Transcallosal Projections Require Glycoprotein M6-Dependent Neurite Growth and Guidance

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The function of mature neurons critically relies on the developmental outgrowth and projection of their cellular processes. It has long been postulated that the neuronal glycoproteins M6a and M6b are involved in axon growth because these four-transmembrane domain-proteins of the proteolipid protein family are highly enriched on growth cones, but in vivo evidence has been lacking. Here, we report that the function of M6 proteins is required for normal axonal extension and guidance in vivo. In mice lacking both M6a and M6b, a severe hypoplasia of axon tracts was manifested. Most strikingly, the corpus callosum was reduced in thickness despite normal densities of cortical projection neurons. In single neuron tracing, many axons appeared shorter and disorganized in the double-mutant cortex, and some of them were even redirected laterally toward the subcortex. Probst bundles were not observed. Upon culturing, double-mutant cortical and cerebellar neurons displayed impaired neurite outgrowth, indicating a cell-intrinsic function of M6 proteins. A rescue experiment showed that the intracellular loop of M6a is essential for the support of neurite extension. We propose that M6 proteins are required for proper extension and guidance of callosal axons that follow one of the most complex trajectories in the mammalian nervous system.

Keywords: axon pathfinding, corpus callosum, Gpm6a, Gpm6b, Plp1

Introduction

During nervous system development, axons actively grow and navigate through a complex environment for a long way to their targets (Raper and Mason 2010). The long navigation path involves many challenging steps, at which the growth cones have to faithfully detect and respond to guidance signals (Tessier-Lavigne and Goodman 1996; Dickson 2002). The presentation of the guidance signals is often transient, and the growth cones themselves may also change their reactivity to guidance signals over time. Therefore, timing is another critical challenge as the growth cones must arrive at the right environment in a particular window of time in order to be guided successfully.

The cortical callosal projection follows one of the most complex trajectories in mammalian axon guidance (Lindwall et al. 2007). This long connection between the two hemispheres involves multiple pathfinding steps. First, radial migration of callosal neurons makes the initial path of their axons directed down to the white matter, where the axons make a sharp turn medially toward the midline (Hatanaka and Yamauchi 2013). After crossing the midline and constituting the corpus callosum (CC), the axons give off collateral branches that innervate the appropriate cortical areas. The entire process is regulated by a complex combination of guidance molecules and various cytoskeletal regulators. Indeed, agenesis of the CC is one of the most common consequences of mutations affecting various signaling molecules (Lindwall et al. 2007).

M6a is a four-transmembrane domain-protein belonging to the proteolipid protein (PLP) family (Yan et al. 1993). Because M6a is highly enriched on growth cones (Sheetz et al. 1990; Baumrind et al. 1992; Lagenaur et al. 1992; Nozumi et al. 2009), a function in axon growth has long been postulated. In vitro experiments have reported complex actions of M6a in axon elongation: Upon overexpression of M6a in primary cultured neurons or neuron-like cell lines, neurite outgrowth is enhanced in some cells (Mukobata et al. 2002; Alfonso et al. 2005; Zhao et al. 2008) but not in others (Sato, Mita et al. 2011). Moreover, anti-M6a antibodies strongly inhibit neurite outgrowth in primary cultured neurons (Lagenaur et al. 1992). However, contrary to the initial interpretation, the antibodies induce growth cone arrest actively rather than suppressing the function of M6a in this context (Sato, Mita et al. 2011). Finally, because microscopic inspection of brain sections of M6a single-knockout mice did not reveal obvious morphological impairment (Sato, Mita et al. 2011; El-Kordi et al. 2013), the physiological function of M6a has remained unknown.

In this study, we followed the hypothesis that M6b, another neuronally expressed member of the PLP family (Yan et al. 1993; Werner et al. 2001), may functionally overlap with M6a and generated M6a/M6b double-null mutant mice. Indeed, the double mutants developed a severe hypoplasia of some axon tracts. In particular, the CC was markedly underdeveloped. The callosal defective phenotype was associated with atypical misrouting of callosal axons and cell-autonomous impairment of neurite outgrowth in culture. Our results therefore reveal for the first time a requirement for M6 proteins in axon growth and guidance in vivo.

Materials and Methods

Animals

Wild-type mice were purchased from Japan SLC, Inc. Mice lacking M6a (official gene name Gpm6a) were generated previously in the C57BL/6 background (Sato, Mita et al. 2011; El-Kordi et al. 2013). Mice lacking M6b (Gpm6b) or PLP (Plp1) were generated previously.
(Klugmann et al. 1997; Werner et al. 2013; Dere et al. 2014) and crossed into the C57BL/6 background for at least 10 generations. Genotyping was performed by PCR amplification of the genomic DNA prepared from the tails with specific primers. M6a/M6b double-knockout mice were obtained by crossing M6a+/− × M6b+/− or M6a−/− × M6b−/− females with M6a+/− × M6b−/− or M6a−/− × M6b−/− males. M6a/M6b/PPlp triple-knockout mice were obtained by crossing M6a−/− × M6b+/− × PPlp−/− females with M6a−/− × M6b−/− × PPlp−/− males. The day on which a vaginal plug was detected was designated as embryonic day 0.5 (E0.5).

The day of birth was designated as postnatal day 0 (P0). All experimental protocols were approved by the Animal Committee of the National Institute of Genetics and carried out according to the guidelines to minimize pain and discomfort in the animals.

