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# Transcriptional Expression of Myelin Basic Protein in Oligodendrocytes Depends on Functional Syntaxin 4: a Potential Correlation with Autocrine Signaling

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**Myelination of axons by oligodendrocytes is essential for saltatory nerve conduction. To form myelin membranes, a coordinated synthesis and subsequent polarized transport of myelin components are necessary. Here, we show that as part of the mechanism to establish membrane polarity, oligodendrocytes exploit a polarized distribution of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery components syntaxins 3 and 4, localizing to the cell body and the myelin membrane, respectively. Our data further reveal that the expression of myelin basic protein (MBP), a myelin-specific protein that is synthesized "on site" after transport of its mRNA, depends on the correct functioning of the SNARE machinery, which is not required for mRNA granule assembly and transport *per se*. Thus, downregulation and overexpression of syntaxin 4 but not syntaxin 3 in oligodendrocyte progenitor cells but not immature oligodendrocytes impeded MBP mRNA transcription, thereby preventing MBP protein synthesis. The expression and localization of another myelin-specific protein, proteolipid protein (PLP), was unaltered. Strikingly, conditioned medium obtained from developing oligodendrocytes was able to rescue the block of MBP mRNA transcription in syntaxin 4-downregulated cells. These findings indicate that the initiation of the biosynthesis of MBP mRNA relies on a syntaxin 4-dependent mechanism, which likely involves activation of an autocrine signaling pathway.**

During myelination, oligodendrocytes (OLGs) express large quantities of myelin proteins and lipids that are subsequently transported from the cell body via processes to myelin membranes, which are wrapped around axons to form the myelin sheath. Like the apical and basolateral plasma membrane domains in polarized epithelial cells, the myelin-like membrane (sheet) and cell body plasma membrane can be considered a reflection of the polarized nature of cultured OLGs (1). Indeed, in previous work, we observed that distinct viral proteins, i.e., the hemagglutinin (HA) of influenza virus and the glycoprotein of vesicular stomatitis virus (VSV G), which are sorted and transported in epithelial cells to the apical and basolateral domains, respectively, also display a distinct and preferential localization in cultured OLGs (2). In fact, vesicular traffic to the myelin sheet relies on a basolateral-surface-like rather than an apical-surface-like transport and sorting mechanism, and basolateral sorting signals similar to those in epithelial cells target proteins to myelin sheets (3–6). Of interest, vesicle-mediated transport does not occur in compact myelin but rather proceeds via lateral membrane diffusion (7), suggesting that once myelin is established, polarization is maintained in a nonvesicular manner.

From studies on polarized epithelial cells, it is known that a distinct membrane composition is established by polarized transport of membrane constituents. The final step in vesicular transport of membrane proteins involves the docking and fusion of a vesicle with its target membrane, which is mediated by a protein family referred to as soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (8–10). The SNARE proteins are integral membrane proteins that are present on both the vesicles (v-SNARE) and the target membranes (t-SNARE). Importantly, the apical and basolateral plasma membrane domains of polarized epithelial cells contain distinct t-SNAREs (11), i.e., syntaxins 3 and 4, which are preferentially localized at the apical and basolateral plasma membrane, respectively (12–17). Both syntaxin 3 and syntaxin 4 are known to be present in OLGs (1, 18, 19), but their functional role in myelin biogenesis is still largely unknown. Therefore, in this work, we examined whether syntaxins 3 and 4 are functionally expressed in rat primary OLGs and involved in myelin biogenesis, thereby focusing on the role of myelin sheet-localized syntaxins and the two major myelin proteins, myelin basic protein (MBP) and proteolipid protein (PLP). These proteins are expressed in a timely fashion and sorted and transported to the myelin membrane by different mechanisms (1, 20, 21). MBP, located at the cytoplasmic surface of myelin membranes, is a basic, membrane-associated adhesive protein and essential for myelination *in vivo* (22–24). Its adhesive properties organize the close apposition of the inner membrane leaflets, leading to myelin compaction (25), essential for saltatory nerve conduction. Furthermore, MBP appears to act as a portal for protein entry into myelin membranes (7). MBP is transported to the myelin sheath in its mRNA form (26, 27), which is thought to circumvent premature adhesion of membranes (1, 22, 24, 25). Although MBP mRNA is assembled in nonmembranous granules (26–28), a causal relationship with the vesicular transport ma-

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chinery is likely, given that membrane trafficking appears to be involved in RNA transport and/or anchoring (29–31). In contrast, PLP is an integral membrane protein that is synthesized at the endoplasmic reticulum and subsequently processed by vesicular transport, reaching the myelin membrane via a transcytotic transport mechanism (1, 32–36). Furthermore, a distinct role for the v-SNAREs vesicle-associated membrane protein 7 (VAMP7) and VAMP3, cognate binding partners of syntaxins 3 and 4, respectively, in PLP trafficking has been recently demonstrated (32). PLP plays a major role in assembly and stabilization of the myelin sheath in that the protein brings about the correct apposition of the extracellular leaflets of the membrane (37, 38).

Here, we report that syntaxins 3 and 4 are functionally expressed in rat primary OLGs and distribute in a polarized manner, syntaxin 3 being largely restricted to the cell body, whereas syntaxin 4 is upregulated during OLG differentiation and locates toward the myelin sheet. Surprisingly, our findings further indicate that MBP mRNA transcription, but not MBP mRNA trafficking, depends on functional expression of syntaxin 4 but not syntaxin 3, whereas trafficking of PLP to the myelin membrane proceeds independently of syntaxin 4. The intimate involvement of syntaxin 4 in initiating MBP mRNA expression in oligodendrocyte progenitor cells (OPCs) is supported by the lack of effect of downregulation of syntaxin 4 in immature OLGs (imOLGs), while the effect was reversed by conditioned medium of developing OLGs. These data are taken to suggest that syntaxin 4-mediated autocrine signaling at the onset of OPC differentiation is necessary for initiating MBP mRNA transcription, preceding its granule-mediated transport to the myelin membrane. This insight will aid in developing novel approaches toward inducing remyelination in demyelinating pathologies, such as multiple sclerosis.

## MATERIALS AND METHODS

**Cell cultures. (i) Primary oligodendrocytes.** Primary OLG cultures were generated by a shake-off procedure as described previously (39, 40). Enriched OPCs were resuspended in SATO medium containing 10 ng/ml platelet-derived growth factor AA (PDGF-AA; Peprotech, Rocky Hill, NJ) and 10 ng/ml fibroblast growth factor 2 (FGF-2; Peprotech). For immunocytochemical studies, OPCs were plated on poly-L-lysine (PLL; 5 μg/ml; Sigma, St. Louis, MO)-coated 13-mm glass coverslips (VWR, Amsterdam, the Netherlands) at 30,000 cells per well (500 μl), and for conditioned medium, quantitative PCR (qPCR), Western blotting, and coimmunoprecipitation (co-IP) analysis, cells were plated on PLL-coated 10-cm dishes (Nalge Nunc International, Roskilde, Denmark) at $10^6$ cells per dish (6 ml or 4.5 ml for conditioned medium). After 48 h, differentiation was induced by growth factor withdrawal, and cells were cultured in SATO medium (40) supplemented with 0.5% fetal calf serum (FCS; Bodinco, Alkmaar, the Netherlands) for 3 days (immature OLGs [imOLGs]) or 10 days (mature OLGs [mOLGs]). Conditioned medium of developing OLGs was collected 3 days after initiating differentiation and used in a 1:1 ratio with fresh medium (SATO with 0.5% FCS).

**(ii) Myelinating cocultures.** Primary rat dorsal root ganglion neurons (DRGNs) were isolated from 15-day-old Wistar rat embryos (Harlan, the Netherlands), as described before, with minor modifications (41). Dissociated DRGNs were plated as 40-μl drops at a density of 60,000 cells on 13-mm coverslips (0.5 ml) that were precoated with PLL (10 μg/ml), followed by growth factor-reduced Matrigel (1:40 dilution; BD Bioscience, Bedford, MA). DRGNs were cultured in 500 μl of neurobasal medium (Invitrogen, Paisly, United Kingdom) supplemented with 2% B27 (Invitrogen) in the presence of nerve growth factor (NGF; 100 ng/ml; Serotec, Kinglington, United Kingdom). Fibroblasts were eliminated with two 48-h cycles of 10 μM 2′-deoxy-5-fluorouridine (FdU; Sigma) 1 and 5 days after plating of the DRGNs. OPCs were seeded onto DRGNs at 14 to 19 days *in vitro* at a 1.5:1 ratio in basal medium Eagle (BME; Invitrogen) supplemented with 1% ITS supplement (Sigma), 0.25% FCS, and D-(+)-glucose (4 mg/ml; Sigma), after which the cocultures were maintained for 14 days. All experimental procedures were approved by the Animal Ethical Committee of the University Medical Center Groningen (UMCG).

