Dymeclin deficiency causes postnatal microcephaly, hypomyelination and reticulum-to-Golgi trafficking defects in mice and humans

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Abstract

Dymeclin is a Golgi-associated protein whose deficiency causes Dyggve–Melchior–Clausen syndrome (DMC, MIM #223800), a rare recessively inherited spondyloepimetaphyseal dysplasia consistently associated with postnatal microcephaly and intellectual disability. While the skeletal phenotype of DMC patients has been extensively described, very little is known about their cerebral anomalies, which result in brain growth defects and cognitive dysfunction. We used Dymeclin-deficient mice to determine the cause of microcephaly and to identify defective mechanisms at the cellular level. Brain weight and volume were reduced in all mutant mice from postnatal day 5 onward. Mutant mice displayed a narrowing of the frontal cortex, although cortical layers were normally organized. Interestingly, the corpus callosum was markedly thinner, a characteristic we also identified in DMC patients. Consistent with this, the myelin sheath was thinner, less compact and not properly rolled, while the number of mature oligodendrocytes and their ability to produce myelin basic protein were significantly decreased. Finally, cortical neurons from mutant mice and primary fibroblasts from DMC patients displayed substantially delayed endoplasmic

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Introduction

Dyggve-Melchior-Clausen syndrome (DMC, MIM #223800) is a severe autosomal-recessive spondyloepimetaphyseal dysplasia associated with microcephaly and intellectual disability, caused by loss-of-function mutations in the DYM gene encoding DYMECLIN, a Golgi protein thought to be involved in intracellular trafficking (1–3). The skeletal manifestations of the disease include short-trunk dwarfism with specific radiological features, severe proximal limb shortening and facial dysmorphism (4). Microcephaly is also consistently observed in DMC along with mild-to-severe intellectual disability, frequently including poor or absent language acquisition (5–9). Interestingly, both skeletal growth defects and microcephaly develop during childhood and are therefore rarely diagnosed at birth. This suggests that the physiological mechanisms affected in DMC involve postnatal processes. Interestingly, Smith–McCort Dysplasia 1 (SMC1, MIM #607326), an allelic spondyloepimetaphyseal dysplasia with skeletal features identical to DMC but with normal intelligence and no microcephaly, also results from mutations in DYM (10,11), whereas Smith–McCort Dysplasia 2 (SMC2, MIM #615222) is caused by homozygous mutations in RAB33B, another Golgi protein involved in trafficking (12,13). Importantly, DYM mutations causing SMC1 consist of missense substitutions that do not lead to protein degradation but may instead result in some residual activity of the protein. In contrast, DMC is associated with truncating mutations predicted to induce a loss of DYM function (1). This suggests that the cerebral phenotype is associated with a complete deficiency of DYMECLIN and that its role in the brain could be different from that in the bone. Biochemical and live-imaging studies indicate that DYMECLIN is associated with Golgi membranes (1,5) and is perpetually shuttled between the Golgi and cytosolic pools in a highly dynamic manner (1). Consistent with this, several Golgi vesicle proteins have been identified as potential DYMECLIN interactors (3,14). These findings argue for a role of DYM in Golgi homeostasis and/or Golgi-associated vesicular trafficking. Mice deficient in Dymeclin develop progressive skeletal abnormalities reminiscent of human DMC/SMC phenotypes (3). However, the brain phenotype of these mutant mice has never been investigated.

In this study, we analyzed the cerebral phenotype of Dym-deficient mice as well as two DMC patients to understand the role of Dymeclin in brain development. We found that Dym-deficient mice developed microcephaly postnataally, with a narrowing of the frontal cortex, a thinner corpus callosum associated with hypomyelinated axons and a reduced number of oligodendrocytes. This was accompanied at the cellular level by an enlarged Golgi apparatus in both mature oligodendrocytes and cortical neurons. While oligodendrocytes showed a reduced capacity to produce the myelin basic protein (MBP), cortical neurons showed a strong impairment of endoplasmic reticulum (ER)-to-Golgi transport, a function also altered in fibroblasts from DMC patients. Furthermore, we found that in addition to postnatal microcephaly, the brains of the two patients with DMC displayed a thinning of the corpus callosum, reinforcing the link between altered Dymeclin function and brain development.