**Section Preparation and General Histology**

Animals were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS). The brains were dissected out and further fixed with 4% PFA overnight. They were then embedded in paraffin and serially cut into 5-µm-thick sections using a microtome (RM2255, Leica Microsystems). Frozen, 20-µm-thick sections were prepared from the OCT-compound-embedded brains as described previously (Sato et al. 1998) and used for immunohistochemistry. After the preparation, some sections were stained with hematoxylin and eosin or with fluoromycin (1:300, Life Technologies).

**Immunostaining**

The sections or cultured cells were incubated with primary antibodies in 10% Tris-HCl, pH 7.4, 130 mM NaCl, 0.1% Tween-20 (TBST) at 4°C overnight, followed by secondary antibodies at room temperature for 2 h as described previously (Hirata et al. 2012). The primary antibodies used are as follows: rat anti-M6a (1:300, MBL); rat anti-M6a monoclonal M6 (1 µg/ml, Developmental Studies Hybridoma Bank); rabbit anti-M6b C-terminal (0.079 µg/ml, Werner et al. 2001); rabbit anti-M6b N-terminal (Werner et al. 2013); mouse anti-neurofilament 2H3 (1:100 hybridoma supernatant, Developmental Studies Hybridoma Bank); mouse anti-5-bromodeoxyuridine (BrdU) (1:100, BD Biosciences); hybridoma supernatant, Developmental Studies Hybridoma Bank); rabbit anti-M6b C-terminal (0.079 µg/ml, Werner et al. 2001); rabbit anti-M6b N-terminal (Werner et al. 2013); mouse anti-neurofilament 2H3 (1:100 hybridoma supernatant, Developmental Studies Hybridoma Bank); mouse anti-5-bromodeoxyuridine (BrdU) (1:100, BD Biosciences); mouse anti-NeuN (1:500, Merck Millipore); mouse anti-microtubule-associated protein (MAP) 2 (1:500, Sigma); rat anti-LI (1:1000, Merck Millipore); rabbit anti-cux1 (M-222, 1:100, Santa Cruz Biotechnology), mouse anti-Satb2 (1:1000, Abcam); rat anti-Ctip2 (1:1000, Abcam); rabbit anti-Thr1 (1:1000, Abcam); rabbit anti-Sox5 (1:1000, Santa Cruz); rabbit anti-GFP (1:500, MBL); mouse anti-neuron-specific tubulin Tuj1-1 (1:1000, Covance); rabbit anti-neuronal class III β-tubulin (1:1000, Covance); rabbit anti-Tau-1 (1:500, Santa Cruz) antibodies. As the secondary antibodies, Cy3-conjugated species-specific antibodies (1:500, Jackson ImmunoResearch) and Alexa488-conjugated species-specific antibodies (1:500, Life Technologies) were used.

**Quantification of Immunolabeling Intensity**

Coronal frozen sections prepared from P0 wild-type mice were double-immunolabeled with rat anti-M6a-specific monoclonal M6 and rabbit anti-M6b-specific C-terminal antibodies, followed by Alexa488-conjugated anti-rat and Cy3-conjugated anti-rabbit Ig antibodies. The sections were cover-slipped with ImmunoSelect Antifading Mounting Medium (Dianova GmbH) and digitally photographed with a CCD camera (DP71, Olympus) at a fixed exposure time to ensure that fluorescent intensities of all images were confined to the submaximal range. The mean intensities for regions of interest were then measured using Image J software (National Institutes of Health). Each average intensity value was calculated from 41 to 61 sections prepared from five individual mice.

**Immunoblot and Deglycosylation Assay**

Cortex lysates were prepared from P6 pups in lysis buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonylfluoride, and Complete Mini Protease Inhibitor Cocktail (Roche). The lysates were treated with peptide N-glycosidase (PNGase F, NewEngland BioLabs) according to the manufacturer’s instruction. Proteins (10 µg) were then separated on a 12% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. M6b was detected with an antiserum against the M6b-specific N-terminus (Werner et al. 2013) on samples that were never heated above 37°C. M6a was detected with an antiserum that recognizes the C-terminus of both M6a and M6b (Werner et al. 2001) on samples that were boiled for 2 min at 95°C, which selectively aggregates M6b at high-molecular weight but does not affect the migration in the gel of M6a.

**Size Measurement of the CC and the Anterior Commissure**

A series of sagittal paraffin sections were prepared from P7 mouse brains. Five sections centered at the midline were selected, stained with hematoxyline and eosin, and digitally photographed with a CCD camera (DP71, Olympus). The cross-section areas of the tracts were measured on the images using Image J software (National Institutes of Health).

**BrdU Labeling**

For birthdate labeling of layer II/III neurons, 4 mg of BrdU was injected intraperitoneally to a pregnant mother at E15.5 of gestation. The BrdU-labeled brains were later dissected and subjected to cryosectioning or dissociation for culturing cortical neurons. The BrdU incorporation into DNA was immunohistochemically detected after DNA denaturation with 2 N HCl for 2 h at room temperature as described previously (Suzuki et al. 2012).

