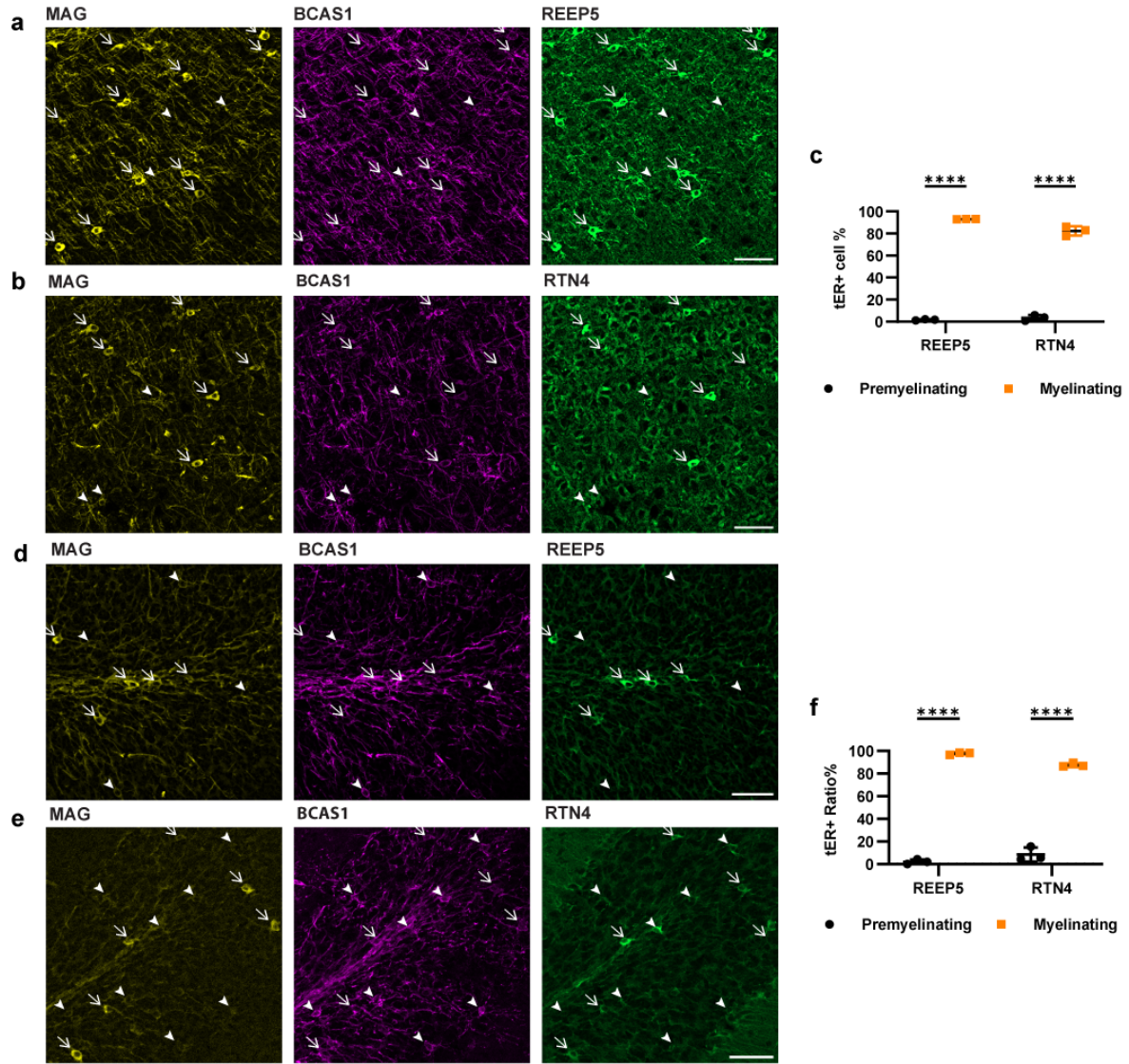
### 3. Results

fluorescence signal, normalized by the myelin marker MBP signal (Fig. 3.5c, d). This observation could be explained by the previously reported finding that the size of the inner tongue decreases as myelination completes. Presumably, the ER in the inner tongue withdraws from the myelin as the inner tongue undergoes closure. To confirm, EM analysis was performed on wildtype mice at postnatal day 28 (P28), a period corresponding to a late stage of developmental myelination. Most myelin sheaths lacked inner tongues or have narrow inner tongues, and the ER was rarely observed (Fig. 3.5e)

In addition, cultured mouse oligodendrocytes were utilized to investigate the subcellular distribution of various ER subdomains. Markers SEC61b and KDEL, indicative of rough ER and ER sheets, respectively (Farías et al., 2019), were predominantly found in the cell body. In contrast, tubular ER markers RTN4 and RTN1 (H. Zhang & Hu, 2016) were distributed more broadly. The calculation of the peripheral to cell body signal ratio demonstrated a predominance of tubular ER over other ER forms, indicating that oligodendrocytes preferentially extend their ER as tubules towards the growing front (Fig. 3.6a).

Furthermore, the requirement of tubular ER for myelination was explored. Knocking down *Rtn4* via RNA interference in primary oligodendrocytes led to a marked reduction in cell size (Fig. 3.6b, c), underscoring the critical role of tubular ER in membrane expansion.

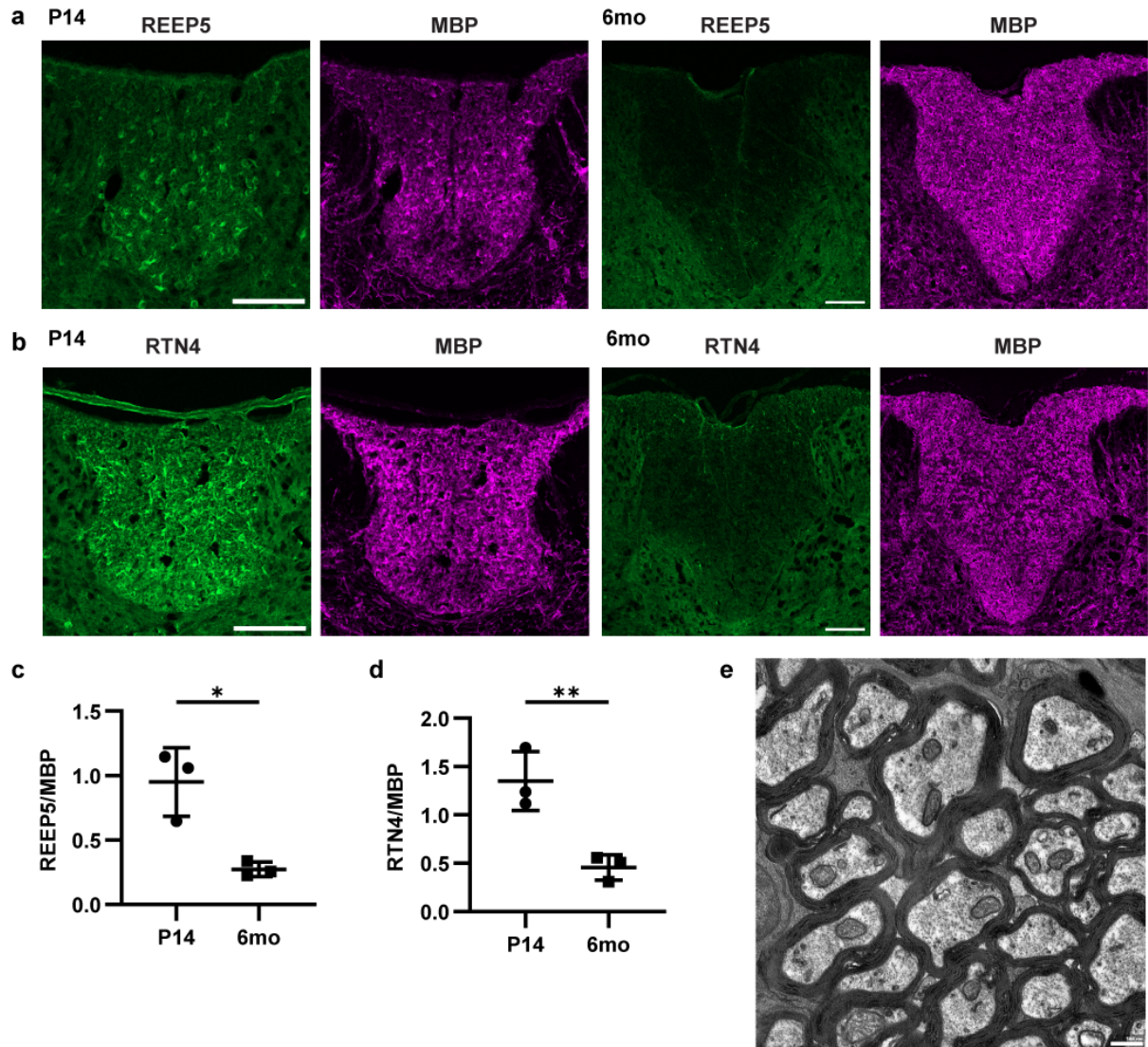
### 3. Results



**Fig. 3.4 | Tubular ER is more abundant in myelinating than premyelinating oligodendrocytes**

**a, b, d, e** Immunohistochemistry of P14 mouse cortex (a, b) and cerebellum (d, e). Arrow heads: premyelinating oligodendrocytes ( $MAG^+BCAS1^+$ ); arrows: myelinating oligodendrocytes ( $MAG^+BCAS1^+$ ). **c** Quantification of (a) and (b) showing mean  $\pm$  SD,  $1.65 \pm 0.55\%$  or  $3.54 \pm 2.60\%$  premyelinating cells are REEP5+ or RTN4+,  $93.04 \pm 0.25\%$  or  $82.34 \pm 4.16\%$  myelinating cells are REEP5+ or RTN4+. (n=3 wildtype P14 mice, Two-way ANOVA followed by Sidak's multiple comparison test). **f** Quantification of (d) and (e) from three P14 mice, showing mean  $\pm$  SD,  $2.04 \pm 2.09\%$  or  $8.40 \pm 6.19\%$  premyelinating cells are REEP5+ or RTN4+,  $97.79 \pm 1.03\%$  or  $87.48 \pm 1.68\%$  myelinating cells are REEP5+ or RTN4+. (n=3 wildtype P14 mice, Two-way ANOVA followed by Sidak's multiple comparison test). Scale bars: 50  $\mu$ m (a, b, d, e). \*:  $P \leq 0.05$ , \*\*:  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$ , \*\*\*\*:  $P \leq 0.0001$