Results

Dym<sup>−/−</sup> mice develop postnatal microcephaly

In humans, microcephalies are congenital, but some occur after birth and become more pronounced during childhood (15). To determine whether the Dym mutation causes microcephaly in mice, we used homozygous Dym-mutant mice lacking normal Dym transcript expression and previously shown to develop chondrodysplasia similar to that of DMC/SMC (3). We first macroscopically examined whole brains from young adult Dym<sup>−/−</sup> and wild-type (WT) mice at P30. Most Dym<sup>−/−</sup> brains appeared smaller in size. The length of the anteroposterior (AP) and dorsoventral (DV) axes was reduced and the frontal part of the cortex was narrowed (Fig. 1A). Brain weight was found to be systematically reduced in both males and females, with an average weight of 0.382 g (±0.005 g) for Dym<sup>−/−</sup> brains versus 0.432 g (±0.003 g) for WT brains. The brain weight of heterozygous animals was similar to that of WT animals (Fig. 1B). To determine whether this weight reduction was already present at birth or appeared postnatally, we compared brain weight at birth (P0), P5 and P30. At P0, the difference in brain weight was hardly noticeable. In contrast, at P5, the brain weight of Dym<sup>−/−</sup> animals was reduced by 10.2% in comparison with those of WT littermates (Fig. 1B). This difference remained stable up to adulthood, as the brain weight of Dym<sup>−/−</sup> animals at P30 was lower by 11.0% compared with WT or heterozygous littermates (Fig. 1B). Again, no sex-related differences were noted (not shown). To measure the difference in brain volume, volumetric magnetic resonance imaging (MRI) was performed. Whole brain tissue volume, estimated from manual masking of the structural MRI, was reduced in Dym<sup>−/−</sup> mice (mean volume in WT: 0.35 ± 0.063 ml; in Dym<sup>−/−</sup>: 0.29 ± 0.022 ml). Deformation-based morphometry (DBM) revealed local volumetric reductions in the frontal cortex, white matter and inferior structures, including the brain stem (Fig. 1C). Together, these data indicate that Dym<sup>−/−</sup> mice display a reduction in brain weight and volume and develop microcephaly during the postnatal period.

Dym<sup>−/−</sup> mice have a smaller frontal cortex and narrowed external capsule and corpus callosum

To determine which parts of the brain are affected in Dym<sup>−/−</sup> mice, we analyzed coronal sections counterstained with cresyl violet at P30. Five reference planes were chosen for comparisons between Dym<sup>−/−</sup> and WT brains (anterior (a) to posterior (e)), based on the Franklin and Paxinos mouse brain atlas (Fig. 2A). Interestingly, the length of the anteroposterior axis was found to be significantly reduced in Dym<sup>−/−</sup> brains as compared with WT brains (by 9.73%, Fig. 2B). The dorsoventral axis was also reduced in the anterior part of the brain (plane a, 4.8%, plane b, 4%). In contrast, no difference in the mediolateral axis was noted between the two groups (not shown). Given that reduced cerebral cortical development is the most common cause of inherited primary microcephaly (16), we next examined the architecture of the cortex, its surface area and its thickness in the five reference planes. The cortical surface area was reduced by 8% in Dym<sup>−/−</sup> brains, but this reduction was restricted to the most anterior part (Fig. 2C), consistent with a
Figure 1. Analysis of brain weight and volume in Dym<sup>−/−</sup> mutant mice. (A) Brains from WT (Dym<sup>+/+</sup>) and Dym-mutant (Dym<sup>−/−</sup>) mice at P30. Dashed lines delimit the rostrocaudal extent of the cerebral cortex (Cx), OB, Olfactory Bulb, Cb, Cerebellum. (B) Brain weight of P0, P5 and P30 WT (Dym<sup>+/+</sup>), heterozygous (Dym<sup>+/−</sup>) and homozygous mutant (Dym<sup>−/−</sup>) mice. Quantitative data are expressed as means ± SEM for WT (n = 9, 31 and 37 at P0, P5 and P30, respectively), heterozygous (n = 17, 58 and 46 at P0, P5 and P30, respectively) and homozygous mutant mice (n = 18, 18 and 23 at P0, P5 and P30, respectively). Results were compared using one-way ANOVA followed by a post-hoc Bonferroni multiple comparison test (**P < 0.01, ***P < 0.001). Brain weight was reduced in all mutant mice from P5 onward. (C) Brain volumetric MRI from adult Dym<sup>−/−</sup> mice (n = 5). Regions of significant volume reduction in experimental mice compared with controls are shown overlaid in blue/green on the MRI template image. Color indicates estimated P-values as shown by the color bar. P-values are uncorrected for multiple comparisons. Note the local volumetric reductions in the frontal cortex (arrowhead), white matter (asterisks) and inferior structures from Dym<sup>−/−</sup> mice, including brain stem (arrows).