**Callosal Axon Labeling in Utero**

An expression plasmid for membrane-anchored green fluorescent protein (pCAGGS-memGFP, a generous gift from Dr F. Suto) was used in utero electroporated into one side of the cortex as described previously (Mizuno et al. 2014). Briefly, after in utero electroporation of the pla9003 sets (TRE-nlsCre [10 ng/µL], CAG-loxP-stop-loxP-TurboRFP-WPRE [1 µg/µL]), brains were harvested from P7 postnatal mice, fixed and sliced into 100-µm-thick sections using a microslicer (DTK-1000, Dosaka EM). The images were taken using a confocal microscope (FV 1000, Olympus).

**Single Neuron Tracing**

The supernova method for sparse neuron labeling was described (Mizuno et al. 2014). Briefly, after in utero electroporation of the plasmid sets (TRE-nlsCre [10 ng/µL], CAG-loxP-stop-loxP-TurboRFP-WPRE [1 µg/µL]), brains were harvested from P7 postnatal mice, fixed and sliced into 100-µm-thick sections using a microslicer (Dosaka EM). For each section, 12–15 z-plane images were taken with a confocal microscope (FV 1000, Olympus) and stacked. The axon and dendrites of each labeled neuron were traced and reconstructed using the software Illustrator (Adobe Systems).

**Primary Neuron Culture**

Neocortices were dissected from E18.5 mouse embryos, treated with 0.1% trypsin, and dissociated into single cells by gentle pipetting. The cells were plated at a density of 6 × 10^5 cells/cm² on a laminin-coated four-well chamber slide (Nalge Nunc) and cultured in Neurobasal medium (Life Technologies) supplemented with N2 and B27 supplements (Life Technologies) at 37°C in a 5% CO₂ atmosphere for 48 or 72 h depending on the experiments. Cerebellar granule cultures were
prepared from P6 to P8 pups essentially as described (Banker and Golshin 1998). In brief, the dissociated cells were plated on poly-D-lysine-coated coverslips with Basal Medium Eagle (Life Technologies) containing 2 ms glutamax (Life Technologies), 0.1 mg/ml gentamicin (Sigma), and 10% fetal calf serum (Life Technologies). On the following day, the medium was changed to DMEM (Lonza) supplemented with 2 ms glutamax, 0.48% glucose, N2 and B27 supplements.

**Transfection of Cultured Cortical Neurons**

The expression plasmids for mouse M6a, M6b, DM20 and some of the M6a truncation forms were described previously (Sato, Mita et al. 2011; Sato, Watanabe et al. 2011). The other two constructs (Δ105–114 and Δ115–128) were similarly generated by PCR with designed primers, so that the deleted sequences were replaced by a nyc-tag sequence. All open reading frames were followed by an internal ribosome entry site (IRES) and EGFP coding sequence and sub-cloned into the pCAGGS vector (Niwa et al. 1991). The expression plasmids were in utero electroporated into the cortex as described earlier, with slightly stronger electric pulses (35 V 50 ms 4 times 1 Hz). When the embryos became E18.5, the brains were dissected and processed for dissociated culture as described earlier.

**Quantification of Neurite Length in Culture**

The cortical neurons were cultured for 72 h and fixed with 4% PFA/ PBS for 1 h at room temperature and immunostained with TuJ-1 monoclonal antibody. The fluorescent images were acquired with the CCD camera. Transfected neurons were determined by co-labeling with anti-GFP antibody. For the detection of BrdU-labeled neurons, rabbit anti-neuronal class III β-tubulin antibody substituted for the TuJ-1 antibody for enabling double-immunolabeling with mouse anti-BrdU antibody. On the fluorescent images, the neurites were manually traced with the plug-in software NeuronJ for ImageJ, and the lengths of axons and dendrites were measured using the built-in tools as described previously (Yamatan et al. 2010). For neurite quantification of cerebellar granule neurons, after 2 days in vitro, the cells were transfected with a dsRed expression construct (kindly provided by Michael Wehr) using Lipofectamine 2000 (Life Technologies) and digitally photographed on the next day. On the fluorescent images, neurites of >200 μm were traced and measured using the plug-in software NeuronJ.

**Results**

**Overlapping Expression of Glycoproteins M6a and M6b in the Central Nervous System**

M6a and M6b are highly homologous proteins with 55% identity in their amino acid sequences (Yan et al. 1993; Gow 1997; Schweitzer et al. 2006; Mobius et al. 2008). Previous studies by in situ hybridization have indicated that M6b is expressed in glial cells additional to neurons whereas M6a is restricted to the latter (Yan et al. 1996). Immunohistochemistry revealed that their protein distributions in neurons were largely similar, as exemplified at E16.5 and P0 (Fig. 1A, B). During developmental and neonatal stages, both proteins were highly concentrated on actively elongating axons. Particularly, the newly forming long axon tracts such as the anterior commissure (Fig. 1A), corticothalamic and thalamocortical pathways (Fig. 1A), and the CC (Fig. 1B) were intensely labeled with both anti-M6a- and anti-M6b-specific antibodies. A closer inspection revealed additional immunolabeling of neuronal cell bodies and dendrites (Fig. 1C). To evaluate co-expression of the two proteins semi-quantitatively, we measured fluorescence intensity of the immunolabeling in the cortex and several major tracts. In all examined regions, M6a and M6b were expressed, although their relative abundance differed moderately (Fig. 1D). The specificity of the antibodies was certified by immunohistochemistry on null-mutant brain sections using the same labeling and imaging conditions (Fig. 1E, F).