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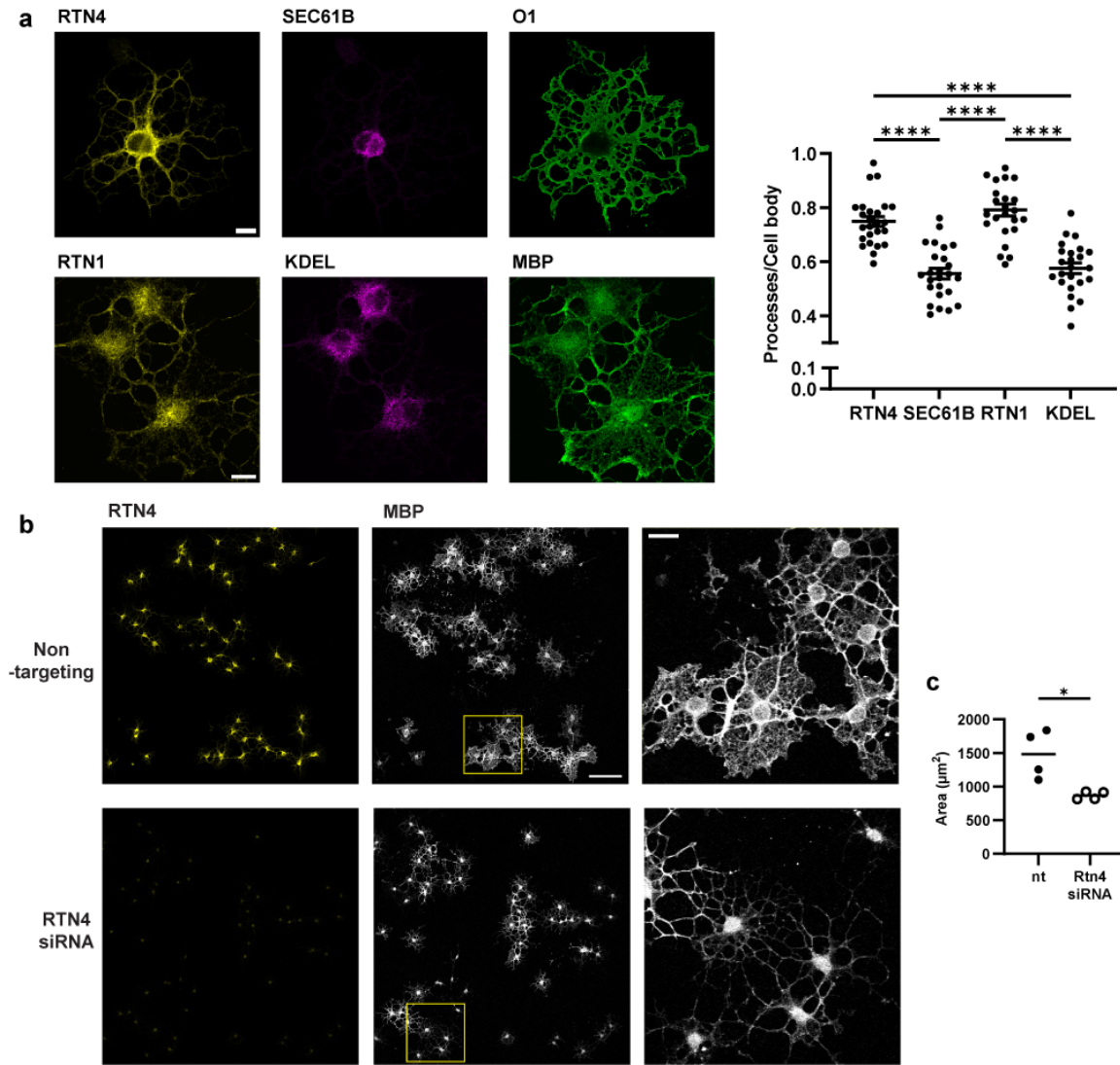


**Fig. 3.5 | Tubular ER is absent in mature myelin**

**a, b** Immunohistochemistry of P14 and adult (6mo) mouse thoracic spinal cord, focusing on dorsal white matter **c, d** Quantification of (a) and (b), respectively: tubular ER (REEP5 or RTN4) fluorescence signal normalized by myelin (MBP) signal, in dorsal white matter of thoracic spinal cord, showing mean  $\pm$  SD, (f) P14:  $0.95 \pm 0.27$ , 6-month-old:  $0.27 \pm 0.06$  (g) P14:  $1.35 \pm 0.30$ , 6-month-old:  $0.46 \pm 0.13$  (n=3 wildtype mice for each time point, Two-tailed unpaired t-test) **e** TEM analysis of wildtype P28 optic nerve. Scale bars: 100  $\mu$ m (a, b), 500 nm (e). \*:  $P \leq 0.05$ , \*\*:  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$ , \*\*\*\*:  $P \leq 0.0001$



### 3. Results



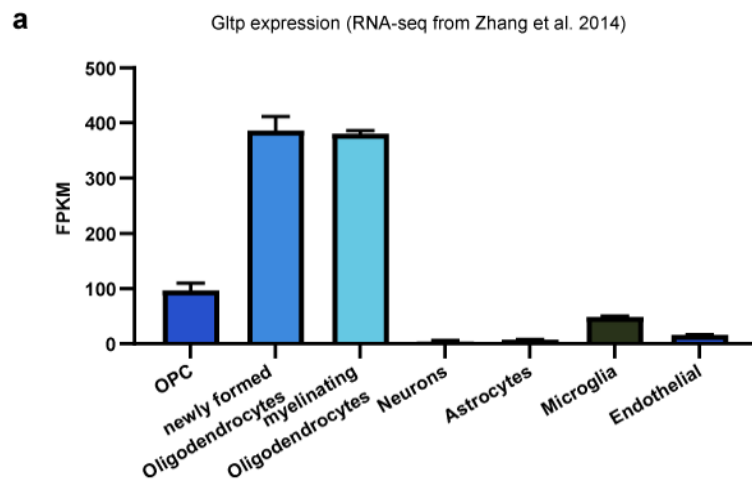
**Fig. 3.6 | Tubular ER supports plasma membrane expansion in cultured oligodendrocytes**

**a** Left: Immunocytochemistry of primary oligodendrocyte culture for tubular ER markers (RTN4 and RTN1), rough ER marker SEC61B, ER sheet marker KDEL (Fariás et al., 2019) and oligodendrocyte marker O1 and MBP for outlining the cells. Right: The ratio of fluorescence signals from different ER subtypes at the cellular processes to that at the cell body, showing mean  $\pm$  SD, KDEL:  $0.58 \pm 0.10$ , RTN1:  $0.79 \pm 0.11$ , SEC61B:  $0.56 \pm 0.10$ , RTN4:  $0.75 \pm 0.09$  (n=24 cells for KDEL/RTN1, 25 cells for SEC61B/RTN4, one-way ANOVA followed by Tukey's post-hoc test) **b** *Rtn4* knockdown in oligodendrocyte culture. **c** Quantification of area based on MBP fluorescence signal from four independent replicates, showing mean  $\pm$  SD, non-targeting siRNA:  $1483 \pm 359.8$ , *Rtn4* siRNA:  $868.1 \pm 60.03$ . (n=4 technical replicates, Two-tailed unpaired t-test) Scale bars: 10  $\mu\text{m}$  (a), 100  $\mu\text{m}$  (b), 20  $\mu\text{m}$  (b zoom in) \*:  $P \leq 0.05$ , \*\*:  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$ , \*\*\*\*:  $P \leq 0.0001$

### 3.3 Glycolipid transfer protein (GLTP) is associated with active myelination

To explore the possibility of nonvesicular lipid transport from the ER to the plasma membrane during myelin development, an in-depth re-analysis of published transcriptome and proteome databases was performed. Glycolipid transfer protein (GLTP) was identified as a good candidate (Sharma et al., 2015; Y. Zhang et al., 2014). GLTP stands out as a highly abundant and specific transcript in oligodendrocytes according to an RNA-Seq (Fig. 3.7a). Further investigation through immunohistochemistry solidified GLTP as a protein abundant within oligodendrocytes and myelin. This was supported by co-staining with BCAS1 and MAG, which indicated a more pronounced presence of GLTP in oligodendrocytes undergoing myelination compared to those in premyelinating stages (Fig. 3.8a, b). Additionally, it was observed that GLTP expression decreased as the myelin reached maturity (Fig. 3.8c, d). A zoom-in view of P14 myelin showed focal staining of GLTP within myelin (Fig. 3.8e), indicating GLTP is present in the inner tongue.

Moreover, immunocytochemical analysis on mouse primary oligodendrocytes showed that GLTP is localized to the oligodendroglial cell body, the processes as well as the growing front, which is akin to neuronal growth cones (Fig. 3.8f).

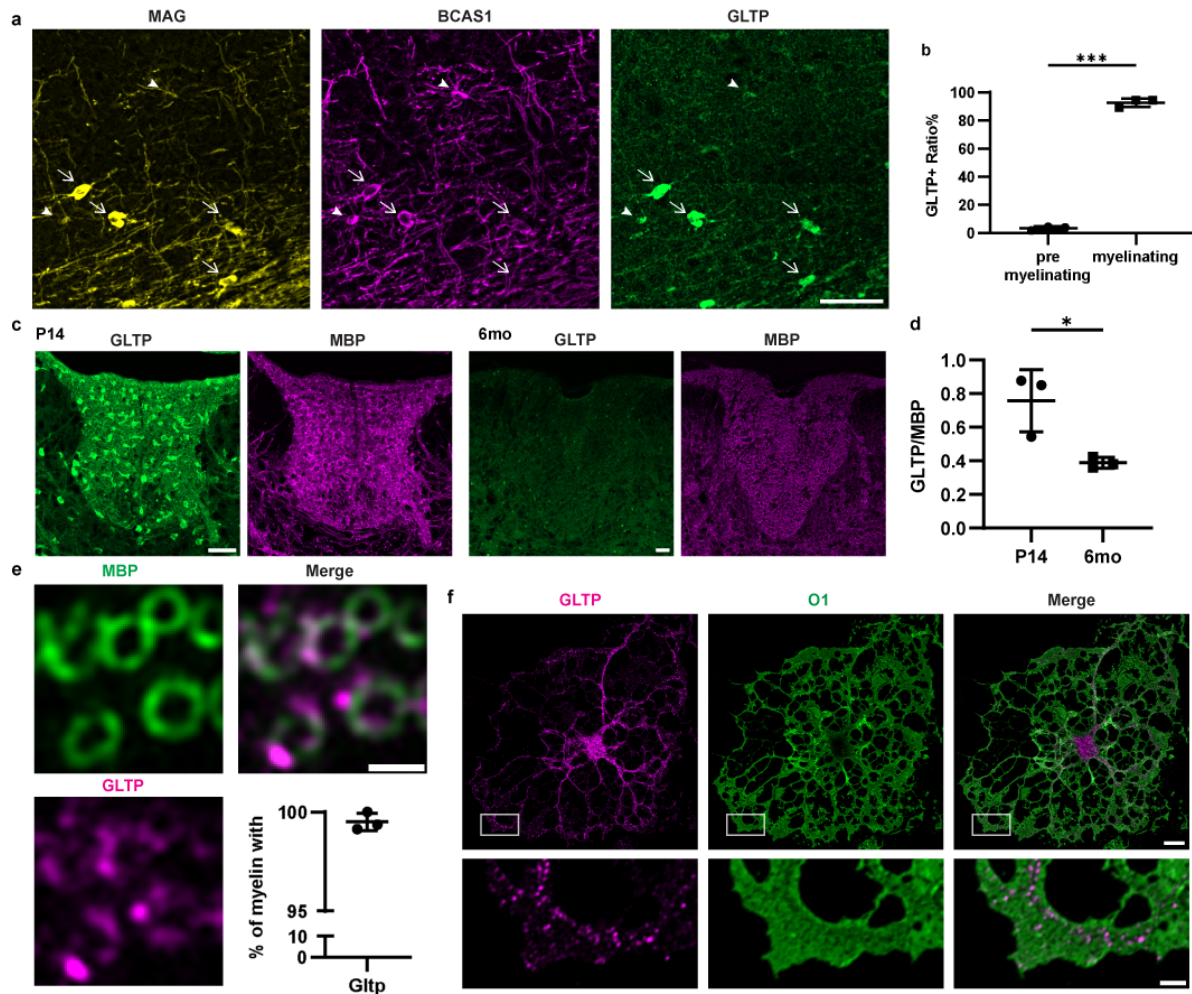


**Fig. 3.7 | Glycolipid transfer protein (GLTP) is associated with active myelination**

**a** RNA-seq analysis from Zhang et al. J. Neuroscience 2014. FPKM reads (two replicates per cell type) OPC: 88.495447, 105.799478; newly formed oligodendrocytes 368.317293, 404.231063; myelinating oligodendrocytes: 375.889082, 384.631206; neurons: 3.025255,

### 3. Results

5.900611; astrocytes: 7.211993, 7.877303; microglia: 49.882491, 47.164643; endothelial cells: 15.936901, 16.717767.