In all planes, suggesting possible defects in the white matter (Fig. 2D).

Myelin is abnormal in Dym<sup>−/−</sup> brains

In keeping with the results above, we next examined the expression of MBP, one of the most abundant components of the myelin membrane in the central nervous system (CNS) and therefore a good marker for white matter. Immunohistochemical labeling of P30 sections with an anti-MBP antibody confirmed the reduction in the surface area of the corpus callosum in Dym<sup>−/−</sup> brains (Fig. 3D and E), and densitometry further revealed a reduction in MBP expression (Fig. 3E). Reduced protein levels were confirmed by western blotting of whole brain extracts (Fig. 3F). Electron microscopic analyses of the white matter revealed thin, poorly compacted and incorrectly rolled myelin sheaths in Dym<sup>−/−</sup> mutants (Fig. 4A). In contrast, the number of axons and their diameters were similar in both groups (Fig. 4B). Measurement of the G-ratio (axon diameter/total fiber diameter) confirmed the strong reduction of the myelin sheath thickness (Fig. 4B). Moreover, the density of oligodendrocytes (the myelin-forming cells in the CNS), labeled with an antibody to Olig2 (expressed at all stages of oligodendrocytic maturation), was significantly decreased in the corpus callosum of Dym<sup>−/−</sup> animals at P30 as compared with their WT littermates (Fig. 4C and D upper panels). A similar decrease in oligodendrocytic density was measured in sections labeled with APC, a marker specific for mature oligodendrocytes (Fig. 4C and D lower panels). While no cell death was detected in adults, at P5, cleaved-caspase 3 labeling revealed an increased amount of apoptotic nuclei in the corpus callosum of Dym<sup>−/−</sup> animals (Fig. 5D). TUNEL and Olig2 co-labeling confirmed that these dying cells were oligodendrocytes (Fig. 5C and D). Together, these data indicate that white matter volume is reduced in Dym<sup>−/−</sup> brains due to a decrease in the number of oligodendrocytes and axonal hypomyelination.

The Golgi apparatus is markedly enlarged in Dym<sup>−/−</sup> neurons and oligodendrocytes

As also observed by Denais and colleagues (14), the Golgi apparatus was markedly disorganized in primary fibroblasts from DMC patients (Supplementary Material, Fig. S1A). In an effort to understand the link between the defective white matter and cortical development on the one hand and Dynein function on the other, we examined the structure and function of the Golgi apparatus in primary neurons and oligodendrocytes from Dym<sup>−/−</sup> mice. In primary cortical neuronal cultures from the telencephalon from E14.5 mouse embryos, co-labeling for GM130, a marker of the cis-Golgi apparatus, and NeuN, a neuronal marker, revealed a markedly enlarged Golgi structure in Dym<sup>−/−</sup> cultures (Fig. 5A and B). In oligodendrocytes from glial-enriched primary cultures prepared from mouse forebrains dissected at P0, the co-labeling of mature oligodendrocytes with GM130 and APC also revealed an abnormally large Golgi apparatus in Dym<sup>−/−</sup> cultures (Fig. 5A and B). Confirming these results, the Golgi apparatus of mature neurons and oligodendrocytes was markedly enlarged in cultures differentiated from Dym<sup>−/−</sup> embryonic stem cells (Supplementary Material, Fig. S1B). Consistent with in vivo observations, co-labeling with the nuclear marker Olig2 and MBP revealed that the number of oligodendrocytes able to express MBP was reduced significantly in Dym<sup>−/−</sup> cultures (Fig. 5C and D). Further, Dym<sup>−/−</sup> oligodendrocytes expressing MBP appeared poorly developed and less branched (Fig. 5C and D). Together, these results indicate that Golgi structure is perturbed in Dym-deficient neurons and oligodendrocytes and could result in maturation or trafficking defects.
Anterograde trafficking is impaired in Dym<sup>−/−</sup> neurons and in human DMC fibroblasts