**Constructs and primers. (i) shRNA.** Syntaxin 3, syntaxin 4, and VAMP3 short hairpin RNA (shRNA) constructs were designed with DSIR (42), resulting in the following target sequences (uppercase letters; lowercase letters depict the added nucleotides necessary for cloning into the retroviral vector): 5′-acaaaGGCGCGCCACGAAAGAAATTGATAA TTAACTCGAGATAATTATCAATTTCTTTCG GTTTTTCCTGCAG-Gcacaa-3′ (shRNA against rat syntaxin 3), 5′-acaaaGGCGCGCCAGGTG TTTGTGTCTAATATAACTCGAGATATATTAGACACAAACACCGT TTTTCCTGCAGGcacaa-3′ (shRNA against rat syntaxin 4), and 5′-acaa aGGCGCGCCACGGAGATGTTCACTTTCTAACTCGAGATAGAAAG TGAACATCTCCGGTTTTTCCTGCAGGcacaa-3′ (shRNA against rat VAMP3). With restriction enzymes AscI and SbfI, shRNA constructs were cloned into the lentiviral vector pHR'trip-eGFP (where eGFP is enhanced green fluorescent protein) (Addgene) or with LR clonase in pLenti-x2 Puro DEST (Addgene). Correct insert of the construct was confirmed by DNA sequencing.

**(ii) Overexpression.** The cDNA encoding syntaxins 3 and 4 were a kind gift of Thomas Weimbs (University of California Santa Barbara, Santa Barbara, CA) (15). For cloning the syntaxin genes in the retroviral vector pLXIN (Clontech Biosciences, Mountain View, CA), an XhoI restriction site at the ATG start codon of the syntaxin 3 and syntaxin 4 genes and an XhoI restriction site after the stop codon of both genes were introduced by PCR. The following primers were used: 5′-CATGTATTCGAA GAGCTCTTCGCACATGG-3′ (forward syntaxin 3), 5′-CTAGGTGATC AAGAGCTCCTAGGGCCCACG-3′ (reverse syntaxin 3), 5′-CGAATAG CTATGAGCTCCATGGTCTAG-3′ (forward syntaxin 4), and 5′-GATC TCCTAGAGCTCACGTAGGGAC-3′ (reverse syntaxin 4). The PCR product was digested with XhoI (Invitrogen) and ligated with the 1.8-kb retroviral vector pLXIN. The orientation and the integrity of the obtained pLXIN constructs were confirmed by DNA sequencing.

**Production of viral particles and cell transduction. (i) Lentiviral particles.** For production of lentiviral particles, the constructs, packaging, and envelope plasmids (pRSV-Rev and pMD.G) were transfected into the HEK293T packaging cell line using calcium phosphate. Two days after transfection, cells were washed with 0.01 M phosphate-buffered saline (PBS), and medium was collected after 24 h. The lentiviral-particle-containing medium was filtered through a polyvinylidene difluoride (PVDF) membrane-based filter (Millipore; 0.45-μm pore size) and either used immediately or stored frozen at −80°C. Cells were transduced before shake-off for coculture experiments or at the indicated developmental stage in monocultures for 6 to 8 h with twofold-diluted lentiviral-particle-containing medium that was supplemented with 4 μg/ml hexadimethrine bromide (Polybrene; Sigma). For qPCR experiments of OLG monocultures, transduced OPCs were selected in SATO medium supplemented with FGF-2, PDGF-AA, and 0.25 μg/ml puromycin (Sigma) G418 for 5 days. After selection, the cells were cultured in SATO with 0.5% FCS for 7 days.

**(ii) Retroviral particles.** The production of retroviral particles and the subsequent infection of OPCs were performed according to reference 43. Briefly, for production of recombination-deficient retroviruses, the constructs were transfected into the GP+E86 packaging cell line (Genetix Pharmaceuticals, Inc., Cambridge, MA), using the FuGENE 6 transfection reagent. Two days after transfection, cells were collected, diluted 5-fold, and cultured under selection in packaging cell medium supplemented with 1 mg/ml G418 until resistant clones appeared (70% confluent). The cells were subsequently washed with PBS, and packaging cell medium without G418 was added. The conditioned medium containing the viral particles was collected after 24 h, filtered (Schleicher and Schuell, Dassel, Germany; 0.45-μm pore size), and either used immediately or stored frozen at −80°C. Transductions were carried out by exposing OPCs to retroviral particles, 8 μg/ml Polybrene, 10 ng/ml FGF-2, and 10 ng/ml PDGF-AA for 16 to 18 h. The cells were cultured for 24 h and then cultured under selection in SATO medium supplemented with FGF-2, PDGF-AA, and 400 μg/ml G418 during 5 days. After selection, the cells were cultured in SATO with 0.5% FCS for 10 days.

**VSV infection.** VSV strain San Juan A was a kind gift from Peter Rottier (University of Utrecht, the Netherlands). OLGs were washed twice with serum-free medium (pH 6.8) before the virus was added. The virus was incubated with the cells for 1 h without $CO_2$. After this, medium was removed and replaced with fresh culture medium (pH 7.4) without serum and incubated at 5 to 7% $CO_2$ for 6 to 8 h.

**qPCR analysis.** Total RNA from cells was isolated using the InviTrap spin cell RNA minikit (Stratec, Berlin, Germany). Total RNA (1 μg) was reverse transcribed in the presence of oligo(dT)12-18 and deoxynucleoside triphosphate (dNTPs; Gibco, Paisley, United Kingdom) with superscript II reverse transcriptase (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. qPCR amplifications were performed on copy DNA using primers specific for rat MBP mRNA with exon II, rat MBP mRNA without exon II, and the hydroxymethylbilane synthase (HBMS) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) housekeeping genes, i.e., 5′-CACATGTACAAGGACTCACAC-3′ (forward exon II-containing MBP mRNA), 5′-GAAGAAGTGGACTACTGGGT-3′ (reverse exon II-containing MBP mRNA), 5′-ACTTGGCCACAGCAAGTACC-3′ (forward exon II-negative MBP mRNA), 5′-TGTGTGAGTCCTTGCCAGAG-3′ (reverse exon II-negative MBP mRNA), 5′-CCGAGCCAAGCACCAGGAT-3 (forward HMBS), 5′-CTCCTTCCAGGTGCCTCAGA-3′ (reverse HMBS), 5′-GACTTGCTCGAGATGTCA-3′ (forward HPRT1), and 5′-ACCACCCTGTTGCTGTAG-3′ (reverse HPRT1). The expression of rat MBP mRNA with and without exon II was measured by real-time qPCR on a StepOnePlus system (Applied Biosystems, Foster City, CA) with Absolute SYBR green ROX mix according to the following conditions: 15 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30s, followed by a melting curve stage. The melting curve stage was cycled first for 15 s at 95°C and then for 1 min at 60°C, after which the temperature was increased by 0.3°C each 15 s to a final temperature of 95°C, which was held for 15 s. The results were analyzed with StepOne software and normalized to the HBMS and HPRT1 housekeeping genes.

**Immunocytochemical analysis. (i) Monocultures.** For live staining of surface components, aspecific binding was blocked with 4% bovine serum albumin (BSA) in PBS for 10 min at 4°C, after which cells were incubated with A2B5 (antigangliosides; a kind gift from Thijs Lopes-Cardozo) for 30 min at 4°C, washed three times with ice-cold PBS, and incubated for 25 min at 4°C with the appropriate tetramethyl rhodamine isocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-conjugated antibodies (1:50; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were fixed with 4% paraformaldehyde (PFA). For (subsequent) staining of internal antigens, fixed cells were either permeabilized with ice-cold methanol (MBP, hnRNP A2) for 10 min or 0.1% Triton X-100 for 30 min (Ranscht monoclonal antibody [R-MAb], 2′3′-cyclic nucleotide phosphodiesterase [CNP], PLP, syntaxin 3, syntaxin 4, VAMP3, VSV G). After a 30-min block with 4% BSA, the cells were incubated for 60 min at room temperature (RT) with antigalactosylceramide (anti-GalC)-sulfatide (R-MAb; a kind gift from Guus Wolswijk, NIN, Amsterdam, the Netherlands) (44), anti-CNP (1:100, Sigma), anti-rat syntaxin 3 (1:500; Synaptic Systems, Göttingen, Germany), anti-rat syntaxin 4 (1:500; Synaptic Systems), anti-VAMP3 (1:500; Synaptic Systems), anti-hnRNP A2 (Novus Biologicals, Cambridge, United Kingdom), anti-MBP (1:100; rat monoclonal antibody; Serotec, Kinglington, United Kingdom), anti-PLP (4C2, 1:10; a kind gift of Vijay Kuchroo, Harvard Medical School, Boston, MA) (45), or anti-VSV G (1:100; Sigma). Next, the cells were washed with PBS and incubated for 30 min with the appropriate Alexa Fluor-, TRITC-, or FITC-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Nuclei were counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). For double staining, cells were sequentially stained with the different antibodies. Coverslips and slides were mounted in Dako mounting medium and analyzed with a conventional fluorescence microscope (Provis AX70; Olympus, New Hyde Park, NY) or with a confocal laser scan microscope (Leica SP8 AOBS CLSM or Zeiss LSM 780). For differentiation assays, the number of CNP, PLP, or MBP of total cells was determined.

**(ii) Cocultures.** Cocultures were fixed in 4% PFA and incubated at RT in 0.5% Triton X-100 in 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA) for 45 min. After being washed with PBS, cells were incubated for 2 h at RT with anti-MBP (1:250), anti-GFP (1:100 Molecular Probes), anti-PLP (1:50), and anti-NF-H antibodies (1:5,000; EnCor Biotechnology Inc., Gainesville, FL) diluted in 2% NGS. Staining was visualized by an incubation for 30 min at RT with appropriate Alexa Fluor-conjugated secondary antibodies diluted in 2% NGS. Coverslips were mounted in Dako mounting medium. All analyses were performed using a confocal laser scan microscope (Zeiss LSM 780). To determine the myelination potential for transduced cells, the ability of 50 GFP-positive cells to establish MBP- or PLP-positive internodes per coverslip was established with at least 3 coverslips per condition, and mock-transduced cells were set at 100%.

