Down-regulation of Polysialic Acid Is Required for Efficient Myelin Formation

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Oligodendrocyte precursor cells modify the neural cell adhesion molecule (NCAM) by the attachment of polysialic acid (PSA). Upon further differentiation into mature myelinating oligodendrocytes, however, oligodendrocyte precursor cells down-regulate PSA synthesis. In order to address the question of whether this down-regulation is a necessary prerequisite for the myelination process, transgenic mice expressing the polysialyltransferase ST8SiaIV under the control of the proteloid protein promoter were generated. In these mice, postnatal down-regulation of PSA in oligodendrocytes was abolished. Most NCAM-120, the characteristic NCAM isoform in oligodendrocytes, carried PSA in the transgenic mice at all stages of postnatal development. Polysialylated NCAM-120 partially co-localized with myelin basic protein and was present in purified myelin. The permanent expression of PSA-NCAM in oligodendrocytes led to a reduced myelin content in the forebrain of transgenic mice during the period of active myelination and in the adult animal. In situ hybridizations indicated a significant decrease in the number of mature oligodendrocytes in the forebrain. Thus, down-regulation of PSA during oligodendrocyte differentiation is a prerequisite for efficient myelination by mature oligodendrocytes. Furthermore, myelin of transgenic mice exhibited structural abnormalities like redundant myelin and axonal degeneration, indicating that the down-regulation of PSA is also necessary for myelin maintenance.

Polysialic acid (PSA),3 a linear homopolymer of N-acetylneuraminic acid, is a posttranslational modification of the neural cell adhesion molecule (NCAM) (1). NCAM occurs in various isoforms that are generated by alternative splicing (2). The three major isoforms of NCAM are the two transmembrane proteins NCAM-180 and NCAM-140 and the glycosylphosphatidylinositol-linked NCAM-120. The latter is the predominant isoform in oligodendrocytes (3). PSA is synthesized in the Golgi apparatus by two sialyltransfereases, ST8SiaIV (4, 5) and ST8SialI (6, 7). Although in the mammalian brain, polysialylated NCAM (PSA-NCAM) is mainly found in neurons, other cell types within the central nervous system synthesize PSA-NCAM as well. Migrating oligodendrocyte precursor cells (OPC) express PSA, which is, however, down-regulated after these cells reach their final destination and start to differentiate into mature myelinating oligodendrocytes (8–10). The presence of PSA seems to be functionally important, since migration of OPC is impaired after endo- neuraminidase treatment, which specifically hydrolyzes PSA (11). More recent data suggest that PSA is not required for cellular motility in general but for directional movement of OPC in response to platelet-derived growth factor (PDGF) (12). In addition to its effect on migration, PSA seems also to inhibit differentiation of OPC into mature myelinating oligodendrocytes, because removal of PSA by endo-neuraminidases accelerates oligodendrocyte differentiation (13).

PSA is also down-regulated in neurons at the time when their axons become myelinated. Axons that remain nonmyelinated in the adult (e.g. mossy fibers in the hippocampus) in contrast remain PSA-positive (14, 15). These observations together with the fact that premature removal of PSA accelerates myelin formation led to the hypothesis that PSA expressed on the axonal membrane is an inhibitor of myelination (16). This is supported by the observation that premature removal of PSA by endo-neuraminidase increases myelin formation in vivo and in vitro (16). The re-expression of PSA in multiple sclerosis lesions (17) and hypomyelinated mice (18) might therefore be one factor inhibiting (re)myelination.

Accelerated oligodendrocyte differentiation and myelin formation by premature removal of PSA suggests but does not prove that the normal postnatal down-regulation of PSA is a prerequisite for myelination. In order to further address this question and to discriminate between PSA-dependent effects in oligodendrocytes and neurons, we have generated transgenic mice overexpressing the polysialyltransferase ST8SiaIV in neurons and oligodendrocytes. In the present study, we asked whether down-regulation of PSA in OPC is a secondary effect of the differentiation process or whether PSA down-regulation is a prerequisite for myelin formation. Using transgenic mice overexpressing the polysialyltransferase ST8SiaIV in oligoden-
Oligodendrocytes via the proteolipid protein (PLP) promoter, we show here that the continuous expression of PSA in mature oligodendrocytes causes hypomyelination.

**EXPERIMENTAL PROCEDURES**

*Generation of PLP-PST Transgenic Mice*—Mouse ST8SiaIV was amplified by PCR using oligonucleotides PSTsense (5′-GCAAGCTTCCATGGCTCAATTAGAAGAAGC-3′) and PSTanti (5′-GCTCTAGATTTGCTCATGCACCTTCC-3′), digested with HindIII and XbaI (introduced restriction sites are underlined in the primer sequences), treated with Klenow enzyme (fill-in reaction), and ligated into the Pmel site of the PLP promoter cassette (19) (kindly provided by W. B. Macklin (Cleveland Clinic Foundation)) (see Fig. 1A). Correct sequence of ST8SiaIV cDNA was confirmed by DNA sequencing. In order to generate transgenic mice, vector sequences were removed from the plasmid by digesting the DNA with NotI and Apal, followed by agarose gel purification. The promoter-cDNA-poly(A) fragment was purified and using Qiaquick gel purification (Schleicher & Schuell). Pronucleus injection into fertilized eggs from F2 C57BL/6 mice (postnatal day 1 or 2) (21, 22). Cells were seeded and maintained in tissue culture flasks, coated with poly-L-lysine (molecular weight >300,000) (Sigma) in Dulbecco’s modified Eagle’s medium with 10% horse serum (Invitrogen) for 8–10 days. Cell suspensions were prepared as described and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum. After 8–10 days, cells reached confluence and were established and maintained on a mixed C57BL/6 x CBA genetic background. Genotyping of mice was done by PCR on genomic tail DNA using oligonucleotides PSTsense and PSTanti as primers, which resulted in a 1090-bp product in the presence of the transgene.