**Fig. 3.8 | Glycolipid transfer protein (GLTP) is associated with active myelination**

**a** Immunohistochemistry of P14 mouse cortex. Arrow heads: premyelinating oligodendrocytes (MAG<sup>-</sup>BCAS1<sup>+</sup>); arrows: myelinating oligodendrocytes (MAG<sup>+</sup>BCAS1<sup>+</sup>). **b** Quantification of (a) showing mean  $\pm$  SD, 3.33  $\pm$  1.36% premyelinating cells are GLTP+, 92.62  $\pm$  3.08% myelinating cells are GLTP+ (n=3 wildtype P14 mice, Two-tailed pair t-test). **c** Immunohistochemistry of P14 and adult mouse thoracic spinal cord, focusing on dorsal white matter. **d** quantification of GLTP fluorescence signal normalized by myelin (MBP) signal, in dorsal white matter of thoracic spinal cord, showing mean  $\pm$  SD, P14: 0.76  $\pm$  0.19, 6-month-old: 0.39  $\pm$  0.03 (n=3 wildtype mice for each time point, Two-tailed unpaired t-test). **e** zoom-in view of P14 spinal cord and ratio of myelin (MBP<sup>+</sup> ring) overlapping with GLTP, showing mean  $\pm$  SD, 99.51  $\pm$  0.44% myelin overlaps with GLTP (n=3 wildtype P14 mice). **f** Immunocytochemistry of primary oligodendrocytes. Magenta: GLTP; Green: oligodendrocyte marker O1 for outlining the cell. Scale bars: 50  $\mu$ m (a), 100  $\mu$ m (c), 1  $\mu$ m (e), 10  $\mu$ m (f upper), 2  $\mu$ m (f bottom). \*: P  $\leq$  0.05, \*\*: P  $\leq$  0.01, \*\*\*: P  $\leq$  0.001, \*\*\*\*: P  $\leq$  0.0001

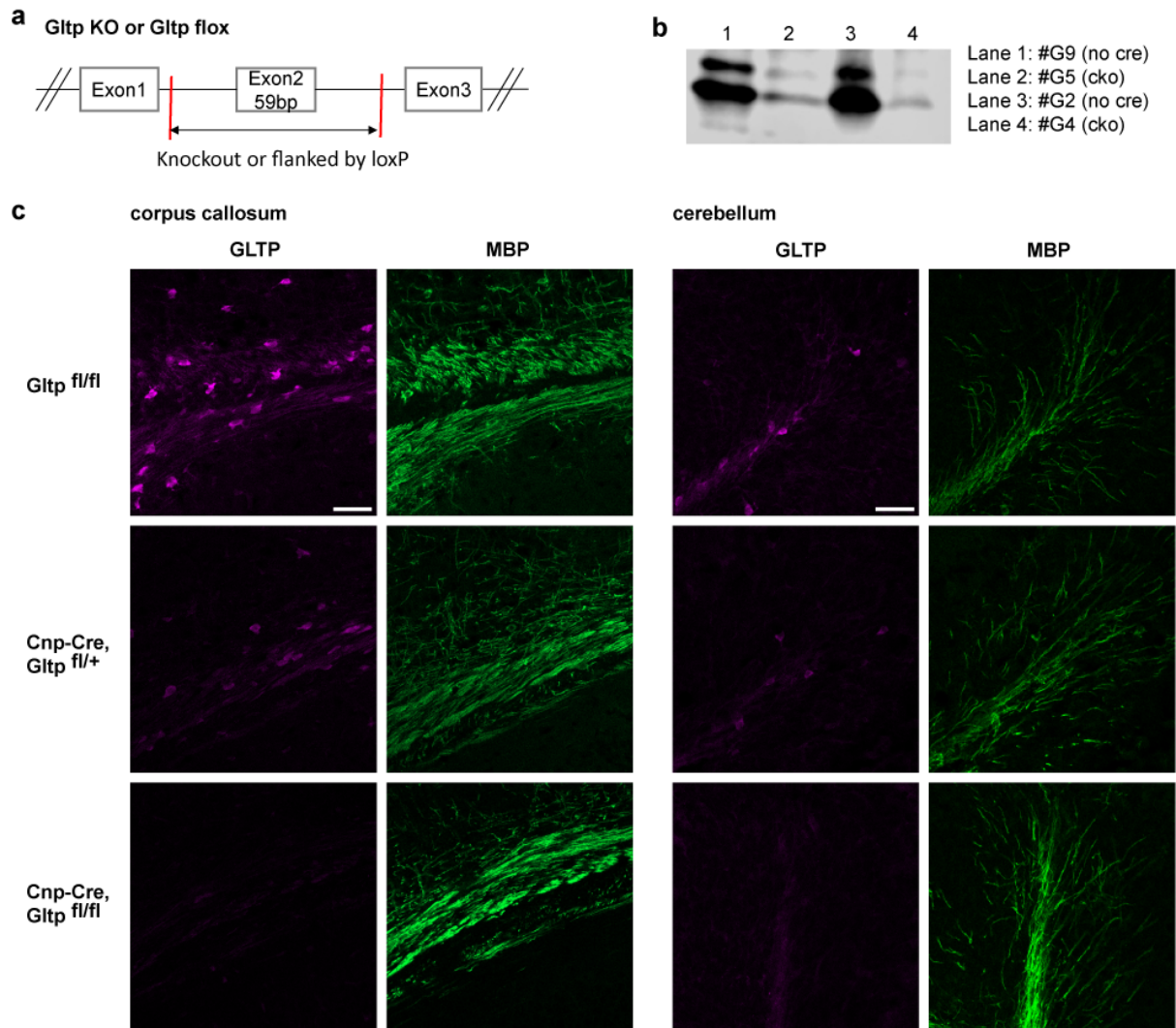
#### 3.4 *Gltph* mutant has ER pathology in myelin at P14

The hypothesis that GLTP is responsible for transferring GalCer from ER to plasma membrane was tested through the creation of *Gltph*-deficient mouse models. To this end, a whole-body *Gltph* knockout (KO) model and a floxed *Gltph* model (Fig. 3.9a) were developed, leveraging the resources of the DZNE facility. The universal *Gltph* KO results in embryonic lethality, necessitating a focus on the conditional KO model, created by breeding *Gltph* floxed mice with *Cnp-Cre* mice (Lappe-Siefke et al., 2003) to drive the oligodendrocyte-specific gene deletion. *Gltph* cKO (*Cnp-Cre*, *Gltph*<sup>fl/fl</sup>) exhibited diminished GLTP expression in both Western blot and immunohistochemistry analyses (Fig. 3.9b, c). Immunohistochemistry further verified the effective deletion of *Gltph* from oligodendrocytes across various brain regions.

Firstly, *Gltph* cKO mice were analyzed by EM at P14. Although these mice could form compact myelin, their myelin displayed unusual membrane ring structures (Fig 3.10a for spinal cord, Fig. 3.10b for optic nerve). These structures were observed across various brain regions in different *Gltph* cKO mice but were absent in the *Cnp-Cre* control littermates, as confirmed by examining over 1500 myelin cross-sections per condition (Fig 3.10c). Variations in the thickness of these rings were observed (Fig 3.10a zoom-in), with some displaying fewer wraps (marked by an arrow), others more wraps (marked by an arrowhead), and a subset presenting with a comet tail-like extension (marked by a star). Furthermore, such ring formations were also identified in the outer tongue (marked by double arrowhead, Fig. 3.10a zoom-in), within the cytoplasm of the cell body (Fig. 3.10d) and on the nuclear envelope (Fig. 3.10e). Volume electron microscopy (ATUM-SEM) unveiled that these rings were long tubes, traceable for up to 11  $\mu\text{m}$  (Movie 2). 3D reconstruction indicated these tubes originated from the ER, first transitioning into a "lumenless" ER, and eventually rolling up to form a tube (Fig. 3.10f, Movie 3, 4). Analysis further revealed that myelin containing these rings typically showed a reduction or absence of ER. Specifically, in regions where rings were present in the inner tongue, there was merely a 40% probability of detecting ER, in contrast to inner tongues without such rings, which exhibited a normal likelihood of ER observation, aligning with wild-type patterns (Fig. 3.10g).



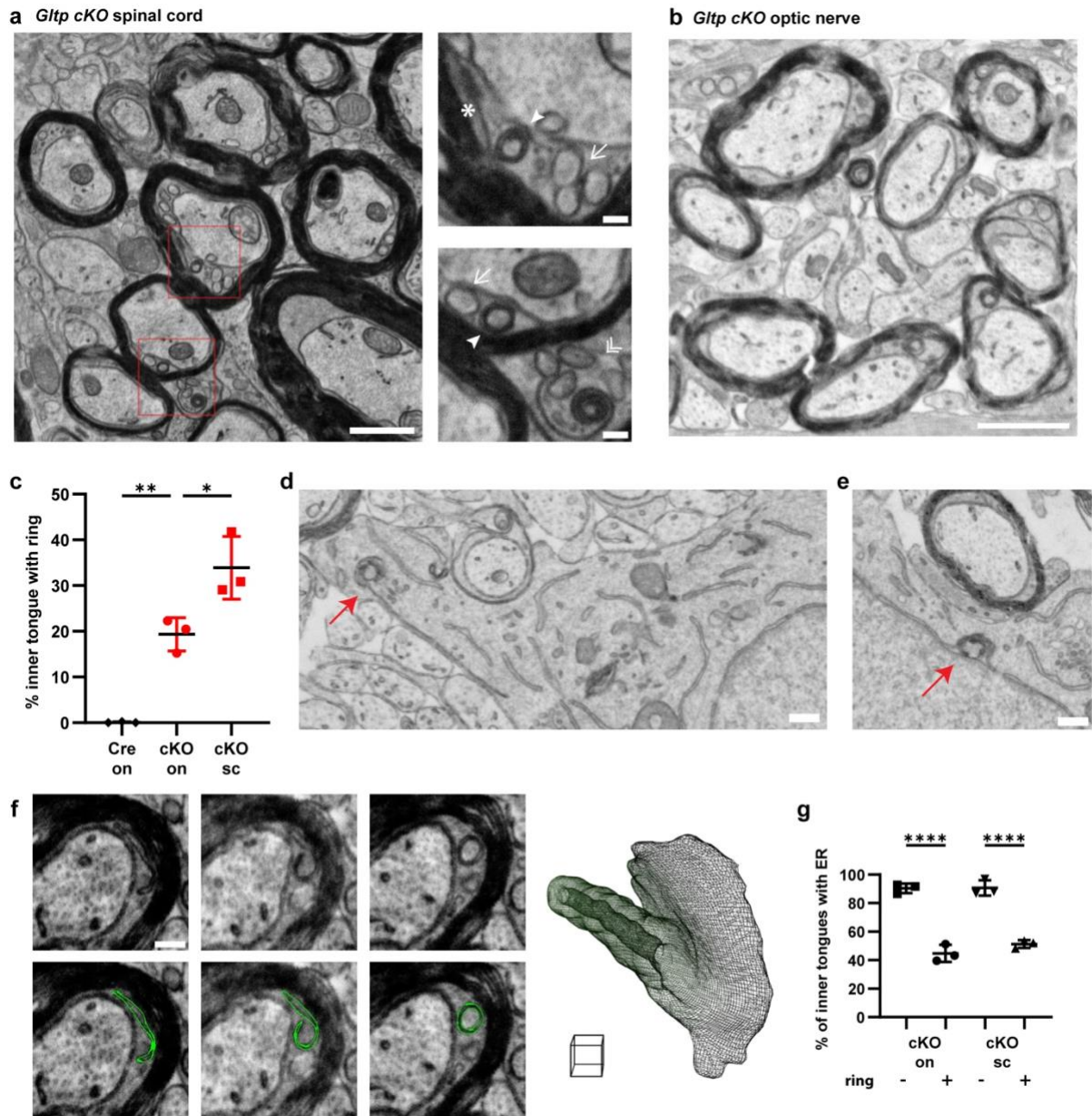
### 3. Results



**Fig. 3.9 | Design and verification of *Gltpr* mutants**

**a** Design of *Gltpr* knockout (KO) or *Gltpr* flox mouse lines. **b** Western Blot analysis of whole brain lysate from four P14 mice, 12 µg protein per lane. **c** Immunohistochemistry of corpus callosum and cerebellum from P14 WT (*Gltpr* <sup>fl/fl</sup>), het (*Cnp-Cre, Gltpr* <sup>fl/wt</sup>) and cKO (*Cnp-Cre, Gltpr* <sup>fl/fl</sup>) Scale bars: 50 µm (c).

