Cell Tissue Res (1997) 289:195–206

Cell & Tissue Research

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Regular articles

Functional analysis in vivo of the double mutant mouse deficient in both proteolipid protein (PLP) and myelin basic protein (MBP) in the central nervous system

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Received: 20 January 1997 / Accepted: 18 February 1997

Abstract. Myelination is an important developmental process of the central (CNS) and peripheral nervous system (PNS). To unravel the functions of the two dominant myelin proteins in the CNS, proteolipid protein (PLP) and myelin basic protein (MBP), we generated and characterized the homozygous double mutant mouse line (*plp*$^{-/-}$, *mbp*$^{-/-}$), which is viable and fertile. Plasma membrane processes of oligodendrocytes deficient in PLP and MBP, but not in myelin-associated glycoprotein (MAG), spirally wrap large diameter axons, tightly adhering at their extracytosolic surfaces and forming a pseudo-compacted myelin. Neuromotor activity and coordination are considerably improved compared to the *shiverer* trait.

Key words: Myelination – Proteolipid protein – Myelin basic protein – Pseudomyelin – PLP knock out mouse – *Shiverer* mouse

Introduction

The proteolipid isoproteins PLP and DM20 are four-helix transmembrane proteins (Folch and Lee 1951; Stoffel et al. 1983, 1984; Weimbs and Stoffel 1992). Together with the myelin basic proteins (MBP), a family of peripheral membrane proteins (Carnegie 1971; Omlin et al. 1982; Takahashi et al. 1985), they are the main protein constituents of CNS myelin and account together for more than 80–90% of the total myelin protein. PLP and MBP are present in normal myelin in balanced, almost stoichiometric amounts. The topology and physical properties suggest that PLP (Boison and Stoffel 1994; Weimbs and Stoffel 1992) and MBP (Omlin et al. 1982; Privat et al. 1979) are responsible for the highly ordered and compact myelin structure, PLP for the tight apposition of the outer surfaces and MBP for the proximity of the cytosolic surfaces of wrapping, tongue-like processes of the plasma membrane of oligodendrocytes. Axoglial contact in the myelin assembly has been attributed to a minor protein constituent of myelin, myelin-associated glycoprotein (MAG) (Arquint et al. 1987).

Gene expression of these myelin proteins occurs in oligodendrocytes during the process of myelination in a both temporally and spatially coordinated fashion. Animal mutants of MBP and PLP leading to inherited disorders of myelination are useful models for studying the function of myelin-specific proteins in the complex process of myelination.

The MBP gene of *shiverer* (*mbp*$^{-/-}$) mice carries a deletion of exons 2 to 7 (Roach et al. 1985; Molineaux et al. 1986) causing an almost complete lack of CNS myelin with severe neurological symptoms and a short life span.

We have generated a *plp*$^{-/-}$ mutant mouse line by gene targeting (Boison and Stoffel 1994). Axons in the CNS of PLP-deficient mice (*plp*$^{-/-}$) are wrapped by a loosely packed membrane system with regular main dense lines but devoid of intermediate dense lines. Despite their completely distorted myelin sheath, PLP-deficient mice – unlike the MBP-deficient *shiverer* mice – have only mild neuromotor defects and a normal life span and reproduction (Boison et al. 1995).

PLP point mutations cause dysmyelinoses like the *jimpy* mouse (Nave et al. 1986), the myelin-deficient rat (Boison and Stoffel 1989), the shaking pup dog (Nadon et al. 1990), or several X-linked human sudanophilic leukodystrophies (Pelizaeus-Merzbacher disease, reviewed by Gow et al. 1994). Their pleiotropic phenotype contrasts that of the PLP-deficient (*plp*$^{-/-}$) mouse. Structurally altered PLP leads to a premature death of oligodendrocytes

This paper is dedicated to Professor Andreas Oksche on the occasion of his 70th birthday, in recognition of outstanding contributions to neuroscience and to the internationalisation of science

The support of the Deutsche Forschungsgemeinschaft, the BMBF (01KS9502) of the Hertie Stiftung, and of the Thyssen foundation is gratefully acknowledged. This work was carried out within the Center of Molecular Medicine (ZMMK). W. St. is responsible for this research project.

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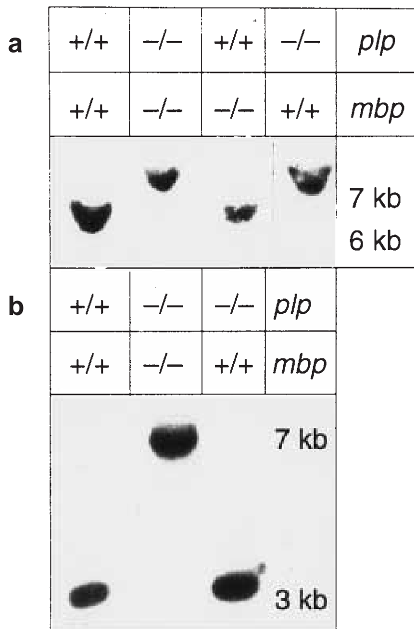


Fig. 1a, b. Genotyping of PLP-/MBP-deficient offspring. Southern blot hybridization analysis of HindIII-restricted DNA obtained from tail biopsies of mice homozygous for the *shiverer* ($mbp^{-/-}$) and $plp^{-/-}$ allele separated on a 0.7% agarose gel, blotted to a GeneScreenPlus membrane, and hybridized with a PLP (**a**) and MBP (**b**)-specific probe. **a** Mice carrying a PLP-knock-out allele can be detected by a 7 kb signal due to the 1 kb neo-insertion present in the HindIII fragment (Boison and Stoffel 1994), whereas mice carrying a wild-type PLP allele are characterized by a 6 kb signal. **b** The deletion of the *mbp* gene (exons II to VII) was verified by Southern blotting of the HindIII-restricted DNA of the $plp^{-/-}$, $mbp^{-/-}$ mouse with the 350 bp PCR fragment obtained with oligonucleotide primers hybridizing to the 5′ intron sequence upstream of the deleted exon II, 5′GAGGCCGCACATCAGCCC-TGATTTTTGCTAAG 3′, and 3′ primer, 5′ CATGTAATGAATG-TTGCATCTTGGGCAATCTATCT 3′, hybridizing to the sequence flanking the deletion downstream of exon VII. The corresponding distance between these primer locations in the wild-type *mbp* gene does not give a PCR fragment with these primers

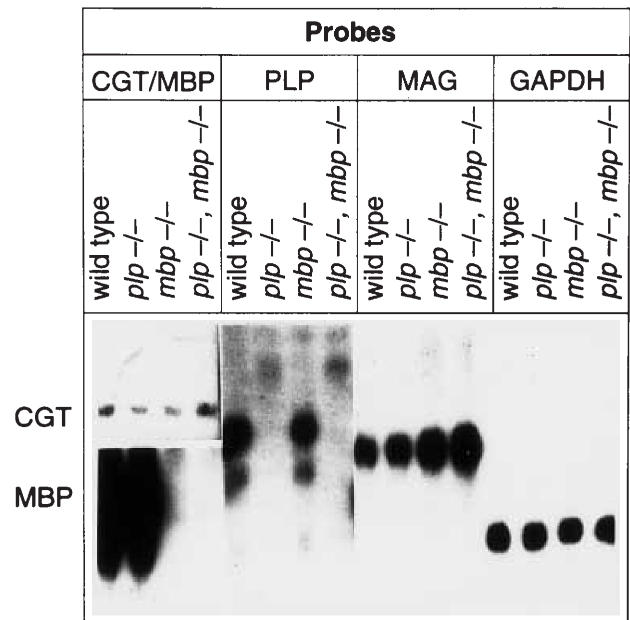


Fig. 2. RNA analysis. Ten microgram of RNA obtained from total brains of 21-day-old mice was analyzed on Northern blots. They were hybridized with probes for PLP, MBP (*bottom left*), CGT (*top left*), MAG, and GAPDH as an internal control (see experimental procedures). Four different genotypes were analyzed: wild type; $plp^{-/-}$: PLP-deficient; $mbp^{-/-}$: *shiverer;* $plp^{-/-}$, $mbp^{-/-}$: PLP-MBP-deficient "double mutant". All blots were overexposed in order to detect even traces of the respective mRNAs

at the end of the myelination period. A similar phenotype can result from overexpression of the PLP gene as transgene (Kagawa et al. 1994; Readhead et al. 1994).

This study was undertaken to assess the impact of the two abundant protein constituents, PLP and MBP, on the assembly of the myelin membrane at the molecular and cellular level in the three genotypes, the *shiverer* $mbp^{-/-}$, the $plp^{-/-}$ and the double mutant ($plp^{-/-}$, $mbp^{-/-}$). The geno- and phenotype of the *shiverer* (Takahashi et al. 1985) and the $plp^{-/-}$ mouse have been characterized (Boison and Stoffel 1994).