**Oligodendrocyte Primary Culture and Immunofluorescence**—Mixed cultures were prepared from the forebrains of newborn mice (postnatal day 1 or 2) (21, 22). Cells were seeded and maintained in tissue culture flasks, coated with poly-L-lysine (molecular weight >300,000) (Sigma) in Dulbecco’s modified Eagle’s medium with 10% horse serum (Invitrogen) for 8–10 days. Cell suspensions were prepared as described and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum. After 8–10 days, cells reached confluence, oligodendrocyte precursor cells grown on top of the astrocyte monolayer were shaken off and seeded on poly-L-lysine-coated glass coverslips at a density of 3–4 × 10³/12-mm coverslip and grown in SATO medium (Dulbecco modified Eagle’s medium supplemented with 10 µg/mL insulin, 0.1 mMputrescine, 0.2 µM progesterone, 0.5 µMtriodothyronine, 0.22 µM sodium selenite, 0.1 mg/mL apotransferrin, 520 nM L-thyroxine, 25 µg/mL gentamycin, 2 mM L-glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin) with 1% horse serum. After 1–4 days, coverslips were fixed in 4% paraformaldehyde in 10 mM sodium phosphate (pH 7.4), 150 mM NaCl (PBS) (10 min). Nonspecific binding sites were blocked for 30 min in 0.2% gelatin in PBS, and cells were permeabilized with 0.3% Triton X-100. Cells were incubated with rabbit antiserum against myelin basic protein (MBP), diluted in 0.2% gelatin in PBS. In some experiments, cells were additionally stained with antibodies directed against PSA (mouse monoclonal antibody 735) (23), l-myelin-associated glycoprotein (MAG) (kindly provided by A. M. Butt, University of Portsmouth, UK), and NCAM (rat monoclonal antibody H28; Chemicon). Additional PSA immunostainings were performed with anti-embryonic NCAM rat IgM antibody (BD Biosciences). Primary antibodies were detected with anti-rat IgM-Alexa Fluor 594, anti-rat Ig-Alexa Fluor 488, anti-rabbit Ig-Cy2, anti-rabbit Ig-Cy3, and anti-mouse Ig-Cy3 (Jackson Immunoresearch/Dianova, Hamburg, Germany) in blocking solution. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). Specimens were mounted in Kaiser’s gelatin and analyzed with an Axiovert 100M microscope (Carl Zeiss, Jena, Germany). In some experiments, deconvolution was applied to z-stacks using the Axiosvision program (Carl Zeiss).

**In Situ Hybridization**—cRNA probes for PLP and PDGF α receptor were synthesized as described recently (20, 25). Mouse ST8SiaIV was subcloned into the pcDNA3 vector (Invitrogen), and cRNA probes for MAG were transcribed from a MAG cDNA in pCR-Blunt II TOPO vector (clone IRAM p995e019Q obtained from RZPD, Berlin, Germany). MBP cDNA was amplified from murine brain RNA by reverse transcription-PCR using oligonucleotides MBPsense (5′-GCCTGGATGTTGATGGCAATC-3′) and MBPanti (5′-AGGTGCTTCTGTCAGCCATA-3′). The PCR product was cloned into the EcoRV site of pBluescript SK(−). Antisense and sense probes were transcribed with T7 or SP6 RNA polymerase and digoxigenin-RNA labeling mix following the instructions of the manufacturer (Roche Applied Science). In situ hybridization on brain sagittal paraffin sections or cryosections was done as described (20). Bound probes were detected with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrates. Sections were examined with a Zeiss Axiovert inverted microscope (Carl Zeiss, Jena, Germany).

**Immunofluorescence of Brain Cryosections**—Brains were removed, immediately frozen in cold isopentane, and stored at −80 °C. Coronal or sagittal cryosections were cut at 10 µm on a cryostat (Leica CM3050S cryotome) at −20 °C and postfixed in 4% paraformaldehyde in PBS for 10 min. Cryosections were stored with desiccant at −80 °C. Sections were permeabilized with 0.5% Triton X-100, and nonspecific binding sites were blocked with 1% bovine serum albumin, 5% fetal calf serum in PBS for 1 h at room temperature. PSA and MBP were stained with antibodies 735 and rabbit MBP antiserum (Chemicon, Hofheim, Germany) in blocking solution. Primary antibodies were visualized with anti-mouse Ig Cy2-conjugate and anti-rabbit Ig Cy3-conjugate in blocking solution. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma). Sec-
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sections were mounted in Kaiser’s gelatin (Merck) and examined with a Zeiss Axiocam fluorescence inverted microscope attached to an AxioCam camera. Photographs were taken with the Axiovision program (Carl Zeiss, Jena, Germany).

Detection of Apoptotic Cells—Apoptotic cells were detected using bromodeoxyuridine incorporation with terminal deoxynucleotidyltransferase using a 5-bromodeoxyuridine triphosphate-FragELT DNA fragmentation detection kit (Merck) according to the manufacturer’s instructions. Brain cryosections were postfixed in 4% paraformaldehyde in PBS, treated with 3% H₂O₂ for 5 min, and incubated with dNTP, 5-bromodeoxyuridine triphosphate, and terminal deoxynucleotidyltransferase in reaction buffer for 30 min at room temperature. After washing, bromodeoxyuridine was detected using anti-bromodeoxyuridine-biotin/streptavidin peroxidase conjugate and 3,3′-diaminobenzidine as substrate.