An extensive ER with dilated cisternae has been reported as a hallmark of Dym deficiency in fibroblasts and chondrocytes from both DMC patients (4,17,18) and Dym<sup>−/−</sup> mice (3). Because the Golgi apparatus is affected in Dym-deficient neurons, we asked whether Dymeclin could be involved in anterograde, i.e. ER-to-Golgi, trafficking. To examine trafficking between these two compartments in cortical neurons, we analyzed the retention and release of a Golgi enzyme, mannosidase II (ManII) using the retention using selective hooks (RUSH) system (19). In this system, the ManII-SBP-GFP fusion protein is initially sequestered in the ER by the KDEL hook until Biotin is added to the culture medium. At steady state, ManII-SBP-GFP was found in the ER as expected in both WT and Dym<sup>−/−</sup> neurons (Fig. 6). One hour after the addition of biotin, which releases SBP from its local hook protein and triggers ManII translocation, we detected ManII-SBP-GFP in the Golgi apparatus in half of the transfected neurons from WT cultures. Two hours after biotin addition, ManII-SBP-GFP was fully translocated to the Golgi apparatus of almost all WT neurons. In contrast, ManII-SBP-GFP was still detected in the ER compartment in the majority of Dym<sup>−/−</sup> neurons even 2 h after biotin addition (Fig. 6). These data show that ER-to-Golgi anterograde trafficking is impaired in Dym-deficient neurons. To check whether this phenomenon could also occur in human DMC cells, we used the same RUSH assay in primary fibroblasts from two patients with previously characterized DYM mutations (2,4). Comparable results were obtained with these DMC fibroblasts, although 40–50% of the transfected cells finally displayed the localization of the reporter in the Golgi apparatus after 2 h of biotin treatment (Fig. 7). To confirm that the delay in anterograde trafficking was due to the deficiency of Dymeclin, we repeated the RUSH assay in mouse neurons or human fibroblasts overexpressing WT Dym-GFP, using a red fluorescent version (mCherry) of the ManII reporter. Two hours after the addition of biotin, ManII-SBP-mCherry was consistently detected in the Golgi apparatus of both Dym<sup>−/−</sup> neurons (Supplementary Material, Fig. S2A) and DMC fibroblasts (Supplementary Material, Fig. S2B) overexpressing Dym-GFP. These data confirm that Dym deficiency results in defective anterograde trafficking and suggests that Dymeclin is required for efficient ER-to-Golgi cargo translocation both in human and mouse cells.

Corpus callosum thickness is reduced in DMC patients

Despite the observation of microcephaly and intellectual deficits associated with DMC, white matter defects have not so far been detected...
Figure 3. Analysis of the expression of MBP in Dym−/− mice. (A) Double immunofluorescence for Cux1 (red, used as a specific marker for cortical layers II–III) and Ctip2 (green, used as a specific marker for cortical layers IV–VI) and Dapi staining (blue) in the anterior cortex (plane a) from WT and Dym−/− mice at P30. Scale bar = 100 µm. (B) Cortical thickness measurements in layers II–III, IV–VI and I–VI (n = 7). Results were compared using a two-way ANOVA followed by a post-hoc Bonferroni multiple comparison test (***P < 0.001). Note that the significant reduction of frontal cortical thickness in Dym−/− mice is associated with a shortening of the deeper layers. (C) Determination of cell density in layers II–III and V–VI (n = 12). Results were compared using a two-way ANOVA followed by a post-hoc Bonferroni multiple comparison test. (D) Immunohistochemical analysis of MBP in the corpus callosum of WT and Dym−/− mice at P30. (E) Measurement of MBP-positive surface and densitometric quantification of MBP labeling intensity in the corpus callosum of WT and Dym−/− mice. Ten to fourteen animals per group were used. Results were compared using the Mann–Whitney U-test. (P < 0.05, **P < 0.001). MBP-positive surface and intensity are reduced in Dym−/− mice. (F) Western blot analysis of total hemi-brains at P30 using an anti-MBP antibody and densitometric analysis of MBP protein expression levels. Results were compared using the Mann–Whitney U-test. (P < 0.05). MBP protein level is reduced in the brain of Dym−/− mice.
documented in DMC patients, most likely because of the rarity of the syndrome and the scarcity of cerebral data available. Given our demonstration of the involvement of Dymeclin in neuronal and oligodendrocytic Golgi function in mice, we next examined the thickness of the corpus callosum in cerebral MRIs of two other unrelated DMC patients with well-characterized DYM mutations. Strikingly, these patients not only exhibited microcephaly with a postnatal onset, the thickness of their corpus...
callosum was markedly reduced (Fig. 8), consistent with our findings in the brain of Dym−/− mice as well as the observation that Dymeclin deficiency impairs Golgi function, and possibly as a consequence, proper myelination. These findings provide additional support to a role for Dymeclin-mediated intracellular transport in normal brain development.