### 3. Results



**Fig. 3.10 | *Gltpr* mutant has ER pathology in myelin at P14**

**a** Membrane rings in inner tongue at spinal cord of P14 *Gltpr* cKO (*Cnp-Cre, Gltpr<sup>flx/flx</sup>*). Arrows: thin rings; arrowheads: thick rings; Star: a comet tail-like structure associated with the ring; double arrowheads: rings in the outer tongue **b** Membrane rings in inner tongue at optic nerve of cKO (*Cnp-Cre, Gltpr<sup>flx/flx</sup>*). **c** Ratio (mean  $\pm$  SD) of inner tongue containing rings: 0.08 $\pm$ 0.13% of inner tongues in optic nerve of *Cnp-Cre* (Cre on), 19.34 $\pm$ 3.65% in optic nerve of *Cnp-Cre, Gltpr<sup>flx/flx</sup>* (cKO on), 33.90 $\pm$ 6.87% in spinal cord of *Cnp-Cre, Gltpr<sup>flx/flx</sup>* (cKO sc) (n=3 mice for each condition, from 436+537+543 inner tongues of “Cre on”, 508+525+531 of “cKO on”, 589+585+664 of “cKO sc”, One-way ANOVA followed by Tukey’s post-hoc test) **d** Ring (red arrow) at cell body of oligodendrocyte. **e** Ring (red arrow) at nuclear envelop of oligodendrocyte. **f** Volume EM of optic nerve from *Gltpr* cKO. Ring membrane is segmented in green, and 3D reconstruction of ring from a 1.5  $\mu$ m-thick stack (31 slices with 50nm interval), from dark green to light green across the stack. Scale cube: 1x1x1  $\mu$ m. **g** Amount of ER

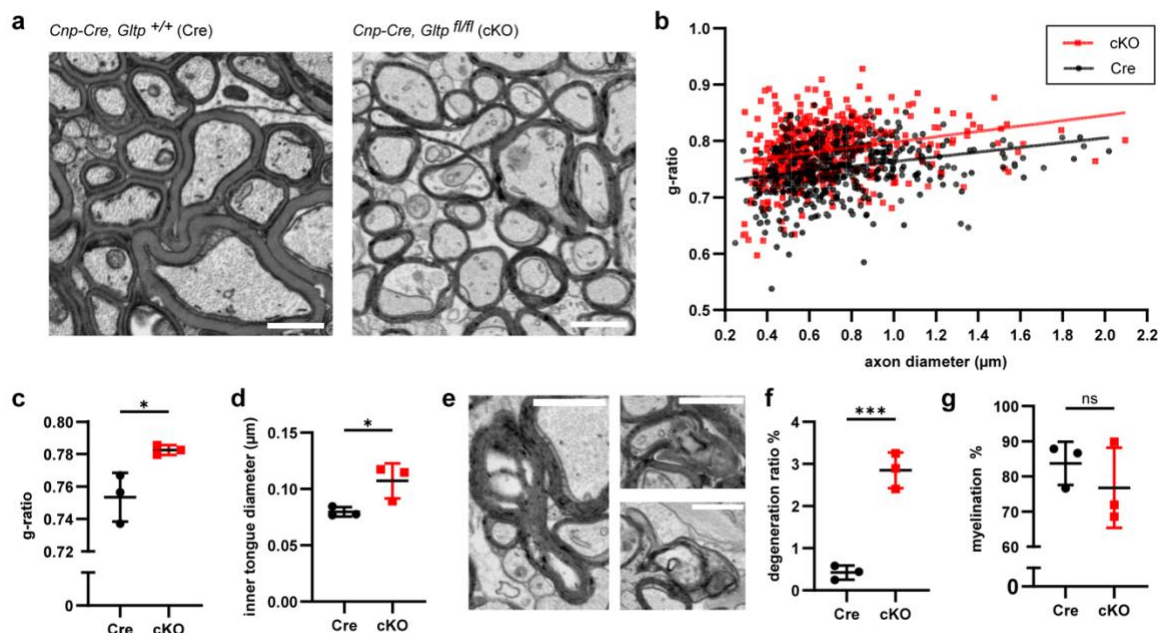
### 3. Results

decrease in the presence of rings, suggested by the ratio (mean  $\pm$  SD) of inner tongue containing the ER:  $90.34 \pm 3.33\%$  of ring- vs.  $44.69 \pm 6.01\%$  of ring+ inner tongues in optic nerve of *Cnp-Cre*, *Gtlp<sup>flox/flox</sup>* (cKO on),  $90.67 \pm 5.47\%$  of ring- vs.  $51.31 \pm 2.78\%$  of ring+ inner tongues in spinal cord of *Cnp-Cre*, *Gtlp<sup>flox/flox</sup>* (cKO sc) (n=3 mice for each condition, One-way ANOVA followed by Sidak's multiple comparisons test) Scale bars: 1  $\mu$ m (a), 150 nm (a zoom-in view), 1  $\mu$ m (b), 100 nm (d, e), 200 nm (f). \*:  $P \leq 0.05$ , \*\*:  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$ , \*\*\*\*:  $P \leq 0.0001$

#### 3.5 *Glt*p mutant exhibits hypomyelination and degeneration at P28

To understand the effects of *Glt*p knockout and the development of membrane rings on myelination, a later time point P28 was examined. At this stage, *Glt*p cKO exhibited hypomyelination, as evidenced by a reduced g-ratio (inner myelin diameter divided by outer myelin diameter) compared to *Cre* littermates (Fig. 3.11a, b, c).

Additionally, myelin inner tongues of the *Glt*p cKO mice were noticeably swollen (Fig. 3.11d), a condition likely stemming from the pathological effects induced by the presence of membrane rings. Moreover, degeneration increases in the *Glt*p cKO (Fig. 3.11e, f). Despite of these changes, the proportion of axons that were myelinated were not significantly reduced in the *Glt*p cKO mice, suggesting that the knockout of GLTP and the membrane rings do not hinder the onset of myelin biogenesis (Fig. 3.11g).



**Fig. 3.11 | *Glt*p mutant exhibit hypomyelination and degeneration**

### 3. Results

**a** Optic nerve of *Gltp* cKO and Cre control mice at P28. **b, c** g-ratio (a measurement of myelin thickness defined as inner diameter of myelin divided by outer diameter), quantification from three mice for each condition. (b) shows g-ratio of individual myelin relative to the enwrapped axon's diameter. (c) shows the mean of myelin g-ratio from each mouse, showing mean of the cohort  $\pm$  SD, Cre:  $0.754 \pm 0.015$ , cKO:  $0.783 \pm 0.003$  (n=3 mice for each condition, Two-tailed unpaired t-test) **d** mean of inner tongue diameter from three mice for each condition, showing mean of the cohort  $\pm$  SD, Cre:  $0.080 \pm 0.004$   $\mu$ m, cKO:  $0.107 \pm 0.016$   $\mu$ m. (n=3 mice for each condition, Two-tailed unpaired t-test) **e** examples of degenerations **f** Ratio of degeneration, showing mean of the cohort  $\pm$  SD, Cre:  $0.424 \pm 0.170\%$ , cKO:  $2.852 \pm 0.425\%$  (n=3 mice per condition, Two-tailed unpaired t-test) **g** Percentage of axons that are myelinated, showing mean of the cohort  $\pm$  SD, Cre:  $83.720 \pm 6.148\%$ , cKO:  $76.780 \pm 11.43\%$  (n=3 mice per condition, Two-tailed unpaired t-test). Scale bars: 1  $\mu$ m (a and e) \*:  $P \leq 0.05$ , \*\*:  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$ , \*\*\*\*:  $P \leq 0.0001$

#### 3.6 Delivery of glycolipid to myelin is impaired in *Gltp* mutants

To elucidate whether GLTP is essential for glycolipid transport during the myelination process, myelin was isolated from individual *Gltp* cKO and Cre control mice at two distinct developmental stages, employing a standard two-round sucrose density gradient centrifugation technique (Erwig et al., 2019). This preparatory step was followed by a shotgun lipidomics analysis to examine the lipid composition in detail, with the nomenclature for the lipidome detailed in Box 1 for clarity.

Principal Component Analysis (PCA) on the lipid species composition of different samples revealed clear separations among the four conditions (Fig. 3.12f). A volcano plot generated from multiple t-tests comparing the *Gltp* cKO and Cre control mice underscored significant lipidomic alterations, with the top 10 downregulated and upregulated lipid species listed (Fig. 3.12a, b, c). Hexosylceramide (HexCer) constitute the majority of downregulated lipids. Unfortunately, the lipidomics analysis cannot distinguish GalCer and glucosylceramide: the two were detected together as HexCer. The reduction in HexCer species was especially pronounced for those with normal fatty acids (NFA), whereas HexCer species with 2-hydroxy fatty acids (HFA) did not exhibit significant fluctuations in the *Gltp* cKO mice (Fig. 3.12d), a phenomenon that may be explained by the compensatory upregulation of HFA-glucosylceramide in GalCer-deficient mutants (Bosio et al., 1996; Coetzee et al., 1996).

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Further grouping of lipid species into classes and subsequent analysis via two-way ANOVA followed by Tukey's post-hoc test revealed that *Glt1* cKO mice exhibited altered myelin lipid composition. This alteration manifested as a decrease in HexCer and ether-linked phosphatidylethanolamine (PE O-), alongside an increase in cholesterol and variable changes in phosphatidylserine (PS) levels (Fig. 3.12e). Further lipidomics report can be found in Fig. 3.13a-p.

To corroborate the lipidomics outcomes, the delivery of GalCer was evaluated using anti-GalCer antibodies in a 2D oligodendrocyte culture system capable of generating myelin-like MBP-positive sheets. This assay involved immunocytochemical measurement of extracellular and intracellular GalCer, before and after permeabilization with Triton X-100, respectively (Fig. 3.12g). Observations indicated a diminished extracellular to intracellular GalCer ratio in oligodendrocytes derived from *Glt1* cKO mice, pointing to a disruption in GalCer delivery (Fig. 3.12g, h). Furthermore, a complementary gene disruption approach, siRNA, was adopted to validate this deficit in GalCer transport (Fig. 3.12g, i). The specificity of the anti-GalCer antibody, *Glt1* knockout and knockdown efficiency in primary oligodendrocyte culture were confirmed (Fig. 3.13q-s).



### 3. Results

#### Box 1: Lipid nomenclature

##### (1) List of reported lipid classes

Some lipid classes (eg. PC) were detected by MSMS mode to obtain subspecies details. In this mode, the lipid was fragmented so the acyl chain composition of the lipid molecule is identified.