**(Co)immunoprecipitation.** Cells were washed and scraped in PBS and lysed on ice for 30 min in TNE-lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and a cocktail of protease inhibitors [Complete Mini; Roche]). For immunoprecipitation of integrins, surface proteins were first biotinylated as described previously (39). Protein concentrations were determined by a Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard. Equal amounts of protein (25 μg) were incubated with 20 μl A/G Plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) in TNE-lysis buffer with the anti-integrin α6 antibody (1:100; Millipore, Chemicon, Bedford, MA) overnight head over head at 4°C. Beads were washed four times with IP wash buffer (TNE-lysis buffer supplemented with 1% NP-40 and 350 mM NaCl), once with PBS, and resuspended in nonreducing SDS sample buffer. After 5 min at 95°C, proteins were separated by SDS-PAGE, followed by Western blotting and detection of (surface) integrin α6 by using infrared (IR) fluorescent dye (IRDye 680)-conjugated streptavidin (Li-Cor Biosciences, Lincoln, NE) and IR detection (see below).

Cells for protein-RNA coimmunoprecipitations were scraped in TNE-lysis buffer supplemented with RNase inhibitors (New England BioLabs, Ipswich, MA), followed by centrifugation for 15 min at 12,000 rpm. Equal amounts of protein (40 to 80 μg) were incubated overnight at 4°C with precleared A/G Plus agarose beads and 4 μg antibody (syntaxin 4, VAMP3). After being washed with IP wash buffer, samples were either resuspended in reducing sample buffer for Western blot analysis or received proteinase K treatment (30 μg proteinase K [Ambion, Life Technologies] in HNTM buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100]) at 50°C for 30 min, after which RNA was isolated with TRI reagent (Sigma) for qPCR analysis.

***In situ* hybridization.** OLGs were hybridized with 48 tetramethylrhodamine (TMR)-labeled 20-nucleotide-long probes designed against rat 14-kDa MBP, the major isoform present in rodent myelin, according to reference 46. Notably, individual probes will bind to other MBP isoforms; however, 30 to 40 probes should bind for visualization, and given that in OPCs little if any probe labeling is observed, visual hybridization with, e.g., golli-MBP can be excluded. Cells were fixed in 4% PFA and opened with ethanol, incubated overnight at 37°C with 1 ng/μl probe mix in 10% formamide-containing hybridization buffer, and washed with SSC (150 mM NaCl, 15 mM sodium citrate). If colabeling with antibodies was required, cells were subsequently blocked with BSA and incubated with primary and secondary antibodies as described above. Coverslips were mounted in Dako mounting medium and analyzed with a confocal laser scan microscope (Leica SP8 AOBS CLSM or Zeiss LSM 780).

**Western blot analysis.** Cells were harvested by being scraped in PBS and centrifuged for 7 min at 7,000 rpm, followed by lysis of the cell pellets

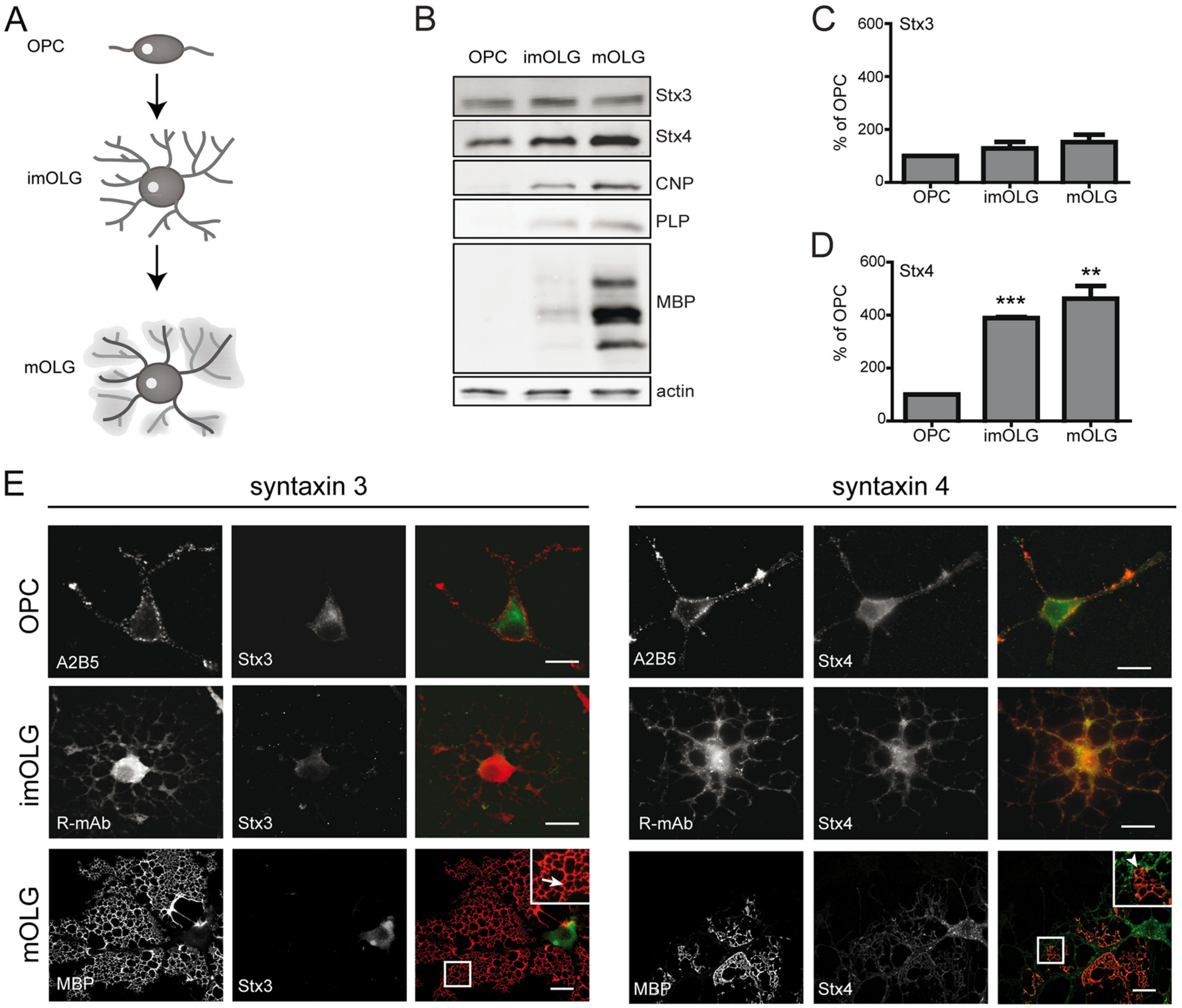


**FIG 1** Upregulation of syntaxin 4 during rat oligodendrocyte development. (A) Schematic overview of oligodendrocyte development and differentiation as reflected by changes in morphology for oligodendrocyte progenitor cells (OPCs), via immature oligodendrocytes (imOLGs) to mature and fully differentiated cells (mOLGs). Mature cultured OLGs form myelin-like membranes, i.e., sheets, the *in vitro* equivalent of (noncompact) myelin sheaths *in vivo*. (B to D) Cell lysates of OPCs, imOLGs, and mOLGs were analyzed for protein expression of syntaxin 3 (Stx3), syntaxin 4 (Stx4), CNP, PLP (2D2), and MBP. Actin served as a loading control. Quantification of protein levels of syntaxin 3 is shown in panel C and of syntaxin 4 in panel D. Expression, as a ratio of actin, was quantified relative to that of OPCs (set at 100%). Bars depict means plus SD. Data were obtained from four independent experiments. Statistical significance between OPC and the other developmental stages is shown (**, $P < 0.01$; ***, $P < 0.001$, one-sample *t* test). Note that with an increase of (im)mature OLG markers CNP, PLP, and MBP, the expression of syntaxin 4 but not syntaxin 3 is upregulated. (E) Localization of syntaxins 3 and 4 (green) in A2B5-positive OPCs, R-MAb-positive imOLGs, and MBP-positive mOLGs. Insets show higher-power magnifications. Note that in mOLGs, syntaxin 4 is more localized toward myelin sheets (inset, arrowhead), whereas syntaxin 3 is more retained to the cell body (inset, arrow). Scale bars are 10 μm.

in TNE-lysis buffer on ice. Equal protein amounts (20 μg) were mixed with SDS reducing sample buffer, heated for 5 min at 95°C or 30 min at 37°C (PLP), and subjected to SDS-PAGE and Western blotting as described previously (47). Primary antibodies used were anti-rat syntaxin 3 (1:1,000), anti-rat syntaxin 4 (1:1,000), anti-VAMP3 (1:3,000; Synaptic Systems), anti-MBP (1:100; rat monoclonal; Serotec, Kinglington, United Kingdom), anti-PLP (4C2 or 2D2; 1:100), anti-CNP (1:250), anti-integrin α6 (1:500), or antiactin antibody (1:1,000; mouse monoclonal; Sigma). The signals were detected using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) and analyzed using Odyssey V3.0 analysis software. The anti-PLP antibody 4C2 is directed against a nonconformational epitope in the first extracellular loop (PLP 50 to 69) and recognizes both PLP and its minor splice variant DM20, whereas anti-PLP antibody 2D2 is directed against an intramolecular region that is absent in DM20 (PLP 100 to 123). Only the band corresponding to PLP is used in the quantitative analysis.