When cortical neurons were dissociated and cultured, the vast majority of them were found to be double-positive for M6a and M6b (Fig. 2A), whereas only a small fraction was preferentially labeled for either M6a or M6b. The proteins were distributed in axons (Fig. 2B) as well as cell bodies and dendrites (Fig. 2C). For M6a, a strong enrichment at membrane edges has been described previously (Sheetz et al. 1990; Baumrind et al. 1992; Lagenaur et al. 1992; Alfonso et al. 2005; Sato, Watanabe et al. 2011). We found that M6b exhibited a similar enrichment at peripheral membrane edges (Fig. 2B, C).

**Impairment of the CC in M6a/M6b Double-Null Mutant Mice**

Previous visual examination did not reveal striking morphological defects in the brains of M6a single-mutant mice (Sato, Mita et al. 2011; El-Kordi et al. 2013). We first suspected compensatory upregulation of M6b protein in M6a-deficient brains, but the abundance of M6b protein was unchanged according to immunoblot (Fig. 3). Likewise, the abundance of M6a was not increased in M6b single-deficient brains, suggesting the absence of compensatory adaptation of M6 proteins at the level of protein quantity. Considering the possibility of overlapping functions for M6a and M6b, we next generated M6a/M6b double-knockout mice. The M6b gene is located on the X chromosome. Both M6a−/− M6b−/− males and M6a− M6b−/− females were born at approximate Mendelian ratios. Around 20% of them died during 3–5 weeks after birth for unknown reasons (Supplementary Fig. 1), but the rest developed to adulthood and was found to be fertile.

Histological analyses of brain sections indicated that some of the axon tracts were considerably thinner in these double mutants (Fig. 4A, B). In particular, the CC appeared thinner compared with that of wild-type mice or the carriers of only one M6a mutant allele (Fig. 4C–F). The necrotic white matter, through which thalamocortical and corticofugal axons course, also appeared underdeveloped in double-mutant mice (Fig. 4A, B). Because the axon phenotypes were not different between M6a−/− M6b−/− Y males and M6a− M6b−/− females were born at approximate Mendelian ratios, we will hereafter refer to these mutants collectively as M6a/M6b double-null mutants.

To quantify genotypic influences on axon tract formation, we measured cross-sectional areas of the CC and the anterior commissure in P7 brain sagittal sections (Fig. 4G). Both tracts exhibited a trend toward a decrease in size in a gene-dose-dependent manner (Fig. 4H, I), although the genotypic differences in the anterior commissure did not reach statistical significance when analyzed by multiple-comparison testing, which is particularly stringent (Fig. 4D). For the CC, however, multiple-comparison testing indicated a significant reduction in the average bundle size of the M6a/M6b double-null mutants compared with that of control M6a single heterozygous littermates (Fig. 4H). A trend to a reduced thickness of the CC in M6a or M6b single-null mutants did not reach significance, suggesting that the functions of M6a and M6b in tract formation are indeed additive.

Developmentally, the callosal axons begin to project around E17 (Ozaki and Wahlsten 1998). In the early
developmental stages by P0, the formation of the CC was not yet visibly defective in double-null mutants (Fig. 5A,B), suggesting that the initial projection of callosal axons occurs relatively normal in the mutants. However, the hypoplasia manifested by P7, as the CC had thickened with accumulating axons (Fig. 4A–F). Even in the fully matured adult brain, the CC bundle was still thinner in M6a/M6b double-null mutants compared with wild-type mice (Fig. 5C,D), indicating that
the impairment of the CC projection in double-null mutants is not simply due to a delay in development. Taken together, these observations suggest that the late growth of the thickness of the CC between P0 and P7 is defective in M6a/M6b double-null mutants.

Neocortical Neurons Differentiate Properly in M6a/M6b Double-Null Mutants

The brain sizes and cortical thicknesses appeared normal in the double-null mutants (Figs 4, 5, and 6). Staining for the axonal marker, L1 visualized the thinner but normal-appearing fasciculation of axons that course through the white matter in mutant cortices (Fig. 6A, B). The somato-dendritic marker MAP2 revealed polarized dendritic processes that perpendicularly extend toward the surface similarly in wild-type and double-null cortices (Fig. 6C, D).

Callosal axons originate from neocortical neurons located in layer II/III (Fame et al. 2011). In addition, a fraction of layer V neurons contribute to the callosal projection. M6a/M6b double-mutant cortices displayed clearly distinct layers that were normally formed in a birth-order-dependent manner (Fig. 6E, F). When the number of neurons in individual layers was counted, the neuronal densities of M6a/M6b double-null mutant and wild-type mice were similar (Fig. 6G). These results suggest that the reduced thickness of the CC in the M6a/M6b double-null mutants is not caused by a reduction in the number of cortical neurons. Furthermore, we found that the expression of layer-specific transcription factors that determine neuron-specific axon projection phenotypes was unchanged in double-null mutant cortices (Fig. 7A, B). Therefore, altered neuronal identities are unlikely to cause the CC hypoplasia in the double-null mutants.

**Figure 2.** Distribution of M6a and M6b proteins in cultured cortical neurons. (A–C) Immunostaining with rat anti-M6a monoclonal M6 (A–C) and anti-M6b C-terminal (A′–C′) antibodies and their merged images (A″–C″) of 48-h-cultured cortical neurons prepared from E18.5 wild-type mouse embryos. M6a and M6b proteins are distributed in both the axon (B) and dendrites (C). Note the concentration of these proteins on shaft and growth cone filopodia (B) and dendritic membrane edges (C). Scale bars: 20 μm (A), 10 μm (B,C).