We now generated a mouse line completely deficient in the main structural proteins PLP and MBP by crossing the *shiverer* $mbp^{-/-}$ locus into homozygous $plp^{-/-}$ mutant mice. The resulting homozygous double mutant ($plp^{-/-}$, $mbp^{-/-}$) mice show normal reproduction and a much longer life span (up to 24 months) than their *shiverer* counterparts (up to 3 months). The shivering symptoms and the number of seizures are less profound in the double mutant. Ultrastructural analysis revealed loosely packed membrane layers, in which an intraperiod line (IL) is visible but no main dense line (MDL) is established, around axons with larger diameter.

MAG is overexpressed in the PLP-MBP-deficient double mutant mouse of the RNA level, although this is not as apparent at the protein level. It is conceivable that MAG, now in the newly formed pseudomyelin, beyond its adhesive function in axonoglial contact, also triggers the adhesion of the outer surfaces of the layers of the pseudo-compacted myelin sheath, sufficient to ameliorate the *shiverer* symptoms but also to improve longevity and fertility.

Materials and methods

Animals

PLP-deficient mice ($plp^{-/-}$) from our transgenic breeding facility were crossed with heterozygous *shiverer* mice ($mbp^{+/-}$) (Jackson Laboratories, Bar Harbor). The resulting F1 generation was intercrossed to obtain mice homozygous for the PLP/MBP deficiency ($plp^{-/-}$, $mbp^{-/-}$).

Genotyping

Genotyping of the PLP allele of the double knockout mice was performed as described (Boison and Stoffel 1994). The deletion in the *mbp* gene (exons II to VII) of the double mutant was verified by Southern blotting of HindIII-restricted DNA of the ($plp^{-/-}$, $mbp^{-/-}$) mice with a 350 bp PCR fragment obtained with oligonucleotide primers hybridizing to a 5′ intron sequence upstream of

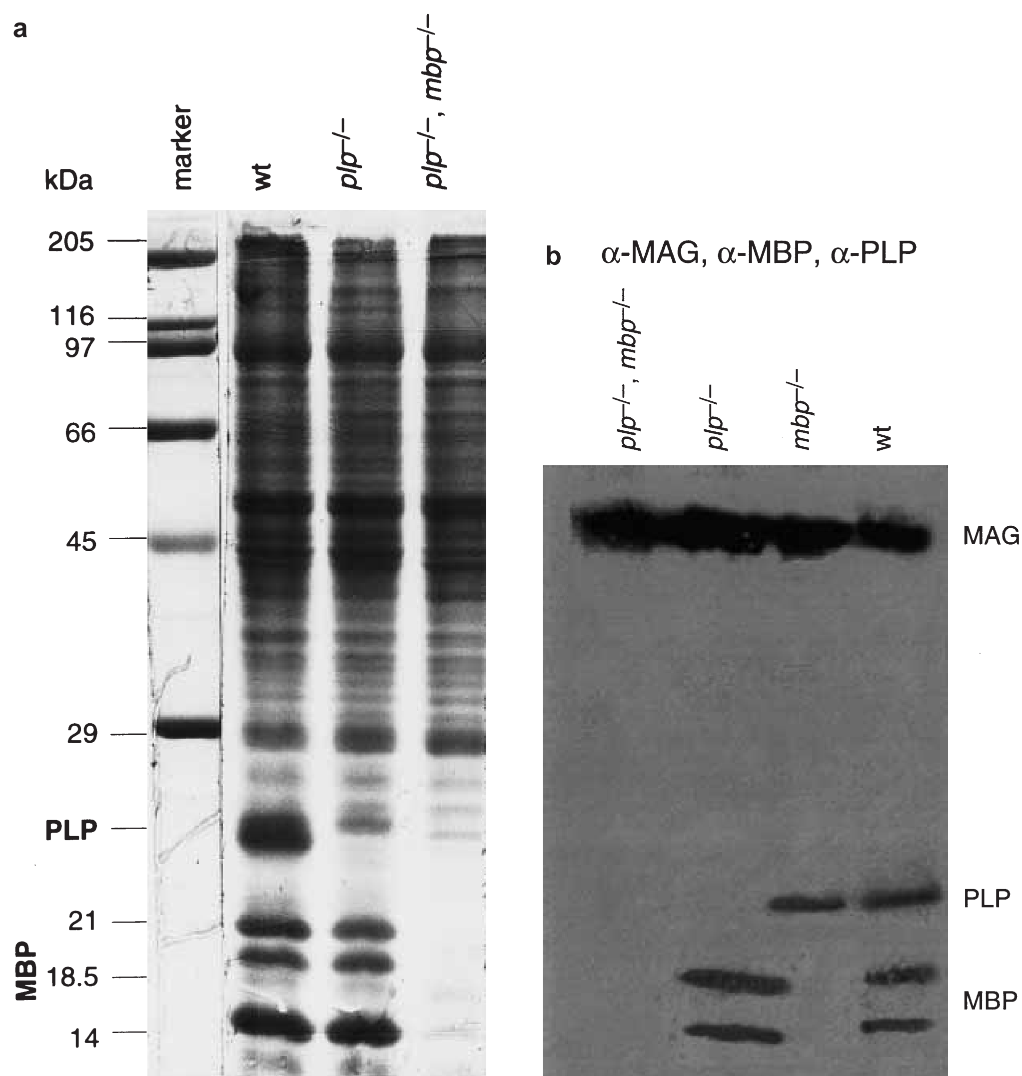


Fig. 3a, b. Analysis of myelin proteins. Myelin proteins (40 µg/lane) isolated from brains of 28-day-old mice (genotypes see legend to Fig. 2) were analyzed by 12% SDS-PAGE. **a** Polyacrylamide gels were stained with Coomassie Blue. **b** Individual proteins (PLP, MBP, and MAG) were visualized by Western blot analysis (Towbin et al. 1979) with their respective antibodies and visualized with alkaline phosphatase-labeled second antibody with bromochloroindolyl phosphate/nitro blue tetrazolium as substrates following established procedures (Harlow and Lane 1988)

the deleted exon II, 5′ GAGGCCGCACATCAGCCCTGATTTTTGCTAAG 3′, and a 3′ primer, 5′ CATGTATGAATGTGCATCTTGGGCAATCTATCT 3′, hybridizing to the sequence flanking the deletion downstream of exon VII. The corresponding sequence between these primer locations in the wild type MBP gene is too long to give a PCR fragment with these primers. This 350 bp probe monitors a 7 kb restriction fragment, which was in the HindIII restriction of the double mutant tail DNA.

RNA analysis

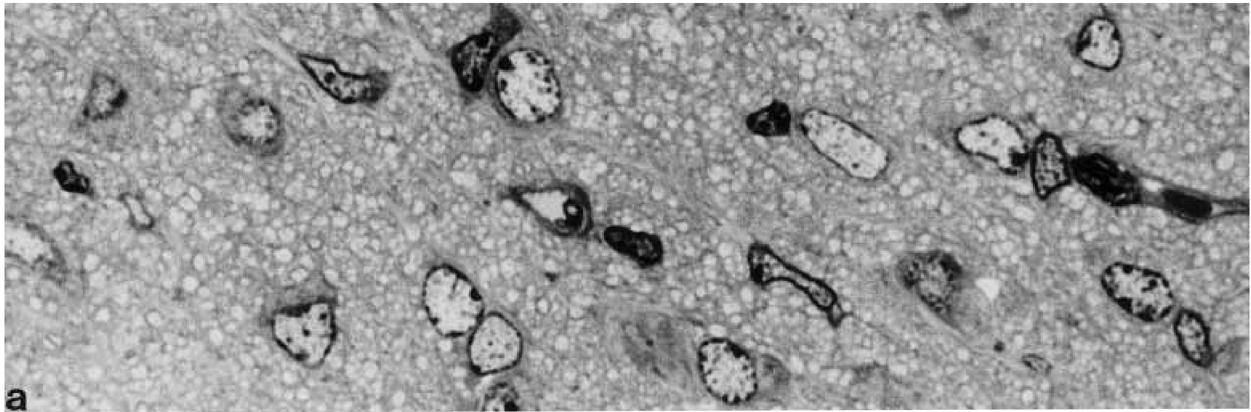
RNA was prepared from total brains of 21-day-old mice (Chomczynski and Sacchi 1987). RNA (15 µg) was separated on 1.5% agarose gels containing formaldehyde and blotted onto nitrocellulose (BA 85 Schleicher & Schuell GmbH, Dassel, Germany) with 20×SSC. After baking for 2 h at 80° C the membrane was prehybridized in 50% formamide, 50 mM phosphate buffer, pH 6.8, 5×SSC, 1×Denhardt's and 100 µg salmon sperm DNA/ml for 4 h at 42° C. After prehybridization, the membrane was hybridized in fresh buffer containing 10% dextrane sulphate and 2×10^5 cpm ^{32}P-labeled probe/ml overnight at 42° C. The membrane was washed 4×5 min in 2×SSC, 0.1% SDS at 50° C, and twice for 15 min in 0.1×SSC, 0.1% SDS at room temperature.