**Statistical analysis.** Data are expressed as means ± standard deviations (SD) and were obtained from at least three independent experiments. Statistical analysis was performed using the one-sample *t* test relative to the control that was set to 100% in each independent experiment. When absolute values of more than two means were compared, statistical significance was calculated by a one-way analysis of variance (ANOVA),

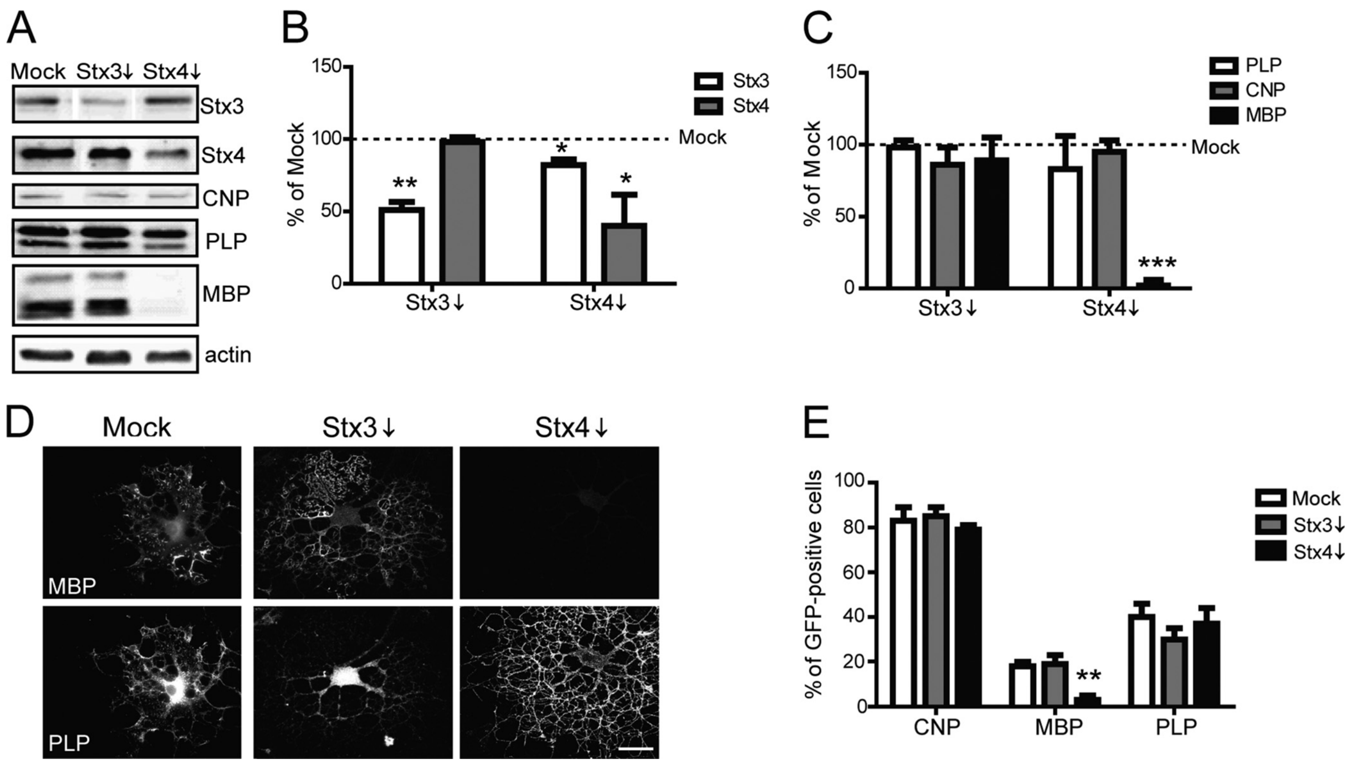


**FIG 2** Downregulation of syntaxin 4 but not syntaxin 3 decreases MBP protein levels in oligodendrocytes. Primary rat oligodendrocyte progenitor cells (OPCs) were transduced with lentiviruses that express vector-only (Mock) or shRNA against either syntaxin 3 (Stx3 ↓ ) or 4 (Stx4 ↓ ). (A to C) After 7 days, cell lysates were analyzed for protein levels of Stx3, Stx4, CNP, MBP, and PLP (4C2). Actin served as a loading control. Quantification of protein levels in panel A is shown in panel B (syntaxins) and panel C (myelin-specific proteins). Expression, as a ratio of actin, was quantified relative to that of mock (set at 100%, horizontal line). Bars depict means + SD. Data were obtained from three (B) and five (C) independent experiments. Statistical differences with mock-transduced cells are shown (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, one-sample *t* test). Note that syntaxin 4 but not syntaxin 3 downregulation decreases MBP protein expression. (D, E) After 7 days, cells were fixed and permeabilized, and double immunocytochemistry was performed for the indicated proteins. (D) Representative images from three independent experiments are shown. Scale bar is 10 μm. Note that syntaxin 3-downregulated cells generated MBP-positive sheets, whereas PLP is more retained in the cell body compared to that in mock-transduced cells. Syntaxin 4-silenced cells show little, if any, MBP staining, whereas PLP is present deep into the processes. The percentages of cells positive for CNP, MBP, and PLP of total GFP-positive cells, i.e., transduced cells, are shown in panel E. At least 500 cells per experiment were analyzed in three independent experiments. Bars depict means plus SD. Statistical differences with mock-transduced cells are indicated (**, $P < 0.01$, one-way ANOVA with Tukey's posttest). Note that in syntaxin 4-downregulated oligodendrocytes, MBP-positive cells were hardly observed.

followed by Tukey's posttest. In all cases, a *P* value of <0.05 was considered significant.

## RESULTS

**Syntaxins 3 and 4 differentially localize to cell body and myelin sheet, respectively, in oligodendrocytes.** Upon development, OLGs undergo a carefully defined process of maturation, during which different morphological features can be discerned. Thus, the cells differentiate from a bipolar progenitor cell (OPC) to a cell with more and branched primary processes (imOLG) and eventually to one with elaborate laminar sheets (mOLG) (Fig. 1A) (48, 49). Not only is this development accompanied by the biosynthesis of myelin-specific proteins, including 2′3′-cyclic nucleotide phosphodiesterase (CNP), MBP, and PLP (Fig. 1B), but also different surface membrane domains are generated and maintained. Thus, in addition to the cell body plasma membrane, constituting the main boundary domain during early development (Fig. 1A, OPC), an elaborate myelin-like membrane domain (sheet) is formed upon full differentiation (Fig. 1E, mOLG, arrow). Given previous observations that the plasma membrane and the myelin sheet are served by cognate apical and basolateral trafficking (2, 3), respectively, and since syntaxins 3 and 4 are known to be localized at and near distinct membrane surfaces in polarized cells (15), we wondered whether these syntaxins also distributed differently in OLGs and whether they could similarly play a prominent role in the sorting and trafficking events involved in the assembly of myelin membranes. We therefore first determined the protein expression pattern of both syntaxins as a function of OLG development. Total lysates obtained at different developmental stages of primary OLGs were analyzed by Western blotting. As shown in Fig. 1B and C, the expression level of syntaxin 3 remained remarkably constant during rat OLG development. Interestingly and in marked contrast, the expression of syntaxin 4 was substantially upregulated during developmental progression (Fig. 1B and D) and, relative to its expression in OPCs, increased more than 4-fold under conditions of avid myelin membrane biogenesis (mOLGs). From these data, it is tempting to suggest that syntaxin 4 might be particularly involved in myelin sheet-directed transport, whereas syntaxin 3 would largely act in docking and fusion of transport vesicles directed toward the cell body plasma membrane. To investigate this possibility, the localization of syntaxins 3 and 4 at the