Lipid Analysis—Lipids were isolated from brain or purified myelin and analyzed as described (25). Phosphoglycerolipids were removed by mild alkaline hydrolysis. Lipids were dissolved in methanol/chloroform (1:1) and applied onto HPTLC silica gel 60 plates (Merck). Sphingomyelin (Sigma) and gangliosides from bovine brain (Calbiochem/Merck) were applied as standards. Gangliosides were separated by TLC using chloroform/methanol, 0.22% CaCl₂ (60:35:8). In some experiments, myelin lipid TLCs were developed in chloroform/methanol/water (70:30:4). Lipids were visualized by spraying TLC plates with cupric sulfate in aqueous phosphoric acid followed by a 10-min incubation at 180 °C.

Myelin Purification—Myelin was isolated from the forebrains of wild-type and transgenic mice at various time points as described by Norton and Poduslo (26). Briefly, brains were homogenized in 10.5% (w/v) sucrose using an Ultra-turrax homogenizer (Janke & Kunkel, Staufen, Germany). Homogenates were centrifuged for 45 min with 17,000 × g. The myelin-containing upper part of the pellet was resuspended in 30% sucrose, overlaid with 10.5% sucrose, and centrifuged for 50 min with 68,000 × g. Myelin was recovered from the 30%/10.5% interphase, washed twice with water, and subjected to a second sucrose gradient in order to remove contaminating axolemma. Purified myelin was again washed twice with water, resuspended in water, and stored in aliquots at −80 °C. In order to determine the total amount of myelin in the brain, the purified myelin was lyophilized, and the dry weight was determined. For protein assays and Western blotting, myelin samples were solubilized in 1% SDS. Protein concentrations were determined using the Bio-Rad DC protein assay. In some experiments, phosphatase inhibitors were included in all solutions: 1 mM sodium ortho-vanadate, 1 mM sodium pyrophosphate, and 1 mM sodium fluoride.

SDS-PAGE and Western Blotting—Forebrains were homogenized in 20 mM Tris·HCl (pH 7.4), 150 mM NaCl (Tris-buffered saline), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. Membranes were isolated by centrifugation of the homogenate at 500 × g for 10 min followed by centrifugation of the supernatant at 100,000 × g for 1 h. SDS-PAGE (7, 10, or 12% acrylamide) was done using standard procedures (27). Protein transfer onto nitrocellulose membranes (pore size 0.45 μm; Schleicher & Schuell) was done with 39 mM glycine, 48 mM Tris as the transfer buffer using the semidyblotting technique as described (27). The following primary antibodies were used: anti-PSA mouse IgG 735, anti-NCAM rat IgG H28 (kind gifts from Dr. R. Gerardy-Schahn, Medizinische Hochschule Hannover, Germany), rabbit anti-Fyn Ig (Santa Cruz Biotechnology, Inc.), rabbit anti-MBP Ig (Chemicon, Hofheim, Germany), and mouse anti-β-actin IgG (Sigma). Non-specific binding sites were blocked with 3% dry milk in 10 mM Tris·HCl (pH 7.4), 150 mM NaCl, and primary antibodies were diluted in the same buffer. Bound primary antibodies were detected using the appropriate peroxidase-conjugated secondary antibodies (Jackson Immunoresearch/Dianova, Hamburg, Germany) followed by chemiluminescence detection with x-ray films, as described (28). In some experiments, lysates were incubated with 20 μg/ml endo neuraminidase Nf (gift from Dr. R. Gerardy-Schahn) for 2 h at room temperature before adding SDS-PAGE sample buffer. Sialic acids were detected with biotinylated lectin (10 μg/ml in Tris-buffered saline, 0.05% Tween 20) from Tritrichomonas mobilensis (Calbiochem/Merck) followed by streptavidin-conjugated peroxidase (Jackson Immunoresearch/Dianova) and chemiluminescence detection. Signal intensities of linear exposures were quantified by densitometry using AIDA software (Raytest, Straubenhardt, Germany). Data were tested for significant differences using Student’s t test.

Histology and Electron Microscopy—Intracardial perfusion with glutaraldehyde, tissue processing, and electron microscopy was done as described (29).

RESULTS

Generation of PLP-PST Transgenic Mice—Transgenic mice expressing the polysialyltransferase ST8SiaIV under the control of the PLP promoter were generated (Fig. 1A). Several transgenic founder mice were identified by Southern blot analysis (Fig. 1B), and two transgenic lines, tg246 and tg281, were established and maintained on a mixed C57BL/6 × CBA genetic background. Expression of the transgene was examined by Northern blotting using a ST8SiaIV-specific cDNA probe (Fig. 1C). These experiments showed a significant up-regulation in the transgene expression between postnatal day 1 (P1) and adult mice (7 weeks). In contrast, the endogenous ST8SiaIV expression was down-regulated. In situ hybridizations of 4-week-old mouse brains with a digoxigenin-labeled ST8SiaIV cRNA probe indicated expression of the transgene in oligodendrocytes in the white matter tracts of forebrain, thalamus, and cerebellum (Fig. 1D), as expected. Detection of the endogenous ST8SiaIV expression required much longer incubation times and is therefore not visible here (data not shown).

Size and morphology of the brain appeared not to be altered in the transgenic mice, and we did not observe changes in the postnatal development of the olfactory bulb, which is reduced in size in NCAM-deficient mice (30, 31).