Hybridization probes

The following genomic and cDNA fragments of PLP, MBP, MAG, CGT, and GAPDH were labeled with the random priming DNA labeling kit (Boehringer Mannheim) following the manufacturer's recommendations: a genomic 700 bp BglII fragment containing sequences of exon III and intron IIIA of the PLP gene, a 620 bp PstI fragment, the complete coding region of rat MBP, a 700 bp NcoI fragment of CGT cDNA (Tuohy and Thomas 1993), a 560 bp (1351–1910) of mouse MAG cDNA fragment and a 550 bp HindIII-XbaI cDNA fragment of human liver glyceraldehyde 3-phosphodehydrogenase (GAPDH). Fragments were purified using the Quiaex gel extraction kit (Qiagen, Chatsworth, Ca.).

Isolation of myelin and protein analysis

Myelin of total brain of 28-day-old mice was prepared by sucrose gradient centrifugation. One brain was homogenized in 16 ml 0.32 M sucrose and centrifuged for 20 min at 4° C. The sediment was suspended in 5 ml 0.17 M sucrose and layered on top of 6 ml 0.68 M sucrose and centrifuged in a SW41 Beckman rotor at 30,000 rpm for 120 min. The myelin-containing interphase (approx. 2 ml) was collected with a bent Pasteur pipette, diluted with 5 ml water, sedimented at 15000×g and washed twice. Myelin aliquots (40 µg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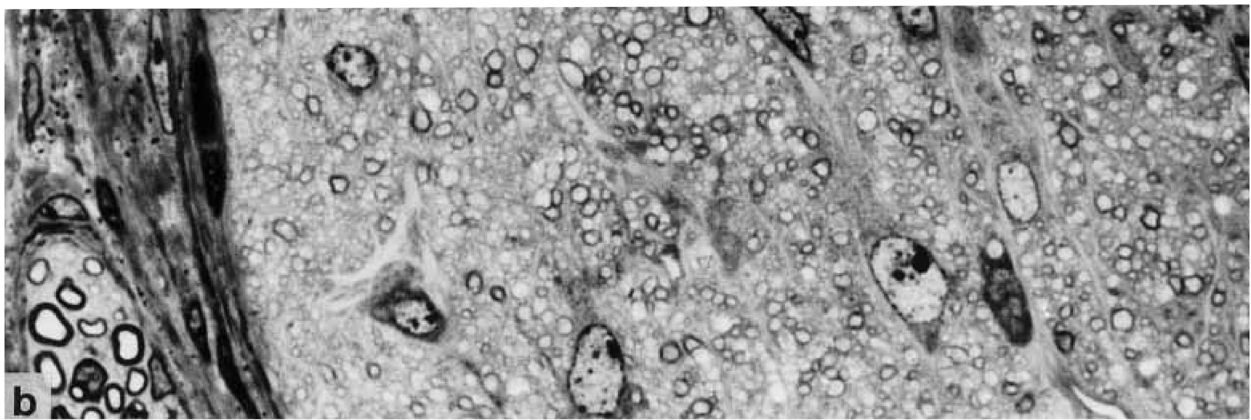
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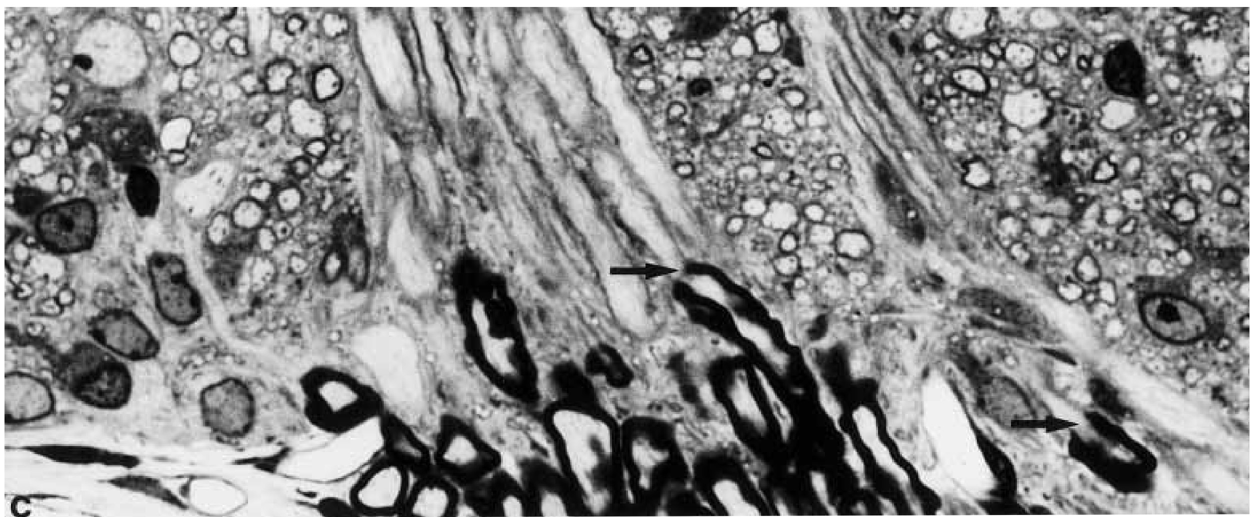
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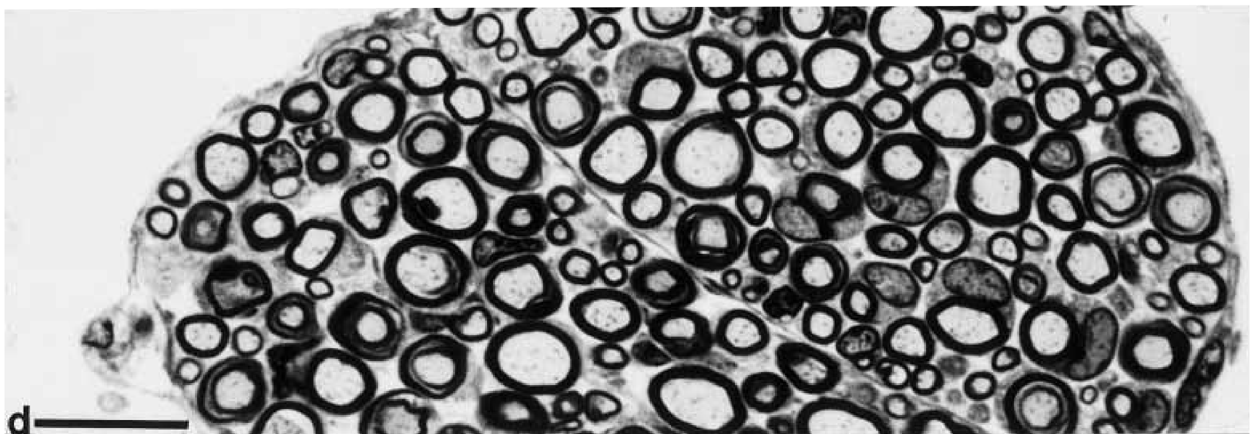
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protein/lane) were separated on 12% SDS-PAGE. Gels were stained with Coomassie blue.

Light and electron microscopy

Twenty-nine-day-old mice were anaesthesized with Nembutal and perfused with 6% glutaraldehyde via the left cardiac ventricle. Cervical segments of the spinal cord and the optic nerve were obtained, postfixed in 1% phosphate-buffered OsO_4 in 0.1 M sucrose, and embedded in Epon 812. The semithin sections were stained with toluidine/pyronin. Ultrathin cross-sections of optic nerve and spinal cord were contrasted with uranylacetate and lead citrate and examined as previously described (Büssow 1978).

Results

Generation and genotyping of mice deficient in both PLP and MBP ($plp^{-/-}$, $mbp^{-/-}$)

The double mutant deficient in PLP and MBP was obtained by introducing the *shiverer* mutation into PLP-deficient mice by cross-breeding female *plp*$^{-/-}$ and heterozygous (*mbp*$^{+/-}$) mice. The *plp*$^{-/-}$ phenotype was detected by the presence of a 7 kb HindIII fragment. The 1 kb neogene inserted into intron 3 extends the 6 kb HindIII fragment of the wild type allele (Fig. 1A, Boison and Stoffel 1994).