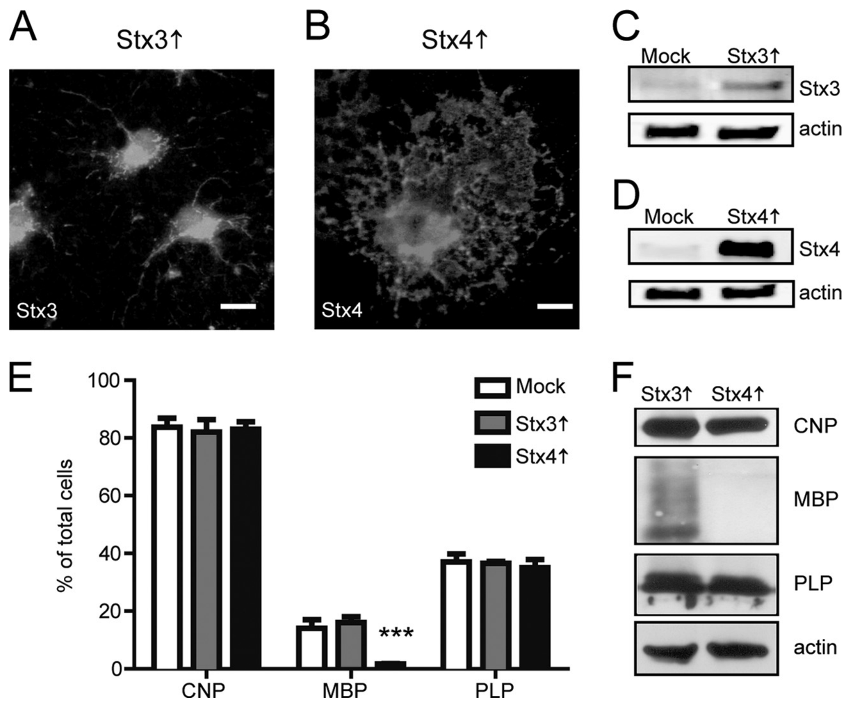


**FIG 3** Overexpression of syntaxin 4 but not syntaxin 3 decreases MBP levels in oligodendrocytes. Primary rat oligodendrocyte progenitor cells (OPCs) were transduced with retroviruses that express vector-only (Mock), syntaxin 3 (Stx3 ↑ ), or syntaxin 4 (Stx4 ↑ ) and, after selection, analyzed 10 days after initiating differentiation. (A) Localization of syntaxin 3 in syntaxin 3-overexpressing mature oligodendrocytes (OLGs). Note that upon its overexpression, syntaxin 3, as endogenous syntaxin 3 (Fig. 1E), does not localize to myelin sheets. (B) Localization of syntaxin 4 in syntaxin 4-overexpressing mature OLGs. Note that in overexpressing cells, syntaxin 4 localized to myelin-like membranes, similarly as observed for the distribution of endogenous syntaxin 4 (Fig. 1E). Scale bars are 10 μm. Representative pictures from three independent experiments are shown in panels A and B. (C) Western blots of syntaxin 3 expression in mock (vector-only)-transduced and syntaxin 3-overexpressing OLGs. Actin served as a loading control. (D) Western blots of syntaxin 4 expression in mock (vector-only)-transduced and syntaxin 4-overexpressing OLGs. Actin served as a loading control. (E) Cells were fixed and permeabilized to perform immunocytochemistry for CNP, MBP, and PLP. The percentages of cells positive for CNP, MBP, and PLP in the overexpressing cells were determined. At least 500 cells per experiment were analyzed in three independent experiments. Bars depict means plus SD. Statistical differences with mock-transduced cells are indicated (***, $P < 0.001$, one-way ANOVA with Tukey's posttest). Note that in syntaxin 4-overexpressing OLGs, MBP-positive cells were hardly observed. (F) Cell homogenates were analyzed for protein levels of CNP, MBP, and PLP (2D2). Actin served as a loading control. Note the virtual absence of MBP expression in syntaxin 4-overexpressing OLGs.

different developmental stages was visualized by immunofluorescence, using antibodies directed against gangliosides (A2B5), the galactosphingolipids GalC and sulfatide (R-MAb), and MBP as markers for OPCs and immature and mature OLGs. In all developmental stages, syntaxin 3 largely localizes to the perinuclear region along with a more punctuate distribution throughout the cytoplasm of the cell body, presumably reflecting its association with vesicular structures (Fig. 1E, left). In fully differentiated MBP-positive cells (mOLGs), no significant localization of syntaxin 3 at the myelin sheet could be observed (Fig. 1E, left, arrow), and the protein localized mainly to the cell body and, occasionally, at primary processes. In A2B5-positive OPCs, syntaxin 4 was located in intracellular vesicles and at the plasma membrane of the cell body. At later stages of development, syntaxin 4 was directed toward the membrane of the processes and, in marked contrast to the localization of syntaxin 3, abundantly localized within the myelin sheet, displaying a more prominent membrane association than seen for syntaxin 3 (Fig. 1E, right, arrowhead). Thus, these data reflect a preferential polarized distribution of the syntaxins in cultured rat OLGs, syntaxin 3 localizing primarily to the cell body, whereas syntaxin 4 shows a preferential association toward the myelin sheet. This apparent polarized distribution could be indicative of potential distinct roles of these syntaxins in myelin biogenesis. Given the upregulation of syntaxin 4 during development (Fig. 1B and D), and its localization at the myelin membrane (Fig. 1E, right), we investigated in particular the role of this syntaxin in the biogenesis of myelin membranes, focusing on myelin-directed transport of the major proteins MBP and PLP.

**Downregulation and overexpression of syntaxin 4 but not syntaxin 3 preclude the expression of MBP.** To assess a functional role for either syntaxin in myelin biogenesis, we downregulated their expression by transducing OPCs using a lentiviral construct that concomitantly expresses shRNA directed against syntaxin 3 or 4 and GFP. Western blot analysis of total cell lysates of mature OLGs revealed that by this approach, which resulted in a transduction efficiency of 60 to 80%, the levels of syntaxins 3 and 4 were reduced by approximately 50 and 60%, respectively (Fig. 2A and B). In addition, upon downregulation of syntaxin 4, it was noted that the levels of syntaxin 3 expression were consistently reduced by approximately 20%. Upon downregulation of either syntaxin 3 or syntaxin 4, the levels of expression of the myelin-specific proteins CNP and PLP in mature OLGs were virtually unaffected (Fig. 2A and C). Intriguingly, downregulation of syntaxin 4 but not syntaxin 3 caused a virtual abolishment of MBP expression (Fig. 2A and C). To verify whether downregulation of either syntaxin affected the intracellular distribution of the inves-

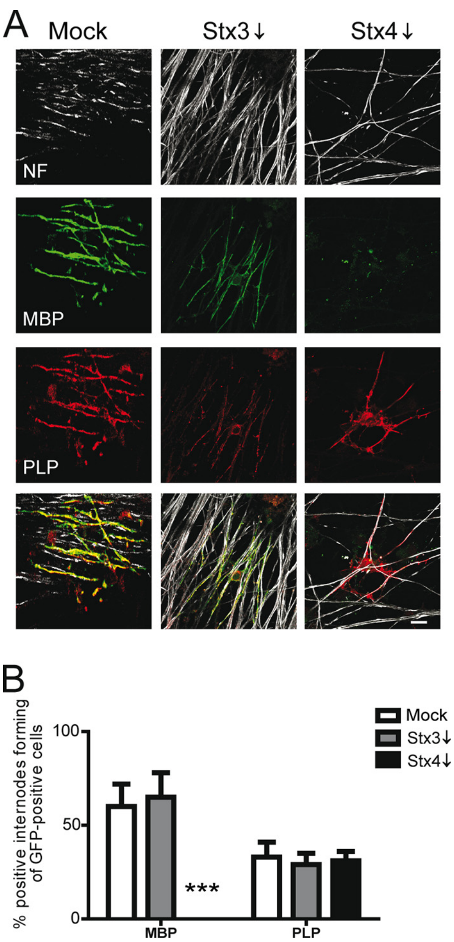


**FIG 4** Downregulation of syntaxin 4 but not syntaxin 3 decreases MBP-positive internodes in oligodendrocyte-DRG neuron cocultures. (A, B) Transduced OPCs were cocultured with dorsal root ganglion neurons (DRGNs) for 14 days, after which the cultures were subjected to triple immunocytochemistry for neurofilament-H (NF; white), PLP (red), and MBP (green). (A) Representative images from three independent experiments are shown. Scale bar is 10 μm. The percentages of GFP-positive cells that produce PLP- and MBP-positive myelin segments are shown in panel B. Bars depict means plus SD. Data were obtained from three independent experiments. Statistical differences with mock-transduced cells are indicated (***, $P < 0.001$, one-way ANOVA with Tukey's posttest). Note that also in oligodendrocyte-DRGN cocultures, few if any MBP-positive myelin segments are observed upon syntaxin 4 but not syntaxin 3 downregulation.

tigated proteins, transduced (i.e., GFP-positive) cells were examined for CNP, MBP, and PLP expression and localization. Consistent with the Western blot analysis, MBP expression was virtually absent in syntaxin 4-downregulated cells (Fig. 2D, top), whereas in syntaxin 3-downregulated cells the distribution of MBP was very similar to that observed in mock-transduced cells. Syntaxin 4 downregulation did not significantly affect the number of CNP- and PLP-expressing cells (Fig. 2E) compared to that of mock (vector-only)-transduced cells (Fig. 2D and E). However, at these conditions, PLP, although still residing in vesicular structures, was more localized to the processes and myelin sheets, likely due to the lack of MBP expression (7). In marked contrast, upon downregulation of syntaxin 3, PLP appears largely retained in the cell body (Fig. 2D). However, syntaxin 3 downregulation did not affect the number of cells that express CNP, MBP, or PLP in the transduced OLGs (Fig. 2E).