Western blot analysis of brain homogenates revealed that PSA was increased in the adult brains of transgenic mice (Fig. 1E), although PSA-expression was also considerable in wild-type mice in accordance with previous observations (32, 33). Reprobing the membranes with NCAM-specific antibodies showed that the NCAM-120 bands were strongly reduced in the transgenic brains because of the molecular mass shift due to
the presence of PSA. Western blot analysis of forebrain total membranes pretreated with endoneneuraminidase (endoN) to remove PSA indicated that the level of all NCAM isoforms was not changed in the transgenic mice (Fig. 1E) and confirmed that most NCAM-120 (which is the major NCAM isoform in oligodendrocytes) in the brain of transgenic mice was polysialylated.

The presence of PSA in oligodendrocytes was further examined by immunofluorescence on coronal and sagittal cryosections of adult (3-month-old) brains using PSA- and MBP-specific antibodies. PSA in the forebrain of wild-type mice was restricted to the subventricular zone around the lateral ventricle (Fig. 2A, wt), where PSA-positive neural precursors are generated in the adult brain (34), but was absent from white matter tracts (e.g. the corpus callosum). In contrast, PSA was strongly expressed in the corpus callosum (Fig. 2A, tg) and other white matter tracts in the striatum (Fig. 2B, tg) and cerebellum (Fig. 2C, tg) of transgenic mice. Thereby, PSA partially co-localized with MBP (Fig. 2D), indicating the presence of PSA-NCAM in myelin. No co-localization of PSA and MBP in wild-type brains was observed. Data shown are from the line tg246; however, similar results were obtained with transgenic mice of the line tg281.

Presence of PSA in Purified Myelin of PLP-PST Transgenic Mice—In order to address the question of whether the presence of PSA interferes with myelination, MBP protein levels were determined in the forebrain of adult (3-month-old) wild-type and transgenic mice (Fig. 3A). Densitometric evaluation clearly showed that compared with wild-type mice, MBP levels were significantly reduced in both transgenic lines. Northern blot analysis of total RNA from brains of transgenic and wild-type mice at different ages indicated that the down-regulation of MBP protein was (at least in part) due to reduced MBP mRNA expression (Fig. 3C). MBP mRNA levels were decreased in young transgenic mice up to 12 weeks of age but reached normal levels in older animals. In contrast, expression of the oligodendrocyte marker cerebroside sulfotransferase (CST), which starts to be expressed earlier during oligodendrocyte differentiation, was similar in wild-type and transgenic mice at all time points analyzed (Fig. 3, C and E).

In order to confirm reduced myelin content in transgenic mice, myelin was purified from the forebrains of transgenic and wild-type mice. Western blot analysis of forebrain total membranes pretreated with endoneneuraminidase (endoN) to remove PSA indicated that the level of all NCAM isoforms was not changed in the transgenic mice (Fig. 1E) and confirmed that most NCAM-120 (which is the major NCAM isoform in oligodendrocytes) in the brain of transgenic mice was polysialylated.
wild-type mice at different time points (between 1 week and 12 weeks of age), and its dry weight was determined (Fig. 4A). We found a significant reduction in the myelin content at P21 and 10–12 weeks. The difference of the mean at P10 was not significant, most likely because of the low myelin content at this age. To examine possible changes in the myelin composition, Western blot analysis was performed. These confirmed the presence of polysialylated NCAM-120 in myelin (Fig. 4B). The total amount of NCAM-120 in myelin was, however, not reduced in the myelin of transgenic mice (Fig. 4C). Examination of several other myelin proteins, Fyn, CNP, MBP, did not reveal obvious differences in the amount of these proteins in purified myelin (Fig. 4D). Furthermore, silver staining of SDS-polyacrylamide gels did not indicate obvious changes in the myelin protein composition between transgenic and wild-type mice (data not shown).

In order to rule out the possibility that overexpression of ST8SiaIV affects other sialylation reactions because of the increased utilization of CMP-sialic acid for polysialylation, brain homogenates, membranes, and purified myelin were examined by Western blotting using the sialic acid binding lectin from Tritrichomonas. No significant differences in the lectin staining pattern were observed (Fig. 5, A and B). Furthermore, we examined gangliosides isolated from the forebrain and purified myelin of adult (8-month-old) transgenic and wild-type mice. Alkaline-stable lipids were separated by HPTLC. In the forebrain samples, we found a significant reduction in the ganglioside GM1 in the transgenic mice. In addition, the levels of two other myelin-specific sphingolipids, galactosylceramide and sulfatide, were decreased (Fig. 5C). In purified myelin, however, the level of galactosylceramide, sulfatide, and ganglioside GM1 was not decreased in transgenic mice as compared with wild-type controls (Fig. 5D). We therefore conclude that the overexpression of ST8SiaIV did not inhibit sialylation of sphingolipids but that the reduced GM1 level in total forebrain samples reflects the hypomyelination and progressive demyelination (see below) observed in ST8SiaIV-transgenic mice.
FIGURE 3. MBP was down-regulated in ST8SiaIV-transgenic mice. A, total homogenates of the forebrain from wild-type and transgenic mice (3 months of age) were analyzed by Western blotting using MBP-specific antiserum. Membranes were reprobed for actin to control for equal loading. B, intensities of MBP bands of Western blots were determined by densitometry. Shown are the mean ± S.D. (n = 3) of MBP levels from three animals per genotype. The mean MBP level of wild type was set to 100%. Asterisks indicate a significant difference of the mean (p < 0.05, t test).