Discussion

Postnatal microcephaly is a hallmark of DMC syndrome, which is caused by loss-of-function mutations in the human DYM gene (1). Our results show that Dym-deficient mice are a suitable model to study the cerebral phenotype observed in DMC patients.
and confirm clinical observations that DYMECLIN deficiency in the human brain induces postnatal-onset microcephaly. In agreement with the progressive appearance of microcephaly that continues during childhood, we observed a marked reduction in the thickness of the corpus callosum in Dym−/− mice, in association with myelination defects, a largely postnatal process (20,21). Interestingly, our study also reveals a marked thinning of the corpus callosum in two unrelated DMC patients, confirming our findings in mutant mice and suggesting that white matter defects are central to the pathophysiology of DMC syndrome. As the principal commissure interconnecting the two cerebral hemispheres, the corpus callosum contains more than 200 million axons, and volumetric alterations to this structure likely reflect more general white matter volume defects. Consistent with this hypothesis, the brain stem volume was also found to be reduced in Dym−/− mice, and the medulla oblongata appeared distinctly narrower than normal in the brain MRI of Patient 2. Our data suggest that volumetric MRI to quantify white matter volume and density should be systematically performed in children with DMC to better characterize these newly identified structural anomalies, which might represent an important feature of the DMC brain phenotype. These findings also provide a relevant explanation for the low IQ and specific language difficulties frequently described in DMC patients, especially as previous studies have evidenced a substantial correlation between general intelligence and white matter volume (22,23).

What is the mechanism underlying this postnatal development of white matter defects and microcephaly? Our data show that Dymeclin deficiency results in thinner, less compact and improperly rolled myelin membranes. In the CNS, myelination is carried out by oligodendrocytes, which enwrap axons to form the myelin sheath (24).

![Figure 6](image.png)

Figure 6. Functional analysis of ER-to-Golgi trafficking using the RUSH system in primary cultures of cortical neurons from WT and Dym−/− mice. Retention and release analysis of the Golgi enzyme ManII-SBP-GFP in the absence (non-treated) or presence of biotin (60 and 120 min incubation). In non-treated cultures, ManII-SBP-GFP (green) is sequestered in the ER (co-localized with the ER marker Calnexin (Calx, red)) by the KDEL hook in both WT and Dym−/− neurons. Upon biotin addition, ManII-SBP-GFP is released into the Golgi apparatus (co-localized with the Golgi marker GM130 (pink)) where it is found in all transfected neurons from WT cultures after 120 min. In neurons from Dym−/− cultures, the Golgi translocation of ManII-SBP-GFP is strongly delayed. For each condition, an average of 40 neurons was counted and neurons were assigned to three groups depending on whether ManII-SBP-GFP was found in the ER, the Golgi apparatus (Golgi) or in both compartments (ER Golgi). Neurons are stained with the neuronal marker MAP2 (red). Results were compared using a two-way ANOVA followed by a post-hoc Bonferroni multiple comparison test (*P < 0.05, **P < 0.01, ***P < 0.001).
Although we did not detect abnormal neuronal cell death at P5 and P30, the possibility that neuronal numbers are also modified, although to a lesser extent than the number of oligodendrocytes or at later stages cannot be ruled out. In addition to the abnormal morphology of the Golgi stacks, we observed slowed ER-to-Golgi trafficking in both primary neurons and DMC fibroblasts that could be completely rescued upon Dym-GFP expression. This observation is in agreement with previous findings showing the delayed relocation of GM130 to the Golgi in Dym−/− mouse fibroblasts recovering from Brefeldin-A treatment, which causes the reversible redistribution of Golgi membranes to the ER (3). Moreover, DMC patient fibroblasts expressing naturally secreted Gaussia luciferase (Gluc), used to assess the efficiency of the secretory pathway (27), displayed reduced secretion capacities, again implicating Dymeclin in anterograde trafficking (data not shown). These results potentially uncover a novel link between the localization of Dymeclin in the Golgi apparatus, and the appearance of structural brain defects including microcephaly.