To obtain further support for the remarkable observation of a syntaxin 4-dependent modulation of MBP expression, the effect of overexpression of syntaxin 4 was examined as well. As previously reported, overexpression of syntaxin leads to assembly of nonfunctional SNARE complexes, thereby mimicking dominant-negative features (13, 50). Thus, syntaxins 3 and 4 were overexpressed in OPCs using a retroviral expression system, followed by selection of the transduced cells. As shown in Fig. 3A, the localization of overexpressed syntaxins 3 and 4 resembled that of the respective endogenous syntaxins (Fig. 1E), syntaxin 3 localizing largely to the cell body, whereas syntaxin 4 was prominently present in the processes and in the sheets. In all cases, the morphology of the cells and/or the appearance of myelin-like membranes were indistinguishable from those of mock-transduced cells. At a roughly 7-fold overexpression of syntaxins 3 and 4 (Fig. 3C and D, respectively), no differences were observed between the overexpressed and mock-transduced cells in terms of the number of cells that expressed CNP and PLP (Fig. 3E). Also, irrespective of the overexpressed syntaxin species, the expression levels of CNP and PLP were indistinguishable (Fig. 3F). However, as observed upon syntaxin downregulation, overexpression of syntaxin 4 but not syntaxin 3 resulted in the virtual absence of MBP expression (Fig. 3F). Similarly, upon visual examination, the number of MBP-positive cells was dramatically reduced, if present at all, upon syntaxin 4 overexpression (Fig. 3E). Hence, both downregulation and overexpression of syntaxin 4 but not syntaxin 3 precluded MBP protein synthesis but not that of the myelin-specific proteins PLP or CNP.

**Downregulation of syntaxin 4 prevents the expression of MBP but not PLP at internodes in myelinating cocultures.** To better appreciate the relevance of these observations, which were recorded in enriched OLG monocultures, we verified these data in mixed myelinating OLG-dorsal root ganglion neuron (DRGN) cultures. Moreover, in this manner, some insight could be obtained into the functional consequences of myelination in the absence of MBP. Thus, syntaxins 3 and 4 were downregulated in OPCs prior to their seeding onto DRGNs. Immunocytochemical analysis of 14-day-old cocultures showed that also in the presence of neurons, MBP, but not PLP, is virtually absent from myelin segments in syntaxin 4-downregulated cells (Fig. 4A and B). Thus, neuron-derived signals were not able to overcome syntaxin 4-mediated downregulation of MBP mRNA transcription. Furthermore, the typical morphology of the myelinated membranes was seriously compromised in syntaxin 4-downregulated cells. In syntaxin 3-downregulated cultures, cells with MBP-positive internodes were readily observed and, in terms of cell number, were indistinguishable from those seen in mock-transduced cells (Fig. 4A and B). In this case, the intensity of PLP in the myelin segments was clearly reduced, along with prominent expression of PLP in the cell body, although the cellular morphology and ability to form myelin membranes were largely retained.

**Downregulation of syntaxin 4 does not affect vesicular delivery of several myelin sheet-directed proteins.** The data presented thus far suggest that downregulation of syntaxin 4 interferes with MBP expression without affecting expression and/or transport of relevant myelin-specific proteins like PLP and CNP, as investigated in this work. To determine whether vesicular transport of other myelin sheet-directed proteins was

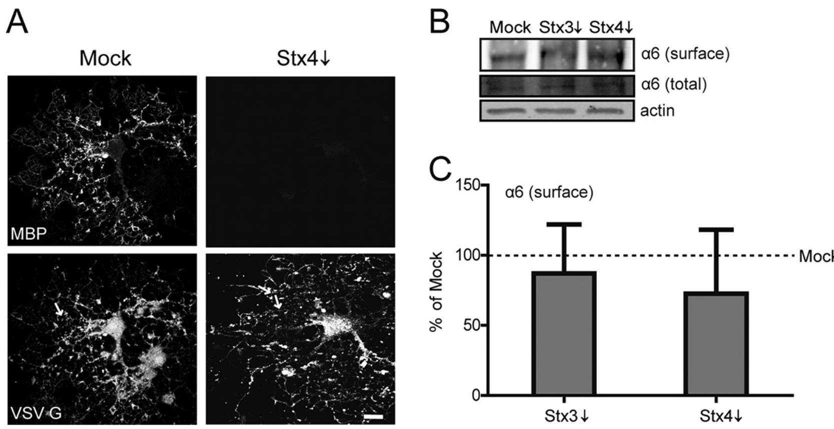


**FIG 5** Downregulation of syntaxin 4 does not affect vesicular delivery of myelin sheet-directed proteins. (A) Primary rat oligodendrocyte progenitor cells (OPCs) were transduced with lentiviruses that express vector only (Mock) or shRNA against syntaxin 4 (Stx4 ↓ ). Cells were infected with VSV after 7 days and labeled for VSV G after 6 h. Note that vesicular transport is not impeded upon syntaxin 4 downregulation, as VSV G still localizes to the myelin sheet (arrow). The scale bar is 10 μm. (B, C) OPCs were transduced with lentiviruses that express vector only (Mock) or shRNA against syntaxin 3 (Stx3 ↓ ) or 4 (Stx4 ↓ ). After 7 days, cells were surface biotinylated, followed by immunoprecipitation for integrin α6. Integrin α6 and actin in total cell lysates served as input controls. Integrin surface expression was quantified relative to that of mock-transduced cells in panel C (set at 100%, horizontal line; $P > 0.05$, nonsignificant, one-sample *t* test). Bars depict means plus SD. Data were obtained from three independent experiments.

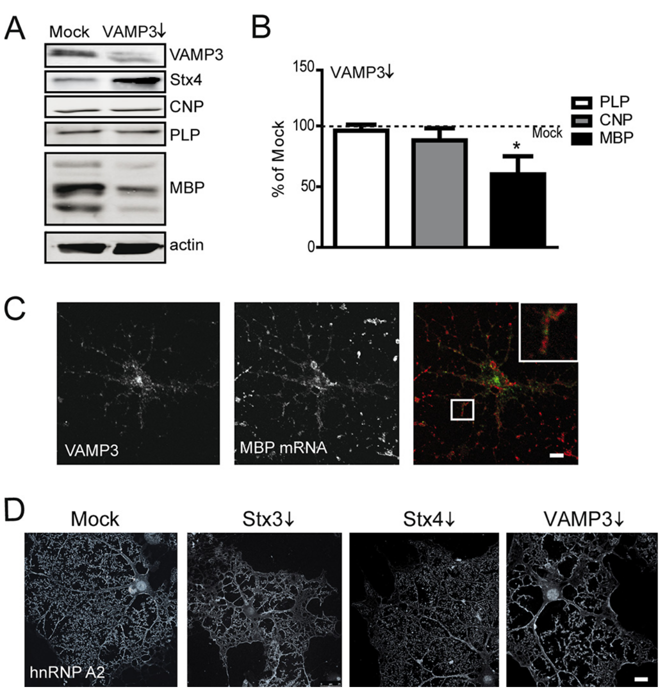


**FIG 6** The SNARE machinery is not involved in MBP mRNA granule transport. (A, B) Primary rat oligodendrocyte progenitor cells (OPCs) were transduced with lentiviruses that express vector only (Mock) or shRNA against VAMP3 (VAMP3 ↓ ) and analyzed 7 days after transduction for protein levels of VAMP3, CNP, PLP (2D2), and MBP. Actin served as a loading control. Quantification of protein levels from panel A is shown in panel B. Expression, as a ratio of actin, was quantified relative to that of mock (set at 100%, horizontal line). Bars depict means plus SD. Data were obtained from three independent experiments. Statistical differences with mock-transduced cells are indicated (*, $P < 0.05$, one-sample *t* test). Note that VAMP3 downregulation decreases MBP protein expression. (C) Nontransduced mature oligodendrocytes were double labeled for VAMP3 and MBP mRNA. Note that VAMP3 hardly if at all colocalizes with MBP mRNA. (D) OPCs were transduced with lentiviruses that express vector only (Mock) or shRNA against VAMP3 (VAMP3 ↓ ), syntaxin 3 (Stx3 ↓ ), or 4 (Stx4 ↓ ) and labeled 7 days after transduction for hnRNP A2, a prominent constituent of mRNA granules. Scale bars are 10 μm.

perturbed, we next analyzed the localization of VSV G, a viral model protein for myelin sheet-directed traffic (2, 3, 51, 52). Also, the transport of VSV G was seemingly unaffected upon downregulation of syntaxin 4, as VSV G is transported and localized to myelin-like membranes in syntaxin 4-downregulated cells, i.e., in the absence of MBP, similar to mock-transduced cells (Fig. 5A, arrow). To verify therefore whether transport of compounds specifically relevant to the regulation of MBP expression might be perturbed, we investigated if trafficking of integrin α6, which localizes to the surface of myelin membranes in mature OLGs (53) and is involved in MBP expression (54), relies on syntaxin 4. Thus, by means of surface biotinylation and immunoprecipitation, we quantified the pools of integrin α6 present on the surface of OLGs in mock-transduced and syntaxin 4-downregulated cells. The data as presented in Fig. 5B and C demonstrate that irrespective of the presence of syntaxin 4, the level of surface association of integrin α6 remained unaltered, and its total expression is similar at all conditions (Fig. 5B). Moreover, these data also emphasize, as revealed by quantitation of the integrin α6 surface pool and localization of VSV G, that effective downregulation of syntaxin 4 has little if any effect on the vesicular delivery of these proteins to the myelin sheets.

To obtain further insight into the underlying mechanism of the syntaxin 4-dependent failure of MBP expression, we took into account the possibility that MBP mRNA granule transport (26, 55) relies on the SNARE machinery and that sheet-localized syntaxin 4 fulfills a crucial role in proper granule docking and concomitant localized MBP expression.