**Figure 3.** Abundance of M6 proteins in M6a and M6b mutant mice. Immunoblot analysis for M6a (upper) and M6b (lower) proteins of cortex lysates prepared from wild-type (M6a+/+ M6b+/Y), M6a single- (M6a−/− M6b+/Y), M6b single- (M6a+/+ M6b−/−), and M6a/M6b double- (M6a−/− M6b−/−) null mutants. One representative case from three independent experiments using different individual mice. Because M6a and M6b are glycoproteins, treatment with peptide N-glycosidase (PNGase F) causes mobility shift of the bands. In the M6a/M6b double-null mutants, both M6 proteins are undetectable. Note that the abundance level of each M6 protein is not increased when the respective other M6 protein is lacking. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected as an internal loading control.
Abnormal Pathfinding of Callosal Axons in M6a/M6b Double-Null Mutants

In order to examine trajectories of callosal axons, prospective callosal neurons in the neocortical layer II/III were GFP-labeled by in utero electroporation at E15.5 (Mizuno et al. 2007). In wild-type brains at P7, a great many labeled axons were found to project medially toward the midline (Fig. 8A). After crossing the midline (Fig. 8C), the axons gave off dense collateral projections into the contralateral cortical plate (CP) (Fig. 8D). In contrast, the same GFP-labeling procedures visualized reduced axonal projections in M6a/M6b double-null mutants (Fig. 8B). Specifically, the labeled axons hardly reached the midline (Fig. 8E), and the few axons that managed to cross the midline failed to collateralize into the CP (Fig. 8F). In various mutant mouse lines, agenesis of the CC has been reported to accompany the formation of Probst bundles, an abnormal swirl of callosal axons that fail to cross the midline (Serafini et al. 1996; Lanier et al. 1999; Bagri et al. 2002; Gu et al. 2003; Menzies et al. 2004; Mendes et al. 2006; Smith et al. 2006; Islam et al. 2009; Niquille et al. 2009).
Interestingly, M6a/M6b double-null mutants never developed this typical callosal misguidance phenotype. Instead, some of them exhibited a dense accumulation of GFP-labeled axons in the ipsilateral striatum (arrow in Fig. 8B; 4 out of 13 double-null mutant mice). Because the cortical neurons born at E15.5 rarely project their axons subcortically in normal situations (Mizuno et al. 2007; Hatanaka et al. 2009; Zhao et al. 2011), these results suggest misrouting of double-mutant axons from the normal callosal pathway.

Single neuron labeling further confirmed the axon misrouting phenotype. Using a sparse cell labeling technique (Mizuno et al. 2014), axons and dendrites of well-isolated layer II/III cortical neurons were traced and reconstituted in wild-type and double-null mutant brains (Fig. 8G–J). For wild types, 27 layer II/III neurons from 3 individual cortices were tracked. Among them, nine axons were successfully followed to cross the midline and penetrate the contralateral side (Fig. 8G). Except for one neuron that had a long axonal branch projecting to the ipsilateral cortex, no axons were found to turn laterally toward the ipsilateral striatum or the subsequent descending pathway. Conversely, in M6a/M6b double-null mutants, the axon trajectories of the layer II/III neurons appeared short and severely disorganized (Fig. 8H). Fifteen neurons from three individual cortices were tracked. Among them, five axons turned medially toward the midline but stopped short of reaching the contralateral cortex (Fig. 8H). Three axons were directed laterally, and two of them actually penetrated the striatum (Fig. 8H). Because it was not possible to track the axons further, it is uncertain whether they indeed terminated in the striatum or passed through it down to the brain stem. Taken together, these results indicate that callosal axons are not simply shorter but are aberrantly misdirected from the callosal pathway in M6a/M6b double-null mutants.

**Axon Development in M6a/M6b/Plp Triple-Null Mutants**

PLP, the third (and last) member of PLP family, is highly enriched in oligodendrocytes, in which it constitutes the most abundant myelin protein (Jahn et al. 2009). Because a low level of Plp-gene activity has been reported in neurons (Timsit et al. 1992; Jacobs et al. 2003; Miller et al. 2003; Sarret et al. 2010), we aimed to determine whether M6a/M6b/Plp triple mutant mice might develop more severe axonal impairment or even be completely acallosal. The Plp and M6b genes are expressed in oligodendrocytes, and the most abundant myelin protein in these cells. However, the role of M6a in axonal development and guidance is not well understood. In this study, we examined the axonal development in M6a/M6b/Plp triple-null mutants and compared them to the axonal development in M6a/M6b double-null mutants. Our results suggest that M6a plays a crucial role in axonal development and guidance, and the loss of M6a results in aberrant axonal misrouting. This is the first report of aberrant axonal misrouting in M6a/M6b double-null mutants, and it highlights the importance of M6a in axonal development and guidance. Additionally, our results suggest that the loss of M6a in M6a/M6b/Plp triple-null mutants leads to more severe axonal impairment compared to the loss of M6a in M6a/M6b double-null mutants.
From 45 breedings, we obtained 4 $M6a/M6b/Plp$ triple-null mutant males, 3 of which were sacrificed embryonically before myelination at E18.5, and the remaining 1 at P90. The major axon tracts were principally present in all of the E18.5 triple-null mutant embryos (Fig. 9A). Although the limitation in the number did not allow a quantitative analysis, the size of the CC in the triple-null mutants was roughly comparable with that of the $M6a/M6b$ double-null mutants (Fig. 5B) or the $M6a$ single heterozygotes (Fig. 5A) at this early stage. The only one adult triple-null male obtained from the breedings also had a clearly recognizable CC (Fig. 9B) notwithstanding that it was smaller, probably related to the additional myelination defect due to combined PLP and M6b deficiency (Werner et al. 2013). In sum, triple mutants lacking all PLP-family members were not acallosal, and we did not observe any obvious contribution of PLP to axon development.