Lipid class	Full name	Structure detail level
Cer	Ceramide	species
Chol	Cholesterol	species
DAG	Diacylglycerol	subspecies
HexCer	Hexosylceramide	species
LPA	lyso-Phosphatidate	species
LPC	Lyso-phosphatidylcholine	species
LPE	Lyso-phosphatidylethanolamine	species
LPE O-	Lyso-Phosphatidylethanolamine (-ether)	species
LPI	Lyso-Phosphatidylinositol	species
LPS	Lyso-Phosphatidylserine	species
PA	Phosphatidate	subspecies
PC	Phosphatidylcholine	subspecies
PC O-	Phosphatidylcholine (-ether)	subspecies
PE	Phosphatidylethanolamine	subspecies
PE O-	Phosphatidylethanolamine (-ether)	subspecies
PG	Phosphatidylglycerol	subspecies
PI	Phosphatidylinositol	subspecies
PS	Phosphatidylserine	subspecies
SM	Sphingomyelin	species

##### (2) Lipid species nomenclature

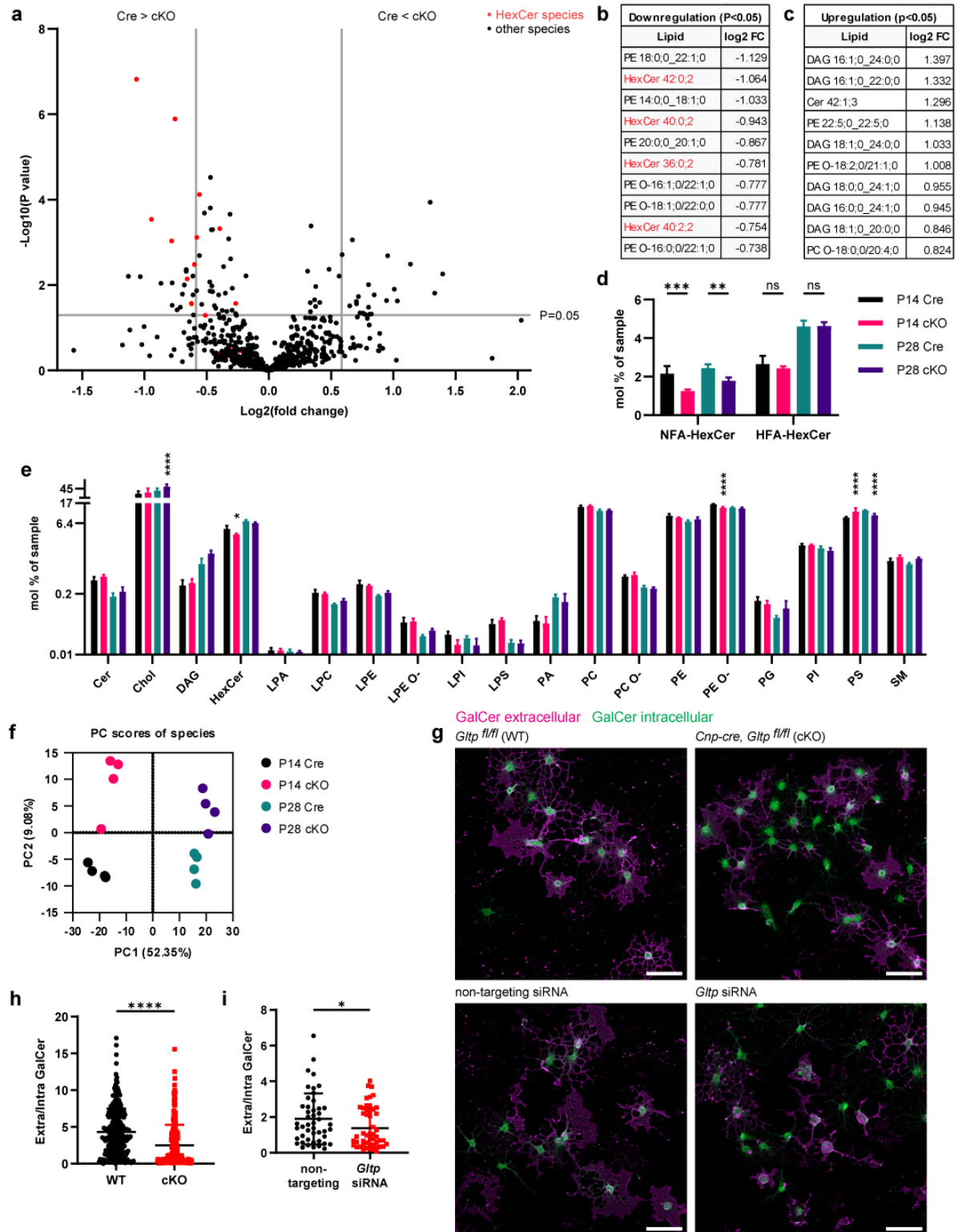
Lipid species are annotated according to their molecular composition as:

Head group <sum of the carbon atoms in the hydrocarbon moiety>:<sum of the double bonds in the hydrocarbon moiety >;<sum of hydroxyl groups>.

For example, HexCer 42:0:2 denotes a Hexosylceramide species with a total of 42 carbon atoms, 0 double bond, and 2 hydroxyl groups in the ceramide backbone.

Lipid subspecies annotation contains additional information on the fatty acid chains and their positions on the glycerol backbone, when known. For instance, PE 18:0;0\_22:1;0 refers to a phosphatidylethanolamine molecule with stearic acid (18:0;0) and docosenoic acid (22:1;0), without distinguishing between the sn-1 and sn-2 positions for these acyl groups (indicated by underscore "\_"). Conversely, PE O-16:1;0/22:1;0 refers to a plasmalogen form of phosphatidylethanolamine, where a plasmenyl chain with 16 carbon atoms and one double bond (O-16:1;0) is ether-linked to the sn-1 position of the glycerol backbone, and a docosenoic acid (22:1;0) is esterified to the sn-2 position of the glycerol backbone (a slash "/" is used to show that the positions of the fatty acid chains on the glycerol can be distinguished).

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**Fig. 3.12 | Delivery of glycolipid to myelin is impaired in *Gltp* mutant**

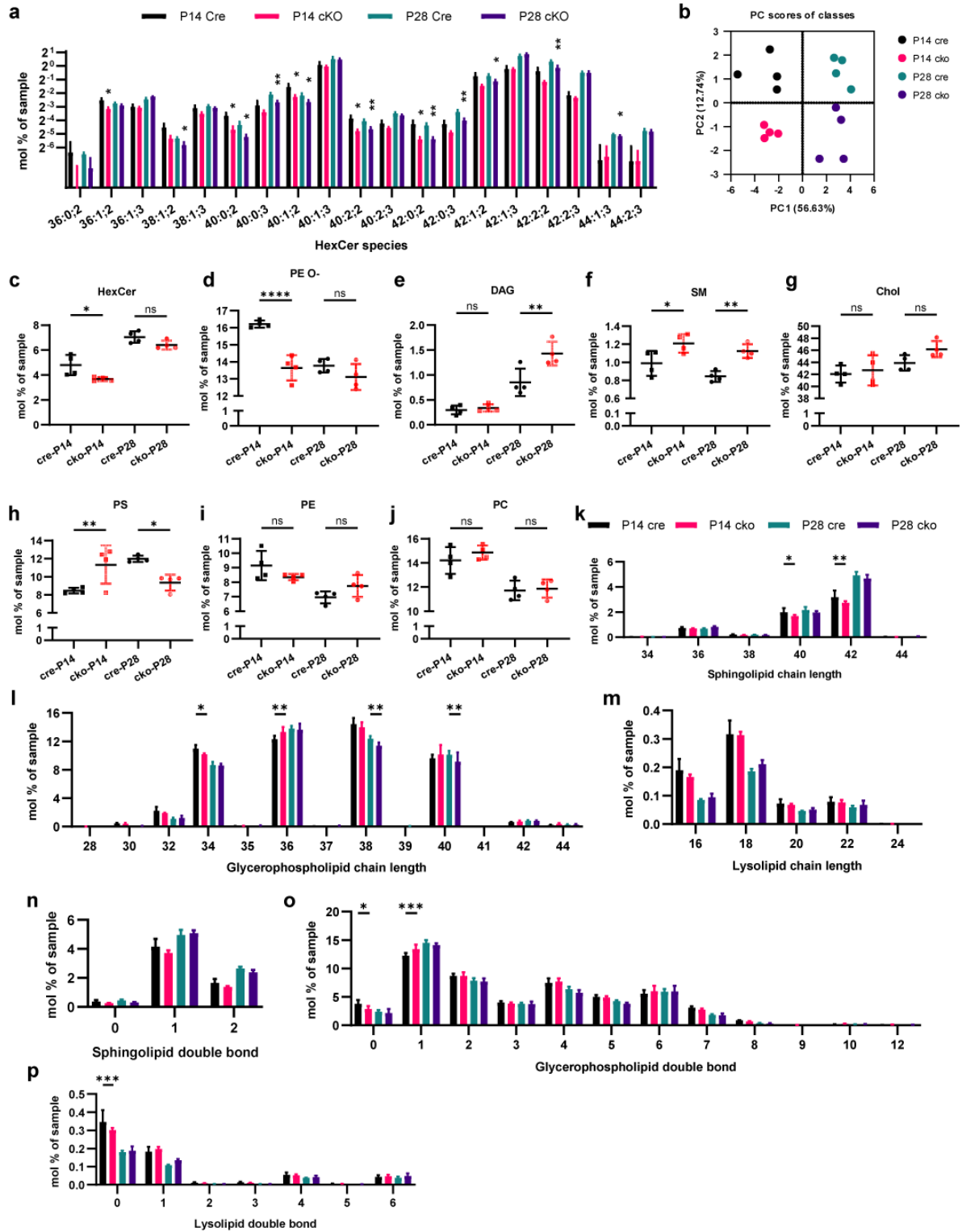
**a-f** Lipidomics analysis of myelin purified from *Gltp* cKO (*Cnp-Cre Gltp<sup>flx/flx</sup>*) and Cre control (*Cnp-Cre*) mice (n=4 mice for each genotype/time point). (a) Volcano plot shows differentiated regulated lipid species in cKO vs. Cre control. Vertical lines mark  $\pm 1.5$  fold change. Data points above horizontal line have p-value  $< 0.05$ . Data were grouped based on genotype (Cre and cKO), and transformed to log2 for two-tailed Welch's t-test. (b) and (c) Top 10



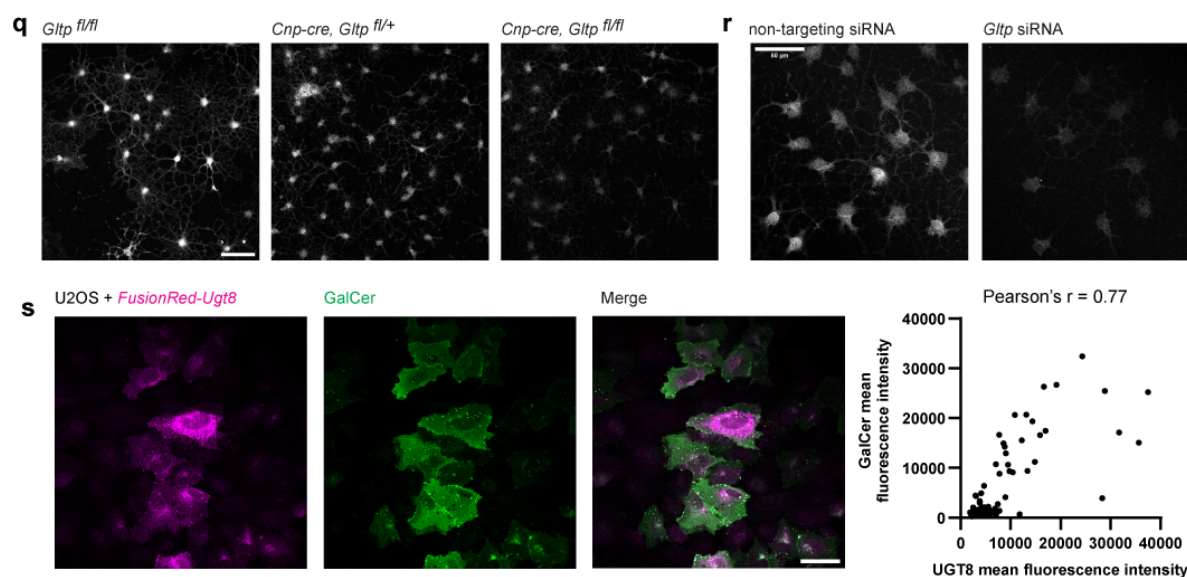
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downregulated and upregulated lipid species sorted by fold changes. (d) Relative amount of HexCer containing normal fatty acid (NFA) or 2-hydroxy fatty acid (HFA), showing mean  $\pm$  SD, P14-Cre-NFA:  $2.15 \pm 0.39\%$ , P14-cKO-NFA:  $1.25 \pm 0.08\%$ , P28-Cre-NFA:  $2.44 \pm 0.19\%$ , P28-cKO-NFA:  $1.78 \pm 0.17\%$ , P14-Cre-HFA:  $2.65 \pm 0.44\%$ , P14-cKO-HFA:  $2.42 \pm 0.11\%$ , P28-Cre-HFA:  $4.61 \pm 0.3\%$ , P28-cKO-HFA:  $4.62 \pm 0.2\%$ . (Two-way ANOVA followed by Tukey's post-hoc tests). (e) Relative amount of various lipid classes, showing mean  $\pm$  SD. Color code is the same as in (d). (Two-way ANOVA followed by Tukey's post-hoc test. Stars indicate significant changes compared to the control of same age. Descriptive statistics and raw data are available in supplementary information) (f) Principal Component Analysis (PCA) of lipid species from the samples. Each sample is myelin from one animal. **g** Mouse primary oligodendrocyte's extracellular(magenta) and intracellular(green) GalCer signal obtained before and after Triton X-100. **h** Quantification of WT and KO cells from three mice for each condition, showing mean  $\pm$  SD, WT (*Gltp*<sup>*fl/fl*</sup>):  $4.32 \pm 3.18$ , cKO (*Cnp-Cre*, *Gltp*<sup>*fl/fl*</sup>):  $2.50 \pm 2.78$ . (n=286 for WT, n=265 for cKO, Two-tailed unpaired t-test). P value is calculated by Student's t-test. **i** Quantification of non-targeting and GLTP siRNA, showing mean  $\pm$  SD, non-targeting siRNA:  $1.90 \pm 1.43$ , *Gltp* siRNA:  $1.38 \pm 1.13$ . (n=47 for nt, n=50 for siRNA, Two-tailed unpaired t-test) Scale bars: 50  $\mu$ m (f) \*:  $P \leq 0.05$ , \*\*:  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$ , \*\*\*\*:  $P \leq 0.0001$