Reduced Numbers of Mature Oligodendrocytes in PLP-ST8SiaIV Transgenic Mice—Reduced myelin content and MBP gene expression could be due to changes in the time course of oligodendrocyte differentiation, the number of mature oligo-

FIGURE 4. Hypomyelination in ST8SiaIV-transgenic mice. A, myelin was purified from the forebrains of wild-type (white columns) and transgenic (black columns) mice and lyophilized, and the myelin dry weight was determined. Shown are the mean ± S.D. (n = 3). Asterisks indicate significant differences between transgenic and wild-type mice (t test; p < 0.05). B, Western blot analysis of purified myelin with PSA-specific antibody 735 indicated presence of PSA-NCAM in the myelin of transgenic mice at all time points analyzed. In contrast, PSA was hardly detectable in the myelin of wild-type mice after the period of active myelination (14 and 24 weeks). C, Western blot analysis of NCAM. Treatment of samples with endoneuraminidase (+ endoN) to remove PSA before SDS-PAGE showed that the amount of NCAM-120 was not reduced in transgenic mice. D, Western blot analysis of purified myelin for various myelin-associated proteins (Fyn, CNP, and MBP) indicated no significant differences between wild-type and transgenic mice. Western blots shown are from 24-week-old mice. Similarly, silver staining of purified myelin did not indicate major differences in the protein composition (data not shown). The asterisks indicate a significant difference of the mean (p < 0.05, t test).
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FIGURE 5. ST8SiaIV overexpression did not reduce sialylation of glycoproteins and glycosphingolipids. A, membranes (membr.) and total homogenates (homog.) of the forebrain (30 μg of protein/lane) of wild-type and transgenic mice (8 months of age) were examined by Western blotting with the sialic acid binding lectin from T. mobilis. B, lectin blot of myelin samples (100 μg/lane) from 3-week-old transgenic (lines tg246 and tg281) and wild-type mice. Spingolipids were isolated from 8-month-old forebrain (C) or purified myelin (D) and analyzed by TLC using chloroform, methanol, 0.22% CaCl2 (60:35:8) as a solvent system. The TLC shown in the upper part of D was developed in chloroform/methanol/water (70:30:4). Lipids corresponding to 1 mg (wt weight) of brain tissue and 100 μg of myelin, respectively, were loaded onto HPTLC plates. Gangliosides (GM1, GD1a, GD1b, and GT1b), galactosylceramide (GalC), sulfatide, and sphingomyelin (SM) from bovine brain served as standards. PE and PL phosphatidylethanolamine and other phospholipids, respectively.

dendrocytes, or formation of the myelin sheath. Because PLP is first expressed in mature oligodendrocytes, we expected that differentiation of oligodendrocytes should not be affected in the transgenic mice. In order to prove this hypothesis, we quantified the number of mature oligodendrocytes in the forebrain at different time points (between 1 and 4 weeks of age) by in situ hybridization of sagittal sections of PLP-PST transgenic mice and wild-type littermates using digoxigenin-labeled PLP cRNA probes (Fig. 6A). Interestingly, the number of PLP-positive cells in the forebrain was significantly reduced in transgenic mice at 1 and 2 weeks of age (Fig. 6B). At 4 weeks of age, reduction in the number of PLP-positive cells was less prominent and was no longer significant in the transgenic line tg281 (Fig. 6B). Additional hybridizations were done with a cRNA probe of the PDGFα receptor as a marker for OPC (Fig. 6C). Here we did not observe a decrease in cell number in the transgenic lines at 1 and 2 weeks of age (Fig. 6D). This indicates that the number of OPC was not reduced in the transgenic mice, which was expected, since the ST8SiaIV transgene should not be expressed at the PDGF receptor-positive stage of differentiation. Because reduced numbers of PLP-positive cells could be due to increased cell death, TUNEL staining was performed in order to detect apoptotic cells. TUNEL-positive cells were rarely detectable in the forebrains of both wild-type and transgenic mice at P14 (Fig. 6E), and an increase in the number of apoptotic cells in the transgenic brains could not be detected. In order to confirm our results, we repeated the in situ hybridizations with another marker for mature oligodendrocytes, MAG. One-week-old transgenic mice (line tg246) and wild-type littermates were hybridized to MAG-specific probes (Fig. 6F). The number of MAG-positive cells in the forebrain was significantly reduced in transgenic mice (Fig. 6G).

The observation of a reduced number of PLP-positive cells in transgenic mice was unexpected, because the ST8SiaIV transgene should only be expressed in PLP-expressing mature oligodendrocytes. One possible explanation is that the initial induction of ST8SiaIV transgene expression inhibits further differentiation from immature to mature oligodendrocytes and thereby further up-regulation of PLP expression such that the PLP mRNA level remains below the detection limit of the in situ hybridization.

In Vitro Culture of Oligodendrocytes from Transgenic Mice—We next asked whether presence of PSA affects morphological differentiation of oligodendrocytes in vitro. Therefore, mixed primary cultures were isolated from newborn mice and grown for 8–10 days before shaking off OPC growing on top of the astrocyte monolayer. OPC were differentiated in SATO medium, and the number of MBP-positive cells (as a marker for mature oligodendrocytes) was determined between 1 and 4 days of differentiation (Fig. 7A). The percentage of MBP-positive cells in transgenic cultures was not significantly different from wild-type cultures (Fig. 7B). Thus, in vitro differentiation of oligodendrocytes was not inhibited or delayed in the presence of PSA. As shown in Fig. 7C, wild-type (wt) mature MBP-positive oligodendrocytes from wild-type mice were PSA-negative. In contrast, oligodendrocytes from transgenic mice were PSA-positive (Fig. 7C, tg). PSA was concentrated around the cell bodies but was also found in the membranous extensions of oligodendrocytes. Transgenic oligodendrocytes displayed a less mature morphology, with fewer processes (Fig. 7C) and less extensive membrane sheaths (Fig. 7A). Co-staining for PSA and NCAM revealed partial co-localization of PSA and NCAM in the membrane extensions of oligodendrocytes (Fig. 8A). However, in the perinuclear region, which showed strong PSA staining, the NCAM signal was very low, and PSA and NCAM did not co-localize, suggesting that other proteins beside NCAM carried PSA in the transgenic oligodendrocytes (Fig. 8B). Since ST8SiaIV is highly overexpressed and exhibits autopolyosialylation activity, it is likely that the perinuclear PSA staining is due to autopolyosialylated ST8SiaIV. We cannot, however, rule out the possibility that other glycoproteins were modified by the overexpressed ST8SiaIV. The distributions of NCAM (Fig. 8A) and L-MAG (Fig. 8C) in the membrane extensions were not significantly altered in the transgenic cells as compared with wild-type controls.