In recent years, several Golgi-resident proteins have been implicated in genetic disorders that involve postnatal-onset microcephaly, including RAB18, RAB1GAP and RAB2GAP in Warburg Micro syndrome (28,29), VPS53 in progressive cerebello-cerebral atrophy type 2 (30) and TRAPPC9 in some idiopathic intellectual disability (31–33). Very interestingly, all these proteins are directly involved in the activation/inactivation cycle of the Rab-GTPases (34) and play a role in the regulation of Golgi vesicle trafficking. Moreover, their loss-of-function has been directly associated with clinical features that include corpus callosum hypoplasia. Hence, our study, in conjunction with the probable link between Dymeclin and RAB33 (12,13), (i) indicates that Dymeclin is involved in the regulation of Golgi vesicle trafficking in several cell types, including oligodendrocytes and neurons and (ii) reinforces the hypothesis that the secretory functions of the Golgi apparatus play a central role in the proper development and maturation of both the white matter and cortex in humans and mice.

In conclusion, our data indicate that Dymeclin deficiency results in postnatal microcephaly through a mechanism involving defective myelination and impaired Golgi trafficking in neural cells. Further work is needed to explore the consequences of defective ER-to-Golgi anterograde transport on myelination and axonal transport, and to understand the precise role of Dymeclin in this process, and more widely the role of the secretory traffic in microcephaly.

Materials and Methods

Immunohistochemistry and immunocytochemistry

Mice were sacrificed and the whole brain was dissected out from the level of the foramen magnum to the most rostral part including olfactory bulbs, immediately weighed, fixed in formalin and embedded in paraffin. Coronal sections 16 μm-thick were mounted on gelatin-coated slides, stained with cresyl violet or immunolabeled with anti-MBP (1:100, MAB382, Millipore), anti-Olig2 (1:200, 18953, IBL), anti-APC (1:2000, OP80, Calbiochem), anti-Cux1 (1:200, sc-13024, Santa Cruz) or anti-Ctip2 (1:500, ab18465, Abcam) antibodies. Immunocytochemistry was performed as described (35) using anti-NeuN (1:500, MAB377, Millipore), anti-GM130 (1:200, 610822, BD), anti-Calnexin (1:2000, C4731, Sigma), anti-APC (1:2000, OP80, Calbiochem), anti-Giantin...
and anti-MAP2 (1:200, ab11268, Abcam).

Histomorphometric analyses

Brains coronal sections from P30 mice were stained with cresyl violet and imaged using a Zeiss SteREO Discovery V12 microscope. Five anteroposterior levels (a–e) were selected based on relevant anatomical landmarks of WT mice (36) (from the interaural line/bregma, respectively, (in mm): 5.22/1.42 (a), 4.90/1.10 (b), 4.06/0.26 (c), 2.46/1.34 (d) and 1.50/−2.30 (e)). For each level, we measured (i) the dorsoventral and mediolateral axes, (ii) the surface area of the entire cortex, (iii) the surface area of the white matter comprising the corpus callosum and/or the external capsule, the boundary of the two structures being defined by the position of the cingulum and (iv) the thickness of the motor and somatosensory cortex. The length of the anteroposterior axis was deduced from the number of sections generated between a and e. Measurements were done using Image-J software and quantitative data expressed as means ± SEM.