### 3. Results



### 3. Results



**Fig. 3.13 | Delivery of glycolipid to myelin is impaired in *Gltp* mutant**

**a** Relative amount of different GalCer species, showing mean  $\pm$  SD. P value is calculated from two-way ANOVA followed by Tukey's post-hoc test. **b** Principal Component Analysis of the samples based their lipid classes. **c-j** One-way ANOVA of major lipid classes in myelin (instead of Two-way ANOVA in Fig. 6d), showing mean  $\pm$  SD. P value is calculated from one-way ANOVA followed by Tukey's post-hoc test. **k-m** Chain length of different functional classes, showing mean  $\pm$  SD. P value is calculated from two-way ANOVA followed by Tukey's post-hoc test. **n-p** Double bond of different functional classes, showing mean  $\pm$  SD. P value is calculated from two-way ANOVA followed by Tukey's post-hoc test. **q** Validation of *Gltp* knockout oligodendrocyte culture. **r** Validation of *Gltp* knockdown oligodendrocyte culture. **s** Validation of anti-GalCer antibody. The GalCer signal is high in cells overexpressing UGT8, the enzyme responsible for GalCer synthesis, and correlation of GalCer and UGT8. Data points represent measurement from individual cells (n=96). Scale bars: 50  $\mu$ m (q, r, s) Descriptive statistics and raw data for lipidomics are available in supplementary information. \*:  $P \leq 0.05$ , \*\*:  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$ , \*\*\*\*:  $P \leq 0.0001$

### 3.7 Supplementary movies

The following movies were uploaded to the cloud. Key frames or maximum intensity projections were shown in figures.

**Movie 1:** Volume EM (ATUM-SEM) stack for 3D reconstruction in Fig 3.1a. 11.25  $\mu$ m-thick (226 slices with 50 nm interval), from P14 wildtype mouse optic nerve.

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**Movie 2:** Volume EM (ATUM-SEM) stack revealed the ring structure is a long tube (arrow), 11  $\mu\text{m}$ -thick (222 slices with 50 nm interval), from P14 *Glt<sub>p</sub>* cKO mouse optic nerve. Scale bar 500nm.

**Movie 3:** Volume EM (ATUM-SEM) stack of rolling-up ring in myelin inner tongue, 1.5  $\mu\text{m}$ -thick (31 slices with 50 nm interval), from P14 *Glt<sub>p</sub>* cKO mouse optic nerve. Related to Fig 3.10f. Scale bar 100nm.

**Movie 4:** Volume EM (ATUM-SEM) stack of rolling-up ring in oligodendrocyte cell body, 2.1  $\mu\text{m}$ -thick (43 slices with 50 nm interval), from P14 *Glt<sub>p</sub>* cKO mouse optic nerve. Related to Fig 3.10d. Scale bar 100 nm.

## 4. Discussion

### 4.1 The working model

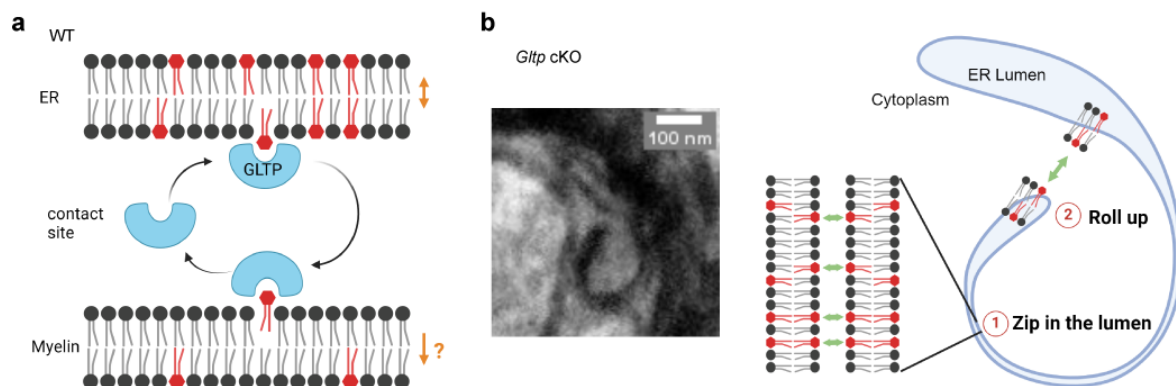
#### 4.1.1 Transport of GalCer in developing myelin

I proposed a model in which GLTP facilitates nonvesicular transport of GalCer to support myelination (Fig. 4.1a). GalCer is synthesized through the addition of galactose to ceramide, a process mediated by the enzyme UDP-galactose:ceramide galactosyltransferase encoded by the *Ugt8* gene in mouse. The enzymatic domain of UGT8 is located at the lumen of the ER. Consequently, GalCer is initially formed in the luminal leaflet, but it has rapid access to the cytoplasmic leaflet (Burger et al., 1996; Xavier Buton et al., 2002; X Buton et al., 1996; van Meer, 2011). GLTP facilitates the transfer of GalCer from the ER to myelin, a specialized domain of oligodendroglial plasma membrane, where a yet-to-be-identified machinery transports GalCer from the intracellular leaflet to the extracellular leaflet. In this scenario, GalCer in the intracellular leaflet of plasma membrane remains low, creating a gradient and establishing the transfer direction as ER-to-plasma membrane. But eventually, the intracellular leaflet also accumulates a significant amount of GalCer, which may be beneficial, serving as molecular glue like MBP and contributing to the formation of major dense line. This explains the swollen inner tongue observed in P28 *Gltp* cKO.

In *Gltp* knockout (Fig. 4.1b), GalCer is accumulated in both luminal leaflet and cytoplasmic leaflet of the ER. Headgroups of GalCer have been shown to interact with each other via carbohydrate-carbohydrate interaction, potentially involving hydrogen bonds and hydrophobic interactions, leading to the adherence of GalCer bilayers in vitro (Boggs, 2014; Kulkarni et al., 1999). As a result, GalCer in the intracellular leaflet aids in zipping up the ER, leading to the observed “lumenless” ER. Additionally, due to the accumulation of GalCer at the cytoplasmic leaflet, the ER adopts a rolled-up configuration. The ER is known to be prone to roll up when its membrane can interact, as seen, for instance, with nonmonomeric GFP on the ER (Snapp et al., 2003). I propose that *Gltp* mutant resembles this case. The observed rings in the mutant further support the idea that GalCer is localized to both leaflets of the ER.

## 4. Discussion

The “lumenless” ER is also observed in wildtype and I think this is the site for active GalCer synthesis (Fig 3.1b). But in wildtype, because the GalCer on cytoplasmic leaflet of ER is actively transported away, the ER never rolls up.



**Fig. 4.1 | Graphical illustration of model showing transport of GalCer in developing myelin.**

**a** In wild type myelin, GLTP transfers GalCer (red) from the ER to myelin, a specialized domain of oligodendroglial plasma membrane. Double-head arrow indicates GalCer reach an equilibrium in ER. Single-head arrow indicates that a yet-to-be-identified machinery transports GalCer to the extracellular leaflet of plasma membrane, keeping GalCer low in the intracellular leaflet of myelin membrane, creating a gradient and establishing the transfer direction as ER-to-plasma membrane. **b** Ring formation in *Glt1* cKO occurs in two steps. Step 1: GalCer attract each other and zip up the ER from the lumen. Step 2: GalCer accumulating in cytoplasmic leaflet of ER make the ER to roll up. Created with BioRender.com

### 4.1.2 The existence of tether

Tethering molecules are required for the formation of all membrane contact sites reported so far. In developing myelin, what bridges the ER and plasma membrane into close proximity? GLTP is a cytosolic protein that can interact with VAP-A (Tuuf et al., 2009), an ER transmembrane protein involved in the formation of various ER-organelle contact sites (Balla et al., 2019). VAP-A forms a dimer (Subra et al., 2023). GLTP can work at VAP-A mediated ER-plasma membrane contact site if one VAP-A subunit binds to a plasma membrane protein while the other binds to GLTP. But it is also possible that myelin is a special case in which nonvesicular lipid transport occurs without a tether. The inner tongue, being a small compartment, allows the ER to be consistently close to the plasma membrane. Observations along the inner tongue reveal a continuous proximity between the ER and the plasma membrane.

## 4. Discussion

This suggests that myelin might resemble the situation of a test tube with high-concentration liposomes, where transfer can occur without a tethering mechanism.

### 4.1.3 Potential floppases

In my working model, GalCer is moved from the intracellular leaflet to the extracellular leaflet on plasma membrane. There are two classes of proteins that can move lipids in this direction: scramblases and floppases. Scramblases move a variety of lipids in both directions. Asymmetric lipid distribution between leaflets is essential for cellular homeostasis (Doktorova et al., 2020), which also applies to myelin – for instance, phosphatidylserine normally resides in the intracellular leaflet of myelin, and when exposed to the extracellular leaflet by scramblases, it shows the “eat me” signal (Djannatian et al., 2023). Therefore, the involvement of floppases is more likely. Floppases facilitate the movement of lipids from the intracellular to the extracellular leaflet and are usually ATP-binding cassette (ABC) transporters, which belong to a large protein family with approximately 50 members in the human genome. These transporters are capable of translocating substrates including lipids and small molecule drugs across cellular membranes. A number of ABC transporters are found in oligodendrocytes (Kim et al., 2008; Wolf et al., 2012).

ABCA2 is an abundant ABC transporter in oligodendrocytes (Tanaka et al., 2003) and is required for myelination in mouse (Mack et al., 2007; Sakai et al., 2007). This transporter is present at the endolysosomal systems. Interestingly, endosomal transport is known to take part in the remodeling of myelin membrane protein, such as proteolipid protein (PLP) (Baron et al., 2015; Feldmann et al., 2011; Winterstein et al., 2008). The possibility that this remodeling extends to lipids, specifically whether GalCer is sorted in the recycling endosome by ABCA2, warrants closer examination.