Ultrastructural Analysis of Myelin in ST8SiaIV-transgenic Mice—Although myelin content was reduced, no obvious severe behavioral deficits were observed in young mice up to an age of about 4 months. Interestingly, however, we observed...
progressive neuromotor coordination deficits and hind limb paralysis in a minor fraction (about 5%) of older transgenic mice of the line tg246. The reason for this variation might be the variable genetic background. We examined the central nervous system of adult (6-month-old) transgenic mice by electron microscopy for myelin deficits (Fig. 9). Structural abnormalities like redundant myelination (Fig. 9D), axonal degeneration (Fig. 9B), and vacuolar degeneration (Fig. 9E) in the optic nerve (Fig. 9) and the spinal cord (data not shown) were observed in both transgenic and wild-type mice. There was, however, no increase in their number in transgenic mice. F, hybridizations of sections from 1-week-old brains with a MAG-specific cRNA probe revealed a significant decrease in the number of MAG-positive cells in the corpus callosum of transgenic (line tg246) mice as compared with wild-type littermates (mean ± S.E.; n = 3) (G). The asterisks indicate a statistically significant difference of the mean between wild-type and transgenic mice (t test; *, p < 0.05). Scale bars, 100 μm (A and F), 50 μm (C), and 40 μm (E).

**DISCUSSION**

Oligodendrocyte differentiation and myelination of axons in the central nervous system are controlled by several positive and negative signals (36). Negative signals are important to prevent the premature differentiation of OPC and myelination before they reach their final destination. Examples for negative regulators of myelination are the sphingolipid sulfatide (37) and...
LINGO-1 (38). Another potential negative regulator of myelination is PSA.

PSA is required for the migration of OPC as shown using endoN treatment or OPC isolated from NCAM-deficient mice (11). More recent observations indicate that PSA is also required for the PDGF-dependent migration of OPC (12). Upon differentiation of OPC into oligodendrocyte precursors and thereafter mature myelin-forming oligodendrocytes, PSA is down-regulated (8, 9). Down-regulation is mainly caused by reduced ST8SiaIV mRNA synthesis (39). Differentiating oligodendrocytes up-regulate PLP. Accordingly, due to the use of the

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**FIGURE 7.** In vitro culture of ST8SiaIV-overexpressing oligodendrocytes. A, purified oligodendrocytes isolated from newborn wild-type (wt) and transgenic mice (tg; line tg246) were differentiated for up to 4 days in SATO medium and stained for MBP by indirect immunofluorescence. Shown are representative pictures of transgenic and wild-type cells at day 3. B, the number of MBP-positive cells was determined every day. Shown are the mean ± S.D. of at least three independent examinations per time point. No significant differences in the number of MBP-positive cells were observed. C, oligodendrocytes (day 3) were stained for MBP and PSA. Scale bars, 40 μm (A) and 20 μm (C). DAPI, 4',6-diamidino-2-phenylindole.

**FIGURE 8.** NCAM and l-MAG expression in cultured oligodendrocytes. A, purified oligodendrocytes isolated from newborn wild-type (wt) and transgenic mice (tg; line tg246) were differentiated for 3 days in SATO medium and stained for PSA (red) and NCAM (green). B, higher magnification of deconvoluted z-stacks of the transgenic cell shown in A revealed significant co-localization of PSA and NCAM in the cell extensions but not in the perinuclear region (areas depicted in B are boxed in A). C, oligodendrocytes differentiated for 3 days were stained with an antiserum directed against l-MAG. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (not shown in B). Scale bars, 20 μm (A and C) and 5 μm (B).
PSA-negative mice have recently been generated by deleting both polysialyltransferases, ST8SiaIV and ST8SiaII (43). In these mice, the density of some myelinated fiber tracks was significantly decreased. However, it was not clear whether this phenotype results from inefficient myelination or reduced axon numbers. The damage of the corpus callosum and other fiber tracts in ST8SiaIV/ST8SiaII double knock-out mice appeared to be secondary to the ventricular dilation (43). Since the phenotype of these PSA-negative mice could be rescued by simultaneously deleting NCAM, it seems that an important role of PSA is to block NCAM function during development (43). If the effect of PSA overexpression on the number of mature (PLP-positive) oligodendrocytes and myelin formation would be due to block NCAM function, NCAM-deficient mice should pharmacologically alter expression of PSA in mice of one transgenic line raises the possibility that the presence of larger amounts of PSA in myelin can interfere with myelin maintenance, although at present, we cannot rule out the possibility of a transgene insertion artifact.

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Moreover, PSA affected maintenance of myelin and axon-supporting function of oligodendrocytes, as indicated by redundant myelination and axonal degeneration. The observed demyelination in mice of one transgenic line raises the possibility that the presence of larger amounts of PSA in myelin can interfere with myelin maintenance, although at present, we cannot rule out the possibility of a transgene insertion artifact.