### Neurite Extension is Cell-Intrinsically Defective in $M6a/M6b$ Mutant Neurons

We examined cellular phenotypes of $M6a/M6b$ mutant neurons in vitro. When cortical neurons were dissociated from the mutant embryos and cultured, axons extended by individual neurons were significantly shorter than those of wild-type neurons (Fig. 10A). Interestingly, axonal length was similarly reduced in either single- and double-null mutant neurons under these culture conditions. Dendrite length was also significantly impaired in null mutant neurons (Fig. 10A), suggesting that neurite growth, in general, is compromised by deleting the $M6a$ and $M6b$ genes. Despite the shortening of neurites, however, mutant neurons still exhibited a clearly polarized morphology with a single axon and multiple dendrites (Fig. 10B).

To test whether the neurite extension defect is specific to callosal neurons, we selectively labeled layer II/III neurons in vivo by BrdU birthdating at E15.5 (Fig. 6E,F). Upon culturing, both BrdU-positive and -negative neurons displayed impaired neurite length (Fig. 10C), indicating that the defective population includes, but is not limited to, layer II/III callosal neurons.

We further tested the generality of the neurite-extension supporting function using another neuron type, cerebellar granule neurons. Although less severe compared with cortical neurons, the neurites of cerebellar neurons prepared from $M6a/M6b$ double-null mutants were significantly shorter than those of wild-type neurons (Fig. 10D). This observation supports the hypothesis that M6 proteins have a general cell-intrinsic supporting function for neurite extension, albeit to different degrees in different neuron types.

### The Second Intracellular Domain of $M6a$ is Required for Efficient Neurite Growth

We attempted to rescue the impaired neurite outgrowth of cultured $M6a/M6b$ double-null neurons by introducing the members of the PLP family. First, we tested transfection of GFP-tagged $M6a$ in wild-type cortical neurons. Its forced expression in the wild-type situation did not enhance neurite length beyond the normal level (Fig. 11A), suggesting that the abundance of M6 proteins in wild-type cortical neurons is sufficient to support normal neurite lengths. In contrast, when the GFP-tagged $M6a$ was transfected into $M6a/M6b$ double-null neurons, their neurite length was reconstituted to a nearly normal level (Fig. 11B). Interestingly, similar neurite lengths

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**Figure 6.** Normal cortical structure in $M6a/M6b$ double-null mutants. (A–D) Immunostaining for the axon marker L1 (A,B) and the dendritic marker MAP2 in the cortex of P7 $M6a+/− /M6b+/*$ wild-type (A,C) and $M6a+/− /M6b+/*$ double-null mutant (B,D) mice. (E,F) Distribution of BrdU-labeled neurons in the cortex of P7 $M6a+/− /M6b+/*$ (E) and $M6a−/− /M6b−/− /Plp+/*$ double-null mutant (F) mice after BrdU injection at E15.5. The distribution patterns of preferentially labeled layer II/III neurons are indistinguishable between the two genotypes. Scale bars: 100 μm. (G) NeuN-positive neuronal densities in individual layers of the somatosensory, visual, and motor areas of $M6a+/+ /M6b+/*$ wild-type (black bars) and $M6a−/− /M6b−/− /Plp+/*$ double-null mutant (white bars) mice at P7. Each value was calculated from 18 sections prepared from 3 different mice. The motor area lacks a clear layer IV.
were observed when double-null neurons were supplemented with GFP-tagged M6b or DM20, which is the PLP splice variant most homologous to M6 proteins (Nave et al. 1987). Considering that the expression of any PLP-family member is sufficient to rescue normal lengths of double-null mutant neurites, either proteolipid can probably adopt similar functions in neurite outgrowth.

Using this rescuing assay, we searched for domains required for efficient neurite outgrowth. M6a is a 278-amino acid four-transmembrane domain-protein with both N- and C-termini protruding into the cytoplasm (Fig. 11C). It lacks a cleavable N-terminal signal sequence, and most mutations retain the protein in the endoplasmic reticulum. Our previous study (Sato, Mita et al. 2011) succeeded in generating intracellular deletion mutants that were present at the cell surface (Fig. 11C). Among them, the mutant constructs lacking either the N-terminal (ΔN) or C-terminal (ΔC) intracellular domain fully restored normal neurite length when introduced in double-null mutant neurons (Fig. 11D). In contrast, the deletion mutants in the intracellular loop did not rescue the impaired neurite length. The complete deletion of the intracellular loop (M6aΔ105–128) slightly dampened cell surface expression when examined in a cultured cell line. However, the two half deletions of the intracellular loop (M6aΔ105–114 and M6aΔ115–128) were compatible with efficient delivery to the cell surface but still failed to restore the normal neurite length (Fig. 11D). This domain does not contain any obvious sequence motif except for a potential site for fatty acid modification (Weimbs and Stoffel 1992) (asterisk in Fig. 11E). M6b and DM20 contain a cholesterol recognition consensus in this domain (Werner et al. 2013), but the critical tyrosine residue is missing in M6a (position 105 in Fig. 11E). M6a also associates with cholesterol-rich membrane domains (Scorticati et al. 2011), but this sequence comparison makes it unlikely that M6a associates with cholesterol by this domain. In sum, the molecular mechanisms are not clear but our results show that the intracellular loop—but not the N- or C-terminal domain—is critical for the function of M6a in neurite outgrowth.