The *shiverer* genotype of the double mutant (*plp*$^{-/-}$, *mbp*$^{-/-}$) was confirmed by PCR using a 5′ oligodesoxinucleotide primer hybridizing upstream of the *shiverer* deletion between intron 2 and exon 7 (Roach et al. 1985) and a 3′ antisense primer downstream of the deletion, which yielded a 350 bp fragment. This 350 bp PCR fragment was used as a probe for Southern blot hybridization of the HindIII-restricted DNA of the offsprings. The 7 kb Hind III fragment confirmed the *shiverer* deletion (Fig. 1B), whereas the wild type released a 3 kb HindIII fragment. The first generation of mice heterozygous for both alleles were healthy, reproduced well, and had an unconspicuous phenotype. Surprisingly, the homozygous double mutant mice were viable and, in contrast to homozygous *shiverer* mice, reproduced normally. Thus, we were able to establish a breeding colony of the double mutant mice (*plp*$^{-/-}$, *mbp*$^{-/-}$) deficient in both PLP and MBP.

Phenotype of the PLP-/MBP-deficient mouse ($plp^{-/-}$, $mbp^{-/-}$)

Shiverer mice (*mbp*$^{-/-}$) exhibit the well-characterized phenotype: a severe, nearly constant whole body tremor accompanied by frequent violent convulsions and seizures. Premature death of the afflicted individuals occurs 3 months after birth. *Shiverer* mice are rarely able to reproduce and rear their offspring.

The PLP-/MBP-deficient mice (*plp*$^{-/-}$, *mbp*$^{-/-}$), however, show only mild shivering, interrupted by phases with no body tremor. No convulsions and seizures are observed. Homozygous double mutant mice reproduce normally between 6 and 12 weeks of age, with normal litter sizes and a normal behavior in rearing the pups. Animals that are now more than 18 months old show a reduced motor activity, staying most of their time in a resting and apathetic position. Our oldest double mutant homozygous mice are now more than 18 months old.

RNA analysis of PLP-/MBP-deficient mice ($plp^{-/-}$, $mbp^{-/-}$)

Gene expression of four specific and well-characterized genes expressed synchronously in oligodendrocytes during myelination was analyzed by Northern blot hybridization. Specific transcripts of the PLP, MBP, and the MAG genes and of the gene of CGT (UDP-galactose-ceramide galactosyl transferase), a key enzyme in the synthesis of the oligodendrocyte-specific galactocerebrosides and sulfatides (Schulte and Stoffel 1993), were probed. RNA from total brains of 21-day-old wild type, PLP-deficient (*plp*$^{-/-}$), MBP-deficient (*shiverer, mbp*$^{-/-}$), and PLP-/MBP-deficient mice (*plp*$^{-/-}$, *mbp*$^{-/-}$) was probed with respective cDNA probes. The intensity of the signals was compared with an internal GAPDH standard (Fig. 2).

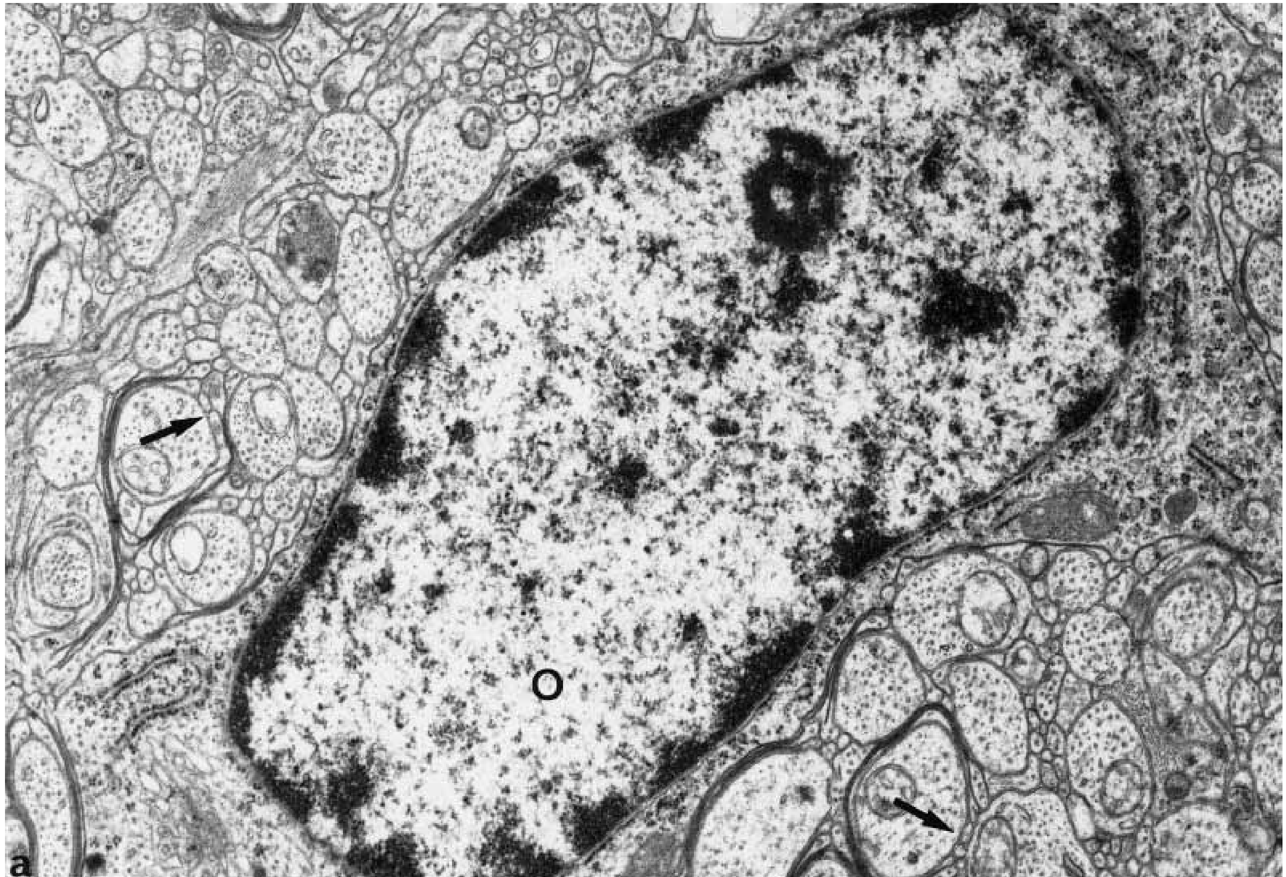
As expected, total RNA of the PLP-deficient mice was completely devoid of the normal *plp* transcripts of 3.2, 2.4, and 1.6 kb. RNA of *mbp* transcripts of *shiverer* mice, and the transcripts of both genes were missing in RNA of brains of PLP-/MBP-deficient mice. The transcription of the CGT gene was not affected by the mutations. However, we observed an approximately twofold overexpression of MAG in *shiverer* mice lacking MBP (*mbp*$^{-/-}$) and an approximately fourfold overexpression in the double mutant RNA (*plp*$^{-/-}$, *mbp*$^{-/-}$), respectively (Fig. 2). RNA blots were overexposed during autoradiography in order to detect even traces of transcripts in the null-allelic mutants.