Human and mouse MRI analyses

Human cerebral MRI was performed as described (37). For mouse MRI, brains (WT = 5, Dym−/− = 5) were imaged on a 9.4 T Varian scanner, using a 3D spoiled gradient echo sequence with a 100G/cm gradient-coil and 33-mm Rapid RF quadrature volume-coil; TR 150 ms, TE 4.6 ms and flip angle 35°. For DBM, a single control mouse was chosen for reference images. All other images were aligned to this reference using affine followed by non-linear registration implemented with the IRTK package (38,39). A reference image was created by taking the intensity average of the aligned images, and three further iterations of registration were performed, using an updated intensity average as the reference image. A measure of local volume change induced by the transformation between each image and the final reference image was obtained from the determinant of the Jacobian operator applied to the transformation. A voxelwise statistical comparison of volume relative to the reference image, represented by the Jacobian determinant, was performed with Randomise, implemented in FSL, v4.1 (40).

Primary cultures and transfection

Primary cortical neurons were extracted from the forebrain of E14.5 embryos using 0.25% trypsin dissociation (Invitrogen), 0.6 mg/ml DNase I digestion (Sigma) and manual dissociation in Minimum Essential Medium (Sigma) supplemented with 10% horse serum, antibiotics, 2 mm glutamine (Invitrogen), 7.5% sodium bicarbonate (Invitrogen) and 0.6% glucose (Sigma). Cells were maintained in Neurobasal medium (Invitrogen) supplemented with B21 (Miltenyi Biotec) and 5 mm glutamine at 37°C in a humidified 5% CO2 incubator. Primary oligodendrocytes were prepared from neonatal mouse brains and cells were maintained...
Electron microscopy

Mice were perfused transaortically with 20 ml saline followed by 100 ml of 2% paraformaldehyde with 2% glutaraldehyde at 4°C. Brains were post-fixed in 2% paraformaldehyde at 4°C. Sagittal sections 70-μm-thick were post-fixed in 1% glutaraldehyde for 10 min, treated with 1% osmium tetroxide for 10 min and dehydrated in an ascending series of ethanol solutions. Sections 10 μm thick were post-embedded on glass slides and cured at 60°C for 48 h. Blocks of the trunk of the corpus callosum close to the AC (Fluka), were treated with propylene oxide, equilibrated in Durcupan ACM (Fluka), flat-embedded on glass slides and cured at 60°C for 48 h. Blocks of the trunk of the corpus callosum close to the midline were cut from the sections and glued to blank cylinders of resin. Ultrathin sections were cut on a Reichert Ultracut S microtome, collected on pioloform-coated single-slot grids, stained with lead citrate and analyzed using a Philips CM120 electron microscope equipped with Morada Imaging System (Olympus).

Western blot analysis

Brains were dissected out from P30 mice (WT = 10, Dym<sup>−/−</sup> = 10), crushed in lysis buffer supplemented with protease inhibitors and centrifuged. Lysates were heat-denatured, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were probed with anti-MBP (1:2000, MAB382, Millipore) or anti-actin antibodies (1:10 000, MAB1501, Millipore). Signal was detected by chemiluminescence and quantified using Image-J.

Analysis of trafficking kinetics of the ManII-SBP-EGFP cargo

Trafficking kinetics between the ER and the Golgi were evaluated using the RUSH system (19). Mouse primary cortical neurons or human fibroblasts transiently expressing KDEL-streptavidin as a hook and the Golgi enzyme ManII-SBP-EGFP (or -mCherry) as a reporter were imaged either before or 60 and 120 min after biotin addition (40 μM). Ten fields were counted (40–100 transfected cells per condition) and each fluorescent cell classified according to the location of the reporter in the ER, ER/Golgi or Golgi. Experiments were repeated three times, independently.

Animals and ethics statement

Dymeclin mice were provided by Dr H.E. Ruley (Nashville, TN). Experimental mice were obtained by mating heterozygous animals, except for primary cultures for which embryos were generated from WT or Dym<sup>−/−</sup> breeders. Genomic DNA from tails was used for genotyping. Mice were housed with a 12 h light/dark cycle with free access to food and water. The experimental protocol (2010–13/676–0017) was approved by the National Debré-Bichat Ethics Committee and applied in agreement with French laws on animal protection.

Patients and ethics approval

Experiments presented in this study were approved by the Inserm Institutional Review Board, and the human primary fibroblasts were derived from skin biopsies following written informed consent from the patients or their parents.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest Statement. None declared.

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