Figure 7. Normal expression of layer-specific markers in M6a/M6b double-null mutant cortices. Cortex sections from P7 M6a+/- M6b+/- control (A) and M6a−/− M6b−/− double-null mutant (B) mice immunostained for Cux1 (layers II–IV), Satb2 (callosal neuron marker in layers II/III and V), Ctip2 (layer VI), Tbr1 (layer VII), and Sox5 (layer VI, though not yet clear at this developmental stage). Distribution patterns of the layer-specific neuron subtypes are indistinguishable between the two genotypes. Scale bars: 100 μm.
Discussion

The present study addressed a long-standing question regarding physiological functions of M6 proteins and provided direct evidence for M6 as a regulator of axon growth and guidance. According to our histological characterization, the in vivo functions of M6a and M6b in neurite growth appear largely overlapping. Furthermore, when introduced in cultured M6a/M6b double-null neurons, M6a, M6b, and even DM20 reconstituted normal neurite outgrowth. Thus, all PLP-family members can entail similar molecular functions, and their individual contributions to neurite outgrowth may be characterized by quantitative rather than qualitative differences. We observed that the axonal phenotypes of M6a/M6b double-null mutants are not visibly enhanced by the additional deletion of the gene
encoding PLP/DM20. This observation may reflect the fact that M6a and M6b but not PLP/DM20 show considerable overlap in neuronal expression in vivo (Yan et al. 1996).

An interesting observation in the in vitro rescuing experiment is that there was an apparent saturation level of M6 proteins for neurite-outgrowth support, as augmentation of M6a did not further promote neurite outgrowth in wild-type cortical neurons. On the other hand, the endogenous abundance level may not be overly high, because M6a or M6b single-null mutations appreciably compromised neurite outgrowth, particularly in cultured cortical neurons. The actual abundance of M6 proteins in wild-type cortical neurons thus appears optimal for proper neurite outgrowth.

The susceptibility to the single-null mutation varies from neuron to neuron. The reduction of neurite extension by the single-null mutations appeared milder when comparing cerebellar granule neurons to cortical neurons. Moreover, neurite outgrowth of cultured hippocampal and olfactory bulb neurons was not significantly influenced by the M6a single-null mutation (Sato, Mita et al. 2011). These differences in susceptibility may be attributable to different expression levels of M6 proteins in distinct types of neurons. For example, the higher abundance of M6 proteins in olfactory bulb axons (LOT in Fig. 1G) may allow these neurons to tolerate a single mutation. However, this possibility is not strongly supported because M6a and M6b abundance levels do not directly correlate with the susceptibility profile of neurons. Yet, varied responses of different neuron types to the deletion of M6 protein expression are in agreement with the previous observations that overexpression of M6 proteins promotes neurite outgrowth in some neuron types but not others (Mukobata et al. 2002; Alfonso et al. 2005; Zhao et al. 2008).

The present study found that cortical callosal axons are not simply shorter but misdirected in M6a/M6b double-null mutants. At this time, it is unclear whether or not this axon misroutting phenotype is related to the observed cell-intrinsic defect in neurite outgrowth. In an extreme scenario, the two phenotypes may even be entirely independent: The mutations might diminish in neurons the machinery for detecting guidance signals as well as that for process extension. Alternatively, axon misrouting may arise secondary to impaired axon growth. The cortical callosal projection involves several critical guidance steps (Lindwall et al. 2007). To be successfully guided, the growth cones have to arrive at each decision region in the right time window. If growth cones are delayed in arrival, they might miss a critical guidance signal in the constantly changing microenvironment. In this case, the misrouting phenotype may be regarded as a consequence of impaired axonal growth.

Many molecules regulate the midline crossing of callosal axons (Lindwall et al. 2007). When the corresponding genes are disrupted in mice, the callosal axons do not cross the midline and typically form a prominent Probst bundle (Serafini et al. 1996; Lanier et al. 1999; Bagri et al. 2002; Gu et al. 2003; Menzies et al. 2004; Mendes et al. 2006; Smith et al. 2006; Islam et al. 2009; Niquille et al. 2009). In humans, agenesis of the CC also often includes an episode of Probst bundle formation (Engle 2010). In contrast, M6a/M6b double-null mutants never developed such an abnormal swirl of axons. Thus, the mechanisms causing hypoplasia of the CC in the M6a/M6b mutants appears different from those in the above-mentioned cases. In M6a/M6b mutants, the presumptive callosal axons went astray individually in various regions in random-appearing directions; some were directed toward the midline and terminate halfway, whereas others were laterally directed even subcortically. This individualistic behavior clearly differs from the collective axonal misrouting in Probst bundles. It may thus not be attributable to one specific error for detecting a specific guidance signal but rather involve several guidance errors. Regarding the projection phenotypes, the laterally directed misrouting of callosal axons is uncommon and has been observed in none of the CC hypoplastic mutants. To our knowledge, the only related previous observation is that forced overexpression
of a repulsive guidance molecule, semaphorin 3A, in callosal neurons misdirected their axons into the lateral direction (Zhao et al. 2011). The integration of these results is not straightforward, because the loss-of-function mutation of this signaling protein reduces the medially directed axon growth but does not reorient the axons laterally (Gu et al. 2003; Hatanaka et al. 2009; Zhao et al. 2011). Probably, the medial ectopic overexpression of semaphorin 3A reversed its normal lateral–high medial–low gradient and thereby reoriented the axons with respect to the mediolateral direction. Likewise, losing the sense of mediolateral orientation in the axons is one feature of the callosal phenotypes induced by \( \text{M6a/M6b}\) mutations.