←

Fig. 4. Semithin cross-sections of optic nerves of *shiverer* (*mbp*$^{-/-}$) (**a**) and double mutant (*plp*$^{-/-}$, *mbp*$^{-/-}$) (**b**) mice. The comparison of the two sections demonstrates the striking difference in hypomyelination. In the MBP-deficient nerve no myelin is detectable, whereas many of the larger axons in the optic nerve of the PLP-/MBP-deficient mouse show myelin sheaths. **c** Transverse section through the spinal cord of the double mutant (*plp*$^{-/-}$, *mbp*$^{-/-}$) in the region of the transitional zone of the ventral root. The *arrows* indicate the transition from thinly myelinated CNS axons to normally myelinated PNS axons in a node of Ranvier. **d** The transverse semithin section through a ventral root shows apparently normally myelinated PNS nerve fibers. *Bar:* 24 μm

→

Fig. 5. Electron micrographs of cross-sections of optic nerve of *shiverer* (*mbp*$^{-/-}$) (**a**) and double mutant (*plp*$^{-/-}$, *mbp*$^{-/-}$) (**b**) mice. In the *shiverer* optic nerve, completely ensheathed axons are rarely seen, whereas in the double mutant, numerous large diameter axons are completely ensheathed with a myelin-like membrane. In both mutants, the oligodendrocytes (*o*) resemble the metabolically active, light-type oligodendrocyte. *Bar*: 1.2 μm



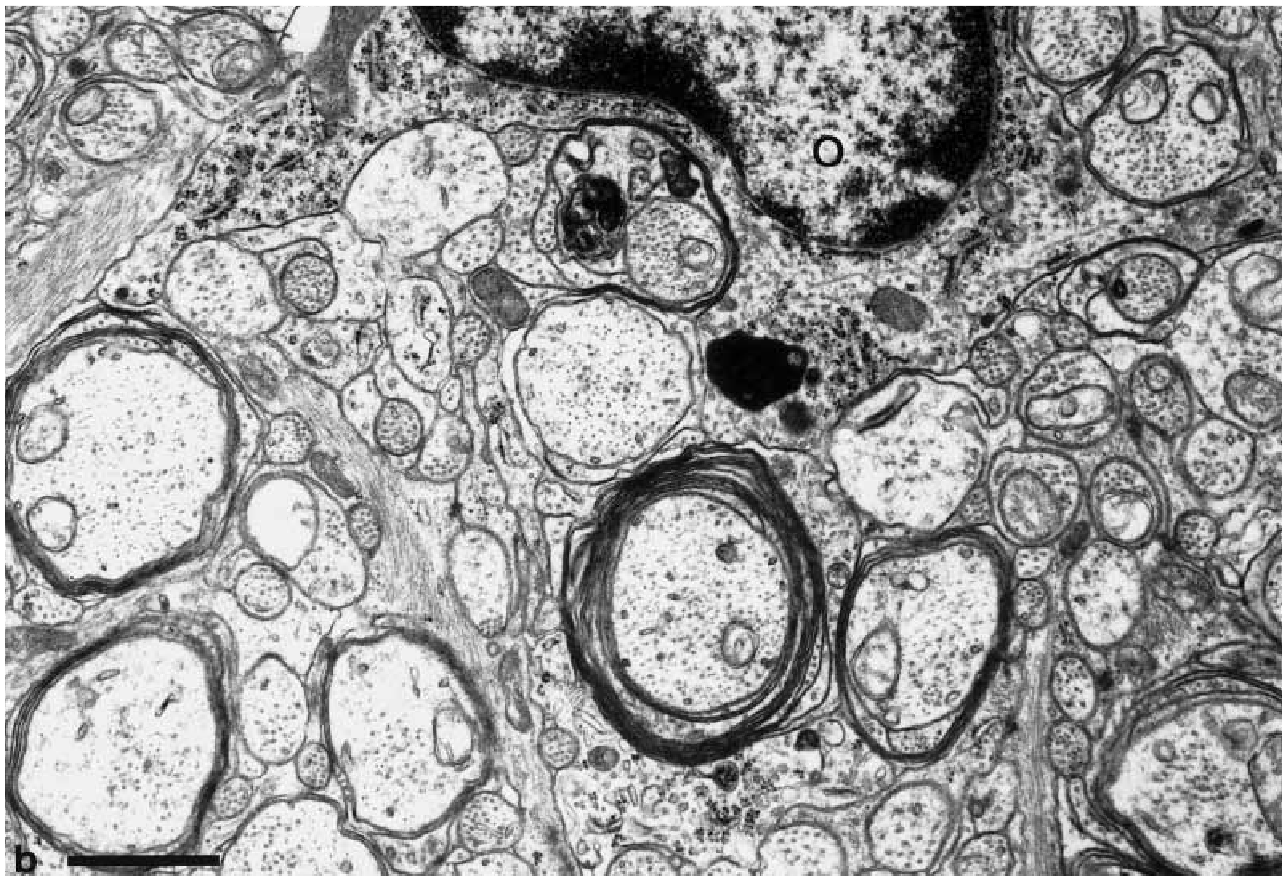
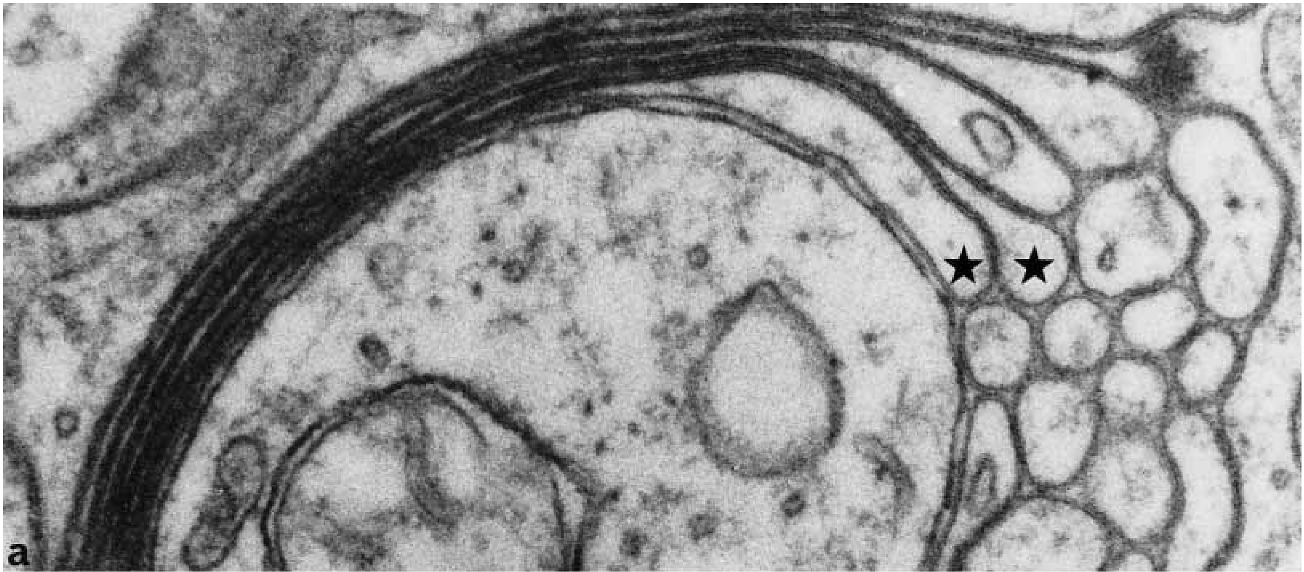
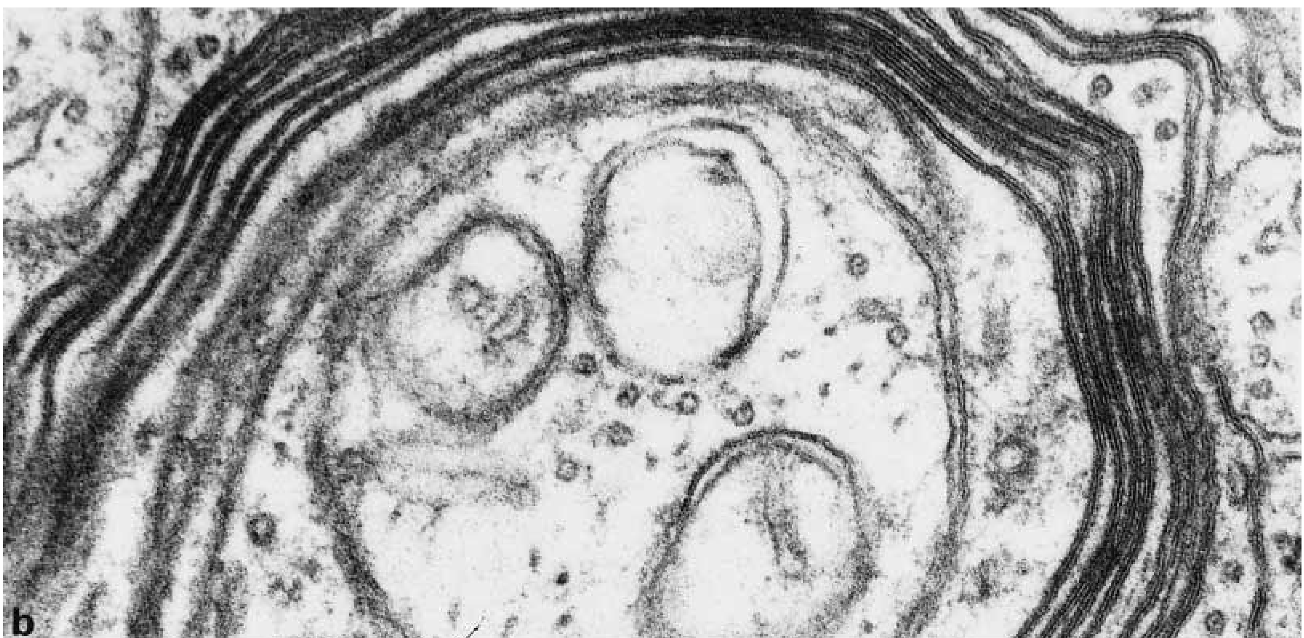
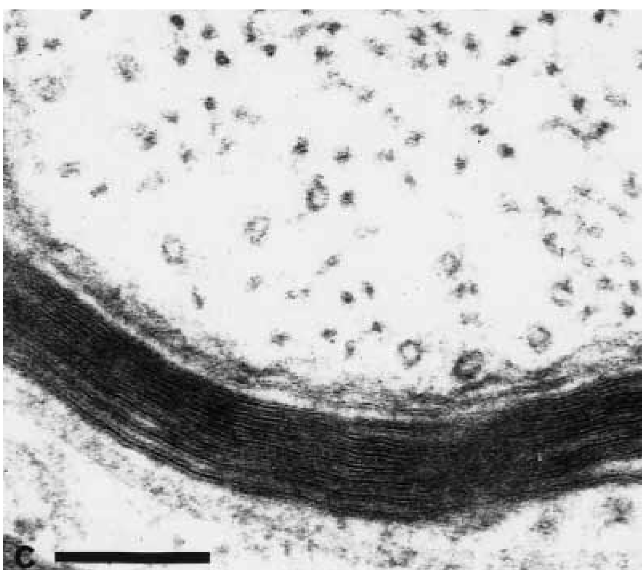