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How could the presence of PSA inhibit myelin formation? Removal of PSA with endoN enhances not only NCAM homophilic interactions but also adhesion between other cell surface molecules, like cadherins, integrins, or L1 (46, 47). According to the proposed “pull/push” model (48), the degree of specific interactions between adhesion molecules and the amount of (nonspecific) repulsive steric forces provided by PSA determine the strength of adhesion and the distance between opposing cells. Johnson et al. (49) showed that the magnitude of repulsion mediated by PSA correlated directly with the reduced homophilic interactions but also adhesion between other cell types strongly increased the alignment of oligodendrocytes with the axonal membranes. In line with this, Meyer-Franke et al. (50) showed that the removal of PSA from axons in neuron/oligodendrocyte co-cultures strongly increased the alignment of oligodendrocytes with the axonal membranes. Similarly, PSA on axonal membranes has been implied as an inhibitor of myelination (16, 17).

Modulation of signal transduction pathways by PSA has been demonstrated recently. Endo neuraminidase treatment of neuronal cultures significantly affected their brain-derived neurotrophic factor-dependent survival, and it was shown that this was due to an inhibition of brain-derived neurotrophic factor signaling after PSA removal (51). Seidenfaden et al. (52) showed that the removal of NCAM from neurons with endoN or galactosidase increased the alignment of oligodendrocytes with the axonal membranes. Similar observations have been made by Franceschini et al. (42). We therefore hypothesize that the presence of PSA on NCAM-120 in oligodendrocytes interferes with signaling pathways required for efficient process extension and wrapping of myelin sheaths around axons. One possible candidate that might be affected by polysialylation is Fyn kinase, which is essential for oligodendrocyte differentiation (53, 54) and up-regulation of MBP gene expression (55) and which moreover co-localizes with NCAM-120 in detergent-resistant membranes (56). In preliminary experiments, we did not observe changes in the tyrosine phosphorylation in purified myelin in transgenic mice. However, it is possible that an inhibition of signal transduction via Fyn kinase or others is only transient during the initiation of myelination.

In addition to its inhibitory effect on oligodendrocyte differentiation (13) and myelin formation, the continuous synthesis of PSA-NCAM also affected myelin in the adult animal. Structural abnormalities of myelin (redundant myelination) and vacuolar degeneration were observed. These observations show that PSA down-regulation is not only necessary for efficient differentiation of oligodendrocytes and myelin formation but that its presence in myelin also interferes with the stable maintenance of the myelin sheath and axonal support by oligodendrocytes.