**The SNARE machinery is not involved in mRNA granule transport.** Interacting binding partners of syntaxin 4 in vesicular transport are v-SNAREs, known as VAMPs. In OLGs, the t-SNARE syntaxin 4 is recognized by VAMP3 (18, 32). To assess, therefore, whether VAMP3 might be involved in facilitating the expression of MBP, we next examined the effect of VAMP3 downregulation on MBP expression. As shown in Fig. 6A and B, an approximately 60% downregulation of VAMP3 resulted in a

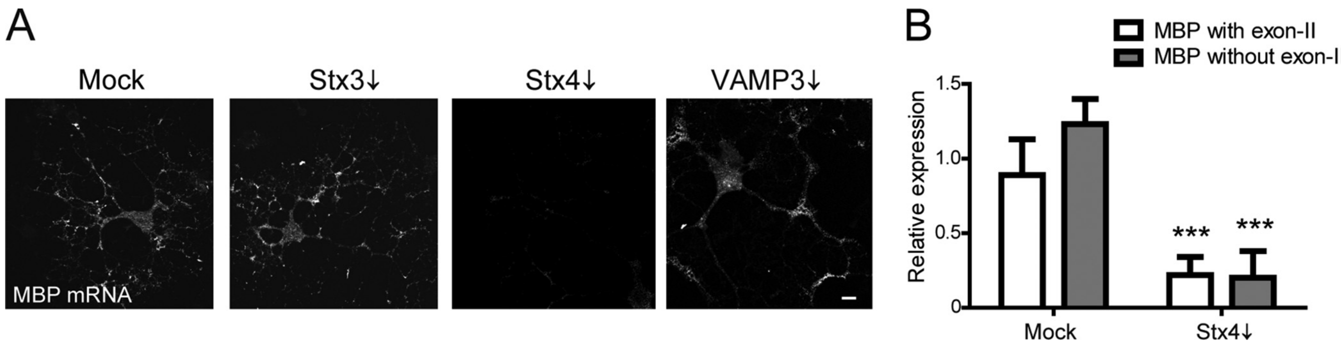


**FIG 7** Downregulation of syntaxin 4 but not syntaxin 3 decreases MBP mRNA levels in oligodendrocytes. (A) Primary rat oligodendrocyte progenitor cells (OPCs) were transduced with lentiviruses that express vector only (Mock) or shRNA against VAMP3 (VAMP3 ↓ ), syntaxin 3 (Stx3 ↓ ), or syntaxin 4 (Stx4 ↓ ) and analyzed 7 days after transduction cells were labeled for MBP mRNA. Scale bar is 10 μm. Note that MBP mRNA is virtually absent upon syntaxin 4 but not VAMP3 downregulation. (B) OPCs were transduced with lentiviruses that express vector only (Mock) or shRNA against syntaxin 4 (Stx4 ↓ ), selected, and 7 days after differentiation subjected to real-time qPCR analysis using specific primers for mRNA for MBP isoforms with and without exon II. mRNA expression was normalized to the HMBS and HPRT1 housekeeping genes. Bars depict means plus SD. Data were obtained from three independent experiments. Statistical differences with mock-transduced cells are shown (***, $P < 0.001$, one-sample *t* test). Note that downregulation of syntaxin 4 results in significantly lower levels of both exon II-positive and -negative mRNA for MBP isoforms.

reduction in MBP expression levels of approximately 30%, compared to those obtained in mock-transduced cells. A slight increase in the expression of syntaxin 4 is observed upon downregulation of VAMP3, which may add to the reduction of MBP expression. The expression levels of CNP and PLP were unaffected. These findings thus suggest that the SNARE machinery could be at least partly involved in regulating MBP expression. However, whether this reduced expression is related to a defect in mRNA transport and/or docking is not apparent from these data. We therefore determined the extent to which VAMP3 and MBP mRNA were colocalizing in the cells, as examined by fluorescence colocalization. To visualize MBP mRNA, 48 short fluorescent probes were employed that specifically bind to the 14-kDa MBP, the major MBP isoform present in rodent myelin (22, 24), and which allows for single MBP mRNA detection (56). As shown in Fig. 6C, VAMP3, although clearly visible as punctate green dots, presumably representing VAMP3-labeled transport vesicles, does not significantly colocalize with MBP mRNA (red). To obtain further support for this notion, coimmunoprecipitation studies were carried out, which revealed no association between MBP mRNA and VAMP3 (data not shown). Finally, we visualized the fate of mRNA granules as such in OLGs by immunocytochemical analysis, employing antibodies against hnRNP A2, a prominent constituent of mRNA granules (57–59). As shown in Fig. 6D, hnRNP A2 was localized deeply into the processes of both mock-transduced cells and syntaxin 4-, syntaxin 3-, and VAMP3-downregulated cells. Also, irrespective of SNARE downregulation, the cellular distribution of granules, as reflected by the hnRNP A2 marker, is indistinguishable from their fate in mock-transduced cells. These data thus suggest that hnRNP A2-containing granules are seemingly properly assembled in the downregulated cells and can be subsequently transported into the processes of OLGs, even in cells with little if any syntaxin 4. Together, these data indicate that the lack of MBP expression is not related to defects in granule assembly or transport *per se*. Rather, the findings obtained so far strongly favor the hypothesis that downregulation of the syntaxin 4 machinery leads to a transcriptional suppression of MBP mRNA, as occurs most efficiently by downregulation of the t-SNARE itself and less so by that of the VAMP3 v-SNARE. To obtain direct support for this hypothesis, the level and localization of MBP mRNA expression were investigated in VAMP3-, syntaxin 3-, and syntaxin 4-downregulated cells.

**The syntaxin 4 machinery regulates MBP mRNA expression.** To examine the presence and distribution of MBP mRNA in syntaxin 3 and syntaxin 4 as well as in VAMP3-downregulated OLGs, RNA *in situ* hybridization experiments were carried out. As shown in Fig. 7A, in mock-transduced cells, MBP mRNA is present in the cell body and penetrates deeply into the primary and secondary processes. Interestingly, upon downregulation of syntaxin 4 but not syntaxin 3, little if any MBP mRNA signal can be detected. In VAMP3-downregulated cells, a seemingly reduced level of MBP mRNA transcripts were detected only in the processes and cell body (Fig. 7A), consistent with a somewhat diminished but not completely abolished level of MBP expression in these cells (Fig. 6A and B). Additional support for a lack of MBP mRNA transcription upon syntaxin 4 downregulation was obtained by real-time qPCR analysis (Fig. 7B). Specifically, transcriptional regulation of MBP mRNA upon downregulation of syntaxin 4 was apparent for transcripts of exon II mRNA-negative MBP isoforms, which are transported to and expressed at the myelin membrane, and for transcripts of exon II mRNA-containing MBP isoforms, which reside mainly in the cytoplasm and nucleus (60, 61). Accordingly, these data indicate that the syntaxin 4-mediated transcriptional suppression of MBP mRNA is a general effect on MBP mRNA transcription and does not appear to be restricted to MBP isoforms, expressed exclusively in the myelin sheet.

If a syntaxin 4-dependent mechanism is required for allowing transcriptional expression of MBP to proceed, we would predict that downregulation of syntaxin 4 at a later developmental state, i.e., after the initiation of MBP transcription, should not affect MBP (protein) expression. To examine this, immature OLGs were lentivirally transduced with shRNA against syntaxin 4. Indeed, downregulation of syntaxin 4 from the imOLG stage onward does not affect the level of MBP (protein) expression, compared to the level obtained in mock-transduced cells (Fig. 8A and B). Also, the localizations of MBP protein and MBP mRNA in mock-transduced and syntaxin 4-downregulated cells appear to be indistinguishable, i.e., both MBP protein and mRNA are present and localize deeply into the processes