Fig. 5a, b







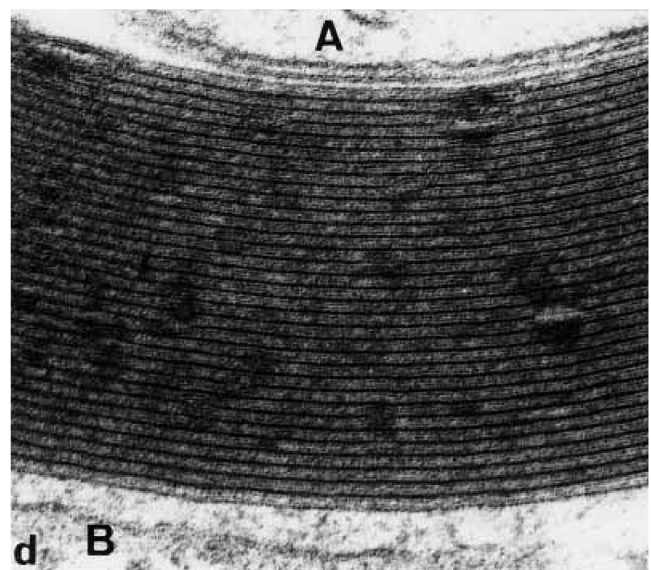


Fig. 6a–d

Myelin proteins of PLP-/MBP-deficient mice ($plp^{-/-}$, $mbp^{-/-}$)

Expression patterns on the RNA level were complemented by the protein analysis of myelins of the wild type and the three genotypes: myelin was prepared from total brains of 28-day-old wild type, $plp^{-/-}$, $mbp^{-/-}$, and ($plp^{-/-}$, $mbp^{-/-}$) mice by sucrose gradient centrifugation (Norton and Poduslo 1973). Myelin of wild type and $plp^{-/-}$ mice accumulates in a distinct white band in the gradient, of *shiverer* mice as a faintly turbid myelin band; however, a distinct opaque myelin band was obtained from the brain of ($plp^{-/-}$, $mbp^{-/-}$) mice.

We analyzed the protein composition of these myelin bands by SDS-PAGE (12%) (Fig. 3A) and Western blot hybridization (Fig. 3B). The pattern of myelin proteins of wild-type mice contains the proteolipid proteins PLP and DM20 (30 and 26 kDa) and the MBP isoforms (21 to 14 kDa). In contrast, in brains of the $plp^{-/-}$ mice, the two isoproteins PLP and DM20 are lacking; however, the pattern of the MBP bands at 21, 18.5, and 14 kDa is identical to the wild type. Brains of *shiverer* mice yield only weak PLP and DM20 bands in SDS-PAGE. The myelin protein pattern of PLP-/MBP-deficient double mutants lacking the PLP and MBP mRNA is completely devoid of PLP, DM20, and MBP. The respective protein deficiencies were confirmed by Western blot analysis with monospecific anti-MAG, rabbit anti-bovine PLP peptide (residency 105–130), and rabbit anti-bovine MBP antibodies (kindly supplied by Dr. David Coleman, Mount Sinai Hospital, N.Y.). The roughly three- to five-fold enhanced expression of MAG is not as distinct in the immunoblot.

Light microscopy

The different degree of hypomyelination in the CNS of *shiverer* mutant ($mbp^{-/-}$) versus double mutant ($plp^{-/-}$, $mbp^{-/-}$) can already be demonstrated at the light-microscopical level.

The cross-section through the optic nerve of the MBP-deficient mouse shows no myelin sheaths (Fig. 4a). In the optic nerve of the PLP-/MBP-deficient mouse, however, one can see disseminated section profiles of myelinated axons (Fig. 4b).

In the region of the CNS-PNS transitional zone of the ventral root, the spinal cord of the double mutant mouse shows a marked difference in thickness between the extremely thin myelin sheath around the CNS part of the axon and the thick PNS myelin on its peripheral portion (↑) (Fig. 4c).

The transverse section through the ventral root of the double mutant ($plp^{-/-}$, $mbp^{-/-}$) shows the light-microscopical appearance of normally myelinated PNS nerve fibers (Fig. 4d).

Electron microscopy

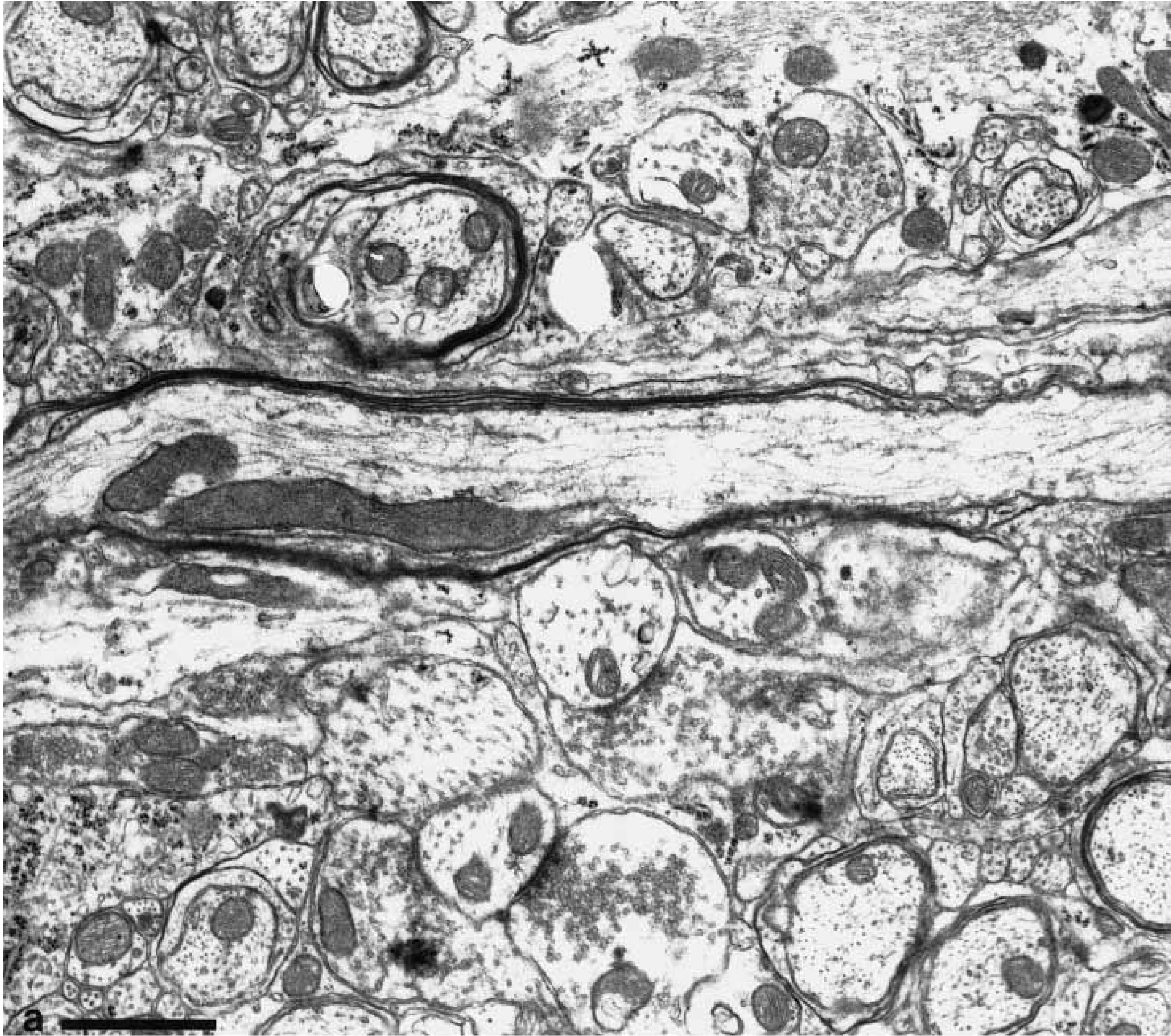
Electron micrographs of cross-sections of the optic nerve of adult *shiverer* ($mbp^{-/-}$) mice at lower resolution (Fig. 5a) show a high degree of hypomyelination of CNS with only few myelin-like sheaths around large-diameter axons. The plasma membrane processes of the oligodendrocytes only seldom completely ensheath one axon. They are usually not spirally wrapped, but pile up with lateral loops accumulating on one side, as indicated by arrows in Fig. 5a.