This study identified the intracellular loop of M6a (compare Fig. 11C) as required for the support of normal neurite outgrowth. Deletion of either non-overlapping half of this domain similarly abolished the rescuing effect, and therefore, we could not further narrow down the functional domain. In PLP/DM20, the homologous intracellular loop is the site of the PLP-specific insertion of 35 amino acids and implicated in conformation folding and membrane insertion (Gow and Lazzarini 1996; Dhaunchak et al. 2011). Considering that this domain contains a potential fatty acid modification site, it is possible that manipulation of this domain in M6a affected membrane interaction and conformation folding. This would lead to nonfunctionality of the truncated products, even though the extracellular antibody epitopes appeared to be sufficiently conformed at the cell surface at least for the half deletion constructs.

An antibody against M6a strongly inhibits axon outgrowth in culture (Lagenaure et al. 1992). Based on analyses involving M6a single-null neurons, we previously proposed that the anti-M6a antibody actively induces axon growth arrest but does not simply inhibit the function of M6a (Sato, Mita et al. 2011). The present study provides further molecular evidence that the antibody action and deletion of M6 proteins are separable events. For neurite-outgrowth inhibition triggered by anti-M6a antibody, a short N-terminal intracellular sequence (amino acid 1–25) is important (Sato, Mita et al. 2011), whereas the same N-terminal sequence was dispensable for rescuing the impaired neurite outgrowth of \( \text{M6a/M6b}\) double-null neurons. Furthermore, the intracellular loop of M6a (amino acid 105–128) was required for rescuing the neurite outgrowth of \( \text{M6a/M6b}\) double-null neurons but not for antibody-induced neurite growth inhibition (Sato, Mita et al. 2011).

Figure 10. Cell-intrinsic defect in neurite extension of cultured mutant neurons. (A) Average length of axons (left), dendrites (middle), and total neurites (right, axons plus dendrites) per neuron as measured in dissociated culture of cortical neurons with genotypes indicated below. Each value was calculated from 290–447 neurons prepared from at least 2 embryos. (B) Double immunostaining of cultured cortical neurons for the axonal marker Tau (magenta) and the soma-dendritic marker MAP2 (green). Both of the wild-type (upper panel) and \( \text{M6a/M6b} \) double-null (lower panel) neurons are clearly polarized. Scale bar: 100 \( \mu \)m. (C) Average length of neurites for BrdU-positive (left) and BrdU-negative (right) cortical neurons. BrdU was injected at E15.5 in vivo so that layer II/III callosal neurons are selectively labeled. Each value was calculated from 64–118 BrdU-positive and 226–329 BrdU-negative neurons prepared from at least 2 embryos. (D) Average neurite length of cerebellar granule cells. Each value was calculated from 370–607 neurons prepared from 3 or 4 pups. \( * P < 0.05 \) by Tukey’s multiple-comparison test when compared with wild-type controls.
Taken together, the neuronal glycoproteins M6a and M6b are required for the proper development of long axons in vivo. Interestingly, previous in vitro studies have reported that M6 proteins may also be relevant at earlier and later steps of neuronal differentiation. For example, M6a is suggested to be involved in the early differentiation of embryonic stem cells into neurons and their migration in vitro (Michibata et al. 2008, 2009). In the present study, however, we did not notice obvious defects in the differentiation or migration of cortical neurons in M6a/M6b mutants. The overexpression of M6a enhances the formation of filopodial spines and membrane protrusions in cultured neurons and non-neuronal cells.
(Alfonso et al. 2005; Fuchsova et al. 2009; Brocco et al. 2010; Fernandez et al. 2010; Huang et al. 2011; Scorticati et al. 2011; Zappia et al. 2012), suggesting a morphogenetic function in late neuronal maturation. These activities require the second extracellular and N-terminal (amino acid 1–62) domains, neither of which overlaps with the intracellular loop identified in this study. Other functions hypothesized for M6 proteins in mature neurons include membrane trafficking; M6a is present in glutamatergic synapses (Roussel et al. 1998; Cooper et al. 2008) and interacts with the G protein-coupled μ-opioid receptor at the transmembrane domain thereby regulating its membrane recycling (Wu et al. 2007; Liang et al. 2008), whereas M6b interacts with the serotonin transporter and promotes its presence at the cell surface (Fjorback et al. 2009). It is not straightforward to discern in vivo those functions of M6 proteins because the developmental impairment of projections in conventional M6a/M6b null mutants is likely to affect subsequent neuronal maturation and synaptic activity. We thus propose that future experiments involving conditional inactivation of M6 genes will be required to test the biological significance of their pleiotropic actions in mature neurons.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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References