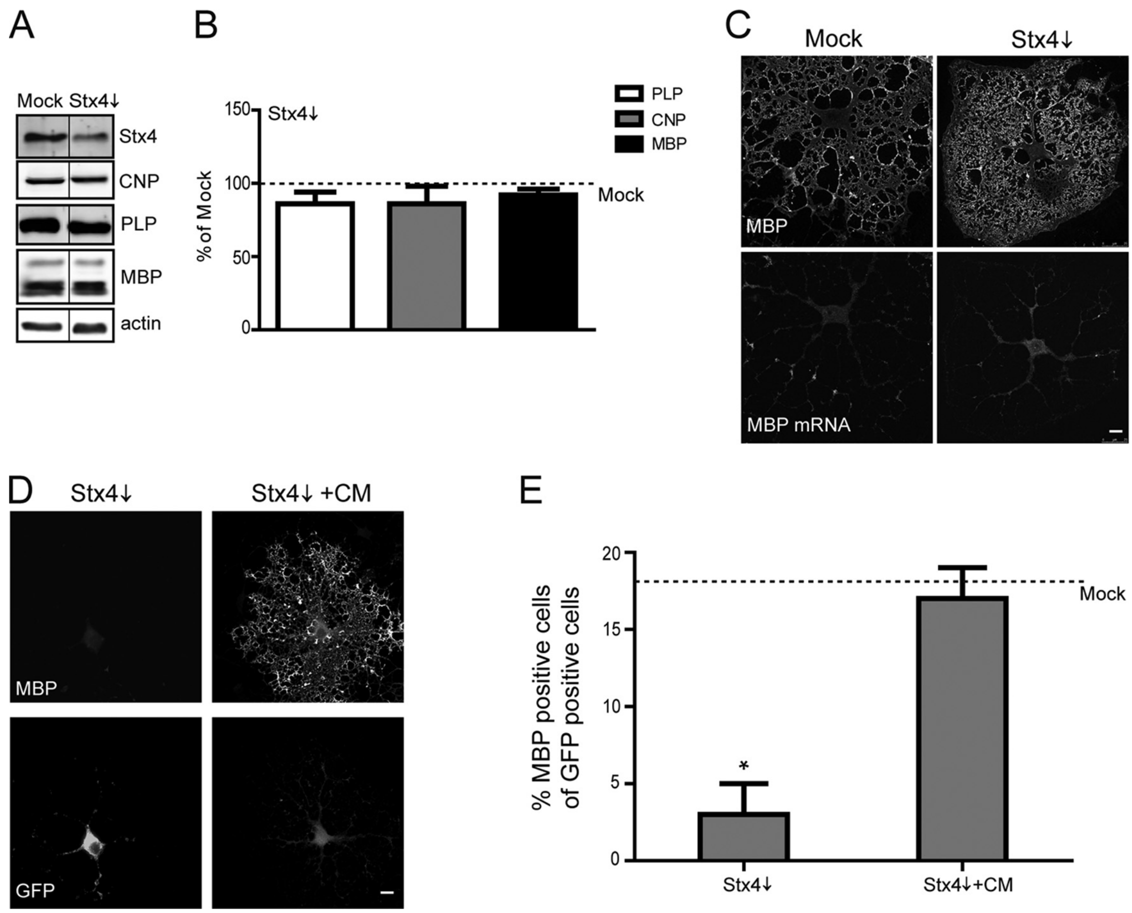


**FIG 8** Conditioned medium of developing OPCs reestablished MBP expression in syntaxin 4-downregulated cells. (A, B) Primary immature rat oligodendrocytes (imOLGs) were transduced with lentiviruses that express vector only (Mock) or shRNA against syntaxin 4 (Stx4 ↓). After 7 days, cell lysates were analyzed for protein levels of Stx4, CNP, MBP, and PLP (2D2). Actin served as a loading control. The cell lysates of syntaxin 4-downregulated and mock-transduced cells are on the same blot. Quantification of protein levels from panel A is shown in panel B. Expression, as a ratio of actin, was quantified relative to that of the control (set at 100%, horizontal line). Bars depict means plus SD. Data were obtained from at least three independent experiments. Statistical differences with mock-transduced cells are shown (*, $P < 0.05$, one-sample *t* test). Note that syntaxin 4 downregulation from imOLGs onward hardly affects MBP protein expression. (C) imOLGs were transduced as described for panels A and B. After 7 days, cells were labeled for MBP protein and MBP mRNA. Representative images from three independent experiments are shown. Scale bar is 10 μm. Note that the appearance of MBP protein and mRNA in syntaxin 4-downregulated cells was indistinguishable from that of mock-transduced cells. (D, E) Primary rat oligodendrocyte progenitor cells (OPCs) were transduced with lentiviruses that express vector only (Mock) or syntaxin 4 shRNA (Stx4 ↓). Upon differentiation, cells were treated with control medium (Mock and Stx4 ↓) or conditioned medium of developing OPCs (Stx4 ↓ +CM). After 7 days, cells were labeled for MBP protein. (D) Representative images from three independent experiments are shown. Scale bar is 10 μm. The percentages of cells positive for MBP of total GFP-positive cells, i.e., transduced cells, are shown in panel E. Bars depict means plus SD. Data were obtained from three independent experiments. Statistical differences with mock-transduced cells are indicated (*, $P < 0.05$, one-way ANOVA with Tukey's posttest). Note that conditioned medium of developing OPCs established MBP expression in syntaxin 4-downregulated cells.

(Fig. 8C). Thus, a syntaxin 4-dependent mechanism is essential for the onset of MBP mRNA transcription. Remarkably, careful examination of the transduced cultures (60 to 80% efficiency) revealed that MBP was reduced in all cells, i.e., in both transduced and nontransduced cells (Fig. 2A). This observation could thus point toward a potential role of a medium-derived factor(s), the presence of which depends on functional active syntaxin 4, that drives MBP mRNA transcription. To obtain experimental support for this possibility, syntaxin 4-downregulated OPCs were differentiated in the presence of conditioned medium derived from developing OLGs, i.e., differentiated from OPCs to immature OLGs, and analyzed after 7 days by immunocytochemistry for MBP expression. Strikingly, in syntaxin 4-downregulated (i.e., GFP-positive cells) treated with conditioned medium, MBP protein expression was reestablished up to the level of mock-transduced cells (Fig. 8D and E). Thus, these findings would support the notion that the initiation of the biosynthesis of MBP mRNA relies on a syntaxin 4-dependent mechanism, which likely involves activation of an autocrine signaling pathway.

## DISCUSSION

SNARE proteins, including syntaxins, are intimately involved in docking and fusion of vesicles, mediating intracellular protein transport, and their ubiquitous presence and distinct identities warrant specificity in vesicle-mediated delivery (8–10). The present study demonstrates that the t-SNAREs syntaxin 3 and 4 are distributed in a polarized fashion in OLGs, in agreement with similar observations reported for more common polarized cells, like epithelial cells (12–17). Moreover, the localization of syntaxin 3 in the cell body and near the plasma membrane on the one hand and that of syntaxin 4 in the myelin sheet on the other is consistent with the apical-surface- and basolateral-surface-like nature, respectively, of these regions in rat OLGs, as previously established (1–3, 21). Interestingly, our data also reveal that functional expression of syntaxin 4, and to a lesser extent that of its binding partner, VAMP3, appears crucial for MBP expression at the transcriptional level but not for MBP mRNA trafficking to the myelin sheet. In fact, our data suggest a role of a syntaxin 4-dependent, autocrine signaling mechanism that regulates MBP mRNA transcription.

Given its role in membrane docking at (basolateral) target domains and its primary localization toward the myelin sheets, we would predict that syntaxin 4 is involved in polarized transport of myelin sheet-directed proteins. Remarkably, its downregulation effectively repressed MBP mRNA transcription. Although a causal relationship between the vesicular transport machinery and mRNA transport and localization has been described (29, 30, 62), our data indicated that neither granule assembly nor its cellular distribution was affected by syntaxin 4 downregulation. Alternatively, a likely explanation for our findings is failure of syntaxin 4-mediated delivery of a molecular factor(s) regulating MBP mRNA transcription. However, quite unexpectedly, effective downregulation of syntaxin 4 did not result in significant downregulation of vesicular transport of the myelin-specific protein PLP, the viral model protein VSV G, or integrin α6, known to regulate MBP mRNA expression (54). Yet, we cannot exclude that in this particular case, sheet-directed transport, mediated by the SNARE machinery, might have exploited alternatives for syntaxin 4, e.g., syntaxin 2, which is present in mature OLGs (18, 19), in a nonpolarized manner (our unpublished observations) or for the VAMP3 v-SNARE, e.g., VAMP2 (18, 19) or VAMP7, which has been implicated in PLP trafficking (32). If so, it should also be emphasized that "t-SNARE substitution" does not apply to the apical machinery, driven by syntaxin 3, as in this case its downregulation did effectively preclude PLP trafficking to the plasma membrane of the cell body, which precedes subsequent transport to the myelin sheet (1, 34, 36, 63–65). Thus, the specificity of the effect of syntaxin 4 is emphasized by similar observations of a reduction of MBP expression upon downregulation of its v-SNARE, VAMP3, the absence of an effect of syntaxin 3, and the apparent inability to potentially maintain MBP mRNA transcription via a syntaxin 2-mediated pathway.

A direct interaction of the syntaxin 4/VAMP3 SNARE machinery with MBP mRNA-containing granules in OLGs could be excluded. The present observations could not be explained by degradation of MBP mRNA, or, in conjunction with that, of MBP. Indeed, downregulation of syntaxin 4 from the immature OLG stage onward does not lower the amount of MBP, nor were changes in MBP protein levels apparent in the presence of the proteasomal inhibitor ALNN (unpublished observations). In fact, the location of syntaxin 4 near the myelin sheet would *a priori* exclude its direct role as a transcriptional activator. In this context, transcription factors such as myelin gene regulatory factor (MRF), required for expression of myelin genes and CNS myelination, are likely also not the target of syntaxin 4, as they usually facilitate the expression of a group of myelination-related genes and do not specifically induce MBP expression as such (66). Furthermore, MRF is also required for the maintenance of mature OLGs and myelin (67), whereas proper functioning of syntaxin 4 is required at the onset of MBP mRNA transcription, its downregulation from the immature OLG stage being without effect on MBP expression. More likely, the absence of MBP as a result of syntaxin 4 downregulation might reflect the inability of OPCs to secrete a factor(s), which is apparently necessary to initiate MBP mRNA transcription, i.e., based upon an autocrine signaling mechanism. Indeed, conditioned medium of developing OLGs, but not neuron-derived signals (Fig. 4), restored MBP protein expression in syntaxin 4-downregulated cells. Given that MBP is essential for the formation of functional myelin (22, 68, 69), it will be important to clarify the identity of this medium constituent(s) and its underlying mechanism in MBP mRNA transcription during development in order to address early (re)myelination defects.

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