The optic nerve of double mutant ($plp^{-/-}$, $mbp^{-/-}$) mice (Fig. 5b), however, contains numerous large-diameter axons completely ensheathed with a myelin-like structured membrane, partly loosely packed and spotwise compacted. Perikarya of oligodendrocytes of the CNS of the two mutants are enlarged and resemble the light type of oligodendrocytes during early stages of myelination.

Electron micrographs of cross-sections of the optic nerve of adult mice at higher resolution (Fig. 6) demonstrate that the compaction of the CNS myelin sheaths of the *shiverer* ($mbp^{-/-}$) (a) and the ($plp^{-/-}$, $mbp^{-/-}$) double mutant (b) and (c) are similarly distorted: no main dense line (MDL) is formed because of the missing MBP. The cytoplasmic surfaces of the plasma membrane are separated by a wide cytosolic cleft. The external surfaces, however, adhere tightly and form a strikingly electron-dense, osmiophilic intraperiod line. In the *shiverer* ($mbp^{-/-}$) mutant (a) axons are covered incompletely by numerous cytoplasmic loops similar to the normal lateral loops of the paranodal complex adjacent to Ranvier's nodes (Fig. 6, ★). Between the loops, thin cytoplasmic processes of oligodendrocytes are oriented parallel to the axon. CNS myelin sheaths of the double mutant (c) occasionally show areas of a "pseudocompaction": electron-dense intraperiod lines alternate with the non-compacted stacked cytosolic clefts. Myelin sheaths of PNS of the double mutant (d) show the characteristic periodicity of the compact myelin sheath. Electron-dense main dense lines alternate with the less dense double lines of the intraperiod line.

Fig. 6. Electron micrographs of cross-sections of optic nerves of *shiverer* ($mbp^{-/-}$) (**a**) and double mutant ($plp^{-/-}$, $mbp^{-/-}$) (**b, c**) mice. At higher resolution, the main dense line (MDL) does not appear to be formed in either mutant. Only the external surfaces of the oligodendrocytic processes adhere tightly and form an unusual electron-dense intraperiod line. In the *shiverer* (**a**) the oligodendrocyte lamellae cover the axon incompletely, forming cytoplasmic loops similar to the lateral loops of the paranode. In the double mutant (**b, c**), however, the oligodendrocytic lamellae form complete myelin-like sheaths, which are mostly loosely stacked with a wide cytosolic cleft separating the membranes. Occasionally, this cytosolic cleft is extremely attenuated (**c**), so that the myelin sheath appears pseudo-compacted. **d** Cross-section through an apparently normally compacted PNS myelin sheath in the ventral root of a PLP-/MBP-deficient mouse. *Bar:* 0.2 μm

Fig. 7. Electron micrographs of cross-sections of the spinal cord of double mutant ($plp^{-/-}$, $mbp^{-/-}$) mice (**a**). Longitudinal sectioned internodes often measure only a few micrometers in length. *Bar:* 1.2 μm. **b** The paranodal complex shows the lateral loops in contact with an innermost oligodendrocytic lamella (↑) forming a so-called pseudonode. *Bar:* 0.35 μm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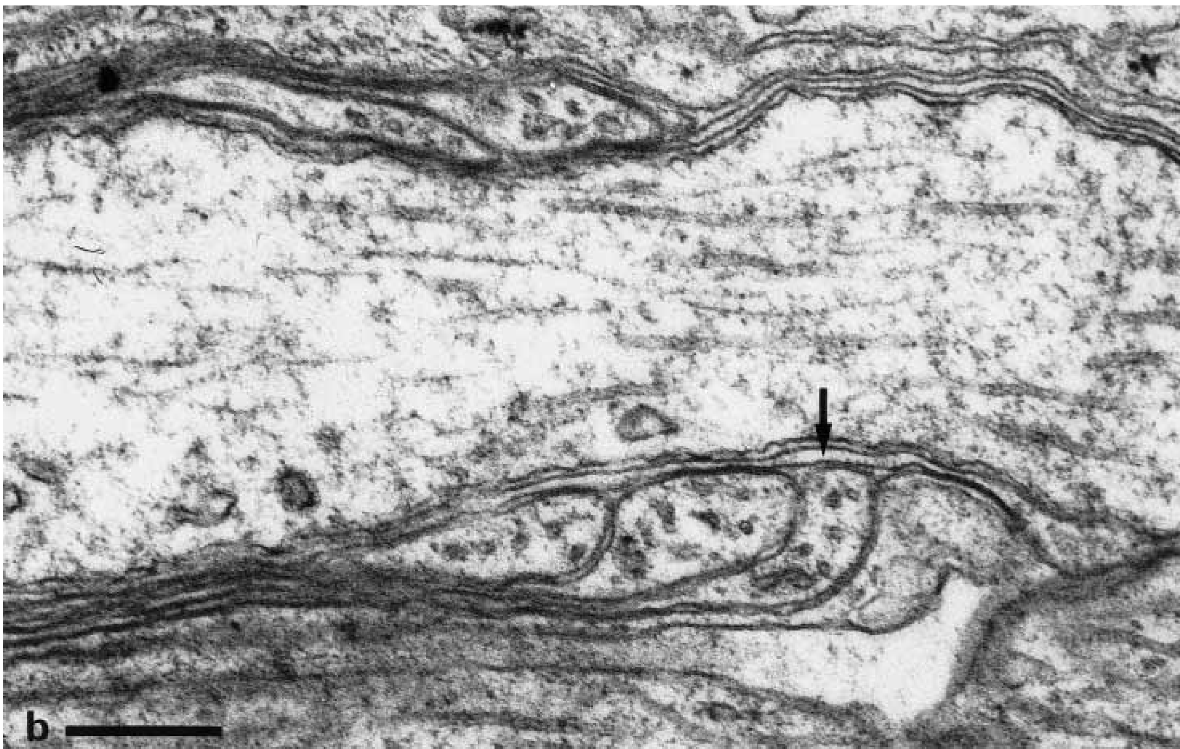
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Longitudinal sections through the myelinated axons in the spinal cord of the double mutant (*plp*$^{-/-}$, *mbp*$^{-/-}$) mice show that the internodes often measure only a few micrometers in length (Fig. 7a). Additionally, the paranodal complex of the PLP-/MBP-deficient oligodendrocytes rarely exhibits the typical axoglial junction between their lateral loops and the axolemma. Instead the lateral loops often establish pseudonodal contacts with a thin oligodendrocyte lamella lying on the axon (Fig. 7b).

Discussion

Oligodendrocytes express in a coordinated, time-regulated fashion myelin-specific proteins during myelination, such as the major structural proteins PLP and MBP, and oligodendrocyte specific enzymes catalyzing the synthesis of lipids, e.g., UDP-galactose ceramide galactosyltransferase (E.C. 2.4.1.45), a key enzyme in the biosynthesis of glycospingolipids (cerebrosides and sulfatides). The expression peaks around the third postnatal week in rodents during the short period of myelination.

MBP is responsible for the cytosolic membrane surface apposition in the myelin sheath, seen as major dense line (MDL) in electron microscopy (Omlin et al. 1982; Rosenbluth 1980b). PLP has been proposed to contribute to the adhesion of the extracytosolic surfaces and the intraperiod line (IL) (Duncan et al. 1987, 1989; Waxman et al. 1990). The PLP-deficient mouse model generated by gene targeting conclusively proved the importance of this integral membrane protein for the compaction of white matter in the intermediate line of CNS myelin (Boison and Stoffel 1994).