## 4.2 Alternative explanations of the results

While my findings support the role of GLTP in ER-to-plasma membrane nonvesicular lipid transport during myelination, the complexity of lipid dynamics suggests that other mechanisms may also contribute to the observed results. Below, I discuss several alternative explanations:



## 4. Discussion

### (1) GLTP transfers GalCer from ER to endosomes

Based on the previous section, an alternative route for GalCer transport could involve its movement from the ER to the endosomes before ultimately reaching plasma membrane. Endosomes are present in developing myelin (Almeida & Macklin, 2023; Krämer-Albers & Werner, 2023). Additionally, existing literatures have reported ER-endosome contact sites and the lipid transfer that occurs between them (Raiborg et al., 2015; Suzuki et al., 2024). Therefore, this alternative explanation presents a plausible route but requires further study for verification.

### (2) GLTP as a shuttle

Like many lipid transfer proteins, GLTP could function as a shuttle. Biochemical studies have confirmed that lipid transfer proteins can transfer lipids between liposomes, while cell biology studies highlight the existence of membrane contact sites, suggesting that non-vesicular transport occurs at these locations. Fully bridging these two types of studies can be challenging, leaving the field open to the possibility that lipid transfer proteins might act as shuttles. In the case of myelin development, given the close proximity of the ER to myelin membrane, GLTP likely does not need to operate over long distances as a shuttle would. However, in adults, where myelin becomes more compact and space is constrained, GLTP emerges as a promising candidate for facilitating lipid turnover within myelin. This is especially relevant given recent suggestions that myelin still undergoes biogenesis in adulthood (Meschkat et al., 2022).

### (3) GLTP affects vesicular trafficking

According to a recent study on HeLa cells (Nurmi et al., 2023), GLTP could also affect vesicular trafficking. However, this conclusion was drawn from a few images without quantification, where mutants appear very similar to controls. Moreover, even if this observation is statistically significant, it could indicate a secondary effect.

I have evidence suggesting this alternative explanation is unlikely. Firstly, as mentioned in the introduction, GalCer transport persists in the absence of vesicular trafficking in both cell lines and cultured oligodendrocytes. Secondly, my lipidomics analysis revealed HexCer as one of the few lipid classes that changed. The fact that

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GLTP's influence is rather specific to certain lipid classes could argue for nonvesicular trafficking since vesicular trafficking is less selective toward lipids.

To further explore this alternative explanation, examining myelin membrane proteins could offer significant insights. Because proteins rely exclusively on vesicular trafficking, unlike lipids, which may utilize nonvesicular pathways, conducting a proteomics analysis of P28 myelin from *Gltp* cKO and *Cre* control appears to be a promising approach to clarify these mechanisms.

However, it's important to keep in mind the challenge of distinguishing direct from indirect influences. The *Gltp* cKO mutant in my study exhibits ER pathology, and with a compromised ER, cellular homeostasis—and consequently, vesicular trafficking—could be affected.

### 4.3 Lipid transport in myelination

#### 4.3.1 Why nature chose galactosylceramide (GalCer) for myelin

It has been postulated that myelin membranes avoid incorporating negatively charged phospholipids at extracellular leaflets to prevent repulsion. Glycolipids, on the other hand, are not only not repulsive, but also attractive due to carbohydrate-carbohydrate interaction (Boggs, 2014). GalCer is one of the few glycolipids synthesized in the ER, so it is produced directly at the growing front and can be transferred directly to the myelin membrane. In contrast, the remaining glycolipids are synthesized in the Golgi apparatus and requires further trafficking to enter the myelin.

It is noteworthy that glucosylceramide is upregulated in *Cgt* mutant mice that are unable to convert ceramide to GalCer (Bosio et al., 1996; Coetzee et al., 1996). While this upregulation could be a compensatory mechanism, it is also possible that, in the wild type, ceramide is predominantly used for GalCer synthesis in the ER, leaving limited precursors for glucosylceramide synthesis in the Golgi.

#### 4.3.2 Specialized lipid distribution in myelin

This study offers a potential explanation for the unsolved puzzle of differential distribution of GalCer and sulfatide in myelin. GalCer is localized to myelin, but once

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sulfated in Golgi apparatus and converted to sulfatide, it is delivered to noncompact myelin fraction enriched with paranode (Maier et al., 2008), and plays a role in paranode formation (Honke et al., 2002). It has been long postulated these two lipids are delivered by different routes (Baron & Hoekstra, 2010; Hayashi & Su, 2004).

My findings shows that the ER extends into the inner tongue of myelin, positioning GalCer synthesis within the myelin sheath itself and facilitating its direct access to the internodal region. In contrast, the paranodal cytosolic space is significantly larger, enabling easier access for the Golgi apparatus and vesicular transport. This suggests that sulfatide is synthesized at the paranode or transported there via Golgi-derived vesicles, supporting the idea of separate lipid delivery mechanisms for internodal and paranodal regions.

### 4.3.3 Other nonvesicular transport for myelination

In this section, I will discuss additional nonvesicular transport families potentially involved in myelination based on three reviews (Y.-J. Chen et al., 2019; Hanna et al., 2023; Saheki & De Camilli, 2017), and list their gene expression in mouse oligodendrocytes relative to other brain cell types in Fig 4.3, based on the RNA-Seq study (Y. Zhang et al., 2014).

The field of nonvesicular lipid transport is still emerging, which raises the possibility that proteins not currently identified for ER-plasma membrane transport could indeed play a role. Additionally, proteins implicated in lipid export from the plasma membrane might also participate in import during myelination, as directionality is not fully clear and may reverse under certain conditions. Also, while I identified GLTP based on its abundance, I believe a low expression of a lipid transfer protein doesn't mean it is not important. This is especially true for bridge-like transfer proteins since one copy is enough to let numerous lipid molecules pass. Therefore, these listed candidates may not be known to be involved in transport to the plasma membrane, or they may not be very abundant. Furthermore, because endosomes are present in developing myelin, I will also include endosome-to-plasma membrane trafficking.

(1) Class II PITP (phosphatidylinositol transfer protein) family. It contains cytosolic proteins Nir2 and Nir3, which act as membrane editors and are unlikely to contribute to bulk transport. Upon phospholipase C activation, they can be recruited to ER-plasma membrane contact sites to clear phosphatidic acids from the plasma

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membrane and to supply new phosphoinositides. Additionally, although Nir1 does not possess the transfer function, it is included in Fig 4.3 due to its reported role as a positive regulator of Nir2 (Quintanilla et al., 2022).

(2) SMP (synaptotagmin-like mitochondrial-lipid binding protein) domain-containing protein family. Four mammalian members from this family have been characterized: Extended synaptotagmin (E-syt) 1/2/3 and TMEM24 (also known as C2CD2L). All of these are ER membrane proteins. Biochemical studies suggested E-syts can transfer glycerolipids and diglyceride (Saheki et al., 2016; Schauder et al., 2014), while TMEM24 prefers to transfer phosphoinositides (Lees et al., 2017). Interestingly, both E-syt1 and TMEM24 operate in different modes dependent on intracellular  $\text{Ca}^{2+}$  level.

(3) OSBP (oxysterol-binding protein) and ORP (OSBP-related protein) family. ORP5 and ORP8 from this family have been shown to transfer phosphatidylserine from the ER to plasma membrane and exchange phosphatidylinositol 4-phosphate (PI4P) from plasma membrane to the ER (Chung et al., 2015).

(4) StART (steroidogenic acute regulatory transfer) and StART-like protein family. There are 15 human StART proteins and a few StART-like proteins. The StART domain can bind various lipids, including sterols and phospholipids, and these proteins are involved in lipid transport across various membrane contact sites, not necessary limited to ER-plasma membrane contact sites (Clark, 2012). Regarding the StART-like proteins, two recent studies (Besprozvannaya et al., 2018; Sandhu et al., 2018) have suggested that GRAMD1/Aster proteins can transfer cholesterol from plasma membrane back to the ER, following the concentration gradient. During myelination, oligodendrocyte synthesize large amounts of cholesterol in the ER, creating an opposite gradient. How GRAMD/Aster proteins operate under this condition warrants further investigations.

(5) Sec22b-Stx1 (syntaxin 1). Sec22b is an ER-anchored SNARE protein and Stx1 is a plasma membrane-anchored SNARE protein. This pair can interact without mediating membrane fusion, thereby stabilizing ER-plasma membrane contacts. Although they lack a lipid transfer function, their associated ER-plasma membrane contact is important for membrane expansion (Gallo et al., 2020; Petkovic et al., 2014).

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(6) Mospd2 (motile sperm domain-containing protein 2). Mospd2 is an ER-anchored protein involved in lipid transfer at a number of membrane contact sites (Di Mattia et al., 2018).

(7) NPC (Niemann–Pick type C). NPC1 and NPC2 are localized to late endosomes and lysosome. They may work to transport cholesterol from endosomes that are present in myelin, to the myelin.

(8) Gltp. Gltp expression is specifically high in oligodendrocytes. Another member from this family, FAPP2, is involved in glycolipid transfer between the ER and Golgi (D'Angelo et al., 2007; Halter et al., 2007), but is expressed at a very low level in the brain.

(9) Bltp (Bridge-like transfer protein). This family is recently characterized to form a hydrophobic tunnel and mediate bulk lipid transport at various membrane contact sites. Some members, such as VPS13s, are present in cells at very low levels. However, due to their efficient operation, they are believed to play an important role in intracellular lipid transport. While proteins from this family remain largely unexplored, there is already some evidence suggesting the involvement of some members in plasma membrane expansion (Cheng & Bezanilla, 2021; Neuman & Bashirullah, 2018; Neuman et al., 2022).

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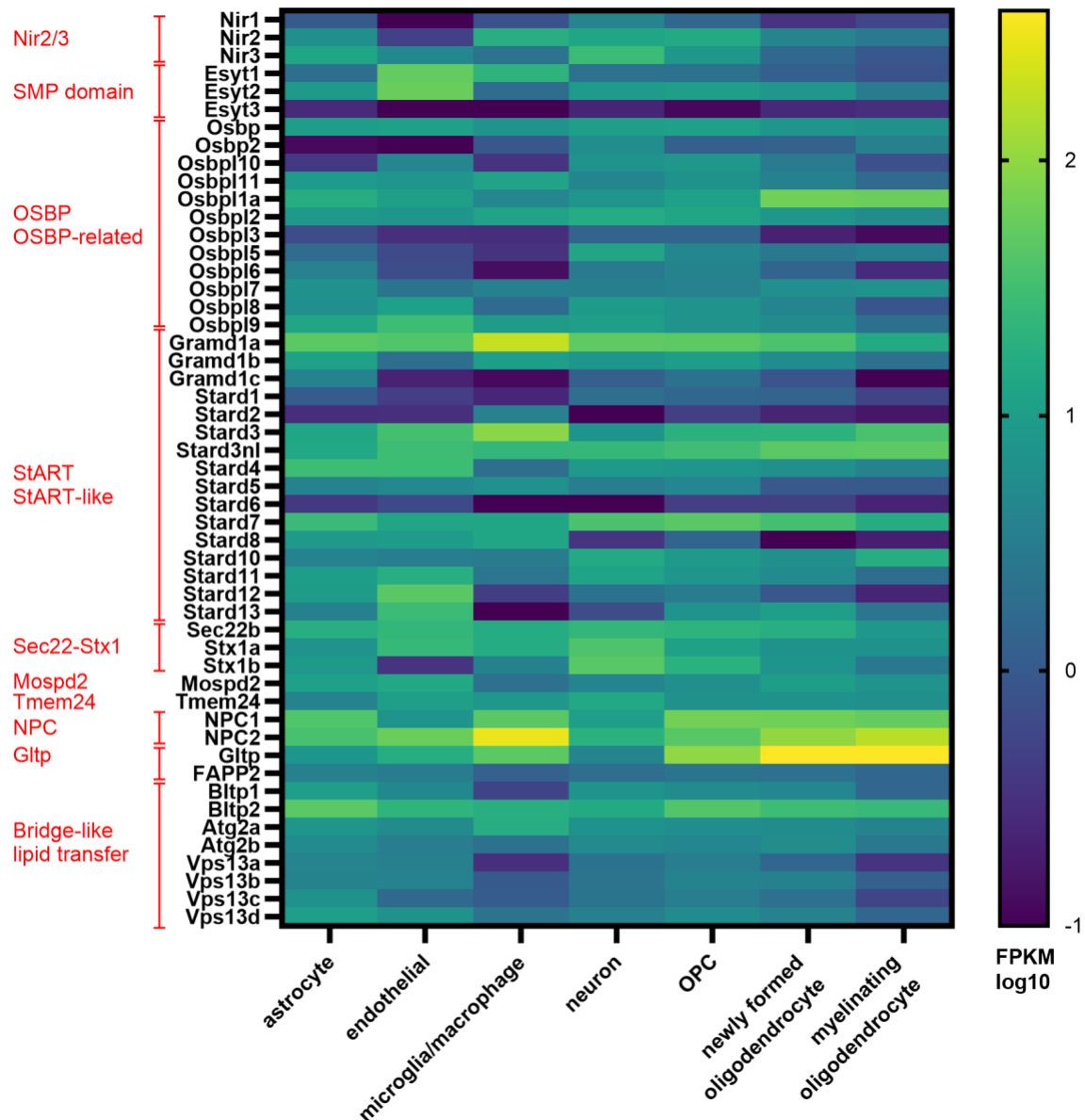


Figure 4.2 Expression pattern of nonvesicular transport protein families across brain cell types in mouse: A heatmap analysis of Zhang et al.'s RNA-Seq data.