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**REFERENCES**

1. Angata, K., and Fukuda, M. (2003) *Biochimie (Paris)* **85**, 195–206
2. Eckhardt, M., Mühlennhoff, M., Bethe, A., Koopman, J., Frosch, M., and Gerardy-Schahn, R. (1995) *Nature* **373**, 715–718
3. Goridis, C., and Brunet, J. F. (1992) *Semin. Cell Biol.* **3**, 189–197
4. Bhat, S., and Silberberg, D. H. (1988) *J. Neurochem.* **50**, 1830–1838
5. Nakayama, J., Fukuda, M. N., Fredette, B., Ranscht, B., and Fukuda, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7031–7035
6. Kojima, N., Yoshiya, Y., Kurosawa, N., Lee, Y. C., and Tsuji, S. (1995) *FEBS Lett.* **366**, 1–4
7. Livingston, B. D., and Paulson, J. C. (1993) *J. Biol. Chem.* **268**, 11504–11507
8. Trotter, J., Bitter-Suermann, D., and Schachner, M. (1989) *J. Neurosci. Res.* **22**, 369–383
9. Bartsch, U., Kirchhoff, F., and Schachner, M. (1990) *J. Neurocytol.* **19**, 550–565
10. Nait-Oumesmar, B., Vignais, L., Duhamel-Clerin, E., Avellanada-Adalid, V., Rougon, G., and Baron-van Evercooren, A. (1995) *Eur. J. Neurosci.* **7**, 480–491
11. Wang, C., Pralong, W. F., Schulz, M. F., Rougon, G., Aubry, J. M., Pagliusi, S., Robert, A., and Kiss, J. Z. (1996) *J. Cell Biol.* **135**, 1565–1581
12. Zhang, H., Vutskits, L., Calaora, V., Durbec, P., and Kiss, J. Z. (2004) *J. Cell Biol.* **163**, 97–103
13. Decker, L., Durbec, P., Rougon, G., and Evercooren, A. B. (2002) *Mol. Cell. Neurosci.* **19**, 225–238
14. Seki, T., and Arai, Y. (1991) *Neurosci. Res.* **12**, 503–513
15. Le Gal La Salle, G., Rougon, G., and Valin, A. (1992) *J. Neurosci.* **12**, 872–882
16. Charles, P., Hernandez, M. P., Stankoff, B., Aigrot, M. S., Colin, C., Rougon, G., Zalc, B., and Lubetzki, C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7585–7590
17. Charles, P., Reynolds, R., Seilhan, D., Rougon, G., Aigrot, M. S., Niezgoda, A., Zalc, B., and Lubetzki, C. (2002) *Brain* **125**, 1972–1979
18. Takikita, S., Fukuda, T., Mohri, I., Yagi, T., and Suzuki, K. (2004) *J. Neuropathol. Exp. Neurol.* **63**, 660–673
19. Fuss, B., Afshari, F. S., Colello, R. J., and Macklin, W. B. (2001) *Mol. Cell. Neurosci.* **18**, 221–234
20. Fewou, S. N., Büssow, H., Schaeren-Wiemers, N., Vanier, M. T., Macklin, W. B., Gieselmann, V., and Eckhardt, M. (2005) *J. Neurochem.* **94**, 469–481
21. McCarthy, K. D., and de Vellis, J. (1980) *J. Cell Biol.* **85**, 890–902
22. Morganti, M. C., Taylor, J., Pesheva, P., and Schachner, M. (1990) *Exp. Neuro* **109**, 98–110
23. Frosch, M., Gorgen, I., Boulois, G. J., Timmis, K. N., and Bitter-Suermann, D. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1194–1198
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Yaghoobtami, A., Gieselmann, V., and Eckhardt, M. (2005) *Eur. J. Neurosci.* **21**, 711–720
26. Norton, W. T., and Poduslo, S. E. (1973) *J. Neurochem.* **21**, 749–757
27. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Eckhardt, M., Fewou, S. N., Ackermann, I., and Gieselmann, V. (2002) *Biochem. J.* **368**, 317–324
29. Büssow, H. (1978) *J. Neurocytol.* **7**, 207–214
30. Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., Barthels, D., Rajewsky, K., and Wille, W. (1994) *Nature* **367**, 455–459
31. Tomaszewicz, H., Ono, K., Yee, D., Thompson, C., Goridis, C., Rut-
ishaufer, U., and Magnuson, T. (1993) Neuron 11, 1163–1174
32. Finne, J., Bitter-Suermann, D., Goridis, C., and Finne, U. (1987) J. Immunol. 138, 4402–4407
33. Eckhardt, M., Bukalo, O., Chazal, G., Wang, L., Goridis, C., Schachner, M., Gerardy-Schahn, R., Cremer, H., and Dityatev, A. (2000) J. Neurosci. 20, 5234–5244
34. Doetsch, F., and Alvarez-Buylla, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14895–14900
35. Spassky, N., Goujet-Zalc, C., Parmantier, E., Olivier, C., Martinez, S., Ivanova, A., Ikenaka, K., Macklin, W., Cerruti, I., Zalc, B., and Thomas, J. L. (1998) J. Neurosci. 18, 8331–8343
36. Coman, I., Barbin, G., Charles, P., Zalc, B., and Lubetzki, C. (2005) J. Neurol. Sci. 233, 67–71
37. Bansal, R., Winkler, S., and Bheddah, S. (1999) J. Neurosci. 19, 7913–7924
38. Mi, S., Miller, R. H., Lee, X., Scott, M. L., Shulag-Morskaya, S., Shao, Z., Chang, J., Thill, G., Levesque, M., Zhang, M., Hession, C., Sah, D., Trapp, B., He, Z., Jung, V., McCoy, J. M., and Pepinsky, R. B. (2005) Nat. Neurosci. 8, 745–751
39. Stoykova, L. I., Beesley, J. S., Grinspan, J. B., and Glick, M. C. (2001) J. Neurosci. Res. 66, 497–505
40. Mühlenhoff, M., Eckhardt, M., Bethe, A., Frosch, M., and Gerardy-Schahn, R. (1996) EMBO J. 15, 6943–6950
41. Close, B. E., and Colley, K. J. (1998) J. Biol. Chem. 273, 34586–34593
42. Franceschini, I., Vitry, S., Padilla, F., Casanova, P., Tham, T. N., Fukuda, M., Rougon, G., Durbec, P., and Dubois-Dalcq, M. (2004) Mol. Cell. Neurosci. 27, 151–162
43. Weinhold, B., Seidenfaden, R., Röckle, I., Mühlenhoff, M., Schertzing, F., Conzelmann, S., Marth, J. D., Gerardy-Schahn, R., and Hildebrandt, H. (2005) J. Biol. Chem. 280, 42971–42977
44. Carenini, S., Montag, D., Cremer, H., Schachner, M., and Martini, R. (1997) Cell Tissue Res. 287, 3–9
45. Chazal, G., Durbec, P., Jankovski, A., Rougon, G., and Cremer, H. (2000) J. Neurosci. 20, 1446–1457
46. Acheson, A., Sunshine, J. L., and Rutishauser, U. (1991) J. Cell Biol. 114, 143–153
47. Fujimoto, I., Bruses, J. L., and Rutishauser, U. (2001) J. Biol. Chem. 276, 31745–31751
48. Rutishauser, U. (1998) J. Cell Biochem. 70, 304–312
49. Johnson, C. P., Fujimoto, I., Rutishauser, U., and Leckband, D. E. (2005) J. Biol. Chem. 280, 137–145
50. Meyer-Franke, A., Shen, S., and Barres, B. A. (1999) Mol. Cell. Neurosci. 14, 385–397
51. Vutskits, L., Djebbara-Hannas, Z., Zhang, H., Paccaud, J. P., Durbec, P., Rougon, G., Muller, D., and Kiss, J. Z. (2001) Eur. J. Neurosci. 13, 1391–1402
52. Seidenfaden, R., Krauter, A., Schertzing, F., Gerardy-Schahn, R., and Hildebrandt, H. (2003) Mol. Cell. Biol. 23, 5908–5918
53. Klein, C., Krämer, E. M., Cardine, A. M., Schraven, B., Brandt, R., and Trotter, J. (2002) J. Neurosci. 22, 698–707
54. Liang, X., Draghi, N. A., and Resh, M. D. (2004) J. Neurosci. 24, 7140–7149
55. Umemori, H., Kadowaki, Y., Hirosawa, K., Yoshida, Y., Yoshida, K., Okano, H., and Yamamoto, T. (1999) J. Neurosci. 19, 1393–1397
56. Krämer, E. M., Klein, C., Koch, T., Boytinc, M., and Trotter, J. (1999) J. Biol. Chem. 274, 29042–29049