MBP is synthesized on free ribosomes, PLP on membrane-bound polysomes (Colman et al. 1982). PLP and MBP occur in myelin in nearly equimolar amounts. Specific intermolecular interactions have been suggested (Edwards et al. 1989) during their assembly with simultaneously synthesized cerebrosides, sulfatides, other phospholipids, and cholesterol in the endoplasmic reticulum membrane for transport to the myelin processes of the plasma membrane.

We have studied the mutual impact of PLP and MBP on the membrane structure, assembly, and oligodendrocyte differentiation in four different genotypes.

The first genotype is heterogeneous on the molecular level and contains point mutations in the coding sequences of the PLP gene in its chromosome X q23 locus. Mutated PLP leads to perturbed myelin membrane structure, hypomyelination, and oligodendrocyte death. The best-known examples of naturally occurring mutants in the PLP gene are the *jimpy* mouse (Morello et al. 1986; Nave et al. 1986; Sidman 1964), the myelin-deficient rat (Boison and Stoffel 1989), the shaking pup dog (Nadon et al. 1990), and several X-linked human sudanophilic leukodystrophies (Pelizaeus-Merzbacher disease, reviewed by Gow et al. 1994). They all have a closely related phenotype during the peak period of myelination. Axial tremor of the body, seizures, and convulsions lead to an early death of jimpy mice and md rat at the age of 3–5 weeks. The nearly complete lack of CNS myelin is due to a premature death of oligodendrocytes (Knapp et al. 1986). We propose that the structurally altered PLP of the PLP mutants is not able to integrate into the oligodendrocyte plasma membrane. This extremely hydrophobic protein, devoid of its specific hydrophobic membrane environment, aggregates within the cell, forming hydrophobic remnant sequences not degradable by proteases. The dominant negative action of abnormal proteolipid proteins in *jimpy* mice could not be reversed by introduction of PLP and DM20 cDNA as transgenes (Nadon et al. 1990). This uncontrolled integration of many copies of PLP minigenes as transgene has led to different phenotypes (Kagawa et al. 1994; Readhead et al. 1994) and to an excess of PLP expression, which prematurely arrests myelin formation and causes oligodendrocyte degeneration similar to the mouse mutants with structurally altered PLP.

The knock-out of the PLP gene expression (*plp*$^{-/-}$) mouse (Boison and Stoffel 1994) contrasts sharply to the pleiotropic phenotype of the point mutations. The most obvious and surprising result was the compatibility of a complete loss of PLP structure and function with oligodendrocyte survival. A distorted, uncompacted myelin sheath with wide spaces between external surfaces of plasma membrane processes has morphologically lost the intraperiod line, but retains a normal main dense line. Apart from these defects and an impaired nerve conductance and neuromotoric coordination, the *plp*$^{-/-}$ mice are healthy and have a normal reproduction rate and the life span of the wild-type mouse. Our results make an essential role of PLP/DM20 for the control of the differentiation of the oligodendrocyte lineage and the survival of oligodendrocytes highly unlikely. A myelin sheath, although structurally severely altered, can be assembled in the absence of PLP. Therefore, the “spiral wrapping principle” in forming the multilayer myelin membrane is conserved and not immanent to the PLP structure and function.

The *shiverer* (*mbp*$^{-/-}$) mouse has a large deletion mutation ranging from intron 2 to 2 kb downstream of the last exon 7 within the MBP gene locus (Molineaux et al. 1986; Roach et al. 1985), which maps to chromosome 18qter (Sidman 1964) and is inherited in an autosomal fashion. MBP deletion in the *shiverer* mouse leads to downregulation of myelination in oligodendrocytes (Rosenbluth 1980b). Unlike the PLP-deficient mouse, the *shiverer* mouse develops neurological symptoms, such as a body tremor, by the end of the second postnatal week and, later on, seizures followed by premature death within about 3 months after birth (Bird et al. 1978; Rosenbluth 1980b). Homozygous mutants are infertile.

The *shiverer* mouse mutation does not interfere with the transcription of other myelin-specific genes. This is documented by the regular transcription of *plp, cgt*, and *mag* (Fig. 2). PLP mRNA translation, however, is strongly reduced and present only as a faint band in the protein pattern of myelin (Fig. 3A). This is also documented by Western blotting (Fig. 3B). It remains to be studied whether the translation rate is reduced or if enhanced PLP degradation occurs.

We suggest that the *shiverer* mouse PLP, synthesized on membrane-bound polysomes, is missing its MBP ligand for forming a heterodimer for transport and targeting to the oligodendrocyte plasma membrane. Instead, membrane synthesis for building the multilayered myelin sheath is inhibited by a hitherto unknown mechanism, which warrants further molecular analysis.

To unravel the mutual impact of the relative PLP and MBP concentrations in oligodendrocytes during myelination, we generated and characterized at the cellular level a new homozygous mouse line, the double mutant (*plp*$^{-/-}$, *mbp*$^{-/-}$). This mouse lacks PLP and MBP, the two main protein constituents of CNS myelin, which are also main antigens of the myelin membrane in CNS. We crossed the *shiverer* (*mbp*$^{-/-}$) into the *plp*$^{-/-}$ locus. Surprisingly, homozygous double mutant mice have ameliorated *shiverer* symptoms and rare seizures. They show a normal development and no premature death of oligodendrocytes and have a normal reproduction rate and a life span of up to now more than 18 months.

Light and electron microscopy reveal that oligodendrocytes of PLP-/MBP-deficient mice are able to ensheath many CNS axons with an atypical myelin containing only few lamellae, which, in contrast to the myelin of *shiverer* mice, spirally wrap axons with a larger diameter. The lamellae form a loose and uncompacted myelin. They lack the major dense lines, but adhere tightly on their extracytosolic surfaces and form distinct electron-dense intermediate lines. The paranodal complexes of the double mutant mice resemble in their morphology the ‘‘pseudonodes’’ described in the CNS of the *shiverer* mutant (Rosenbluth 1980a). The PNS myelin of the double mutant exhibits a normal periodic structure like the nearly normal PNS of MBP-deficient mice (Chernoff 1981).

The expression of other myelin-specific genes other than *mbp* and *plp*, e.g. *cgt*, was unaltered. MAG, normally present as a minor constituent in CNS myelin, is overexpressed in the double mutant at the mRNA level. The MAG mRNA signal in the Northern blot analysis is roughly three- to fivefold stronger than that of the wild type (Fig. 2). However, this overexpression of MAG at the mRNA level is not reflected in the protein pattern of CNS myelin proteins of the double mutant in Coomassie blue-stained polyacrylamide gels. In Western blot analysis, the intensity of the stained MAG band is comparable in the double mutant, the *plp*$^{-/-}$ mutant, and wild-type mouse. However, due to the lack of PLP and MBP in the ‘‘pseudomyelin’’ total-membrane proteins, MAG becomes a major constituent among the residual myelin proteins.

MAG is considered to provide the proper axoglial contact. It is tempting to speculate that, in the (*plp*$^{-/-}$, *mbp*$^{-/-}$) mouse, MAG additionally takes over a basic function of proteolipid protein in maintaining a reduced number of spirally wrapped layers compacted within the intermediate line between extracytosolic surfaces of oligodendrocyte membrane processes.

The PLP-/MBP-deficient mutant mouse line described here might be a valuable model for further studies on oligodendrocyte function during myelination. It will also be a valuable model for future investigations regarding the interactions with other myelin proteins, i.e., myelin-associated glycoprotein (MAG), myelin-oligodendrocyte glycoprotein (MOG), and oligodendrocyte-myelin glycoprotein (OMgp).

Extensive studies have been carried out with complete MBP and derived peptides as potential antigens (Kies 1965; Kies and Alvord 1959; Kies et al. 1965; Lees and Cambi 1985; Lumsden et al. 1966; Trotter et al. 1987) and likewise with PLP and derived peptides (Amor et al. 1993; Endoh et al. 1986, 1990; Greer et al. 1992; Linington et al. 1990; Satoh et al. 1987; Trotter et al. 1987; Tuohy et al. 1988a, b, 1989, 1992; Tuohy and Thomas 1993; Whitham et al. 1991) in the induction of experimental allergic encephalitis (EAE). The double mutant devoid of the two main autoantigens provides a mouse model that might facilitate and expand future studies on the antigens involved in the autoimmune response.

Acknowledgements. We thank Jutta Adam and Christa Franken for their excellent technical assistance in electron microscopy and Maria Düker and Rolf Müller for their skilled biochemical assistance.

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