### 4.3.4 Nonvesicular lipid transport for remyelination

GLTP translation is increased during remyelination in a cuprizone-induced mouse model for Multiple Sclerosis, detected by Ribo-Seq screen (Voskuhl et al., 2019). Like developmental myelination, remyelination may also involve nonvesicular transport. Given its superiority in terms of efficiency, boosting nonvesicular transport could potentially enhance remyelination in neurodegenerative diseases.



### 4.3.5 Other lipid transport for myelination

Beyond vesicular and nonvesicular transport, a recent report (Plochberger et al., 2020) showed that Apolipoproteins may directly fuse with the membrane. Although myelin mainly grows from the inside at the inner tongue, it would be interesting to test this possibility. Perhaps astrocytes or other cell types can facilitate the burden on oligodendrocytes in making myelin by supplying cholesterol directly to the myelin.

## 4.4 Cell biology of myelination

### 4.4.1 Other roles the ER might play in myelin

One critical function of the ER is to serve as a reservoir for calcium storage. Many studies have shown calcium is important for myelination (Baraban et al., 2018; Krasnow et al., 2018). It is very likely ER is playing a role here.

### 4.4.2 Cell biology of cytoplasmic channels

Developing myelin contains cytoplasmic channels that serve as highways for the transport of materials necessary for myelin biogenesis. These channels are highly curved and complex, raising many intriguing cell biology questions. For instance, how can microtubules sustain such high curvature? How are the ER and other organelles transported along microtubules? Additionally, how are extracellular vesicles released in developing myelin? These questions could be interesting directions for future investigations.

## 4.5 Limitation of the study

Although confocal images of immunocytochemistry provide some evidence that GLTP is adjacent to the plasma membrane, a limitation of this study is the absence of unambiguous evidence demonstrating GLTP's operation between ER-plasma membrane contact sites. This is challenging for many cytosolic lipid transfer proteins. For example, the lipid transfer protein, Oxysterol-binding protein (OSBP) is well-established to function at ER-Golgi contact sites, yet its distribution is largely cytosolic (Mesmin et al., 2013). One potential method to address this is by generating a mutated GLTP that halts in the working state: mutating the glycolipid binding site of GLTP may enhance its interaction with the lipid, thereby allowing

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visualization of where GLTP acquires lipids. However, such a mutation has yet to be identified, and its effectiveness remains uncertain.

Another limitation involves the methodology for testing if tubular ER is required for myelination. In this study, *Rtn4* mutation is used to investigate the influence of tubular ER. But RTN4 may have functions beyond stabilizing tubular ER. A more precise approach would involve preventing tubular ER from entering developing myelin, for instance, by mutating adaptors on the ER that link the ER to motor to prevent the tubular ER from entering the myelin.

In general, the limitations of this study stem from the technical challenges inherent in myelin research. Obtaining primary oligodendrocyte cultures is difficult, with cells that are challenging to culture, transfect, and mutate. In this study, I utilized cell-permeable RNAi, but these reagents are costly, and not every knockdown is effective. For example, attempts to knock down *Reep5* were unsuccessful. To circumvent these issues, I conducted what I refer to as "in-cell reconstitution" experiments, by overexpressing *Ugt8*, the enzyme responsible for GalCer synthesis, in a cell line such as U2OS (Fig 3.13s). At the organismal level, challenges are even greater. Attempts to use AAVs for labeling and mutating oligodendrocytes in mouse pups were particularly challenging (data not shown). A zebrafish model could offer a better alternative, which I have begun to explore, with results presented in Appendix.

As a cell biologist, I aspire to conduct more comprehensive biochemical and cell biological studies on oligodendrocytes. I am hopeful that the development of more sophisticated tools in the future will enable deeper investigations into the molecular mechanisms underlying myelin.

### 4.6 Significance of the study

This study, for the first time, systematically characterizes the ER in developing myelin using volume EM and examines its association with myelination. Furthermore, it unveils the involvement of a novel protein, GLTP, in myelination. This study brings a 3D perspective and cell biology concepts to the field of myelination and lipid biology. A special achievement is, as a study using mouse models, it has implications at the molecular scale, such as insights into membrane leaflets.

#### 4. Discussion

Although GLTP is one of the first lipid transfer proteins identified, its function *in vivo* remains elusive. The ambiguity can be attributed largely to the lack of studies within its natural environment. By examining GLTP in oligodendrocytes, a cell type expressing substantial amount of GLTP, my study sheds light to the long-standing mystery regarding GLTP's function.

The findings of this study are novel not only in neuroscience but also in cell biology, aligning with my aspirations to contribute to both fields during my PhD.

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## Appendix I. Using zebrafish to investigate oligodendroglial ER during live myelination.

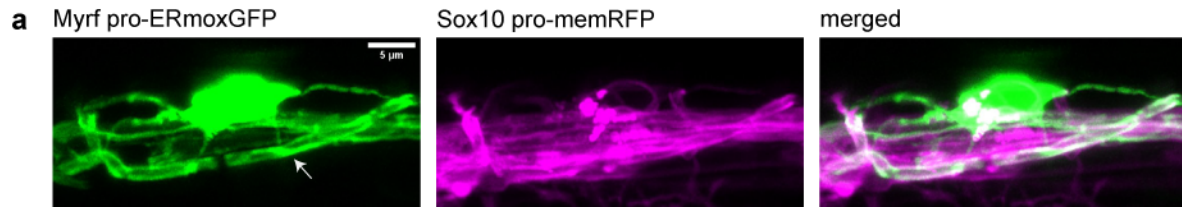
### Method

All zebrafish experimental protocols involving zebrafish were approved and conducted in compliance with the guidelines set by the District Government of Upper Bavaria. The zebrafish were maintained in the DZNE fish facility located in Munich, adhering to local guidelines for animal welfare.

To visualize myelin, I utilized transgenic line Tg(Sox10:mRFP). To express ER markers, I generated the following pDestCG2 Tol2 plasmids: (1) “JWC099”: Myrf promoter-ERmoxGFP. The construction is based on pERmoxGFP (Addgene #68072); (2) “JWC101”: Myrf promoter-mEGFP-Sec61b; (3) “JWC106”: Myrf promoter-Rtn4b-mEGFP; (4) “JWC107”: Myrf promoter-Reep5-mEGFP. The process of microinjection into zebrafish involved the injection of a 1:1 mixture of plasmid DNA (25 ng/μl) and transposase mRNA (ranging from 25 to 200 ng/μl) directly into early-stage embryos. To inhibit pigmentation and enhance imaging, embryos designated for imaging were treated with PTU starting from 8 to 24h post-fertilization (hpf). Injected eggs were analyzed 3–5 days post-fertilization.

### Results

I utilized zebrafish to investigate oligodendroglial ER during live myelination. The oligodendroglial ER was visualized by ER-luminal, monomeric and oxidizing environment-optimized GFP, which was expressed under the control of the Myrf promoter. Myelin was marked by membrane-targeted RFP, which was expressed under the Sox10 promoter. The oligodendroglial ER was in the oligodendroglial cell body, cell processes, and extended into the developing myelin, where it appeared spiral. This spiral appearance indicates its localization within the cytoplasmic channels (Fig A1, Movie A1). Similar results were observed with other ER markers driven by the Myrf promoter, such as mEGFP-Sec61b, Rtn4b-mEGFP, and Reep5-mEGFP (data not shown). Consistent with findings in mice, the ER extends into developing myelin in zebrafish as well, highlighting a conserved mechanism across species.



**Fig A1 Oligodendroglial ER during live myelination**

**a** Maximum intensity projection of z stack of myelinating oligodendrocyte in zebrafish at 4 days post fertilization (4 dpf). Green: oligodendroglial ER-luminal monomeric GFP; Magenta: oligodendroglial membrane-targeted RFP; Arrow: ER-moxGFP appears spiral in myelin. Scale bar: 5  $\mu$ m (a)

**Movie A1:** 3D stack of Oligodendroglial ER in live myelination, 8.88  $\mu$ m-thick (38 slices with 240 nm interval) related to Fig A1.1.

## Discussion

Employing zebrafish as a model organism offers a unique opportunity to observe oligodendroglial ER dynamics in real-time during the process of myelination. However, the ER signal did not show the typical fine structure due to the lack of resolution. A high-resolution microscopy would greatly improve this study.

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<b>Title</b>	CNS MYELINATION INVOLVES NONVESICULAR GLYCOLIPID TRANSPORT FROM ENDOPLASMIC RETICULUM TO PLASMA MEMBRANE IN OLIGODENDROCYTES	<b>Institution Name</b>	LMU Munich
		<b>Expected Presentation Date</b>	2024-06-15
<b>Instructor Name</b>	Mikael Simons		

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<b>Volume / Edition</b>	584	<b>Publication Date of Portion</b>	2010-05-03
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## Declaration of author contributions

We hereby declare author contributions as follows:

Majority of the contents in the results section has been submitted as a research article:

Jianping Wu, Georg Kislinger, Ayşe Damla Durmaz, Benedikt Wefers, Karsten Nalbach, Christian Behrends, Wolfgang Wurst, Martina Schifferer, Mikael Simons. “Nonvesicular lipid transfer drives myelin growth in the central nervous system”

Jianping Wu (J.W.)’s detailed contributions are:

- J.W. proposed to study nonvesicular lipid transport and GLTP, and co-designed experiments with Mikael Simons (M.S.).
- J.W. conducted experiments except for the following:
  - (1) Electron microscopy samples were processed and imaged by SyNergy EM facility (Georg Kislinger and Martina Schifferer).
  - (2) J.W. trained master student Ayşe Damla Durmaz and conducted experiments together for Fig 3.2.1a-g, Fig 3.2.2 a-c, Fig 3.3.1a-d.
  - (3) Shotgun lipidomics analysis were performed by Lipotype.
  - (4) Karsten Nalbach and Christian Behrends provided technical support to search for GLTP interactors.
  - (5) The *Gltp* KO and *Gltp* flox mouse lines were generated by DZNE transgenic facility (Benedikt Wefers and Wolfgang Wurst).
- J.W. quantified all resulting data and generated all the figures.
- J.W. and M.S. together interpreted the results and wrote the manuscript.

The discussion section of this dissertation primarily reflects J.W.’s personal views, with some inputs from M.S. The working model and the graph depicting GalCer distribution across various leaflets in both wildtype and knockout samples were developed by J.W.

Yours truly,

Jianping Wu

Mikael Simons